Investigating the biology of IL23 in monocyte-derived macrophages in enthesitis related arthritis

Corinne Rhiannon Fisher

Department of Medicine

University College London

A thesis submitted for the Degree of Doctor of Philosophy

2021

Declaration

I Corinne Fisher declare that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm this has been indicated in the thesis.

Abstract

Enthesitis related arthritis (ERA) is a subtype of juvenile arthritis with many similarities to the adult spondyloarthropathies (SpA). The innate immune system, intracellular stress responses (including the unfolded protein response (UPR)) and IL23/IL17 pathway are implicated in the pathogenesis of adult SpA. However, these systems remain relatively unexplored in ERA. Novel treatments for SpA which inhibit the IL23/IL17 pathway are now available but their role in the treatment of ERA remains unclear.

The aim of this thesis was to study the biology of IL23 in the innate immune system (in particular in monocyte-derived macrophages (MDMs)) and other relevant cytokines in patients with ERA, compared to a group of age-matched controls and also adult patients with SpA. A bioassay was developed to produce MDMs which were then stimulated with lipopolysaccharide (LPS). Cytokine production at the protein and RNA level and phosphorylation of p38 MAP kinase were used as outputs for this assay. The effect of induction of the UPR on cytokine production was also studied, as well as a number of serum biomarkers known to be relevant in adult SpA.

Key differences were observed in the production of IL23 and other cytokines from LPS-stimulated MDMs with higher levels from patients with ERA compared to healthy controls. Enthesitis, HLA B27 and male sex were especially associated with high levels of cytokine production. Induction of the UPR was not associated with increased cytokine production with the exception of GMCSF and there was no evidence of UPR upregulation in patients with ERA or adult SpA. Higher levels of biomarkers including CRP, calprotectin and Dkk1 were observed in patients with ERA compared to healthy controls.

The results obtained in this thesis indicate important differences in the biology of IL23 and other cytokines in patients with ERA compared to healthy controls which warrants further study.

Impact Statement

Research in to the pathogenesis of ERA is scarce in comparison to adult SpA. Few studies comparing ERA and adult SpA have been published and although the IL23/IL17 pathway and intracellular stress responses (including the UPR) have been studied extensively in adult SpA, this is not the case for ERA.

IL23 plays a key role in enthesitis, the primary pathological feature of SpA across the ages. Findings in this thesis highlight the importance of IL23 and other pro-inflammatory cytokines in the pathogenesis of ERA and identify key features including enthesitis, HLA B27 and male sex as being associated with particularly high levels of IL23. These findings may allow future stratification of patients to earlier or different treatment pathways including the possibility of treatment with IL23 blockade in some patients.

High levels of IL23 were also found in patients treated with TNF inhibitors. This suggests a paradoxical upregulation of the IL23/IL17 pathway and warrants further investigation as this may contribute to the mechanism by which radiographic progression occurs in SpA despite treatment with TNF inhibition. Further, findings in this thesis indicate IL23 and GMCSF production correlate strongly which is important given that GMCSF promotes a pro-inflammatory macrophage phenotype, therefore further enhancing IL23 production. This identifies GMCSF as a potential novel therapeutic target in ERA.

The finding of lower IFNγ production from patients with ERA, compared to healthy controls and patients with polyarticular JIA, provides another mechanism for the upregulation of the IL23/IL17 pathway. The difference seen between the study groups may provide one explanation as to why the IL23/IL17 axis is so important in the pathogenesis of SpA compared to other forms if inflammatory arthritis.

The absence of evidence for UPR upregulation or enhanced cytokine production with UPR induction in MDMs from patients with ERA suggests this may not be the mechanism by which HLA B27 is associated with disease. Thus the investigation of other potential mechanisms is important.

Biomarkers for the diagnosis and prognosis of ERA would be invaluable. This thesis identifies three biomarkers which were higher in patients with ERA compared to healthy controls: CRP, calprotectin and Dkk1. Factors including male sex and HLA B27 were associated with even higher levels of some biomarkers indicating a potential use in patient stratification. However, further longitudinal study is important to confirm these findings. Lower levels of Dkk1 levels in patients with evidence of SIJ fusion identifies another potential mechanism for radiographic progression in patients with SpA and ERA, despite treatment with TNF inhibitors, which warrants further investigation.

Initial results from a pilot study adding oestrogen to the bioassay showed a median decrease in IL23 production and a trend towards increased IFNγ production in patients with

ERA and SpA. This potentially identifies a mechanism for the differences in cytokine levels between males and females but needs further investigation with larger numbers of patients.

This thesis significantly adds to the knowledge of the pathogenesis of ERA, highlights important pathogenic mechanisms for future study and identifies potential novel therapeutic targets for patients with ERA.

Acknowledgments

Firstly, I would like to thank my supervisor, Dr John Ioannou, for the opportunity to undertake this PhD and for his encouragement, support and patience throughout. I would also like to thank my secondary supervisor Dr Despina Eleftheriou for her wisdom and guidance. I would like to express my gratitude to Versus Arthritis for funding this work and for their support and flexibility during this project.

I am extremely grateful to my clinical colleagues, Maria Leandro and Coziana Ciurtin for their constant encouragement and support. I would particularly like to thank Debajit Sen for always believing in me and for reading this thesis. In addition, I would like to mention Lucy Wedderburn who has offered many words of wisdom.

I would not have managed any of the experiments in this thesis without the guidance and teaching of Ania Radziszewska in the lab. I am extremely grateful for her knowledge and patience. I am also extremely grateful to the other members of the lab who made each day enjoyable, in particular Hannah Peckham, Lauren Bourke, Laura Hanns and Kate Webb. I am also very grateful to Hannah Lom and Judith Smith for sharing their knowledge and primer sequences.

I would like to acknowledge all the patients who took part, donating their blood, without which, none of this research would have been possible. I would also like to thank our research nurses Linda Suffield and Frankie Josephs who collected the samples for me and identified suitable patients.

Finally, I would like to thank my family for their unwavering support throughout this PhD, especially my husband and children, who I suspect did not believe this thesis would ever be finished.

Publications

Fisher C, Ciurtin C, Leandro M, Sen D, Wedderburn LR. **Similarities and Differences Between Juvenile and Adult Spondyloarthropathies.** *Front Med (Lausanne). 2021 May 31;8:681621. doi: 10.3389/fmed.2021.681621*

Table of Contents

List of Figures

List of Tables

Abbreviations

AS	Ankylosing spondylitis
BiP	Binding immunoglobulin protein
CHOP	CCAAT/enhancer binding protein homologous protein 10
CCL	Chemokine ligand
CD	Cluster of differentiation
CRP	C reactive protein
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
Dkk1	Dickkopf 1
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERA	Enthesitis related arthritis
GMCSF	Granulocyte macrophage colony stimulating factor
HLA	Human leucocyte antigen
HSP	Heat shock protein
IFN	Interferon
IL	Interleukin
JIA	Juvenile idiopathic arthritis
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCSF	Macrophage colony stimulating factor
MDM	Monocyte derived macrophage
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MRP	Myeloid related protein
NK	Natural killer
NLR	NOD-like receptor
NOD	Nucleotide oligomerisation domain
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
SpA	Spondyloarthropathy
STAT	Signal transducers and activators on transcription
TLR	Toll-like receptor
TM	Tunicamycin
TNF	Tumour necrosis factor
UPR	Unfolded protein response
XBP1	X-box binding protein 1

CHAPTER 1: INTRODUCTION

Overview of Chapter 1

This chapter will start with a discussion about the clinical features of adult SpA and ERA and highlight similarities and differences between these conditions as well as the current gaps in knowledge, in particular for ERA. It will also give an overview of the pathogenesis of SpA focussing on the role of the innate immune system, IL23/IL17 pathway and intracellular stress responses.

The chapter will be divided into two sections comprising a section on the classification criteria, clinical features and current treatments for SpA and ERA (section 1.1). This will be followed by a section on the pathogenesis of SpA and ERA, which initially focusses on aspects of the immune system relevant to this subject and also to subsequent topics covered in this thesis (section 1.2). It ends with a section on novel treatments for SpA and their relevance to patients with ERA.

1.1 Adult and juvenile-onset spondyloarthropathy

1.1.1 Introduction

The concept of spondyloarthropathy (SpA) was first established in 1974 by Moll and colleagues (Moll, Haslock et al. 1974) and comprises a heterogeneous group of inflammatory arthritides occurring in adults and children. SpA can affect the spine (axial SpA (axSpA)) and peripheral joints (peripheral SpA). In adults, axSpA includes the prototypical ankylosing spondylitis (AS) and also nr-axSpA, differentiated from AS by the absence of SIJ changes on plain radiographs. SpA also includes psoriatic arthritis, enteropathic arthritis, reactive arthritis and undifferentiated SpA (Figure 1.1). There is a strong association with HLA B27 across the ages and common clinical features including enthesitis, dactylitis, axial and peripheral arthritis which occur in adults and children. Extra-articular manifestations may include acute anterior uveitis, inflammatory bowel disease and psoriasis. In contrast to other forms of inflammatory arthritis, new bone formation is a key feature, in addition to bone loss associated with inflammation (Dougados and Baeten 2011).

Juvenile-onset SpA is defined by different terminology encompassed by the juvenile idiopathic arthritis (JIA) classification criteria and includes enthesitis related arthritis (ERA), juvenile psoriatic arthritis and juvenile enteropathic arthritis. It shares many of the defining features of SpA with some important differences in clinical presentation and outcome (Ramanathan, Srinivasalu et al. 2013).

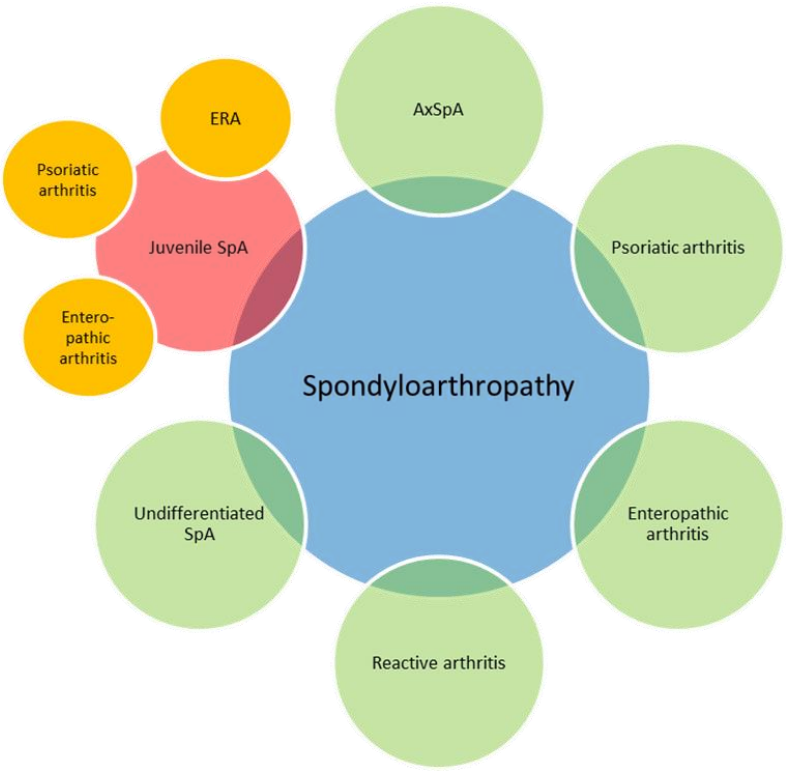


Figure 1.1 The Spectrum of Adult and Juvenile-Onset Spondyloarthropathies

1.1.2 Enthesitis related arthritis

ERA is one of the juvenile forms of SpA and is classified as a subtype of JIA which is the most common form of inflammatory arthropathy in childhood, affecting around 10 000 to 15 000 children in the UK. The term JIA is used to describe any form of inflammatory arthritis of unknown cause occurring in a child or young person before their 16th birthday. It encompasses a broad spectrum of conditions, divided into 7 subtypes according to the pattern of joint involvement and extra-articular features at disease onset which are defined by the International League of Associations for Rheumatology (ILAR) classification criteria. Some forms of JIA have corresponding similar adult-onset arthritides (such as ERA and adult SpA) but others are quite separate diseases with no parallel adult form. Apart from ERA, other JIA subtypes include psoriatic JIA, oligoarticular JIA (subdivided into persistent or extended), polyarticular JIA (subdivided into rheumatoid factor negative and positive disease), systemic JIA with systemic features in addition to arthritis (including fever, rash, lymphadenopathy and organomegaly) and undifferentiated JIA defined as having features not fitting any of the categories above or fitting more than one category (Petty, Southwood et al. 2004). Disease course, treatment and prognosis are dependent on subtype but over half of those affected have on-going disease in to adulthood (Minden 2009).

ERA accounts for 11-16% of all JIA (Flato, Hoffmann-Vold et al. 2006) but in adolescence the prevalence increases to around 35%. It is characterised by arthritis, enthesitis and an

association with HLA B27 as defined by the ILAR classification criteria (table 1.1). ERA has a varied clinical phenotype that shares features with adult SpA. Involvement of the axial skeleton eventually occurs in around 60% (Fisher, Ioannou et al. 2012, Goirand, Breton et al. 2018) but in early disease enthesitis and peripheral lower limb arthritis are more common clinical features in comparison to adult SpA (Jadon, Ramanan et al. 2013).

Enthesitis related arthritis (ERA)	
Arthritis AND enthesitis OR	
Arthritis OR enthesitis with at least two of	presence of or a history of sacroiliac joint tenderness and/or inflammatory lumbosacral pain presence of HLA B27 antigen onset of arthritis in a male > 6 years old acute anterior uveitis history of AS, ERA, sacroiliitis with inflammatory bowel disease, reactive arthritis or acute anterior uveitis in a first degree relative
Exclusions	psoriasis or a history of psoriasis in the patient or a first degree relative presence of immunoglobulin M rheumatoid factor on at least two occasions 3 months apart presence of systemic JIA in the patient

Table 1.1 International League of Associations for Rheumatology (ILAR) classification criteria for ERA (Petty, Southwood et al. 2004).

1.1.3 Epidemiology of SpA and ERA

The prevalence of adult SpA varies widely depending on geographical area which may reflect the prevalence of HLA B27, but also other factors (Mathieu, Paladini et al. 2009). The highest prevalence is reported in North America (1.35%) and Europe (0.54%) compared to South East Asia (0.20%) and South Asia (0.22%). The prevalence of ERA is not well reported. Studies examining the prevalence of AS and psoriatic arthritis only demonstrate a higher prevalence in Europe (0.25% and 0.19% respectively) (Stolwijk, van Onna et al. 2016). Historically, SpA in adults was reported more frequently in males than females but with new definitions including nr-axSpA, the prevalence is almost equal (Rusman, van Vollenhoven et al. 2018). Most patients with SpA have symptoms before the age of 45 years and the median age of onset for axSpA is around 25 years (Boel, Lopez-Medina et al. 2022).

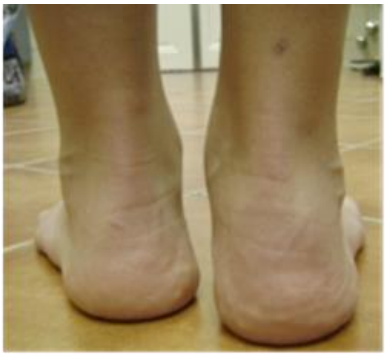
In ERA, there remains a significant male predominance of around 70-80% as females are more likely to be classified as oligoarticular JIA due to the inclusion of being male in the JIA classification criteria for ERA (Butbul Aviel, Tyrrell et al. 2013, Guo, Cao et al. 2015). The median age of onset is later than some other subtypes of JIA at between 10 and 12 years (Mistry, Patro et al. 2019). HLA B27 is found in 60-80% of patients and is associated with

higher active joint count, sacroiliitis and higher overall disease activity (Gmuca, Xiao et al. 2017, Mistry, Patro et al. 2019). A survey of adult patients with SpA found that 8.6-11 % reported onset of disease in childhood which equates to a prevalence of 11-86 per 100 000 people (Gomez, Raza et al. 1997).

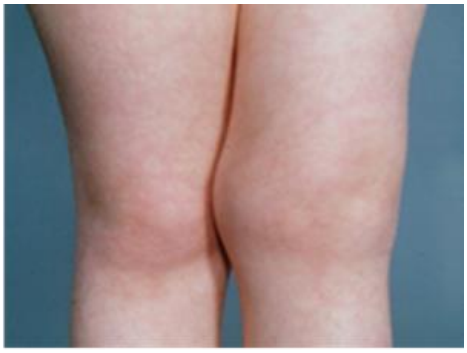
1.1.4 Clinical features of adult SpA and ERA

In adults, the most common types of SpA are axSpA (including AS) and psoriatic arthritis. Chronic back pain is the predominant symptom for those with axial disease, usually with pronounced stiffness and improvement with exercise. The peripheral arthritis associated with SpA often involves the larger lower limb joints in an asymmetric distribution, although psoriatic arthritis may manifest in different patterns including a small joint arthropathy of the hands and feet. Enthesitis associated with SpA is also more common in the lower limbs, often affecting the heel including the insertion of the Achilles tendon and plantar fascia in to calcaneus (Terenzi, Monti et al. 2018).

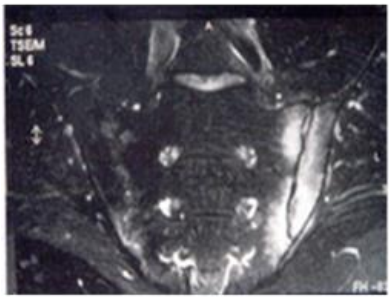
Similarly, the clinical features of ERA typically include asymmetrical, lower limb arthritis and enthesitis and also inflammation of the spine and SIJs in some patients (figure 1.2) which can occur at the time of disease onset or later on in disease (after median 2.5 years, unpublished data, University College London Hospital cohort of 125 patients followed up over a median of 5.5 years) (Goirand, Breton et al. 2018) and often around the time of puberty. Early symptoms may wax and wane and therefore there is often a delay in diagnosis of months or even years (Jadon, Ramanan et al. 2013).



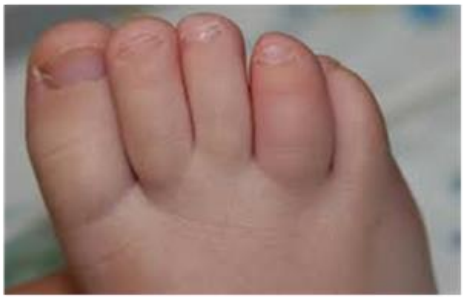
Enthesitis



Peripheral Arthritis



Axial Arthritis



Dactylitis

Figure 1.2 Clinical Features of ERA

In ERA, the most commonly involved joints include knee (40-50%), ankle (25-40%) and hip (30-40%) (Butbul Aviel, Tyrrell et al. 2013, Guo, Cao et al. 2015). Enthesitis also commonly affects the lower limb and is present in more than half of patients with ERA. The most commonly affected sites include the inferior pole of the patella, plantar fascial insertion into the calcaneum, Achilles tendon and the tibial tuberosity (Weiss, Klink et al. 2011, Gmuca, Xiao et al. 2017, Rumsey, Guzman et al. 2018). Clinical examination is likely to underestimate the presence of enthesitis with one study of 30 patients with ERA detecting enthesitis in half of the patients but subsequent ultrasound identifying enthesitis in 83% (Shenoy and Aggarwal 2016). Enthesitis is a key feature of ERA, especially in early disease and the lack of sensitivity of clinical examination may contribute to diagnostic delay.

As with adult SpA, some patients with ERA have peripheral arthritis and enthesitis only and some also develop axial disease. Various predictors of axial disease in ERA have been identified. In one study of 59 patients with ERA, 30 % had developed SIJ involvement a median of 1 year 3 months after diagnosis. The number of active joints and active entheses at onset were early predictors of axial disease (Pagnini, Savelli et al. 2010). Another study of 143 patients with juvenile SpA (including those with psoriasis) found that those with hip joint involvement were more likely to develop sacroiliitis (Stoll, Bhore et al. 2010). HLA B27 and elevated CRP are also associated with sacroiliitis in ERA (Weiss, Xiao et al. 2016).

Identifying those with axial involvement is important as the prognosis may be worse (Bryan and Rabinovich 2014), there is a greater requirement for treatment with TNF inhibitors and lower chance of achieving drug free remission (Fisher, Radziszewska et al. 2015). There is also a reasonably high prevalence of 'silent sacroiliitis' in ERA with positive findings on magnetic resonance imaging (MRI) in the absence of symptoms (Bollow, Braun et al. 1998, Weiss, Xiao et al. 2016).

In common with adult SpA, extra-articular manifestations, including acute anterior uveitis and inflammatory bowel disease, are relatively common, especially in those who are HLA B27 positive (Fisher, Suffield et al. 2014, Gmuca, Xiao et al. 2017, Sen and Ramanan 2017). Family history of HLA B27-associated disease is reported in around 15% (Gmuca, Xiao et al. 2017).

1.1.5 Classification criteria for SpA and ERA

Historically, several different sets of classification criteria have been used for adult SpA. Initially developed in the 1970s and modified in the 1980s, the New York criteria for the diagnosis of AS focus only on axial disease and rely on grading of sacroiliitis using plain radiographs (van der Linden, Valkenburg et al. 1984). In the early 1990s, two sets of criteria were proposed: the Amor criteria (Amor, Dougados et al. 1990) and the European SpA Study Group (ESSG) criteria (Dougados, van der Linden et al. 1991). Both encompassed the whole SpA disease group including back pain and peripheral symptoms but had some disadvantages and therefore the Assessment of SpA International Society (ASAS) developed a new set of criteria including but separating axial and peripheral SpA (Table 1.2) (Rudwaleit, van der Heijde et al. 2011).

<table>
<tr><th colspan="3">Axial SpA</th><th>Peripheral SpA</th></tr>
<tr><td colspan="3">-for patients with ≥3 months back pain (with/without peripheral manifestations), aged <45 years</td><td>- for patients with peripheral manifestations only</td></tr>
<tr><td>Sacroiliitis on imaging plus ≥1 SpA feature</td><td>OR</td><td>HLA B27 plus ≥2 other SpA features</td><td>Arthritis or enthesitis or dactylitis

plus
≥1 SpA feature:
Uveitis
Psoriasis
Crohn’s disease/ Ulcerative Colitis
Preceding infection
HLA B27
Sacroiliitis on imaging</td></tr>
<tr><td colspan="3" rowspan="2">SpA features:
Inflammatory back pain
Arthritis
Enthesitis (heel)
Uveitis
Dactylitis
Psoriasis
Crohn’s disease/ Ulcerative Colitis
Good response to NSAIDs
Family history of SpA
HLA B27
Elevated CRP</td><td>OR</td></tr>
<tr><td>≥2 other SpA features:
Arthritis
Enthesitis
Dactylitis
Inflammatory back pain ever
Family history of SpA</td></tr>
</table>

Table 1.2. ASAS Classification criteria for axial SpA and peripheral SpA (NSAIDs = non-steroidal anti-inflammatory drugs)

The ASAS criteria focus on the different clinical features of SpA (including both axial and peripheral arthritis, enthesitis and extra-articular manifestations), HLA B27, and also encompass MRI assessment of the SIJs enabling much earlier diagnosis of axial disease which is not detected on plain radiographs until a late stage (Dougados and Baeten 2011).

The ILAR classification criteria for ERA (table 1.1) were developed in recognition of the distinct clinical presentation of childhood disease compared to adult SpA. These criteria replaced others including those described by Rosenberg and Petty in 1982, which defined the syndrome of seronegative enthesopathy and arthropathy (SEA). This identified a group of children and young people who had enthesopathy and arthralgia or arthropathy, negative rheumatoid factor and anti-nuclear antibody who were predicted to go on to develop SpA (Rosenberg and Petty 1982). Other terms which were also previously in use include juvenile AS and juvenile SpA. A number of different classification systems used for adult SpA were applied to children and young people but did not capture the spectrum of juvenile disease well. The ILAR criteria were therefore designed to highlight the typical clinical features of SpA in children and young people, specifically peripheral arthritis and enthesitis. A study of adult patients diagnosed with ERA in childhood found that 95% fulfilled the classification criteria for adult SpA (Oliveira-Ramos, Eusebio et al. 2016).

Compared to the ASAS criteria for adult SpA, the ILAR criteria lack differentiation between axial and peripheral disease and do not include inflammatory markers or MRI assessment in the classification criteria for ERA. Patients with psoriatic arthritis and with a family history of psoriasis in a first degree relative are specifically excluded from the ILAR ERA category, even though these patients often have a disease phenotype closely resembling ERA and adults with psoriatic arthritis are considered as having a SpA. In practice, the overlapping nature of ERA and psoriatic JIA, results in a high proportion of patients who would meet the ASAS criteria for adult SpA being classified in the undifferentiated JIA group (Weiss and Roth 2020). There is a male predominance in ERA which may in part be explained by the inclusion of male sex in the ILAR classification criteria which increases the chance of diagnosis in males. This leads to more females being classified as other JIA subtypes such as oligoarticular JIA which may not be appropriate given the evidence in adult SpA of the reduction in male predominance in recent years (Rusman, van Bentum et al. 2020). For these reasons and the fact that those with ERA and axial arthritis are not recognised separately from those with peripheral arthritis only (with different disease course and outcome), questions have arisen over the suitability of the ERA classification criteria and have resulted in a resurgence of the use of the term juvenile SpA (Hofer, Mouy et al. 2001, Colbert 2010). This controversy over classification and terminology and lack of alignment with adult SpA criteria has significantly hampered the development of collaborative research studies in this field resulting in relatively few studies examining the clinical patterns, outcomes and pathogenesis of the whole age spectrum of SpA, in particular ERA.

1.1.6 Diagnosis of ERA

As with adult SpA, the diagnosis of ERA is made clinically but imaging techniques such as ultrasound and MRI are useful adjuncts to confirm the extent and severity of disease. However, plain radiographs of the SIJs in children and young people are unhelpful and may lead to misclassification of disease with frequent false negative and false positive results (Weiss, Xiao et al. 2018). Ultrasound may be useful to confirm the presence or absence of enthesitis. However, variability in entheseal thickness and lack of a ‘normal range’ in children and young people poses problems in clinical practice. One study in healthy children revealed a correlation between entheseal thickness and age (Chauvin, Ho-Fung et al. 2015). Another pilot study noted a correlation with weight and that boys tended to have thicker entheses than girls. However, significant variability was noted even between sides in the same subject and therefore further study is needed to better characterise ultrasound findings in healthy children and young people and in those with ERA and other conditions affecting the entheses (Lin, Diab et al. 2015).

MRI of the SIJs and spine is not included in the classification criteria for ERA but is considered the standard investigation for the diagnosis of sacroiliitis in children and young people. Features of sacroiliitis are comparable to those found in adults (Weiss,

Maksymowych et al. 2018) but standard MRI images can be difficult to interpret in children and young people. Features of normal marrow and cartilage maturation such as bilateral symmetrical metaphyseal high signal intensity and cortical irregularities along the ilial margin of the SIJ can be mistaken for sacroiliitis (Vleeming, Schuenke et al. 2012, Chauvin, Xiao et al. 2019). A multicentre study of patients with ERA and psoriatic JIA found significant variation in the reporting of inflammatory and structural SIJ lesions with frequent false positive results (Weiss, Brandon et al. 2020). Another single centre study in children and young people demonstrated low to moderate inter-reporter reliability in the interpretation of SIJ MRI (Orr, Andronikou et al. 2018). The development of novel MRI techniques may help to distinguish true sacroiliitis from the changes associated with normal maturation but further study is needed (Bray, Vendhan et al. 2016).

1.1.7 Assessment of disease activity in SpA and ERA

Clinical assessment of disease activity in adult SpA is heavily dependent on the use of patient-reported outcomes and thus subjective measures. The Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (Garrett, Jenkinson et al. 1994) has questions about fatigue, axial symptoms, peripheral symptoms, enthesitis, and morning stiffness and is commonly used alongside the Bath Ankylosing Spondylitis Functional Index (BASFI) which assesses the degree of functional limitation (Calin, Garrett et al. 1994). The AS disease activity score (ASDAS) (van der Heijde, Lie et al. 2009) was therefore developed to include some objective measures in addition to patient-reported outcomes and includes inflammatory markers (either ESR or CRP) as well as four questions from the BASDAI. The ASDAS is now widely used for assessing disease activity in SpA with validated classifications for inactive, low, high or very high activity (Machado, Landewe et al. 2011).

Disease severity in adult SpA (in particular axSpA) can be assessed by the Bath Ankylosing Spondylitis Metrology Index (BASMI) which measures spinal movement and the modified Stoke AS Severity Score (mSASSS) which evaluates radiographic severity. These measures focus predominantly on axial disease, reflect late effects of disease and therefore are not sensitive for early or peripheral SpA (Jones, Porter et al. 1995, van der Heijde and Landewe 2005). Imaging, in particular MRI, can be used to assess disease activity and severity in axial disease, peripheral arthritis and enthesitis. MRI features of axial SpA include inflammatory lesions such as bone marrow oedema and osteitis and structural lesions such as erosions, sclerosis and bony ankylosis but these features alone lack specificity and therefore must be interpreted together with the clinical picture (Braun, Baraliakos et al. 2018, Lukas, Cyteval et al. 2018, Jones, Bray et al. 2019). Other imaging modalities such as ultrasound are helpful to assess enthesitis which is often difficult to detect clinically although various clinical scores exist (Braun, Baraliakos et al. 2018, Kaeley 2020).

In ERA, different disease assessment tools are used, which reflect the classification of ERA as a subtype of JIA and also the differences in clinical features in ERA compared to adult SpA.

Disease activity in JIA is assessed using the juvenile arthritis disease activity score (JADAS) which measures four domains: physician global assessment, patient global assessment, active joint count and an inflammatory marker (either CRP or ESR) (Consolaro, Ruperto et al. 2009). Alternatively, the JIA core outcome variables can be used to assess change in disease activity. These include physician global assessment, patient/ parent global assessment, functional status, inflammatory marker (ESR), number of joints with active arthritis and number of joints with restricted range of movement (Giannini, Ruperto et al. 1997). However, these measures do not assess enthesitis or axial arthritis and are therefore likely to underestimate disease activity in many patients with ERA. Measures for adult SpA such as the BASDAI have been applied in ERA but focus more on axial disease and less on peripheral arthritis and enthesitis and so are unlikely to capture disease activity well. The BASDAI but not the ASDAS has been validated in children and young people (Zanwar, Phatak et al. 2018). More recently, the juvenile SpA disease activity (JSpADA) index has been developed. This assesses 8 domains including active joint count, enthesitis count, patient pain assessment, an inflammatory marker (either CRP or ESR), morning stiffness, clinical evaluation of sacroiliitis, uveitis and back mobility measured by the modified Schober's test. It has undergone preliminary validation and appears to be superior to other JIA disease activity measures in assessing disease activity in ERA (Weiss, Colbert et al. 2014). It has subsequently been validated in other patient populations (Zanwar, Phatak et al. 2018).

1.1.8 Patient outcomes in adult SpA and ERA

Historically, patients with adult SpA and hip arthritis or three or more of the following were said to have a worse prognosis: persistently raised erythrocyte sedimentation rate (ESR), unresponsiveness to NSAIDs, limitation of the lumbar spine, dactylitis, oligoarthritis or onset younger than 16 years (Amor, Santos et al. 1994). As discussed above, adult SpA encompasses several disease subtypes which are often studied separately for the purposes of disease outcome. The most common and thus frequently studied is axSpA and in particular AS. A recent systematic literature review comparing burden of disease and treatment in axSpA and nr-axSpA found that axSpA was more common in men, smokers and those with longer disease duration and was associated with more diagnostic delay. AxSpA was also associated with higher levels of CRP and ESR, more structural changes such as syndesmophytes and ankylosis on MRI, higher BASMI and more uveitis. No significant differences were noted in BASDAI, BASFI, quality of life measures and treatment modalities between the axSpA and nr-axSpA. Peripheral manifestations including arthritis and enthesitis were more common in nr-axSpA (Lopez-Medina, Ramiro et al. 2019). It is clear that axSpA (including nr-axSpa) has a much wider impact on patient outcomes including effects on work productivity (Healey, Haywood et al. 2011) and mood. Higher disease activity scores correlate with anxiety and depression (Martindale, Smith et al. 2006, Brionez, Assassi et al. 2010), fatigue (Aissaoui, Rostom et al. 2012) and other physical and psychosocial health related quality of life measures (Packham 2018). However, studies have

demonstrated that treatment of axSpA, in particular with biologic therapies, can significantly improve these outcome measures (Boonen, Patel et al. 2008, van der Heijde, Revicki et al. 2009, van der Heijde, Deodhar et al. 2014).

Studies of clinical outcomes in JIA are rarely divided by subtype and there are therefore relatively few studies of patient outcomes in ERA. Historically, patients with ERA have one of the lowest remission rates in adulthood and a poor prognosis compared to other subtypes of JIA. This may be due, in part, to the frequent delay in diagnosis because of the fluctuating nature of early symptoms (Jadon, Ramanan et al. 2013). The presence of HLA B27 in a study of patients with JIA was associated with an increased chance of developing inflammatory lower back pain and, in males, enthesitis and older age of disease onset (Berntson, Damgard et al. 2008). In a follow up study, patients who were HLA B27 positive had a lower chance of attaining clinical remission after 8 years of disease (Berntson, Nordal et al. 2013).

A study before the routine use of biologics found that patients with ERA had a remission rate of 18% at a median of 16.5 years compared to oligoarticular JIA (54%) and systemic JIA 47% (Minden, Niewerth et al. 2002). Another study in the pre-biologic era reported lower levels of physical functioning, poorer physical health status and more bodily pain compared to other JIA subtypes and healthy controls after a median of 15.3 and 23 years follow-up. Male sex, ankle and hip arthritis and persistently raised inflammatory markers were associated with a worse prognosis (Flato, Hoffmann-Vold et al. 2006). Two studies comparing juvenile and adult SpA reported a greater requirement for hip arthroplasty in patients with juvenile SpA (Gensler, Ward et al. 2008, Jadon, Shaddick et al. 2015).

In the 'post-biologic era', similar findings have been reported. The Childhood Arthritis Rheumatology Research Alliance (CARRA) registry found that patients with ERA had more frequent pain, higher pain intensity and impaired function compared with other JIA subtypes (Weiss, Beukelman et al. 2012). Another study found that patients with ERA reported worse well-being and pain (Boiu, Marniga et al. 2012) and a study of patients with JIA found that ERA was associated with worse body pain, quality of life and physical functioning compared to other subtypes of JIA (Taxter, Wileyto et al. 2015). An explanation for this may be that those with ERA treated with tumour necrosis factor (TNF) inhibitors appear to have a lower chance of achieving inactive disease compared to other JIA subtypes (Donnithorne, Cron et al. 2011). In a study of 118 patients with juvenile SpA, nearly a quarter of patients were in remission off treatment after 4 years observation. However, 57 % had ongoing active disease despite treatment and the risk of this was higher in those with axial symptoms and who were HLA B27 positive (Weiss, Minden et al. 2017). A small study showed patients with refractory hip arthritis were less likely to respond to TNF inhibitors and progression of sacroiliitis occurred despite treatment at a median of 7.2 years follow up (Hugle, Burgos-Vargas et al. 2014). As in adult SpA, SIJ fusion occurs despite treatment with TNF inhibition (Bray, Lopes et al. 2019).

1.1.9 Biomarkers to aid diagnosis, determine disease activity and predict progression in SpA

There has been great interest in identifying biomarkers to aid diagnosis and predict prognosis in adult SpA. As discussed above, diagnostic delay is common in SpA and also in ERA and therefore identifying biomarkers which would aid diagnosis is vital. In addition, prognostic markers, for example to predict those who will develop axial arthritis in ERA are important to guide treatment and improve outcome (Reveille 2015).

Biomarkers proposed for aiding diagnosis and predicting disease activity include C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) but these are raised in less than half of all patients with SpA and therefore lack sensitivity and specificity (Ruof and Stucki 1999, Maksymowych 2017). However, elevated CRP is associated with more extensive SIJ inflammation and worse spinal radiographic changes and progression (Bredella, Steinbach et al. 2006, Poddubnyy, Rudwaleit et al. 2011, Ramiro, van der Heijde et al. 2014, Poddubnyy, Protopopov et al. 2016). CRP may also predict response to treatment with TNF inhibitors (Sieper, van der Heijde et al. 2015) and therefore is helpful in the minority of patients who have a raised CRP.

Studies investigating serum levels of pro-inflammatory cytokines in SpA have not produced consistent results but there are difficulties with measuring serum and plasma levels of cytokines which can be influenced by various factors including diurnal rhythm, sample collection and storage (Aziz 2015). Interleukin (IL)6, IL23 and IL17 have all been reported as elevated in the serum of patients with SpA especially with active disease (Bal, Unlu et al. 2007, Wendling, Cedoz et al. 2007, Romero-Sanchez, Robinson et al. 2008, Melis, Vandooren et al. 2010, Mei, Pan et al. 2011, Chen, Chang et al. 2012). However, other studies have not shown differences in levels between patients and healthy volunteers or associations with disease activity (Andersen, Rasmussen et al. 2012, Sveaas, Berg et al. 2015).

Chemokines have also been studied in the context of SpA. Several matrix metalloproteinases (MMPs) have been proposed as potential biomarkers including MMP3, MMP8 and MMP9. MMP3 levels have been shown to be higher in patients with SpA compared to healthy volunteers (Gao, Zhang et al. 2015) and in some studies reflected disease activity and also predicted structural damage (Maksymowych, Landewe et al. 2007). In ERA, MMP3 levels were found to be no different to healthy volunteers but did correlate with disease activity (Viswanath, Myles et al. 2011). MMP8 and 9 have been shown to be closely associated with disease activity in SpA in one study (Mattey, Packham et al. 2012).

Calprotectin is another acute-phase protein expressed during monocyte and macrophage infiltration of inflamed tissues. Faecal calprotectin is an established biomarker of disease activity in inflammatory bowel disease (Lopez, Leach et al. 2017). Synovial fluid (SF) and serum calprotectin have been shown to be elevated in other forms of inflammatory arthritis (Abildtrup, Kingsley et al. 2015). Serum levels are significantly increased in SpA and fall

following treatment with TNF inhibitors (Turina, Yeremenko et al. 2014, Gupta, Bhattacharya et al. 2016). One study has suggested levels may correlate with disease activity (Jarlborg, Courvoisier et al. 2020). In JIA, elevated serum calprotectin levels predicted flare on withdrawal of methotrexate treatment (Foell, Wulffraat et al. 2010) but this was not confirmed in a recent prospective trial of patients with JIA on anti-TNF treatment (Hinze, Foell et al. 2019). In SpA, elevated levels at baseline may also predict radiographic progression (Turina, Sieper et al. 2014).

Other biomarkers proposed for predicting radiographic progression and prognosis include circulating protein fragments from cartilage and connective tissue degradation (Maksymowych 2015). Levels of one such marker, C-terminal cross-linking telopeptide of type II collagen (CTX-II), correlated with markers of disease activity, radiological damage and progression in one study (Vosse, Landewe et al. 2008). Other markers related to type II and type III collagen include two MMP-generated neo-epitopes, C2M and C3M, both have been found to be elevated in patients with AS and C3M appears to correlate with radiographic progression (Bay-Jensen, Wichuk et al. 2013).

Biomarkers related to the process of new bone formation in SpA have also been investigated to identify factors which may predict radiographic progression. Bone remodelling is thought to involve an interaction between chronic inflammation and the Wnt pathway (Lories and Haroon 2014) and appears to be sequential with new bone formation occurring on resolution of inflammation (Tseng, Pitt et al. 2016). The Wnt signalling pathway is involved in many aspects of cell function and promotes osteoblastogenesis and therefore new bone formation. Inhibitors of this pathway include Dickkopf 1 (Dkk1) which has been studied in patients with AS and found to be lower in most cohorts (Daoussis, Liossis et al. 2010, Kwon, Lim et al. 2012, Klingberg, Nurkkala et al. 2014). Interestingly, patients with higher levels do not have evidence of syndesmophyte formation (Yucong, Lu et al. 2014), suggesting that the presence of Dkk1 may inhibit new bone formation in patients with AS. Bone formation is also promoted by bone morphogenic proteins (BMPs) through stimulation of osteoblast differentiation and proliferation. Sclerostin inhibits BMPs and also the Wnt pathway (Wendling and Claudepierre 2013). Variable levels have been reported in patients with AS. However, low sclerostin levels have been found in some studies compared to healthy controls and this was associated with bony progression (Saad, Ribeiro et al. 2012, Klingberg, Nurkkala et al. 2014). Other potential biomarkers predicting outcome and radiographic progression include vascular endothelial growth factor (VEGF) and osteoprotegrin (OPG). Elevated levels of VEGF have been found in serum from patients with AS, especially those with active disease and with greater radiographic progression (Drouart, Saas et al. 2003, Pedersen, Sorensen et al. 2011). OPG is a member of the TNF receptor superfamily and acts by blocking RANKL binding to its receptor (RANK) on osteoclasts, therefore preventing osteoclastogenesis and bone loss. RANKL expression is upregulated by pro-inflammatory cytokines thus explaining the bone loss seen with inflammatory arthritis (Ruscitti, Cipriani et al. 2015). OPG levels have been variably reported in SpA, with some

studies reporting higher levels and some lower levels compared to controls (Reveille 2015) and others reporting an association with patient reported disease activity measures (de Andrade, de Castro et al. 2014).

Thus, there are numerous biomarkers with the potential for aiding diagnosis and predicting prognosis in SpA but currently none with proven sensitivity. Studies of biomarkers in ERA are limited and it is unclear whether the same markers studied in SpA will also be helpful in patients with ERA.

1.1.10 Current management of SpA and ERA

In both adults and children, multidisciplinary management of SpA is key to improving function and quality of life. In adults with SpA, physical therapy is of proven benefit and physiotherapy intervention either as supervised exercises or an individual home-based exercise programme may be equally effective (Dagfinrud, Kvien et al. 2008, Liang, Zhang et al. 2015). There are no studies in patients with ERA, however, the impact of diagnosis and symptoms (as well as delayed diagnosis), is often significant on psychosocial well-being as well as education and physical functioning.

NSAIDs are the first line pharmacological therapy in adult SpA and result in improved back pain and stiffness in 70-80% of patients with AS (Song, Poddubnyy et al. 2008) but only around a third of those with early active axSpA achieve partial remission on NSAIDs alone (Sieper, Lenaerts et al. 2014). NSAIDs are also of benefit in adults with peripheral SpA where conventional disease modifying anti-rheumatic drugs (DMARDs) such as methotrexate and sulphasalazine may be effective (Braun, Zochling et al. 2006, Fagerli, van der Heijde et al. 2014). There is no evidence that these conventional DMARDs are effective in axSpA (Haibel, Brandt et al. 2007, Chen, Lin et al. 2014).

In some studies, NSAIDs have been shown to reduce CRP in adult patients with axSpA (Barkhuizen, Steinfeld et al. 2006, Sieper, Klopsch et al. 2008) but this is not consistent and NSAIDs do not appear to improve SIJ inflammation on MRI (Baraliakos, Kiltz et al. 2017). However, there is some evidence from early studies that prolonged continuous use of NSAIDs may slow radiographic progression in AS (Boersma 1976, Wanders, Heijde et al. 2005) although this was not confirmed in a more recent prospective study (Sieper, Listing et al. 2016).

There is good evidence for the efficacy of TNF inhibitors in adult SpA with agents including etanercept (Davis, Van Der Heijde et al. 2003), adalimumab (van der Heijde, Kivitz et al. 2006), infliximab (van der Heijde, Dijkmans et al. 2005), golimumab (Inman, Davis et al. 2008) and certolizumab (Landewe, Braun et al. 2014) all effective in reducing disease activity in axSpA (including nr-axSpA). Although recommended for patients with persistently high disease activity calculated by BASDAI or ASDAS, not all patients respond. Clinical trials of TNF inhibitors in axSpA report around 60 % of patients achieving a response rate of 20 %

(Noureldin and Barkham 2018) although real-world studies suggest the response rates are lower at around 50 % (Jones, Dean et al. 2020). Early treatment may be more beneficial with evidence of significant reduction in SIJ inflammation on MRI (Poddubnyy, Listing et al. 2016) and the possibility of partial remission. One study of adult patients with early sacroiliitis and less than 3 years symptoms found that 55.6% of patients achieved partial remission defined by ASAS criteria compared to around 20% of those treated with established disease (Barkham, Keen et al. 2009). Sustained remission without treatment is not seen following initiation of treatment with TNF inhibitors, even in those treated early, with most patients relapsing within a year of treatment cessation (Baraliakos, Listing et al. 2005, Song, Althoff et al. 2012, Sieper, Lenaerts et al. 2014). In those who do not respond to their first TNF inhibitor, a second agent may be effective but response rates are lower (around 50%) and drug survival may be shorter (Glintborg, Ostergaard et al. 2013). Although no difference is noted in the efficacy of the TNF inhibitors for the treatment of axial and peripheral arthritis in SpA, the monoclonal antibodies (adalimumab, infliximab, golimumab, certolizumab) are more effective in the treatment of extra-articular manifestations such as acute anterior uveitis and inflammatory bowel disease and therefore may be used in preference to etanercept (Levy-Clarke, Jabs et al. 2014, Levin, Wildenberg et al. 2016).

It is evident that TNF inhibition produces significant improvements in CRP and SIJ inflammation on MRI in adult patients who respond. However, several studies have suggested similar reductions are not seen in radiographic progression with continued new bone formation (and therefore loss of function) despite treatment (van der Heijde, Landewe et al. 2008, van der Heijde, Landewe et al. 2008, van der Heijde, Salonen et al. 2009). More recent studies have shown slowing of radiographic progression following treatment with TNF inhibitors, especially if used early in disease, suggesting a potential early window of opportunity for treatment (Haroon, Inman et al. 2013, Molnar, Scherer et al. 2018, Fernandez-Carballido, Tornero et al. 2020, Koo, Oh et al. 2020). A recent meta-analysis clearly demonstrates that TNF inhibitors do delay spinal structural progression compared to no treatment but the effect is only seen with long term follow up of more than 4 years (Ajrawat, Touma et al. 2020).

The relatively high proportion of adult patients not responding to TNF inhibition has prompted the investigation of other biologic treatments for SpA, in particular axSpA. Several agents proved ineffective in clinical trials including IL6 and IL1 receptor inhibitors (Haibel, Rudwaleit et al. 2005, Sieper, Porter-Brown et al. 2014), abatacept (Song, Heldmann et al. 2011) and rituximab (Song, Heldmann et al. 2010). Other pathways were identified as key to the pathogenesis of SpA, in particular the IL23/IL17 axis. Biologics inhibiting this pathway were therefore investigated with much anticipation in patients with SpA. These will be discussed further following the next section detailing the current knowledge of the pathogenesis of SpA.

Until recently, treatment regimens for ERA followed those of other JIA subtypes without any stratification between axial and peripheral disease, or reference to the treatment of adult SpA. As with most outcome studies in JIA, clinical trials of treatment in JIA were rarely subtype-specific and focussed on the more prevalent subtypes such as polyarticular JIA. Compared with adult studies, clinical trials in the paediatric population often include small numbers of patients and are relatively short. This is in part due to the lower incidence of disease in children and young people and the ethics around testing new drugs and giving placebo in this population. In the last 10 years, however, a number of prospective clinical trials including two randomised controlled trials of treatment in ERA have been published in addition to several retrospective and registry based studies.

As with adult SpA, first line treatment is usually with NSAIDs and over 80% of patients with ERA received NSAIDs in two large multicentre studies (Gmuca, Xiao et al. 2017, Weiss, Minden et al. 2017). There is no evidence as to the possible long term effects of NSAIDs on disease progression in ERA. Local or systemic corticosteroids may be effective for those with significantly raised inflammatory markers and as a holding measure before further treatment with either DMARDs or TNF inhibitors. Conventional DMARDs are commonly used in ERA because of the prevalence of peripheral arthritis and enthesitis. Although methotrexate is the most commonly used DMARD for JIA (including ERA) and is undoubtedly effective for peripheral arthritis (Wulffraat 2017), there is no specific evidence for the use of methotrexate in ERA. Sulfasalazine may be preferred because of the potential benefits for treating inflammatory bowel disease which is present in a proportion of patients with ERA. A randomised double blind placebo controlled trial of sulfasalazine in 33 patients with juvenile SpA demonstrated significant improvement in physician and patient scores in the sulfasalazine-treated group after 26 weeks, although a significant improvement was also noted in the placebo group (Burgos-Vargas, Vazquez-Mellado et al. 2002).

TNF inhibitors are introduced if patients fail to adequately respond to one DMARD or if axial arthritis is the predominant clinical feature of ERA. There is good evidence for the effectiveness of TNF inhibitors in ERA. Etanercept has been studied in a double-blind placebo-controlled trial in 49 patients with ERA and improvement was seen in JADAS after 24 weeks in 87% of patients with significant reduction in flare in the etanercept-treated group after 48 weeks (Horneff, Foeldvari et al. 2015). An open label, prospective clinical trial (the CLIPPER study) also demonstrated the efficacy and safety of etanercept in patients with ERA and juvenile onset psoriatic arthritis with significant clinical improvement after 3 months treatment which was maintained after two years (Horneff, Burgos-Vargas et al. 2014) and ongoing efficacy was shown in the extension study after 6 years of treatment (Foeldvari, Constantin et al. 2019). Adalimumab has also been studied in ERA with a randomised double-blind placebo-controlled trial in 46 patients demonstrating significant improvement in active joint count and enthesitis score after 10 months treatment (Burgos-Vargas, Tse et al. 2015). Adalimumab is also effective in axial disease with good efficacy demonstrated in a 24 week placebo-controlled double-blind study in patients diagnosed

with juvenile onset AS (Horneff, Fitter et al. 2012). In addition, adalimumab has proved effective in treating the extra-articular manifestations of ERA and juvenile onset psoriatic arthritis including uveitis (Ramanan, Dick et al. 2017), inflammatory bowel disease (Faubion, Dubinsky et al. 2017) and psoriasis (Papp, Thaci et al. 2017). Of the other TNF inhibitors, only Infliximab has been studied in juvenile onset SpA in one randomised trial with improvement in various clinical parameters including arthritis and enthesitis (Marino, De Souza et al. 2020).

Long term follow up studies of patients with ERA on TNF inhibitors are scarce and as described above those with ERA have a lower chance of achieving inactive disease compared with other subtypes of JIA (Donnithorne, Cron et al. 2011). One study of 16 patients with ERA on either etanercept or infliximab found that 38 % had flared a median of 3.5 years after starting treatment despite an initial response rate of 83 % (Hugle, Burgos-Vargas et al. 2014). This study also demonstrated progression of sacroiliitis despite treatment which was confirmed in another observational study which showed a significant positive association between time since start of treatment with TNF inhibition and rates of SIJ fusion (Bray, Lopes et al. 2019). A retrospective registry study of 22 patients with ERA showed inactive disease was achieved in 32% of patients after 3 months of TNF inhibition but no patients were able to achieve sustained remission off treatment during the duration of the study (Otten, Prince et al. 2011). However, as with AS, it appears early treatment with TNF inhibitors may be beneficial. A retrospective study of 217 patients with ERA in the first year after diagnosis found that those treated with TNF inhibitors had a greater improvement in disease activity and lower reported pain scores compared to those who received conventional DMARDs alone or no treatment (Weiss, Xiao et al. 2018). Despite this promising study, remission rates remain low and therefore identifying potential new targets for treatment in ERA is essential to improve outcomes.

1.1.11 Summary of the similarities and differences between adult SpA and ERA

As highlighted in the sections above, differences in the nomenclature between adult SpA and juvenile onset SpA have resulted in difficulties comparing these conditions and therefore undertaking research across the age range of SpA. The adult classification criteria group all SpA together whereas the JIA criteria separate ERA and psoriatic arthritis and those with overlapping clinical phenotype. Assessment of disease activity is often carried out with different scoring systems, and adult disease activity scores may not capture active disease in ERA well. Studies have highlighted the distinct clinical presentations between adult SpA and ERA, with significantly more peripheral enthesitis and arthritis reported in ERA (Chen, Chen et al. 2012, Jadon, Ramanan et al. 2013). In addition, greater delay to diagnosis has been noted in those with juvenile-onset disease (Jadon, Ramanan et al. 2013) and higher levels of orthopaedic surgery requirements in cohorts with long-standing disease (Gensler, Ward et al. 2008, Jadon, Shaddick et al. 2015). Similar demographics in terms of

gender and ethnicity are reported between SpA and ERA and also similar frequency of extra-articular manifestations (Jadon, Ramanan et al. 2013). Adult patients with SpA have more axial arthritis, radiographic disease, especially in the lumbar spine and worse functional status (O'Shea, Boyle et al. 2009). Axial disease has been reported as developing in around a third of patients with ERA in some studies but as this can occur years after the onset of arthritis (Pagnini, Savelli et al. 2010, Stoll, Bhore et al. 2010), this figure is likely to be higher (Fisher, Suffield et al. 2014, Goirand, Breton et al. 2018). Factors influencing the distinct presentations of adult and juvenile onset disease may include changes in the immune system and gut microbiome which occur with age (Yatsunenko, Rey et al. 2012, Carr, Dooley et al. 2016). In addition, musculoskeletal development is associated with increased mechanical strain, which may explain the higher rates of peripheral arthritis and enthesitis in juvenile onset disease. SIJ development is influenced by mechanical loading and significant differences in SIJ orientation are found between children, adolescents and adults with the potential impact on the development of sacroiliitis over time (Vleeming, Schuenke et al. 2012, Coudert, Dube-Cyr et al. 2019). Further research is required to determine the importance of these factors on disease phenotype in ERA compared to adult SpA and their potential importance for future treatment stratification.

In the next section, the pathogenesis of SpA will be discussed. In keeping with clinical studies, most research is undertaken in adults with SpA but the existing knowledge of the pathogenesis of ERA will also be discussed, including differences which may influence the distinct presentation of disease in children and adolescents compared to adults.

1.2 Pathogenesis of SpA

1.2.1 Introduction

Significant advances have been made in recent years to determine the pathogenesis of SpA in adults, especially in well-defined groups, such as AS and psoriatic arthritis. This has resulted in important advances in the understanding of the pathways involved in pathogenesis in particular the IL23/ IL17 axis, leading to promising new treatments for SpA (Yeremenko, Paramarta et al. 2014). Juvenile forms of SpA, including ERA, are presumed to share the same pathogenesis as SpA because of similarities in the clinical phenotype and genetic association with HLA B27. However, with some key differences and relatively little research in to the pathogenesis of ERA, it remains unclear whether the pathways involved are the same, or if SpA and ERA are in fact two distinct disease entities (Conway and O'Shea 2012). It is evident that both the innate and the adaptive immune systems are implicated in the pathogenesis of both ERA and SpA and these will be discussed further in the next sections.

1.2.2 The innate immune system

The innate immune system is the first line of defence against pathogens and is key in preventing infection in the host (Rees 2010). It plays a critical role in activating the adaptive immune response but has also been implicated in the pathogenesis of numerous inflammatory diseases (Turvey and Broide 2010). Cells involved in the innate immune response include monocytes and macrophages, dendritic cells, natural killer (NK) cells, neutrophils, innate lymphoid cells (ILCs), eosinophils and mast cells. There is an epithelial and humoral component to the innate immune system comprising cells lining the skin, gut, genitourinary and respiratory tracts and also circulating innate immune proteins such as C-reactive protein (CRP), lipopolysaccharide (LPS) binding protein and complement proteins (Turvey and Broide 2010). The innate immune system is activated through a limited number of genetically determined receptors (pattern recognition receptors (PRRs)) including Toll-like receptors (TLRs) and nucleotide oligomerisation domain (NOD)-like receptors (NLRs) which recognise conserved essential components of microbes (pathogen-associated molecular patterns (PAMPs)) (Janeway 1989). In addition, the biological consequences of microbial infection or inflammation such as heat shock proteins (HSPs), uric acid and high mobility group box protein 1 are recognised by the innate immune system as damage-associated molecular patterns (DAMPs) (Bianchi 2007). The innate immune system is also triggered when components of normal cells are missing; for example the absence of major histocompatibility complex (MHC) class 1 molecules on microbes and infected cells causes activation of the immune response through NK cells (Shifrin, Raulet et al. 2014).

1.2.2.1 Macrophages

Macrophages are a vital component of the innate immune system as antigen presenting cells with the ability to recognise and phagocytose potential pathogens. Macrophages may be resident in the tissues or recruited from a pool of circulating-blood monocytes to replenish tissue resident macrophages where there is a high turnover of cells or as a result of infection or inflammation (Martinez and Gordon 2014). Evidence from studies in mice suggests that tissue resident macrophages derive from embryonic precursor cells and it is assumed that this is also the case in humans although this hypothesis is difficult to test (Orecchioni, Ghosheh et al. 2019). In mice, early macrophages are found in yolk-sac blood islands which spread in to the tissues as the circulatory system develops (Ginhoux and Guilliams 2016). It is unclear to what extent these foetal macrophage populations persist in to adulthood but evidence suggests, in the absence of inflammation, these cells slowly turnover throughout life (Lavin, Mortha et al. 2015). The proportion of tissue resident and recruited macrophages varies depending on the tissue and in humans is yet to be determined but the function of recruited macrophages appears to be distinct from those that are tissue resident (Guilliams, Mildner et al. 2018). Macrophages derived from monocytes recruited as a result of inflammation differentiate and acquire different

properties depending on the tissue, congregating at sites where pathogens are most frequently encountered (Gordon, Pluddemann et al. 2014). Macrophages synthesise a range of inflammatory mediators and thus co-ordinate the host's response to infection and activate the adaptive immune system (Murray and Wynn 2011). In the context of inflammatory diseases, macrophage responses may not always be beneficial and the production of inflammatory mediators plays an important role in the pathogenesis of these conditions (Murray and Wynn 2011).

Macrophages exhibit great heterogeneity depending on activating or inhibitory factors within their environment and have the ability to polarise to an inflammatory or suppressor phenotype although these phenotypes often coexist (Martinez and Gordon 2014). Terminology defining macrophage polarisation as either M1 or M2 was introduced in 2000 based on mouse studies identifying increased nitric oxide and IL12 production by M1 macrophages (thus promoting a type 1 T helper cell (Th1) or inflammatory cellular response) and increased arginase production by M2 macrophages (and thus a type 2 T helper cell (Th2) or suppressor cellular response, promoting wound healing and homeostasis) (Mills, Kincaid et al. 2000). In vitro, polarisation can be induced by interferon (IFN)γ resulting in classically activated macrophages which have an inflammatory phenotype (Nathan, Murray et al. 1983) and IL4 giving rise to alternatively activated macrophages with a suppressor phenotype (Stein, Keshav et al. 1992). Other cytokines such granulocyte-macrophage colony stimulating factor (GMCSF) and macrophage colony stimulating factor (MCSF) are also capable of polarising macrophages in vitro and in vivo with GMCSF leading to an inflammatory phenotype and thus the production of high levels of pro-inflammatory cytokines including TNFα, IL12 and IL23 and MCSF resulting in a suppressor phenotype producing anti-inflammatory products to aid tissue repair such as IL10 and transforming growth factor (TGF)β (Lacey, Achuthan et al. 2012). The differences seen between GMCSF and MCSF- differentiated macrophages reflect the fact that MCSF is produced in a steady state to maintain homeostasis in macrophage numbers in the tissues (Stanley, Berg et al. 1997) whereas GMCSF is produced at a low basal level which increases in response to infection and inflammation (Metcalf 1995). Skewed macrophage polarisation towards either an inflammatory or suppressor phenotype is critical to the development and outcome of many disease states such as infections, inflammatory conditions and cancer (Sica, Erreni et al. 2015). Macrophage phenotype is not only influenced by the local environment but may be influenced systemically, for example by the gut microbiome and the release of microbial products (Abdel-Haq, Schlachetzki et al. 2019).

1.2.2.2 Other cells of the innate immune system

Whilst macrophages are key in recognising and phagocytosing pathogens, other cells involved in the innate immune response have similar and equally important roles. Neutrophils provide the fastest response to pathogen exposure, resulting in phagocytosis of

smaller pathogens and cell death within minutes (Kruger, Saffarzadeh et al. 2015). Larger pathogens are degraded by granule release or by neutrophil extracellular traps (NETs) (Brinkmann, Reichard et al. 2004). Dendritic cells, like macrophages can recognise, take up and present antigen components to T cells thus play an important role in initiating the adaptive immune response (Steinman 2012). Two main subsets of dendritic cell exist: plasmacytoid dendritic cells and classical dendritic cells. Further classification can be made according to location, in to tissue or blood dendritic cells and, as with macrophages this can also influence function (Itano, McSorley et al. 2003). ILCs, found predominantly at mucosal sites and in non-lymphoid tissue, also play a key role in the innate immune response and are categorised in to three subsets (ILC1, 2 and 3) according to cytokine production aligning with the classification of Th1, Th2 and Th17 cells (Spits, Artis et al. 2013). ILC3s also have the ability to phagocytose pathogens and present antigens, thus initiating the adaptive immune response (Hepworth and Sonnenberg 2014). NK cells are members of the ILC1 family and provide an immediate response to virally infected cells through cytotoxicity and cell lysis in addition to pro-inflammatory cytokine production (Abel, Yang et al. 2018). NK cell function is regulated by MHC class 1 molecules mainly through killer cell immunoglobulin-like receptors (KIRs) to prevent attacks on healthy 'self' tissue (Elliott and Yokoyama 2011). Together with macrophages, the cells of the innate immune response function to produce cytokines and chemokines which cause T cell differentiation and modulate T cell function thus further protecting the host against infection.

1.2.2.3 Toll-like receptors

TLRs are transmembrane receptors, crucial for the function of the innate immune system each recognising a number of distinct microbial components resulting in the induction of appropriate signalling pathways within the cell, thus an inflammatory cascade and ultimately the production of pro-inflammatory cytokines and induction of adaptive immune responses (Ospelt and Gay 2010). In pathological conditions, TLRs may also be activated by endogenous ligands resulting in an inflammatory response without the presence of a microbial stimulus (Yu, Wang et al. 2010). To date, ten functional TLRs have been described in humans (McCormack, Parker et al. 2009).

TLRs signal through Toll/interleukin 1 receptor (TIR)-domain containing adaptors including myeloid differentiation primary response protein 88 (MyD88), TIR-domain containing adaptor protein (TIRAP), TIR-domain containing adaptor inducing IFN-β (TRIF) and TRIF related adaptor molecule (TRAM) (Kawai and Akira 2007) to activate specific transcription factors and thus appropriate inflammatory pathways depending on the TLR activated. All TLRs except TLR 3 utilise the MyD88-dependent response pathway, resulting in pro-inflammatory cytokine production via the activation and nuclear translocation of nuclear factor kappa light chain enhancer of activated B cells (NFκB) and activating protein 1 (AP-1) through a series of factors including interleukin 1 receptor (IL1R) associated kinase (IRAK),

tumour necrosis factor (TNF) receptor associated factor 6 (TRAF6) and mitogen-activated protein kinases (MAPK) (Yu, Wang et al. 2010). For TLRs 2 and 4, this pathway is mediated by TIRAP (Yamamoto, Sato et al. 2002). The MyD88 independent response pathway utilised by TLRs 3 and 4 is mediated by TRIF and also results in the activation of NFκB and IFN regulatory factor 3 (IRF3) and thus the production of pro-inflammatory cytokines and type 1 IFN (IFNα and β) (Yamamoto, Sato et al. 2002). For TLR 4, this pathway is also mediated by TRAM (Yamamoto, Sato et al. 2003).

TLR 4 is one of the best studied TLRs. Forming a complex with CD14 and myeloid differentiation protein (MD)-2, it recognises LPS which is a key component of the cell wall of gram negative bacteria (Kayagaki, Wong et al. 2013). TLR 4 activation via either the MyD88 dependent or independent response pathway results in the release of pro-inflammatory cytokines and chemokines through NFκB, IRF3 and MAPKs (Pitha 2004) (figure 1.3).

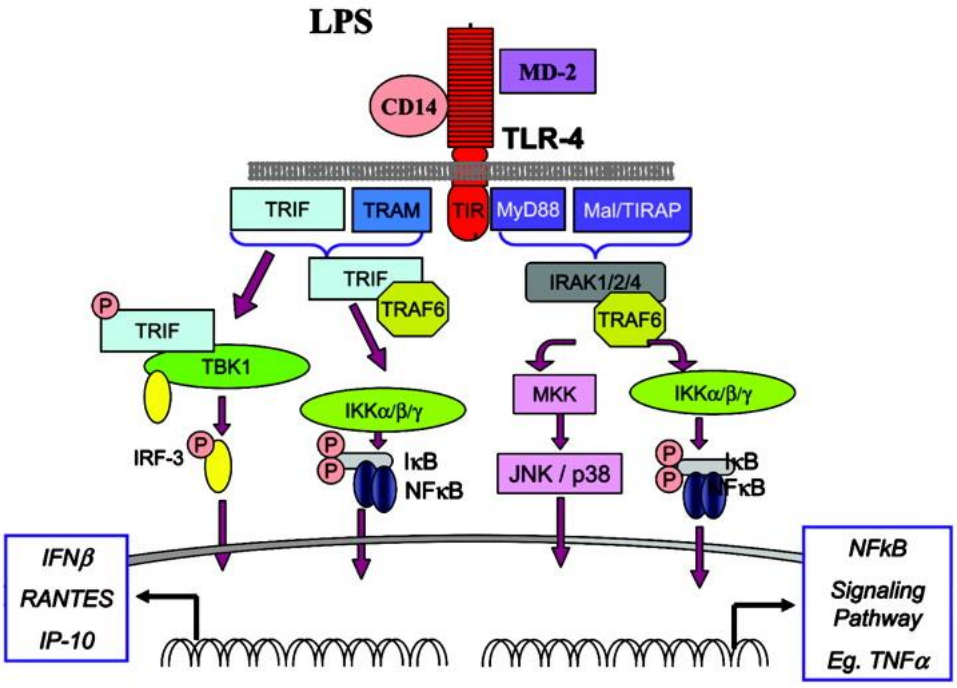


Figure 1.3 The TLR 4 signalling pathway. LPS binds TLR 4 which causes it to dimerise and recruit downstream molecules such as MyD88. This results in the activation of TRAF 6 and subsequently the IκB complex, which leads to the translocation of NFκB to the nucleus. In addition to this, MAPK kinases (MKKs) are activated resulting in signalling through p38 and JNK. Both of these pathways result in the release of pro-inflammatory cytokines including IL6, TNFα, IL12, IL1β and chemokines such as CXC chemokine ligand (CXCL) 10 and 11 as well as antigen presenting molecules (MHC, co-stimulatory molecules) (Pitha 2004).

TLRs have been implicated in the pathogenesis of various inflammatory conditions including inflammatory arthritis (McCormack, Parker et al. 2009). TLR expression is upregulated in the

synovium of patients with rheumatoid arthritis, especially with active synovitis (TLRs 2, 3 and 4 (Radstake, Roelofs et al. 2004, Brentano, Schorr et al. 2005, Ospelt, Brentano et al. 2008)); in the synovium and peripheral blood mononuclear cells (PBMCs) of patients with SpA (TLRs 2, 4 and 5 (De Rycke, Vandooren et al. 2005, Yang, Liang et al. 2007, Assassi, Reveille et al. 2011)) and in patients with ERA (TLRs 2 and 4 (Myles and Aggarwal 2011)). The upregulation of TLRs may be a non-specific indicator of activation of the inflammatory response in patients with inflammatory conditions giving rise to increased pro-inflammatory cytokine production (Tan and Farheen 2011). However, the pathogenic mechanism through which upregulation of TLRs occurs is unclear. In the inflammatory arthritidies such as SpA, gut flora including Gram negative bacteria may play a role as illustrated by animal models (Taurog, Richardson et al. 1994) and also the fact that a large proportion of patients with SpA have either overt inflammatory bowel disease or microscopic gut inflammation (Gilis, Mortier et al. 2018) which could result in TLR 4 activation. Endogenous TLR ligands are also thought to play a role with increased expression of ligands such as tenacin C, an extracellular matrix glycoprotein (Midwood, Sacre et al. 2009), and the S100 proteins (including myeloid related protein (MRP) 8 and 14/ calprotectin) (Vogl, Tenbrock et al. 2007) in patients with inflammatory arthritis. In animal models of experimental arthritis, TLR 4 blockade results in less severe manifestations of arthritis (Abdollahi-Roodsaz, Joosten et al. 2007). However, despite clinical trials of TLR4 antagonists in humans, none have been approved and safety has been a concern (Gao, Xiong et al. 2017).

1.2.2.4 NOD-like receptors

NLRs are another family of PRRs, vital to the innate immune response through their recognition of PAMPs and DAMPs (Ting, Lovering et al. 2008). In humans, 22 NLRs have been identified which can be grouped by function according to whether they are involved in inflammasome formation, signal transduction, activation of transcription or autophagy (Motta, Soares et al. 2015). NLRs are implicated in numerous inflammatory conditions including inflammatory arthritis, inflammatory bowel disease especially Crohn's disease and the autoinflammatory diseases because of their broad involvement in innate immunity, (Zhong, Kinio et al. 2013).

In contrast to TLRs, NLRs are cytoplasmic receptors but activation of NOD 1 and 2 also results in nuclear translocation of NFκB and induction of MAPKs and IRF3, thus the production of pro-inflammatory cytokines (Girardin, Tournebize et al. 2001, Sabbah, Chang et al. 2009). Inflammasome formation is another function of NLRs causing the production of pro-inflammatory cytokines through the activation of caspase 1 and release of IL1 β and IL18. NLRs may also be negative regulators of pro-inflammatory cytokine production (Cui, Li et al. 2012, Schneider, Zimmermann et al. 2012). Autophagy (the process of self-degradation and regeneration of cellular components) may be induced by NOD1 and 2 (Travassos, Carneiro et al. 2010) but inhibition of autophagy by NLRs may also occur (Jounai, Kobiyama

et al. 2011). In addition, NLRs are necessary for the expression of MHC molecules and thus activation of the adaptive immune response (Kobayashi and van den Elsen 2012).

1.2.2.5 NFκB, MAPK and IRF3 signalling

Activation of both TLRs and NLRs results in signalling cascades which can induce NFκB, IRF3 and MAPK signalling. NFκB is a family of transcription factors including RelA, RelB, cRel, NFκB1 (p50) and NFκB2 (p52) which exist in the cytoplasm in their inactive form bound to inhibitory (I)κBs. On activation, various combinations of homodimers and heterodimers are formed which undergo nuclear translocation and bind to the promoter regions of target genes (Hayden and Ghosh 2008). NFκB signalling occurs through either a canonical or non-canonical pathway (Sun 2017). The canonical pathway, activated by a large number of receptors including TLRs and NLRs, involves degradation of IκBs by the IκB kinase (IKK) complex and is characterised by a rapid but transient response resulting in enhanced production of pro-inflammatory cytokines such as TNFα and IL1 (Shi and Sun 2018). In inflammatory disease states, TNFα and IL1 also activate NFκB creating a positive feedback loop for pro-inflammatory cytokine production (Lawrence 2009). The canonical pathway has a key role in innate immune function and regulation (Hayden and Ghosh 2012).

The non-canonical pathway is distinct from the canonical pathway because it is activated by a limited number of receptors belonging to the TNF receptor (TNFR) superfamily such as cluster of differentiation (CD) 40, B cell activating factor (BAFF) and lymphotoxin β receptor (LTβR) (Sun 2012). Instead of IKK, the non-canonical pathway relies on NFκB induced kinase (NIK) and is characterised by a slow response reliant on protein synthesis (Sun 2017). In addition to B cell maturation, osteoclast differentiation and lymphoid development, the non-canonical pathway has also been implicated in the pathogenesis of inflammatory and autoimmune disease (Hayden and Ghosh 2012).

Constituents of the NFκB pathway also communicate with other signalling pathways including the MAPK and IRF pathways (Oeckinghaus, Hayden et al. 2011). Conversely, certain MAPKs such as transforming growth factor beta activated kinase 1 (TAK1) and MAP/ extracellular signal-regulated kinase (ERK) kinase kinase (MEKK3) activate and regulate the canonical NFκB pathway (Yang, Lin et al. 2001, Yao, Kim et al. 2007).

MAPK pathways are vital for normal cellular function and are induced by numerous cell stresses, including TLR signalling and in response to pro-inflammatory cytokines (Cuenda and Rousseau 2007). Comprising three subfamilies: ERKs, c-JUN N-terminal kinases (JNK) and p38, MAPKs mediate cellular processes such as growth, differentiation and apoptosis, in addition to inflammatory and immune responses (Dong, Davis et al. 2002). Common to all these subfamilies is a three-tiered signalling system involving MAPK kinase kinases (MKKKs), MAPK kinases (MKKs) and MAPKs. Upon activation, phosphorylation of MKKKs occurs which

activates MKKs and subsequently MAPKs leading to the upregulation of transcription factors involved in a broad range of cellular activities (Cuenda and Rousseau 2007). Regulation of different MAPKs occurs through distinct MKKs such as MKK1 and MKK2 for ERK1 and ERK2, MKK4 and MKK7 for JNKs and MKK3 and MKK6 for p38 (Arthur and Ley 2013). As discussed above, there is significant communication between the NFκB and MAPK signalling pathways via MKKs and MKKKs such as TAK1 which not only regulates the activation of both JNK and p38 but also IKKs and thus NFκB (Yao, Kim et al. 2007).

The IRF3 pathway is another key regulator of the innate immune response through the production of type 1 IFN (Tamura, Yanai et al. 2008). The inactive form of IRF3 is found in the cytoplasm and activation is mediated by TRAF family member associated NFκB activator (TANK) binding kinase 1 (TBK1) and IKK which cause phosphorylation of IRF3 and thus translocation to the nucleus (Fitzgerald, McWhirter et al. 2003). There, IRF3 binds conserved IFN stimulated response elements (ISREs) leading to the upregulation of type 1 IFN genes and also other cytokines and chemokines such as CXC motif chemokine 10 (CXCL10), regulated on activation, normal T cell expressed and secreted (RANTES) and IL12p35 (Goriely, Molle et al. 2006). IRF3 may also act as a negative regulator of the inflammatory response (Honda and Taniguchi 2006). Together, the NFκB, MAPK and IRF3 pathways play a key role in immune and inflammatory responses and also in cell survival, proliferation and differentiation thus dysregulation of these pathways may result in numerous disease states including inflammatory and autoimmune conditions.

1.2.3 Mechanisms Initiating the Adaptive Immune Response

The adaptive immune response is highly specific to the inducing pathogen and also provides a long-lasting defence through the creation of immunological memory (Janeway, Travers et al. 2005). It comprises the humoral response, which is elicited through antibody production from B cells and the cell mediated response which occurs through T cell interaction with antigen presented on the surface of a host cell (Iwasaki and Medzhitov 2010). It relies upon highly specific surface receptors such as immunoglobulins on B lymphocytes and T cell receptors (TCRs) on T lymphocytes to recognise a vast array of antigens and thus the initial adaptive immune response is slower than that of the innate immune system. The ability to generate immunological memory results in a more rapid response on subsequent exposure to pathogen (Nicholson 2016).

1.2.3.1 Contribution of T lymphocytes

The adaptive immune response is triggered by components of the innate immune response, initially through antigen presentation causing activation of the TCR and clonal expansion of antigen-specific T cells. Secondly, through upregulation of co-stimulatory molecules on

antigen presenting cells which results in a lower threshold for TCR activation and thus differentiation and proliferation of specific T cell subsets. Thirdly, the production of cytokines by innate immune cells provides an environment that enhances T cell differentiation and thus protection against a given pathogen (Jain and Pasare 2017). T lymphocytes may be categorised as cytotoxic (CD8+) which bind to MHC class I molecules or helper (CD4+) which bind to MHC class II molecules. Cytotoxic T cells act by the release of cytokines such as TNFα and IFNγ and cytotoxic granules which ultimately result in apoptosis of target cells (Barry and Bleackley 2002). Helper T cells can be divided into subsets which include Th1, Th2 and Th17 according to cytokine production as a result of polarisation by various stimuli. Th1 cells are predominantly activated by antigen presentation of microbes and secrete IL12, IFNγ and TNFα (Romagnani 1994). Activation of Th2 cells occurs through the production of IL4 and leads to the release of further IL4 as well as IL5, IL13, IL21 and IL25. Th17 cells are key in the immune response against fungi and extracellular bacteria but are also implicated in pathogenesis of chronic inflammatory conditions including SpA, psoriasis, inflammatory bowel disease (IBD) and multiple sclerosis (Smith and Colbert 2014). Th17 cells not only produce IL17 (A and F) but also other pro-inflammatory cytokines including TNFα, IL6, GMCSF, IL21 and IL22 (Wilson, Boniface et al. 2007, Miossec and Kolls 2012). Th17 cells are highly plastic and may further differentiate in to regulatory T cells (Tregs) and follicular helper T cells (Tfh) depending on local cytokine production (Nistala, Adams et al. 2010). A subset of pathogenic Th17 cells also secretes IFNγ (Boniface, Blumenschein et al. 2010). Activated innate immune cells such as macrophages and dendritic cells produce IL23 which is essential for the proliferation and terminal differentiation of Th17 cells (McGeachy, Chen et al. 2009). Ablation of IL23 in animal models of autoimmune diseases including collagen-induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE) and IBD leads to a reduction of Th17 cells and an amelioration of disease (Bianchi and Rogge 2019). Conversely, the production of GMCSF by Th17 cells results in stimulation of IL23 production from antigen presenting cells resulting in an enhanced inflammatory response because of this positive feedback loop (El-Behi, Ciric et al. 2011).

1.2.3.2 Major histocompatibility complex molecules and co-stimulatory molecules

The innate immune response initially triggers the adaptive immune response through the presentation of antigen to T cells via the interaction of MHC molecules with TCRs. There are two classes of MHC molecule: class I are found on almost every cell type and present endogenous antigens originating from self-proteins or foreign proteins produced within the cell (for example viral proteins) and class II which only occur on antigen presenting cells (including macrophages, dendritic cells and B lymphocytes) and present exogenous antigens (Janeway, Travers et al. 2005).

MHC class I molecules are formed of a heavy chain produced by MHC genes and a light chain (β_2-microglobulin). Assembly occurs within the endoplasmic reticulum (ER) where newly synthesised MHC molecules undergo folding, binding to β_2-microglobulin and subsequently their peptide cargo. Peptides or antigens are presented in the binding pocket of the folded heavy chain which is flexible and can therefore accommodate different peptides of around 8-10 amino acids in length (compared to antibodies and T cell receptors which have much more specific targets). The MHC class I molecule moves to the Golgi where it enters a secretory vesicle which enables it to fuse to the cell membrane and therefore interact with CD8+ T cells (Antoniou, Powis et al. 2003).

In contrast to MHC class I, MHC class II molecules comprise two membrane spanning chains of similar size both produced by MHC genes and have an open binding pocket which can accommodate larger peptides of up to 20 amino acids derived from endosomal or lysosomal proteins internalised from the extracellular environment (Suri, Lovitch et al. 2006). MHC class II molecule interaction with CD4 T cells results in their initial activation and polarisation in to either a memory Th cell if the antigen has been encountered before or effector Th cell subsets (Rock, Reits et al. 2016).

There are hundreds of different variants of MHC genes each expressed from three different gene regions on chromosome 6 which encode human leucocyte antigen (HLA) alleles (MHC class I: HLA A, HLA B, HLA C and MHC class II: HLA DR, HLA DP and HLA DQ) (Marsh, Albert et al. 2010, Wieczorek, Abualrous et al. 2017) which are highly polymorphic although individuals only inherit around 9 of these from their parents (Nicholson 2016). Each different MHC molecule presents a unique selection of antigens from the same underlying proteins allowing the immune system to discriminate between self-proteins and lymphocytes and invading pathogens. However, these genetic variations may have an effect on MHC molecule assembly, including heavy chain folding and peptide binding (Neisig, Wubbolts et al. 1996).

In addition to binding MHC molecules, a second signal is required for activation of both CD8+ and CD4+ T cells. Costimulatory molecules, such as CD28 on CD4+ T cells, bind to either CD80 or CD86 on the antigen presenting cell which lowers the threshold for TCR activation, initiating T cell proliferation and resulting in clonal expansion of T cells recognising the specific antigen encountered (June, Ledbetter et al. 1987). Other costimulatory molecules include members of the TNFR superfamily such as OX40 and CD40 (Croft 2003). The signalling cascade downstream of TCR activation integrated with signals from co-stimulatory molecules determine subsequent T cell function and responses (Chen and Flies 2013).

1.2.3.3 Influence of innate cytokines on the adaptive immune response

In addition to TCR activation and co-stimulatory molecules, cytokine production by innate immune cells has a key role in influencing T cell responses. Signals from PRRs and factors resulting in cellular stress such as misfolding proteins lead to increased production of pro-inflammatory cytokines. The cytokine milieu surrounding activated CD4+ T cells determines the type of effector T cell which differentiates and therefore the secondary cytokines produced (as described above for Th1, Th2 and Th17 cells). For CD8+ T cells, cytokines have a more fundamental role as without a specific cytokine signal, effector functions do not develop and survival is poor (Mescher, Curtsinger et al. 2006).

Pro-inflammatory cytokines including IL12, IL23, IL6, TNFα and IL1β from antigen presenting cells such as macrophages and dendritic cells act on cells of the adaptive immune system including T lymphocytes. Activated T lymphocytes produce secondary cytokines such as IFNγ, IL17, IL22 and IL21 which in turn act on effector cells including macrophages, neutrophils and B cells to elicit a range of functions such as killing and removal of pathogens, tissue repair and antibody production. Macrophages can therefore act by secreting cytokines to promote a T cell response initially but also subsequently respond to cytokine production by T lymphocytes (for example IFNγ) to produce a strong phagocytic defence against a pathogen (Iwasaki and Medzhitov 2015). This illustrates that the innate and adaptive immune systems are not separate entities but collaborate closely to produce an inflammatory response and eliminate pathogens (Delves and Roitt 2000, Delves and Roitt 2000).

The next section will focus on the pathogenesis of adult SpA and ERA, with particular reference to HLA B27, the IL23/IL17 axis and environmental factors including the gut microbiome and mechanical stress.

1.2.4 Genetic influences on the pathogenesis of SpA

The MHC class I allele HLA B27 is strongly associated with SpA and in particular with AS in which over 90% of patients have the allele compared to around 6% of the general population (Caffrey and James 1973). Despite the strong link, less than 5% of those who have the HLA B27 allele develop SpA (van der Linden and Khan 1984) although the concordance rate of AS in monozygotic twins is 63% and risk of developing AS in first degree relatives is 8.3% (Brown, Laval et al. 2000). HLA B27 is also associated to a lesser extent with other forms of SpA (50-75% HLA B27 positive) (Sims, Wordsworth et al. 2004) and in ERA is found in around 75-85% of patients (Thomson, Barrett et al. 2002, Flato, Hoffmann-Vold et al. 2006). To date 167 subtypes of HLA B27 have been identified which differ by only a few amino acids. The most common of subtype is HLA B27:05 which is associated with increased risk of AS across all races and ethnicities. This is also the case for patients with ERA (Stanevicha, Eglite et al. 2010). Other HLA B27 subtypes tend to confer increased risk of AS

in certain populations such as HLA B27:02 in Mediterranean populations, HLA B27:04 in Eastern Asians and HLA B27:08 in Southern Asians. Two subtypes, HLA B27:06 and HLA B27:09, do not appear to be associated with AS (Dashti, Mahmoudi et al. 2018). Those HLA B27 subtypes that are associated with AS appear to modify MHC molecule assembly, including heavy chain folding and peptide binding (Neisig, Wubbolts et al. 1996). Despite the strong association with SpA and in particular AS, HLA B27 is only estimated to account for around 20% of the total heritability of AS which is thought to be over 90% (Brown, Kennedy et al. 1997, Bowness 2015, Smith 2015, Ellinghaus, Jostins et al. 2016).

Non-HLA B genes have also been identified as having a strong association with SpA. After HLA B27, genetic variations in endoplasmic reticulum aminopeptidase (ERAP) 1 are the most commonly associated but only in HLA B27 positive individuals (Evans, Spencer et al. 2011). The main function of ERAP1 is to trim peptides to 8-9 amino acids in the ER for loading on to MHC class I molecules for antigen presentation. The single nucleotide polymorphisms (SNPs) identified in ERAP1 downregulate function and therefore affect peptide trimming and thus antigen presentation to T cells suggesting that aberrant peptide processing and unusual antigen presentation are key factors predisposing to AS (Keidel, Chen et al. 2013). The effect of inefficient peptide trimming on the HLA B27 MHC molecule may be to alter its stability allowing the formation of aberrant structures such as homodimers which interact with NK receptors at the cell surface (Tran, Hong et al. 2016). The interaction between HLA B27 and ERAP1 appears to be HLA B27 subtype-specific affecting only those subtypes known to predispose to AS such as HLA B27:05 and HLA B27:04 and not HLA B27:06 and HLA B27:09 (Haroon, Tsui et al. 2012). A protective variant of ERAP1 has been identified (Alvarez-Navarro and Lopez de Castro 2014). Genetic variations in ERAP1 have also been found in patients with ERA (Hinks, Martin et al. 2011).

Other SNPs associated with AS have been found through genome-wide association studies (GWAS) and many of these SNPs appear to be associated with particular inflammatory pathways especially the IL23/ IL17 axis. Variations in the IL23 receptor (IL23R) gene are strongly associated with AS and other related conditions such as psoriasis and IBD (Wellcome Trust Case Control, Australo-Anglo-American Spondylitis et al. 2007). The IL23R, upregulated on activated Th cells, is crucial for IL23 signalling and results in the differentiation and expansion of Th17 cells (McGeachy, Chen et al. 2009). Other cytokine and cytokine receptor genes involved in the IL23/ IL17 pathway have been identified as increasing the risk of developing AS. These include polymorphisms in the IL12B, IL6R, IL1R and IL27 genes. In addition, genetic variations associated with AS have been identified in factors involved in the signalling cascade downstream of the IL23R such as tyrosine kinase 2 (TYK2), signal transducer and activator of transcription 3 (STAT3) and Janus kinase 2 (JAK2). SNPs of genes involved in the innate immune response and thus the production of IL23 and other inflammatory cytokines have also been implicated in AS (including NF-κB, TNFR superfamily member 1A (TNFRSF1A) and caspase recruitment domain family member 9 (CARD9)), as well as G protein-coupled receptors (GPR) (including GPR65) and those

involved in both CD8+ and CD4+ T cell development and activity (Davidson, Liu et al. 2011, Cortes, Hadler et al. 2013, Smith 2015). Differentially expressed genes in the MAPK pathway have been identified in both adult and juvenile SpA (Lamot, Borovecki et al. 2014, Ding, Guan et al. 2018, Wang, Han et al. 2018). Thus, the polymorphisms linked to AS and related diseases have provided important clues as to the pathways involved in pathogenesis of these conditions.

1.2.4.1 Theories on the role of HLA B27 in the pathogenesis of SpA

Despite the long-recognised association between HLA B27 and SpA, the mechanism by which HLA B27 predisposes to disease remains unclear although several theories exist. Three major theories have arisen from the observations that, firstly, HLA B27 misfolds within the ER, leading to an accumulation of protein and the production of pro-inflammatory cytokines including IL23 (Colbert, DeLay et al. 2010). Secondly, HLA B27 forms homodimers at the cell surface which leads to recognition by cells expressing KIRs (including Th17 and NK cells) promoting cell survival and therefore the production of IL17 (Allen, O'Callaghan et al. 1999, Bowness, Ridley et al. 2011). Thirdly, it has been proposed that HLA B27 presents unique arthritogenic peptides (self-peptides resembling pathogenic peptides) to CD8+ T cells, thus initiating a cytotoxic T cell response (Benjamin and Parham 1990).

To address the first theory, Colbert et al were the first to demonstrate slow folding of HLA B27 in the ER compared to other HLA A and B alleles (Mear, Schreiber et al. 1999). They showed that by altering the B pocket within the peptide binding groove of the HLA B27 heavy chain, normal protein folding occurred. The cause of the abnormal folding was found to be an unpaired cysteine residue (Cys 67) on the edge of the HLA B27 B pocket which caused aberrant disulphide bonds to form and therefore a tendency for HLA B27 heavy chains to aggregate within the ER (Allen, O'Callaghan et al. 1999). A further study found that nearly two thirds of newly formed HLA B27 heavy chains were unfolded or misfolded within the ER (Dangoria, DeLay et al. 2002). These findings were confirmed by the observation that binding immunoglobulin protein (BiP), an ER chaperone protein, was found to be associated with HLA B27 in a steady state (Colbert, DeLay et al. 2010). BiP usually binds transiently with newly synthesised MHC class I molecules and only forms a stable association with proteins that misfold (Nossner and Parham 1995). Therefore the observation that HLA B27 appears to have a prolonged interaction with BiP implies heavy chain misfolding.

ER stress occurs when unfolded or misfolded proteins accumulate within the ER and therefore when ER homeostasis becomes dysregulated. ER stress induces the unfolded protein response (UPR) which aims to increase the capacity of the cell to fold, secrete and degrade protein. If successful, homeostasis is restored but if not then apoptotic pathways are induced (Dufey, Sepulveda et al. 2014). The UPR involves the activation of three pathways (figure 1.4) resulting in the transduction of signals to the nucleus. Three proteins:

inositol requiring enzyme 1α (IRE1α), pancreatic ER eIF2α kinase (PERK) and activating transcription factor 6α (ATF6α) are bound to BiP in their inactive state. They are released and therefore activated when BiP binds to misfolded proteins. IRE1α and PERK form homodimers and autophosphorylate whereas ATF6α is released from the ER to the Golgi where it is cleaved and active protein released in to the cytosol (Dufey, Sepulveda et al. 2014). IRE1α increases the production of proteins involved in ER chaperoning and protein folding, as well as inducing splicing of the transcription factor X box protein 1 (XBP1), which results in the activation of pro-inflammatory signalling pathways (via MAPK and NFκB) and cytokine release. Activated PERK causes the phosphorylation of eIF2α which inhibits protein synthesis and also leads to translation of ATF4 inducing the expression of genes such as CCAAT/enhancer binding protein homologous protein 10 (CHOP) which may trigger apoptotic or autophagy pathways. ATF6α activates transcription of target genes such as XBP1 and BiP (Dufey, Sepulveda et al. 2014).

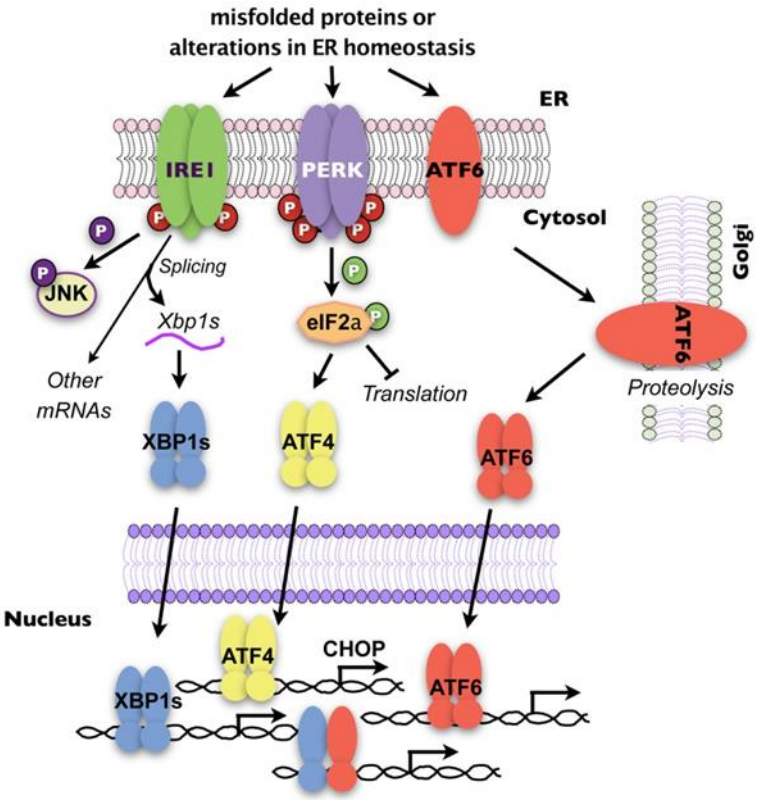


Figure 1.4 The Unfolded Protein Response. Misfolded proteins accumulate within the ER resulting in ER stress. This induces the UPR via 3 pathways with the aim of restoring ER homeostasis by increasing the cells ability to fold, secrete and degrade protein. (From Kim DW, Department of Anatomy, Chungnam National University School of Medicine, Daejeon, Republic of Korea)

There is a clear link between the UPR and the augmentation of pro-inflammatory cytokine production (Li, Schwabe et al. 2005) and components of the UPR are required for the full expression of certain cytokines such as IL6 and TNFα (Martinon and Glimcher 2011). Importantly for SpA, mouse-derived macrophages treated with LPS and an inducer of UPR exhibited strong upregulation of IL23 and in particular the IL23p19 subunit (Colbert, DeLay et al. 2010). These results were replicated by Goodall et al in human monocyte derived dendritic cells from healthy volunteers and the transcription factor CHOP was found to be essential for IL23 production by binding to the IL23p19 promoter (Goodall, Wu et al. 2010). UPR pathways may influence cytokine production by interacting with innate immune signalling pathways at any level, from PRRs to transcription factors regulating cytokine production such as NFκB, MAPK, AP1 and IRF3 (Smith 2018). In addition, the UPR is crucial to the differentiation and survival of certain cell types including B cells, dendritic cells and eosinophils (Iwakoshi, Lee et al. 2003, Iwakoshi, Pypaert et al. 2007, Bettigole, Lis et al. 2015).

In the context of SpA, animal studies have provided some evidence that the UPR may contribute to disease pathogenesis. A summary of animal models of HLA B27 related disease is found in table 1.3. Animal studies are commonly used to investigate the pathogenesis of human disease but animal models may not exactly mirror the human disease and therefore results must be interpreted with caution. Rat models of SpA include HLA B27 and β_2-microglobulin transgenic animals which develop SpA-like inflammatory disease spontaneously, dependent on a high transgene copy number. Lines with lower HLA B27 transgene levels do not develop disease (Taurog, Maika et al. 1999). In a study of macrophages from HLA B27 transgenic rats with inflammatory disease, certain UPR target genes were found to be upregulated compared to wild type rats and HLA B7 transgenic rats. These included BiP, CHOP and XBP1. The effect was enhanced by the addition of IFNγ to the assay and an accumulation of BiP-bound HLA B27 was noted in indicating the presence of heavy chain misfolding in HLA B27 transgenic rats but not wild type of HLA B7 transgenic rats (Turner, Delay et al. 2007). Further studies by the same group in macrophages and lamina propria cells from HLA B27 transgenic rats demonstrated evidence of UPR activation which resulted in increased IL23 production and subsequent enrichment of Th17 cells (DeLay, Turner et al. 2009). Interestingly, when HLA B27 transgenic rats were bred with a healthy line, carrying an extra human β_2-microglobulin, significant reduction of HLA B27 misfolding and UPR activation was noted and the rats did not develop colitis. However, severe arthritis and spondylitis did occur (with characteristic features of human SpA) suggesting that HLA B27 misfolding and UPR activation is not critical to the development of HLA B27-associated SpA (Tran, Dorris et al. 2006).

HLA B27 transgenic mice usually remain healthy (Kievits, Ivanyi et al. 1987). However, two HLA B27-associated mouse models of SpA are described and include a line with naturally occurring ankylosis and enthesopathy of the hind-paws, the 'ANKENT ' model, which exhibits increased incidence of disease in the presence of HLA B27 (Weinreich, Eulderink et

al. 1995). The other is an HLA B27 transgenic/ β_2-microglobulin knockout mouse which also develops spontaneous arthritis of the hind-paws as well as hyperkeratotic nails despite only expressing HLA B27 heavy chains on the cell surface (instead of mature HLA B27) because of the lack of β_2-microglobulin. Disease pathogenesis appears to be related to heavy chain presentation of exogenous antigen or autoantigen and inhibition of HLA B27 heavy chains by an antibody ameliorates disease (Khare, Hansen et al. 1996, Khare, Bull et al. 1998). In both of these examples, disease predominates in male mice and requires exposure to conventional bacterial flora. However, the UPR has so far not been implicated in disease pathogenesis in mouse models.

	Rats	**Mice**	
Lines	Lewis and Fisher	ANKENT	HLA B27/B2M knockout
HLA B27 transgene expression/ copy no.	High (55-150 copies)	-	?
Articular manifestations	Peripheral arthritis and enthesitis, axial inflammation	Peripheral enthesitis of hind limbs	Peripheral arthritis of hind limbs
Extra-articular manifestations	Psoriasis (nail and skin) Bowel inflammation Uveitis, Conjunctivitis Orchitis		Hyperkeratotic nails
Role of bacterial flora in disease manifestation	Important	Important	Important
Mechanism of SpA	HLA B27+ antigen presenting cells such as dendritic cells, monocyte/macrophages	Unclear ?T cell independent mechanism (?BMP associated)	HLA B27 heavy chains
References	(Taurog, Maika et al. 1999)	(Weinreich, Eulderink et al. 1995)	(Khare, Hansen et al. 1996)

Table 1.3 Animal models of HLA B27-associated disease adapted from (Hacquard-Bouder, Ittah et al. 2006) B2M=β_2-microglobulin, BMP=bone morphogenetic protein

The evidence for UPR involvement in human SpA is also inconsistent. One study demonstrated upregulation of UPR genes including XBP1 and CHOP in monocytes from HLA B27 positive patients with AS compared to HLA B27 negative patients and healthy controls (Feng, Ding et al. 2012). Increased expression of BiP was also found in macrophages from synovial fluid from patients with AS compared to patients with osteoarthritis. In contrast, although Zeng et al found enhanced IL23 production from monocyte-derived macrophages (MDMs) from patients with AS compared to healthy controls, there was no evidence that the UPR was responsible for this with no upregulation of three UPR genes including BiP and CHOP (Zeng, Lindstrom et al. 2011). This was confirmed in a study comparing UPR and

cytokine gene expression in MDMs from patients with HLA B27 positive SpA, HLA B27 negative SpA and healthy controls. No difference was observed in UPR gene expression in patients with SpA compared to healthy controls despite enhanced IL23p19, IL12/23p40 and IL12p35 expression in patients with SpA (Ambarus, Yeremenko et al. 2018). Similarly, despite the finding of misfolded HLA B27 in gut biopsies of patients with AS, no evidence of enhanced UPR gene expression was found. Instead, upregulation of genes involved in autophagy was noted (Ciccia, Accardo-Palumbo et al. 2014). Thus, confirmation that the UPR is responsible for the pathogenesis of HLA B27-associated disease in humans has not yet been proven. As discussed above, in transgenic rats, although the presence of HLA B27 is sufficient to induce an inflammatory disease akin to SpA, this requires very high transgene numbers (Hammer, Maika et al. 1990). Therefore the relevance of these models to human disease where only a maximum of two copies of the HLA B27 gene are present is unclear (Smith 2018). This, coupled with the fact that not all patients with SpA and even AS are HLA B27 positive, may explain why the evidence for UPR involvement in human SpA is less convincing (Bowness 2015).

The second theory also relates to the observation that HLA B27 aberrantly folds resulting in the formation of homodimers (Allen, O'Callaghan et al. 1999) and β_2-microglobulin-free heavy chains (Bird, Peh et al. 2003) mediated by disulphide bond formation through the unpaired cysteine residue at position 67. In contrast to the misfolded proteins in the ER, these homodimers and free heavy chains are expressed at the cell surface and are recognised by innate immune receptors. These receptors include KIRs and leucocyte immunoglobulin-like receptors (LILRs) which are found on NK cells, macrophages, dendritic cells, CD4+ and CD8+ T cells and play an important role in the promotion of T cell survival and differentiation of innate immune cells (Colonna, Nakajima et al. 1999). KIRs can be differentiated by the presence or absence of a long cytoplasmic tail and include KIR three domains, long cytoplasmic tail (KIR3DL)1 and KIR3DL2 which are found on both NK cells and also CD4+ T cells. Interaction between MHC class I molecules and KIRs with a long tail enables the transduction of inhibitory signals resulting in modulation of cytokine production and the expression of anti-apoptotic signals, thus promoting cell survival (Beziat, Hilton et al. 2017). HLA B27 homodimers and free heavy chains have a different conformation from and thus bind with different affinity to these KIRs compared to classical, heterodimeric HLA B27 molecules. HLA B27 homodimers bind with particular affinity to KIR3DL2, causing the expansion and increased survival of NK cells and KIR3DL2+CD4+ T cells which does not occur with other HLA class I molecules (Chan, Kollnberger et al. 2005, Wong-Baeza, Ridley et al. 2013). Populations of these cells are increased in patients with SpA compared to healthy controls, especially those who are HLA B27 positive. This is also the case for KIR3DL2+CD4+ T cells in HLA B27 positive patients with ERA (Chan, Kollnberger et al. 2005). Further studies have shown that the expanded KIR3DL2+CD4+ T cells polarise to a Th17 phenotype, leading to increased IL17 production in patients with SpA thus providing a potential mechanism by which HLA B27 is linked to disease pathogenesis (Bowness, Ridley et al. 2011). Further study

to validate this theory is needed to investigate the effect of inhibiting the interaction between HLA B27 homodimers and KIR3DL2 in animal models and by correlating expression of HLA B27 homodimers and free heavy chains with disease activity and progression in patients with SpA (Bowness 2015).

The third theory centres on the function of HLA B27 and other MHC class I molecules to present peptides to CD8+ cytotoxic T cells. It was proposed that HLA B27 has the ability to bind and present a pathogenic self or environmental peptide within the joints thus activating CD8+ T cells resulting in an inflammatory response (Benjamin and Parham 1990). The arthritogenic peptide theory has been extensively investigated and is supported by the finding of HLA B27-restricted CD8+ T cells recognising both bacterially infected and uninfected cells within the synovial fluid of patients with reactive arthritis and AS (Hermann, Yu et al. 1993). This theory was further supported by the detection of Chlamydia and Yersinia-specific CD8+ T cells in the synovial fluid of patients with reactive arthritis (Ugrinovic, Mertz et al. 1997, Appel, Kuon et al. 2004). However, despite this, the corresponding autoantigen has not been found and no convincing candidates for the arthritogenic peptide with a specific HLA B27-restricted CD8+ T cell clone have been identified (McHugh and Bowness 2012). This theory is supported by the involvement of ERAP1 in the pathogenesis of SpA in HLA B27 positive individuals highlighting the role of peptide processing and presentation as central to disease pathogenesis (Bowness 2015). In addition, the effect of HLA B27 subtype on development of disease also supports the arthritogenic peptide theory with peptide-binding ability affected by different conformations of HLA B27. It has been suggested that although the arthritogenic peptide has yet to be identified, gut microbiota may be responsible and HLA B27 has been shown to affect the gut microbiota of transgenic rats (Lin, Bach et al. 2014, Asquith, Davin et al. 2017). However, animal models do not fully support the arthritogenic peptide theory with studies demonstrating that CD8+ T cells are not required for disease pathogenesis in HLA B27 transgenic rats (May, Dorris et al. 2003, Taurog, Dorris et al. 2009). In addition, in a mouse model, expression of IL23 alone was sufficient to induce SpA-like disease by its effect on specific entheseal resident T cells (Sherlock, Joyce-Shaikh et al. 2012). Therefore, the arthritogenic peptide theory also remains unproven.

Other theories linking HLA B27 to the pathogenesis of SpA include those related to the gut microbiome which has been shown to be altered in patients with AS (Costello, Ciccia et al. 2015, Breban, Tap et al. 2017) and ERA (Di Paola, Cavalieri et al. 2016, Stoll, Kumar et al. 2016, Aggarwal, Sarangi et al. 2017) compared to healthy controls. In HLA B27 transgenic rats, SpA-like disease does not develop if the rats are raised in a germ-free environment further implicating the gut microbiome in pathogenesis (Taurog, Richardson et al. 1994) and as described above HLA B27 has been shown to influence the gut microbiome in animal models. A recent study of HLA alleles in AS and rheumatoid arthritis demonstrated that changes in the gut microbiome in these conditions are at least partially due to the effects of HLA B27 and HLA DRB1 respectively, thus supporting the theory that HLA alleles increase

the risk of these diseases through their effect on gut microbiota (Asquith, Sternes et al. 2019). This requires further investigation in humans but it seems likely that this, along with contributory factors linked to the other theories above may explain susceptibility to disease in those who are HLA B27 positive.

1.2.4.2 Male sex

Sex hormones, particularly oestrogen but also progesterone and testosterone, strongly influence the immune response. Differences in cytokine production from effector cells, immunoglobulin production from B cells and the activity of cells such as granulocytes and NK cells are seen between sexes. Genetics and the X chromosome may also play a role (Oertelt-Prigione 2012). These differences influence the clinical manifestations, outcomes and response to treatment in many inflammatory and autoimmune diseases including SpA. Increased incidence of SpA-like disease is found in mouse models of disease as discussed above (Weinreich, Eulderink et al. 1995, Khare, Hansen et al. 1996). In human disease, male sex is associated with more extensive bone marrow oedema of the SIJs (Van Praet, Jans et al. 2014) and a worse radiographic outcome in SpA (de Carvalho, Bortoluzzo et al. 2012, Landi, Maldonado-Ficco et al. 2016, Webers, Essers et al. 2016). However, female sex is associated with higher disease activity and pain scores (de Carvalho, Bortoluzzo et al. 2012, Tournadre, Pereira et al. 2013, van der Horst-Bruinsma, Zack et al. 2013, Landi, Maldonado-Ficco et al. 2016, Kilic, Kilic et al. 2017) but less severe radiographic progression (Lee, Reveille et al. 2007, Vosse, Landewe et al. 2008, Baraliakos, van den Berg et al. 2012, van Tubergen, Ramiro et al. 2012, Maas, Spoorenberg et al. 2015, Landi, Maldonado-Ficco et al. 2016). Differences have also been reported in response to treatment with significantly lower efficacy of TNF inhibitors in females compared to males (Arends, Brouwer et al. 2011, van der Horst-Bruinsma, Zack et al. 2013, Gremese, Bernardi et al. 2014, Lubrano, Perrotta et al. 2017).

Genetic factors may play a role in these differences. A study by Gracey et al found some similarities in the genetic profiles of male and female patients with SpA but also differences including the upregulation of IL17RA expression in male patients. This was in addition to increased levels of IL17 and Th17 cells in the peripheral blood of male patients with AS compared to healthy controls, suggesting that the IL17 axis was important in male patients in particular. The same study also showed increased expression of innate immune sensing genes such as Mediterranean fever gene (MEFV) and nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing (NLRP)12 and autophagy-related genes in males compared to females (Gracey, Yao et al. 2016). Another study identified different variants of the progressive ankylosis protein homolog (ANKH) gene in males and females with AS. The ANKH protein regulates levels of inorganic phosphate important for normal osteogenesis and mice with mutations in the ANKH gene at the 3' end develop severe ankylosis (Ho, Johnson et al. 2000). Males with AS more frequently have variants of

the ANKH gene near the 3' end compared to females where variants are more frequent at the 5' end of the gene, suggesting a mechanism for the more severe radiographic progression seen in men with AS (Tsui, Inman et al. 2005). A SNP in a gene encoding another regulator of inorganic phosphate metabolism, tissue non-specific alkaline phosphatase (TNAP), was found to be associated with AS in males but not females (Tsui, Inman et al. 2007).

Hormonal factors are also likely to contribute to the differences between males and females in SpA. In a mouse model of SpA, treatment with 17β-oestradiol suppressed the development of inflammatory arthritis and also reduced the production of pro-inflammatory cytokines including TNFα, IL6, IL17 and IFNγ (Jeong, Bae et al. 2017). However, studies on the effect of oestrogen in humans have produced inconsistent results. One small study of 17 female patients with AS found lower oestrogen levels in patients with active disease compared to healthy controls and patients with inactive disease. In the same study, 7 patients took oral oestrogen and an improvement in all clinical measures of disease activity was noted (Jimenez-Balderas, Tapia-Serrano et al. 1990). A larger, more recent study did not find any significant differences in disease severity between women with AS who had ever taken the oral contraceptive pill (OCP) compared to those who had not although those on the OCP were diagnosed earlier (Mahendira, Thavaneswaran et al. 2014). Other sex hormones such as testosterone and progesterone have not been shown to be significantly different between patients with SpA and healthy controls (Jimenez-Balderas, Tapia-Serrano et al. 1990, Giltay, Popp-Snijders et al. 1998, Straub, Struharova et al. 2002).

Differing levels of pro-inflammatory cytokines between the sexes may be explained by the effect of oestrogen on innate immune cells. In the study by Gracey et al, increased serum TNFα was found in male patients compared to female patients with AS, in addition to elevated levels of IL17 in males (Gracey, Yao et al. 2016). Another study also demonstrated higher serum levels of TNFα and higher IL18 levels in male patients compared to female patients with AS (Huang, Tso et al. 2012). High levels of oestrogen such as those found during pregnancy or around the time of ovulation have been shown to inhibit the production of pro-inflammatory cytokines including TNFα, IL1β and IL6 and stimulate the production of anti-inflammatory cytokines including IL4, IL10 and TGFβ and also the upregulation of Tregs (Prieto and Rosenstein 2006, Straub 2007). However, lower levels of oestrogen result in enhanced TNFα, IL1β and IFNγ and stimulation of NK cells therefore although females with SpA have lower levels of pro-inflammatory cytokines which may explain some differences in the clinical manifestations, they are not protected from disease. Hormonal factors have not been studied in ERA but with the most common age of onset in late childhood and early adolescence (around the time of puberty) and the occurrence of sacroiliitis in the adolescent years, hormones may play a significant role.

1.2.5 The IL23/IL17 axis

The IL23/IL17 axis has been strongly implicated in the pathogenesis of adult SpA by genetic studies identifying associations with several genes involved in the IL23 signalling pathway, animal models and translational studies. Further evidence for the involvement of the IL23/IL17 axis in SpA came from positive results in initial clinical trials of agents blocking IL23 (Poddubnyy, Hermann et al. 2014) but later trials did not show efficacy in AS (Baeten, Ostergaard et al. 2018). However, IL17 blockade is an effective treatment for AS (Baeten, Sieper et al. 2015) and will be discussed later in this chapter. Clinical trials of IL17 inhibition are now underway in patients with ERA but the evidence for the involvement of the IL23/IL17 axis is less well documented.

1.2.5.1 The IL12/23 cytokine family

IL23 is part of the IL12 family of cytokines which comprises three α subunits: IL23p19, IL12p35 and IL27p28 and two β subunits: IL12/IL23p40 and Ebstein Barr virus induced gene 3 (Ebi3). IL12 and IL23 share the β subunit IL12/IL23p40, with IL23 also comprising the unique α subunit IL23p19 and IL12 the α subunit IL12p35. IL27 is formed of IL27p28 and Ebi3 and IL35 shares the IL12p35 subunit with IL12 in addition to Ebi3 (Croxford, Kulig et al. 2014). Another cytokine, IL39 (consisting of IL23p19 and Ebi3), has been identified in murine studies but has not yet been detected in humans (Bridgewood, Alase et al. 2019). Both IL23 and IL12 are pro-inflammatory cytokines produced by activated antigen presenting cells such as macrophages and dendritic cells and their production is tightly regulated by the expression of the α subunits IL23p19 and IL12p35 (Croxford, Mair et al. 2012). Animal models of inflammatory arthritis and EAE, a murine model of multiple sclerosis, have helped define the key roles of IL12 and IL23. IL12, which induces the differentiation of naïve T cells in to Th1 cells and thus enhances the production of IFNγ, was thought to be the driver of EAE (Adorini 1999). However, later studies demonstrated that mice lacking either IL12/23p40 or IL23p19 were fully resistant to disease but those deficient in IL12p35 were not, thus implicating IL23 rather than IL12 in the pathogenesis of EAE (Cua, Sherlock et al. 2003). IL23 is crucial for promoting the expansion of pathogenic IL17 and GMCSF-producing Th17 cells and is also implicated in murine models of inflammatory arthritis such as CIA, where mice deficient in IL23p19, thus lacking IL23 and consequently Th17 cells, are protected against developing arthritis whereas IL12p35 deficient mice are not (Murphy, Langrish et al. 2003).

In contrast to IL12 and IL23, IL27 has a more immunoregulatory function and in conjunction with IL12 and IL2 enhances the production of IFNγ from T cells and NK cells (Pflanz, Timans et al. 2002). It also has an inhibitory effect on the production of GMCSF by T cells (Codarri, Gyulveszi et al. 2011) and activation of the inflammasome within antigen presenting cells (Mascanfroni, Yeste et al. 2013). Contrary to the other cytokines within the IL12 cytokine

family which are secreted by antigen presenting cells, IL35 is mainly produced by Tregs (Collison, Workman et al. 2007) and B cells (Shen, Roch et al. 2014) and also has an immunoregulatory function with reduced expression leading to loss of the suppressive function of Tregs (Collison, Workman et al. 2007).

1.2.5.2 IL23 in the pathogenesis of SpA- evidence from animal models

With the exception of IL35, the IL12 family of cytokines has been firmly implicated in the pathogenesis of SpA by genetic studies (Smith 2015) and IL23 in particular appears to be key. Enthesitis, which is thought to be the primary pathological process in SpA and which differentiates SpA from other forms of inflammatory arthritis (Watad, Cuthbert et al. 2018), was shown to be IL23-dependent in a mouse model. In this study, a novel population of resident entheseal T cells was identified which expressed the IL23R and the Th17 marker retinoic acid receptor-related orphan receptor γt (RORγt) but neither CD8 nor CD4. When IL23 was over-expressed, these cells were activated and produced IL17, IL6 and IL22 at the entheses but also the aortic root and the uvea resulting in enthesitis and inflammation at other sites associated with extra-articular manifestations in SpA (Sherlock, Joyce-Shaikh et al. 2012). In SKG mice, where SpA-like disease is induced by the injection of curdlan, axial spondylitis, enthesitis and peripheral arthritis were shown to be IL23 dependent (Benham, Rehaume et al. 2014). Another study of non-obese diabetic mice demonstrated that intravenous delivery of IL23 via an adenovirus vector induced psoriatic arthritis with typical skin lesions and significant synovial hypertrophy. Further analysis showed activation of Th17 cells and IL17-producing γδ cells and that treatment with an IL17 inhibitor reduced disease severity (Flores, Carbo et al. 2019). Mice deficient in IL23p19 do not develop arthritis or exhibit only a mild disease phenotype in CIA mouse models, with reduction in Th17 and γδ T cells demonstrating the importance of IL23 in disease severity and initiation of disease (Murphy, Langrish et al. 2003, Cornelissen, Mus et al. 2009). A summary of the evidence from animal models demonstrating the role of IL23 in the development of arthritis is found in table 1.4.

Animal arthritis model	IL23 expression	Effect	Reference
Mouse CIA	IL23p19 deficient mice	No arthritis	(Murphy, Langrish et al. 2003)
	Anti-IL23p19 antibody	Reduction of disease severity if administered 15 days before clinical onset of disease	(Cornelissen, Asmawidjaja et al. 2013)
Rat CIA	Anti-IL23p19 antibody	Prevention of inflammation and bony destruction	(Yago, Nanke et al. 2007)
Mouse AIA	IL23p19 deficient mice	Milder arthritis and reduced structural damage	(Cornelissen, Mus et al. 2009)
HLA B27 transgenic rats	Anti-IL23 receptor (prophylactic or therapeutic treatment)	Prevention of spondylitis and arthritis with prophylactic treatment but not therapeutic treatment	(van Tok, Na et al. 2018)
Non obese diabetic mice	Adenoviral vector encoding single chain of IL23	Skin lesions akin to psoriasis, synovial hypertrophy, intervertebral disc degeneration	(Flores, Carbo et al. 2019)
B10.RIII mice	IL23 overexpression by hydrodynamic delivery of an IL23 minicircle	Enthesitis and entheseal new bone formation, aortic root and uveal inflammation	(Sherlock, Joyce-Shaikh et al. 2012)
SKG mice (injected with curdlan)	Mice treated with anti-IL23	Reduced severity of axial spondylitis, peripheral arthritis, enthesitis and ileitis	(Benham, Rehaume et al. 2014)

Table 1.4 Animal models highlighting the role of IL23 in arthritis (CIA= collagen-induced arthritis, AIA = methylated BSA antigen-induced arthritis)

In HLA B27 transgenic rats, prophylactic treatment with an anti-IL23R antibody prevented the development of SpA whereas therapeutic treatment with the same anti-IL23R antibody did not. Significant suppression of IL17 expression was seen with prophylactic treatment but not with therapeutic treatment (van Tok, Na et al. 2018). A murine model of CIA demonstrated that the inhibition of IL23p19 was effective in reducing disease severity if administered after the initial arthritic stimulus but before the development of disease. However, if IL23 blockade was administered after CIA had developed, no clinical improvement was seen (Cornelissen, Asmawidjaja et al. 2013). Interestingly, Th17 cells are already present in the pre-clinical stages of CIA but are absent in mice lacking IL23 (Murphy, Langrish et al. 2003).

1.2.5.3 IL23 in the pathogenesis of SpA and ERA - evidence from human studies

In human disease, the presence of pathogenic Th17 cells in the preclinical phase has been proposed as an explanation for the lack of efficacy of IL23 blockade in patients with multiple sclerosis (Croxford, Kulig et al. 2014). Early treatment with IL23 inhibition is also supported in human studies of psoriatic arthritis which demonstrate efficacy for the treatment of enthesitis in clinical trials (Araujo, Englbrecht et al. 2019). A study of patients with psoriasis where nearly half had evidence of asymptomatic entheseal inflammation at baseline showed resolution after 12 weeks treatment with IL12/23p40 inhibition and no recurrence during 52 weeks of treatment (Savage, Goodfield et al. 2019).

IL23 has been further implicated in the pathogenesis of SpA by the finding described above of enhanced production from LPS-stimulated MDMs in patients with SpA compared to healthy volunteers (Zeng, Lindstrom et al. 2011, Ambarus, Yeremenko et al. 2018). In addition, some studies have reported elevated IL23 levels in the serum, peripheral blood and synovial fluid of patients with SpA (Melis, Vandooren et al. 2010, Mei, Pan et al. 2011, Chen, Chang et al. 2012, Ugur, Baygutalp et al. 2015). Appel et al demonstrated that IL23 was found in much higher concentrations in the inflammatory lesions of facet joints in patients with AS compared to osteoarthritis and that the source of this IL23 was myeloid precursor cells and CD68+ CD163+ macrophages (Appel, Maier et al. 2013). In view of this, the negative findings in clinical trials of IL23 inhibition in SpA were unexpected but may be explained by the studies above suggesting that IL23 blockade is only effective in early disease and for certain clinical features, such as enthesitis.

IL23 is less well studied in ERA. One study demonstrated higher levels of IL23-producing intermediate monocytes in patients with ERA (Gaur, Myles et al. 2017) but serum levels have not been found to be different between patients and healthy controls (Gaur, Misra et al. 2016).

IL23 is strongly associated with IBD and IL23 inhibition is an effective treatment (Deepak and Sandborn 2017). Overt IBD develops in around 10% of patients with SpA (van der Horst-Bruinsma and Nurmohamed 2012) but a much higher proportion have subclinical microscopic gut inflammation (Mielants, Veys et al. 1988). IL23 expression is enhanced from both infiltrating monocytes and Paneth cells in patients with SpA (Ciccia, Bombardieri et al. 2009). Interestingly, IL17 is not increased in these tissues in patients with SpA but is in Crohn's disease. There is, however, enhancement of a subset of NK cells producing IL22 which may be protective against the development of overt inflammatory bowel disease in patients with SpA (Ciccia, Accardo-Palumbo et al. 2012) although IL22 has been implicated in the new bone formation associated with SpA (Sherlock, Joyce-Shaikh et al. 2012, El-Zayadi, Jones et al. 2017). Despite the overlap between IBD and SpA, differences are also seen in treatment response between these conditions with IL23 inhibition effective in Crohn's

disease and IL17 blockade ineffective and in some cases associated with disease flare in IBD (Hueber, Sands et al. 2012, Feagan, Sandborn et al. 2016, Feagan, Panes et al. 2018).

1.2.5.4 IL27 in the pathogenesis of SpA

The evidence for the involvement of IL27 in the pathogenesis of SpA comes from genetic studies (Cortes, Hadler et al. 2013) and animal models (table 1.5) demonstrating reduced expression of IL27 in HLA B27 transgenic rats (Fert, Cagnard et al. 2014). IL27 promotes the differentiation of Th1 cells resulting in increased IFNγ expression and inhibits the development of Th17 cells (Stumhofer, Laurence et al. 2006). The anti-inflammatory properties of IL27 were demonstrated in EAE where mice lacking the IL27 receptor were more susceptible to disease correlating with increased levels of Th17 cells (Vanden Eijnden, Goriely et al. 2005). In another study, mice deficient in the IL27 receptor developed SpA-like features of axial arthritis with intervertebral disc ossification (Dibra, Xia et al. 2018).

Animal model of arthritis	**IL27 expression**	**Effect**	**Reference**
HLA B27 transgenic rats	Downregulated	Downregulation of IL27 from dendritic cells	(Fert, Cagnard et al. 2014)
EAE mice	IL27 receptor deficiency	More susceptible to disease, increased Th17 cells	(Vanden Eijnden, Goriely et al. 2005)
IL27 receptor knockout mice	IL27 receptor deficiency	SpA-like arthritis with axial inflammation and intervertebral disc ossification	(Dibra, Xia et al. 2018)

Table 1.5 Animal models highlighting the role of IL27 in inflammatory disease

Low levels of IL27 are found in the SF of patients with ERA also correlating with enhanced levels of Th17 cells (Gaur, Misra et al. 2016). Downregulation of IFNγ genes is also found in B27 transgenic rats and in MDMs from patients with SpA which may explain the finding of low levels of IL27 (Fert, Cagnard et al. 2014). IL27 may also be important in bone remodelling as it suppresses RANKL-mediated osteoclastogenesis (Furukawa, Takaishi et al. 2009) in contrast to IL23 which induces RANKL expression and thus osteoclast formation (Li, Kim et al. 2010).

1.2.5.5 Th17 cells and IL17 production in the pathogenesis of SpA

The role of IL23 in Th17 cell expansion and survival (and thus IL17 expression) is clear although IL23 cannot initiate the production of Th17 cells because of the lack of IL23R on naïve CD4+ T cells (Ghoreschi, Laurence et al. 2010). Other cytokines, such as IL1β, IL6 and

TGFβ are also important for Th17 cell development but differences exist between animal models and humans, with the conditions required for human Th17 cell differentiation less well defined. In addition to IL23, IL1β has been shown to play a key role in Th17 cell development in both murine and human studies (Chung, Chang et al. 2009, Revu, Wu et al. 2018). The roles of TGFβ and IL6 have been demonstrated in animal studies but may not be crucial for Th17 development in humans (Annunziato, Cosmi et al. 2008, Ghoreschi, Laurence et al. 2010, Hirahara, Ghoreschi et al. 2010, Sallusto, Zielinski et al. 2012, Zuniga, Jain et al. 2013). The binding of IL23 to the IL23R on Th17 cells and other IL17-producing cells induces signalling through the phosphorylation and activation of JAK2 and TYK2. STAT3 and STAT4 subsequently bind to the receptor complex and are phosphorylated resulting in their dissociation from this complex and translocation to the nucleus. This induces the transcription of IL17 and other genes (Raychaudhuri and Raychaudhuri 2017).

Th17 cells are the main source of IL17 and are characterised by the expression of RORγt, IL17, IL22, and GMCSF. Th17 responses are associated with chronic inflammation tissue and a diverse range of autoimmune diseases. IL17 induces the production of other cytokines including IL6, IL8, TNFα, GMCSF and chemokines, MMPs and receptor activator of NFκB ligand (RANKL) from various cell types including fibroblasts, macrophages, dendritic cells, chondrocytes and osteoblasts (Jovanovic, Di Battista et al. 1998, Smith and Colbert 2014). Th17 cells also secrete other pro-inflammatory cytokines including IL6, IL21 and TNFα (Miossec and Kolls 2012). Therefore, the Th17 response is a key driver of many chronic inflammatory conditions but also provides tissue homeostasis in certain situations and is not always pathogenic (Wu and Wan 2020). One of the key features of pathogenic Th17 cells is their ability to co-produce GMCSF and IL17 (Codarri, Gyulveszi et al. 2011, El-Behi, Ciric et al. 2011, Shiomi and Usui 2015). GMCSF enhances pathogenicity via a positive feedback loop acting on macrophages and dendritic cells to increase IL23 production causing further expansion of Th17 cells and thus increased GMCSF production (Codarri, Gyulveszi et al. 2011).

In adult SpA, increased frequency of Th17 cells in the peripheral blood and synovial fluid has been reported (Jandus, Bioley et al. 2008, Shen, Goodall et al. 2010) and in particular, higher numbers of GMCSF+Th17 cells are found in patients with SpA compared to healthy volunteers and those with rheumatoid arthritis (Al-Mossawi, Chen et al. 2017). In addition, a specific subset of Th17 cells expressing the KIR3DL2 receptor which interacts with HLA B27 homodimers and promotes Th17 cell survival is increased in patients with SpA compared to healthy controls. The blockade of the KIR3DL2-HLA B27 homodimer interaction in a cell line using antibodies to HLA B27 or KIR3DL2 results in an attenuation of IL17 production (Bowness, Ridley et al. 2011, Wong-Baeza, Ridley et al. 2013). A small study in patients with ERA did not show increased numbers of Th17 cells in the peripheral blood compared to healthy controls (Mahendra, Misra et al. 2009) but CD4+ T cells expressing the KIR3DL2 receptor are enhanced in patients with ERA (Chan, Kollnberger et al. 2005). In JIA, IL17-

producing cells are enriched in the joints compared to the peripheral blood of patients and are higher in patients with a more severe disease phenotype (Nistala, Moncrieffe et al. 2008). However, this study did not include patients with the ERA.

The IL17 family of cytokines consists of six members including IL17A and IL17F which have both been linked to chronic inflammatory disease in overlapping but also distinct roles (Iwakura, Ishigame et al. 2011). IL17A and IL17F are structurally similar and can even form heterodimers (Gaffen 2009). IL17A is the prototypical member of the IL17 family (also referred to simply as IL17) and is the most frequently studied. It has been implicated in animal models of inflammatory arthritis (Lubberts, Joosten et al. 2002, Koenders, Lubberts et al. 2005) and has been found at higher concentrations in the serum and synovial fluid of patients with active SpA (Singh, Aggarwal et al. 2007, Wendling, Cedoz et al. 2007). IL17A and F are not only secreted by Th17 cells but are also produced by CD8+ T cells, γδ T cells, invariant natural killer cells (iNKT), ILC3s including lymphoid tissue inducer (LTi) cells, NK cells, mast cells, macrophages, and neutrophils (Isailovic, Daigo et al. 2015). In a study of patients with JIA which included patients with ERA, an expansion in ILC3s was noted in SF and levels correlated with disease activity measures as well as increased numbers of other IL17-producing cells including CD4+ T cells, CD8+ T cells and γδ T cells (Rosser, Lom et al. 2019). In the facet joints of patients with SpA, IL17 is highly expressed in subchondral bone marrow cells from inflammatory lesions and the source is mainly myeloid precursor cells and neutrophils but not Th17 cells (Appel, Maier et al. 2011). This suggests innate immune production of IL17 is also important in the pathogenesis of SpA and the finding of increased IL17 production mainly from mast cells in the synovium of patients with SpA compared to those with rheumatoid arthritis also supports this (Noordenbos, Yeremenko et al. 2012). In addition, increased numbers of IL23R+γδ T cells are associated with enhanced IL17 secretion in patients with SpA compared to healthy controls (Kenna and Brown 2013).

In a mouse model over expressing IL23, the main producers of IL17A at the enthesis were γδ T cells, which increased under inflammatory conditions and accumulated at the aortic root and adjacent to the ciliary body in addition to the enthesis (Reinhardt, Yevsa et al. 2016). A recent study of human-derived entheseal cells (from spinous process entheseal tissue) also demonstrated IL17 production from γδ T cells following stimulation. Interestingly, one subset of these γδ T cells was unresponsive to IL23 stimulation (and lacked IL23R) but produced IL17 in response to other forms of stimulation including phorbol 12-myristate 13-acetate (PMA) and ionomycin (Cuthbert, Watad et al. 2019). This supports the emerging theory of IL23-independent IL17 production which may occur from innate IL17-producing cells (Bridgewood, Sharif et al. 2020). iNKT cells are another such cell which have been found to produce IL17 in the joints of patients with SpA (Venken, Jacques et al. 2019). Thus, IL23 appears to be important in 'priming' or early disease but once IL17 responses are established then IL23 may no longer be necessary for continued IL17 production.

1.2.6 Other inflammatory pathways in SpA

Genetic studies have implicated other pro-inflammatory pathways in the pathogenesis of SpA (Hreggvidsdottir, Noordenbos et al. 2014). GWAS have identified SNPs in cytokines, their receptors and associated pathways. Further evidence for the involvement of other pro-inflammatory pathways comes from animal models of disease, expression of cytokines in human SpA and therapeutic effects of pro-inflammatory cytokine blockade. The following sections highlight a number of key pro-inflammatory cytokines thought to be important in the pathogenesis of SpA.

1.2.6.1 TNFα in the pathogenesis of SpA and ERA

Inhibition of TNFα is a highly effective treatment for both SpA and ERA and high levels of TNFα expression are found in the inflammatory infiltrates of sacroiliac joints (SIJs) of patients with AS (Braun, Bollow et al. 1995). Animal models over expressing TNFα develop a variety of manifestations of SpA including enthesitis, axial inflammation and new bone formation (Kontoyiannis, Pasparakis et al. 1999, Uderhardt, Diarra et al. 2010, Vieira-Sousa, van Duivenvoorde et al. 2015). These are summarised in table 1.6 below.

Animal model of arthritis	**TNFα expression**	**Effect**	**Reference**
TNF transgenic mice (hTNFtg mice) (with blockade of Dkk1)	Overexpression of human TNFα	Peripheral arthritis and sacroiliitis (+ SIJ fusion with blockade of Dkk1)	(Uderhardt, Diarra et al. 2010)
TNFdeltaARE-mice	Overexpression of mouse TNFα by deletion of AU-rich elements in TNF locus	Destructive polyarthritis, enthesitis and ileitis	(Kontoyiannis, Pasparakis et al. 1999)
tmTNFtg mice	Overexpression of transmembrane TNF	Inflammatory infiltration of peripheral and axial joints, ankylosis	(Vieira-Sousa, van Duivenvoorde et al. 2015)

Table 1.6 Animal models highlighting the role of TNFα in SpA

Blockade of TNFα in human SpA results in reduction in inflammatory cell infiltrates on synovial biopsy (Baeten, Kruithof et al. 2001) and a significant improvement in symptoms but without consistent inhibition of new bone formation (van der Heijde, Ramiro et al. 2017). This may be explained by evidence that TNFα inhibition paradoxically upregulates the IL23/IL17 pathway with increased numbers of pathological Th17 cells seen an animal model of inflammatory arthritis treated with TNFα blockade (Notley, Inglis et al. 2008). This was confirmed in a study of Th17 cell subsets in patients with SpA treated with TNFα blockade which showed a significant increase in levels after 12 months of treatment regardless of clinical response (Andersen, Ostgard et al. 2019). Recent studies demonstrate that blockade

of IL17 may be more effective at inhibiting new bone formation (van Tok, van Duivenvoorde et al. 2019) but this needs confirmation in human SpA. The combination of TNFα and IL17 blockade has been investigated in animal models of CIA and has been found to more effective than inhibition of either TNFα or IL17 alone (Alzabin, Abraham et al. 2012) without increased risk of serious infection (Shen, Verma et al. 2019) but this needs further study in human SpA.

1.2.6.2 IL1 in the pathogenesis of SpA and ERA

Genetic associations between IL1 and AS have been identified by GWAS (Monnet, Kadi et al. 2012) and IL1 gene polymorphisms have also been identified in patients with ERA (Aggarwal, Srivastava et al. 2012) but evidence of increased expression of IL1 in patients with SpA is lacking (Bal, Unlu et al. 2007, Romero-Sanchez, Robinson et al. 2008). An animal model of unopposed IL1α and IL1β expression (by knocking out IL1R antagonist (IL1Ra) which competes for the IL1R) exhibits erosive polyarthritis, psoriasis and aortitis but not enthesitis, gut inflammation or new bone formation (Horai, Saijo et al. 2000) thus not fully mimicking SpA. Inhibition of IL17 in this mouse model prevents disease (Koenders, Devesa et al. 2008). In addition, when IL1α and IL1β are knocked out of TNF transgenic mice, no bone or cartilage destruction is noted suggesting that IL1 mediates TNF-induced bone and cartilage erosion in this model of inflammatory arthritis (Zwerina, Redlich et al. 2007). Animal models of arthritis highlighting the role of IL1 are summarised in table 1.7.

Animal model of arthritis	IL1 expression	Effect	Reference
IL1Ra knockout mice	Overexpression of IL1	Erosive arthritis, aortitis, psoriasis-like disease	(Horai, Saijo et al. 2000)
hTNFtg mice, IL1 knockout	IL1 deficiency	Synovial inflammation but no bone or cartilage erosions	(Zwerina, Redlich et al. 2007)

Table 1.7 Animal models highlighting the role of IL1 in inflammatory arthritis

In human disease, the inhibition of IL1 has not proved to be clinically effective in SpA (Haibel, Rudwaleit et al. 2005). Despite this, IL1 may still contribute to the pathogenesis of SpA by enhancing GMCSF production from pathogenic Th17 cells. In EAE, the combination of IL1β and IL23 significantly increases GMCSF and IL17 expression over and above that seen with IL23 alone but TNFα does not (El-Behi, Ciric et al. 2011). This mechanism needs further investigation in the context of SpA and ERA.

1.2.6.3 IL6 in the pathogenesis of SpA

IL6 is also implicated in the pathogenesis of SpA through GWAS identifying polymorphisms in the IL6 receptor (Cortes, Hadler et al. 2013). Animal models implicating IL6 (summarised

in table 1.8), include a model with sustained IL6 signalling where SpA-like disease develops with a male-predominant arthritis in weight-bearing joints and gut inflammation (Ernst, Inglese et al. 2001).

Animal model of arthritis	IL6 expression	Effect	Reference
Mice with point mutation of gp130 subunit of IL6 receptor	Overexpression of IL6	Rheumatoid arthritis-like disease	(Atsumi, Ishihara et al. 2002)
Mice with deletion of STAT3 binding sites on IL6 receptor	Overexpression of IL6	Male-predominant arthritis in weight-bearing joints, intestinal ulceration	(Ernst, Inglese et al. 2001)

Table 1.8 Animal models highlighting the role of IL6 in inflammatory arthritis

A study comparing SF cytokine levels in patients with ERA and polyarticular JIA demonstrated higher levels in patients with ERA (Saxena, Aggarwal et al. 2005). Increased serum levels of IL6 are found in patients with AS but are not consistently associated with disease activity (Bal, Unlu et al. 2007, Pedersen, Sorensen et al. 2011). IL6 is also found in the inflamed SIJs of patients with AS (Francois, Neure et al. 2006). However, clinical trials of IL6 blockade in SpA have failed to show clinical benefit (Sieper and Poddubnyy 2016) suggesting that IL6 in SpA may be present as a downstream response to other pro-inflammatory cytokines and therefore blockade is ineffective for inhibition of disease.

1.2.6.4 GMCSF in the pathogenesis of SpA

GMCSF has emerged as a key cytokine in chronic inflammatory conditions and is implicated in the pathogenesis of inflammatory arthritis (Wicks and Roberts 2016). GMCSF is a haemopoietic growth factor but mainly functions as a pro-inflammatory cytokine activating monocytes and dendritic cells and promoting the polarisation of macrophages to a pro-inflammatory phenotype (Lacey, Achuthan et al. 2012). It was one of the first pro-inflammatory cytokines to be detected in synovial fluid in inflammatory arthritis (Xu, Firestein et al. 1989) and clinical trials suggest GMCSF blockade may be an effective treatment in rheumatoid arthritis (Burmester, McInnes et al. 2018).

Studies in animal models have advanced the understanding of the role of GMCSF in inflammatory arthritis (table 1.9). In mice, GMCSF deficiency results in protection against developing CIA (Campbell, Rich et al. 1998) and blockade of GMCSF ameliorates disease (Cook, Braine et al. 2001). Animal models also suggest that blockade of GMCSF may be more effective than IL17 blockade (Shiomi, Usui et al. 2014) and the combination of both GMCSF and IL17 blockade has a synergistic effect in CIA (van Nieuwenhuijze, van de Loo et al. 2015).

Animal model of arthritis	GMCSF expression	Effect	Reference
Mouse CIA	GMCSF deficient	No arthritis	(Campbell, Rich et al. 1998)
	Anti-GMCSF antibodies	Amelioration of arthritis, lower levels of TNFα and IL1	(Cook, Braine et al. 2001)
	GMCSF administration	Exacerbation of arthritis	(Campbell, Bendele et al. 1997)
Mouse CIA, IL17 knockout	Anti-GMCSF antibodies	Enhanced therapeutic effect of blocking both GMCSF and IL17	(Plater-Zyberk, Joosten et al. 2007)
SKG mouse (zymosan treated)	Anti-GMCSF antibodies	Inhibited progression of arthritis	(Shiomi, Usui et al. 2014)

Table 1.9 Animal models highlighting the role of GMCSF in inflammatory arthritis (CIA= collagen-induced arthritis)

GMCSF is essential for the pathogenicity of Th17 cells and thus their ability to drive inflammation (McGeachy 2011). One of the mechanisms for this involves the synergistic relationship between GMCSF and IL23 with the production of GMCSF induced by IL23 resulting in further enhancement of IL23 expression from antigen presenting cells (Codarri, Gyulveszi et al. 2011, El-Behi, Ciric et al. 2011). Similarly, IL1β induces GMCSF production from CD4+ T cells and γδ cells (Lukens, Barr et al. 2012) and in turn GMCSF increases production of IL1β from monocytes (Khameneh, Isa et al. 2011). In SpA, higher numbers of GMCSF+Th17 cells are found in the peripheral blood of patients compared to healthy volunteers and patients with rheumatoid arthritis and are further increased in synovial fluid (Al-Mossawi, Chen et al. 2017), suggesting GMCSF plays an important role in the pathogenesis of SpA. In this study, GMCSF production was not only enhanced in CD4+ T cells but also in CD8+ T cells, γδ cells and ILCs and occurred independently of IL17 production. Interestingly, GMCSF production from CD4+ T cells was significantly higher in patients on TNF inhibitors perhaps reflecting the upregulation of the IL23/IL17 pathway with TNF blockade. In addition, an association was found between GMCSF expression and a G-protein coupled receptor (GPR65) and silencing this receptor reduced GMCSF. GPR65, which has been associated with AS through GWAS studies (Cortes, Hadler et al. 2013), is a proton-sensing receptor and acidity increased GMCSF production in this study corresponding to the acidic environment reported to be associated with inflamed synovium (Goldie and Nachemson 1969). GPR65 may therefore be important in regulating GMCSF production in the inflamed joint. Therapeutic trials of GMCSF are ongoing in AS. GMCSF has also been implicated in the pathogenesis of JIA with increased levels of T cells expressing GMCSF found in the SF compared to peripheral blood of patients with oligoarticular and polyarticular course disease (Piper, Pesenacker et al. 2014). However, GMCSF has not been studied in patients with ERA.

1.2.6.5 IFNγ in the pathogenesis of SpA

Another cytokine implicated in the pathogenesis of SpA is IFNγ but in contrast to those above, is downregulated in those with disease compared to healthy controls (Smith, Barnes et al. 2008, Fert, Cagnard et al. 2014). IFNγ is secreted by Th1 cells, CD8+ T cells, NK cells, macrophages and ILC1s but also a subset of pathogenic Th17 cells from which it may be co-produced with IL17 and GMCSF (Ghoreschi, Laurence et al. 2010). In some circumstances, IFNγ can inhibit IL17 production by controlling the activation and development of Th17 cells and thus plays a regulatory role in the inflammatory response (Kim and Moudgil 2017). Clinical trials of IFNγ in patients with rheumatoid arthritis in the 1980s and 1990s showed benefit without significant side effects but the administration of recombinant IFNγ has not become a standard treatment for inflammatory arthritis (Lemmel, Brackertz et al. 1988, Cannon, Pincus et al. 1989, Veys, Menkes et al. 1997).

In animal models of inflammatory arthritis including CIA (summarised in table 1.10), deficiency of IFNγ results in increased IL17 and increased severity of arthritis (Vermeire, Heremans et al. 1997, Guedez, Whittington et al. 2001). Treatment with IFNγ reduces IL17 levels, severity of arthritis and facilitates recovery (Nakajima, Takamori et al. 1991, Chu, Swart et al. 2007, Satpute, Rajaiah et al. 2009). Studies in animal models and in vitro demonstrate that IFNγ can also inhibit GMCSF and IL1β secretion providing a mechanism for IL17 inhibition (Alvaro-Gracia, Yu et al. 1993, Kelchtermans, Schurgers et al. 2009, Page, Smale et al. 2010).

Animal model of arthritis	**IFNγ expression**	**Effect**	**Reference**
Mouse CIA	IFNγ deficiency	Increased severity of arthritis, increased IL17 production	(Vermeire, Heremans et al. 1997, Guedez, Whittington et al. 2001, Chu, Swart et al. 2007)
	IFNγ knockout	Treatment with IL17 antibody prevented arthritis	(Kelchtermans, Schurgers et al. 2009)
Rat AIA	Overexpression of IFNγ	Reduced severity of arthritis, reduced IL17	(Kim, Chi et al. 2008, Satpute, Rajaiah et al. 2009)
	Treatment with recombinant IFNγ	Reduced severity of arthritis	(Nakajima, Takamori et al. 1991)

Table 1.10 Animal models highlighting the role of IFNγ in inflammatory arthritis (CIA= collagen-induced arthritis, AIA = methylated BSA antigen-induced arthritis)

IL27 secretion from dendritic cells and macrophages is enhanced by IFNγ further contributing to its immunoregulatory function (Rajaiah, Puttabyatappa et al. 2011). It is therefore significant that studies in SpA show dysregulation of IFNγ genes. A study by Smith

et al found lower expression of genes normally upregulated by IFNγ and higher expression of genes normally downregulated by IFNγ in macrophages from patients with AS compared to healthy controls. These findings correlated with lower expression of the IFNγ gene and were reversed by the administration of IFNγ to the assay implying lower production of IFNγ by macrophages in patients with AS although this was not tested in this study (Smith, Barnes et al. 2008). A later study examined IFNγ related gene expression in dendritic cells from HLA B27 transgenic rats and found under-expression of these genes compared to controls which was present at disease onset, persisted over time and was associated with downregulation in IL27 expression (Fert, Cagnard et al. 2014). Lower levels of IFNγ are found in the synovium of patients with SpA compared to those with rheumatoid arthritis (Canete, Martinez et al. 2000) and a lower frequency of IFNγ expressing T cells has been reported in HLA B27 positive patients with AS. In this study HLA B27 positive healthy controls were also found to have lower levels of IFNγ+ T cells compared to HLA B27 negative healthy controls indicating that dysregulation of IFNγ may be related to HLA B27. The only study reporting IFNγ levels in patients with ERA noted higher levels in SF compared to patients with polyarticular JIA (Saxena, Aggarwal et al. 2005) and therefore further study in both SpA and ERA is key to understanding the role of IFNγ in the pathogenesis of these conditions.

1.2.7 Environmental Factors involved in the pathogenesis of SpA

1.2.7.1 The Gut Microbiome

Evidence for the involvement of gut flora in the pathogenesis of SpA dates back many years and has been clearly illustrated in HLA B27 transgenic rats which remain asymptomatic in a germ free environment but develop features of SpA with exposure to common gut bacteria (Taurog, Richardson et al. 1994). Previous studies have implicated gram negative bacteria in the pathogenesis of SpA (Keat, Maini et al. 1978, Granfors, Jalkanen et al. 1990, Merilahti-Palo, Soderstrom et al. 1991, Rashid and Ebringer 2007) and this is also the case in ERA (Singh, Singh et al. 2011). Patients with SpA have a high prevalence of gut inflammation (Mielants, Veys et al. 1988, Van Praet, Van den Bosch et al. 2013) and GWAS have demonstrated a clear overlap between SpA and Crohn's disease (Jostins, Ripke et al. 2012). Increased expression of IL23p19 is found from infiltrating monocytes in the terminal ileum of patients with both AS and Crohn's disease (Ciccia, Bombardieri et al. 2009). In SpA, gut inflammation correlates with more severe bone marrow oedema in the SIJs of patients (Van Praet, Jans et al. 2014).

Recent advances in sequencing techniques have allowed analysis of the composition of the gut microbiome in healthy individuals and in various disease states. Several studies have shown differences in the gut microbiome between patients with SpA and healthy controls. A

study by Costello et al found differences in several families of bacteria from terminal ileal biopsies from patients with AS with increases in Lachnospiraceae, Ruminococcaceae, Rikenellaceae, Porphyromonadaceae and Bacteroidaceae and decreases Veillonellaceae and Prevotellaceae compared to healthy volunteers (Costello, Ciccia et al. 2015). A more recent study demonstrated a significant increase in Ruminococcus gnavus in faecal DNA from patients with SpA compared to those with rheumatoid arthritis and healthy volunteers which correlated with disease activity in those with a history of IBD (Breban, Tap et al. 2017). A large Norwegian cohort study revealed increased Proteobacteria, Enterobacteriaceae, Bacilli, Streptococcus species, and Actinobacteria, but lower abundance of Bacteroides and Lachnospiraceae (Klingberg, Magnusson et al. 2019). In this study no association was found between the composition of faecal microbiota and HLA B27 but in other studies HLA B27 appears to significantly affect the gut microbiome (Lin, Bach et al. 2014, Asquith, Davin et al. 2017, Asquith, Sternes et al. 2019). The microbiome has also been studied to a lesser extent in ERA with one study of Indian patients showing increases in Bacteroidaceae and Enterobacteriaceae families and a reduction in the Prevotellaceae family in patients compared to healthy controls. Another study found that a strain of the anti-inflammatory bacterial family Faecalibacterium prausnitzii was reduced in both patients with ERA and SpA. The same study demonstrated a higher abundance of Bacteroides in patients with ERA compared to SpA (Stoll, Weiss et al. 2018). Higher abundance of this species has also been found in other JIA subtypes (Tejesvi, Arvonen et al. 2016).

Microbes found in the gut shape host immune response from an early age (Cho and Blaser 2012). Maintenance of microbial homeostasis is important for health with disturbances in the gut microbiome (dysbiosis) implicated in immune dysregulation and conditions such as IBD (Round and Mazmanian 2009). Studies have suggested that patients with AS and their first degree relatives have increased gut permeability thus potentially allowing greater systemic exposure to gut microbes and activation of the innate immune system (Martinez-Gonzalez, Cantero-Hinojosa et al. 1994, Vaile, Meddings et al. 1999). In addition, evidence from animal models suggests that the gut microbiome influences the activation of IL17-producing cells including Th17 cells (Ivanov, Atarashi et al. 2009, Goto, Panea et al. 2014). Links between the gut and joint have also been shown, for example the finding of increased expression of a subset of CD163+ macrophages in the synovium and the colonic lamina propria in patients with SpA (Baeten, Demetter et al. 2002). Another study using a cell line demonstrated increased affinity of gut lymphocytes to synovial endothelial cells supporting the theory of a gut-joint axis with translocation of immune cells (Salmi, Andrew et al. 1995). Therefore, the evidence suggests that the gut microbiome and dysbiosis could be implicated in the pathogenesis of SpA but further investigation is needed into the mechanisms involved.

1.2.7.2 Enthesitis and mechanical stress

The effect of dysbiosis may be far-reaching with activation of the innate immune system resulting in inflammation of distant tissues such as the enthesis (Lories and McInnes 2012, Watad, Bridgewood et al. 2018). Enthesitis, the inflammation of the site where ligaments and tendons attach in to bone, is one of the primary lesions in SpA, followed by synovitis and osteitis later in disease (Watad, Cuthbert et al. 2018). Animal models have highlighted the importance of enthesitis in the pathogenesis of SpA and the inflammatory pathways involved locally at the enthesitis which include IL23/IL17 and TNFα. Sherlock et al demonstrated that over expression of IL23 in a mouse model resulted in activation of a unique subset of RORγt+CD3+CD4–CD8– entheseal resident T lymphocytes which induced IL6, IL17 and IL22 production resulting in enthesitis (which was IL17-dependent) and new bone formation which was IL22-dependent (Sherlock, Joyce-Shaikh et al. 2012). Enthesitis and ileitis were both found to be IL23-dependent in the SKG mouse model of SpA and enthesitis was also IL17-dependent (Benham, Rehaume et al. 2014). Subsequently, Reinhardt et al showed that γδ T cells constituted the majority of RORγt+ IL23R+ entheseal resident T lymphocytes and were the main producers of IL17. With IL23-induced inflammation, increased numbers of γδ T cells were found at the enthesis and also aortic root, and adjacent to the ciliary body thus linking sites affected by extra-articular manifestations in SpA (Reinhardt, Yevsa et al. 2016). TNFα is also important in enthesitis which develops in mice over-expressing TNFα (Jacques, Lambrecht et al. 2014). Early treatment with TNF inhibition prevents signs of enthesitis in HLA B27 transgenic rats (Milia, Ibba-Manneschi et al. 2011).

Studies of the human enthesis are more challenging (Benjamin and McGonagle 2001) but innate immune cells are clearly implicated. Macrophages are present at the enthesis in the early stages of SpA-related enthesitis (McGonagle, Marzo-Ortega et al. 2002). More recent studies have demonstrated a population of CD14+ myeloid cells which, when stimulated with LPS, are the dominant producers of IL23, TNFα and IL1β in the normal human spinal enthesis (Bridgewood, Watad et al. 2019). Further study of cells from human interspinous process entheses demonstrated the presence of ILC3s which possess the IL23R and also an abundant population of γδ T cells. In this study of healthy tissue, IL23R+ILC3s did not express IL17 but did express TNFα. When stimulated with IL23 and IL1β, upregulation of IL17 was seen in addition to increased IL22 production which may contribute to new bone formation following inflammation (Cuthbert, Fragkakis et al. 2017). Studies to characterise the γδ T cells present at the enthesis confirmed the presence of two subsets mirroring the populations seen in peripheral blood. The more numerous Vδ2 subset and the less frequent Vδ1 subset both produced IL17 following stimulation with PMA but the Vδ1 subset appeared to lack the IL23R and therefore did not respond to IL23 stimulation (Cuthbert, Watad et al. 2019). Further studies of the enthesis in human SpA are important to determine the significance of these findings in the pathogenesis of SpA.

The enthesis is subject to enormous mechanical strain and repetitive micro trauma which in susceptible individuals leads to changes within the enthesis resulting in chronic inflammation (Watad, Bridgewood et al. 2018). Bone stress injuries, including SIJ lesions similar to those seen in SpA occur in healthy individuals in response to intense physical activity. Studies have shown asymptomatic elite military recruits undergoing intensive physical training, elite hockey players and recreational runners have evidence of bone stress injuries in particular bone marrow oedema of the SIJs (Kiuru, Niva et al. 2005, Weber, Jurik et al. 2018). In patients with SpA, mechanisms such as higher levels of background inflammation (IL23/17 pathway activation), genetic predisposition (related to HLA B27 and other SpA susceptibility genes) and gut dysbiosis may increase the likelihood of persistent inflammation following these bone stress injuries (Watad, Bridgewood et al. 2018).

The sites affected in SpA tend to be those under the most mechanical stress such as the lower limb and the spine and studies have shown more severe disease in those with manual jobs and with certain types of exercise (Ward, Reveille et al. 2008, Ramiro, Landewe et al. 2015). ERA usually develops in late childhood and early adolescence, a period of significant growth and therefore strain on the musculoskeletal system. Anecdotal evidence from our large cohort of patients with ERA suggests that a significant proportion take part in sport to a high level compared to other JIA subtypes (unpublished data UCLH cohort) and this was confirmed in another cohort of patients with ERA (Lin, Liang et al. 2009). Extra-articular manifestations of SpA are also found at sites under mechanical strain such as the aortic root and anterior uveal tract thus providing an explanation for inflammation at these sites in SpA (Benjamin and McGonagle 2001).

Studies in mouse models of inflammatory arthritis including CIA and mice over-expressing TNFα clearly demonstrate that mechanical stress exacerbates disease whereas relieving mechanical strain limits the development of enthesitis and also prevents new bone formation in the CIA model. Interestingly, mice deficient in T and B cells still develop worse disease in response to mechanical stress suggesting adaptive immunity is not required for this response (Jacques, Lambrecht et al. 2014, Cambre, Gaublomme et al. 2018). Although some immune cells are present, entheses comprise mostly of tenocytes which play a key role in sensing and responding to mechanical stress through stretch-sensitive ion channels, focal adhesions and mechanical stress-induced cytokines. Excessive mechanical stress results in tenocyte death and the release of pro-inflammatory cytokines including IL1β, IL6 and TNFα and DAMPs leading to the recruitment of innate immune cells and eventual healing in healthy individuals. In SpA, perpetuation of the inflammatory response at the enthesis occurs resulting in aberrant tissue healing and eventual new bone formation (Gracey, Burssens et al. 2020). The mechanisms behind this require further investigation in the context of SpA but mechanical stress may explain the differences in presentation of SpA across the ages with peripheral disease more prevalent in juvenile onset disease and axial disease increasing with age and particularly with puberty corresponding to changes in body musculature and composition.

1.2.8 Summary of the similarities and differences in pathogenesis between adult SpA and ERA

There are relatively few studies focussing on the pathogenesis of ERA and it has been assumed that the pathogenesis is similar to or the same as SpA. Studies in JIA commonly include all JIA subtypes and generally low numbers of patients with ERA. Studies of SpA usually include only adult patients. However, studies including both patients with ERA and SpA are rare. One such study characterised the features of synovial biopsies in patients with 'juvenile onset SpA' (which encompassed patients with ERA and also juvenile onset psoriatic arthritis) compared to patients with adult SpA. Although some similarities were found, there were marked differences in lining layer hyperplasia and the number of infiltrating CD163+ macrophages which meant juvenile SpA failed to classify in the SpA group by class prediction analysis. Instead, there was partial overlap with other JIA subtypes (Kruithof, Van den Bossche et al. 2006) which was perhaps due to the mixed nature of the juvenile onset SpA group. As highlighted in the sections above, some studies suggest a similar pathogenesis of ERA and adult SpA, but in others differences are observed.

Thus, further studies are required to determine whether the pathogenesis of ERA is the same as SpA or if there are differences which may explain the differences in the clinical presentation between these conditions. This has important implications for future treatment strategies for patients with ERA.

1.2.9 Novel treatments for SpA

In light of the extensive investigation in to the pathogenesis of adult SpA as described above, and in particular the importance of the IL23/IL17 axis, biologics inhibiting this pathway were greatly anticipated as novel treatments for SpA. Ustekinumab is a monoclonal antibody specifically targeting the common IL12/IL23 subunit IL12/23p40 and an initial open-label study in patients with active AS found that 75% of patients achieved 20% response by ASAS criteria after 24 weeks of treatment and significant improvement in inflammation on MRI (Poddubnyy, Hermann et al. 2014). However, in two subsequent randomised phase III trials, which included patients with AS with inadequate response to TNF inhibition and patients with nr-axSpA, ustekinumab did not show any benefit over placebo and the studies were terminated (Mease 2019). It has proven efficacy in psoriatic arthritis (Kavanaugh, Puig et al. 2016) including improvements in BASDAI and ASDAS suggesting some effect on axial arthritis in this group. Ustekinumab has also been shown to be superior to TNF inhibition in the treatment of enthesitis in patients with psoriatic arthritis (Araujo, Englbrecht et al. 2019). Similar findings have been shown for other inhibitors of IL23 including guzelkumab which significantly improves enthesitis in patients with active psoriatic arthritis (Mease, Gladman et al. 2020). Guzelkumab inhibits the IL23 specific subunit, IL23p19. Other biologics blocking IL23p19 include tildrakizumab and risankizumab and although effective treatments for psoriasis, have not demonstrated any benefit in the

treatment of axSpA (Baeten, Ostergaard et al. 2018). Given that enthesitis is an early feature of SpA (Watad, Bridgewood et al. 2018), it is possible that there is an early window for treatment with IL23 blocking agents but loss of efficacy once disease becomes more established and structural progression occurs.

Treatments blocking IL17 have proved more efficacious in SpA and for axSpA in particular. Secukinumab, a monoclonal antibody targeting IL17A, was shown to produce a 20% improvement according to ASAS criteria in 59% of patients with active AS after 6 weeks of treatment in an initial study. Significant improvement in spinal and SIJ inflammation on MRI was also seen (Baeten, Baraliakos et al. 2013) and confirmed on a subsequent study (Braun, Baraliakos et al. 2017). Sustained response has recently been demonstrated in the phase III study 5 year extension results and secukinumab is now an established treatment for SpA (including psoriatic arthritis) in both TNF inhibitor naïve patients and those previously treated with TNF inhibition (Baraliakos, Braun et al. 2019). Other agents blocking IL17 include ixekizumab (which targets IL17A) and brodalumab (which targets IL17RA) both of which have proved effective in phase III clinical trials for axSpA and psoriatic arthritis (Nash, Kirkham et al. 2017, Dougados, Wei et al. 2020, Mease, Helliwell et al. 2020) although the brodalumab trial in axSpA has so far only been published in abstract form. A trial comparing the TNF inhibitor adalimumab and ixekizumab in psoriatic arthritis showed that the IL17 inhibitor was superior in treating skin disease but produced similar outcomes after 52 weeks for the musculoskeletal manifestations (Smolen, Mease et al. 2020).

Given the involvement of IL17 in osteoblast differentiation and proliferation (Jo, Wang et al. 2018), it was proposed that IL17 blockade may slow radiographic progression to a greater extent than TNF inhibition. The phase III clinical trial of secukinumab in active AS found no significant progression in nearly 80% of patients after 4 years treatment (Braun, Baraliakos et al. 2019). In addition to IL17A, IL17F is also implicated in osteoblast differentiation (Wang, Kim et al. 2018) and therefore the blockade of both IL17A and F has been investigated in vitro resulting in enhanced suppression of new bone formation compared to the blockade of IL17A and F individually (Shah, Maroof et al. 2020). Bimekizumab, a novel biologic agent which selectively inhibits both IL17A and F, has completed phase II clinical trials in active AS and produced promising results with around 60% of patients achieving a 40% response rate by ASAS criteria. However, further longitudinal study is required to determine whether IL17 inhibition will be superior to TNF blockade in slowing new bone formation in axSpA and in particular whether dual blockade of IL17A and F will enhance this effect.

JAK inhibitors are another emerging treatment for SpA with different agents selectively blocking JAK1, JAK2, JAK3 and TYK2 which inhibit a broad range of cytokines implicated in the pathogenesis of SpA. Tofacitinib, a potent inhibitor or JAK3 and JAK1 and minor inhibitor of JAK2, has been shown to be effective in active AS with over 60% of patients achieving a 20% improvement by ASAS criteria after 12 weeks treatment and significant improvement in SIJ and spine inflammation on MRI (van der Heijde, Deodhar et al. 2017). Efficacy has also

been demonstrated in psoriatic arthritis (Mease, Hall et al. 2017). Filgotininb, a JAK1 inhibitor, has proven efficacious in a phase II trial in patients with active AS (van der Heijde, Baraliakos et al. 2018) and psoriatic arthritis (Mease, Coates et al. 2018). Other emerging treatments for SpA in early phase trials include monoclonal antibodies targeting GMCSF which have been shown to be effective in rheumatoid arthritis (Burmester, McInnes et al. 2018).

The number existing biologic agents and future treatments available for adult patients with SpA is growing but only a small number of 'head to head' studies and therefore direct comparisons between agents are available so far. Meta-analyses have been published in an attempt to determine whether one group is superior to another (Deodhar, Chakravarty et al. 2020) but at present TNF inhibition remains the first choice of biologic DMARD in patients with adult SpA.

It has been extrapolated that drugs effective for the treatment of adult SpA will also be of benefit in ERA and juvenile onset psoriatic arthritis. Clinical trials of the IL17 inhibitor secukinumab and the JAK inhibitor baricitinib are underway but drugs not found to be effective in adult SpA such as tocilizumab, abatacept and rituximab have not been studied. A case series of five patients with ERA treated with Ustekinumab demonstrated an improvement in disease activity in all but one patient (Mannion, McAllister et al. 2016). It could be hypothesised that as enthesitis is an important early feature in SpA and is particularly prevalent in patients with ERA, IL23 blockade may be more effective in children and adolescents than in adults with SpA. Further clinical trials are needed to identify novel treatments, enhance treatment pathways and allow better treatment stratification to improve outcomes for patients with ERA.

1.2.10 Introduction Summary

This introduction has given a summary of the current knowledge of the clinical aspects and pathogenesis of adult SpA and ERA. A summary diagram of the existing knowledge in ERA is included below (figure 1.5). The similarities and differences between adult SpA and ERA have been discussed and areas where further research is needed, especially for ERA, have been highlighted. It is evident that research across all aspects of ERA is sparse compared to that of adult SpA. Further study of ERA is vital to understand whether research from adult SpA can be applied across the ages or whether there are important unique features of ERA which warrant separate trials of different therapeutic interventions in the future.

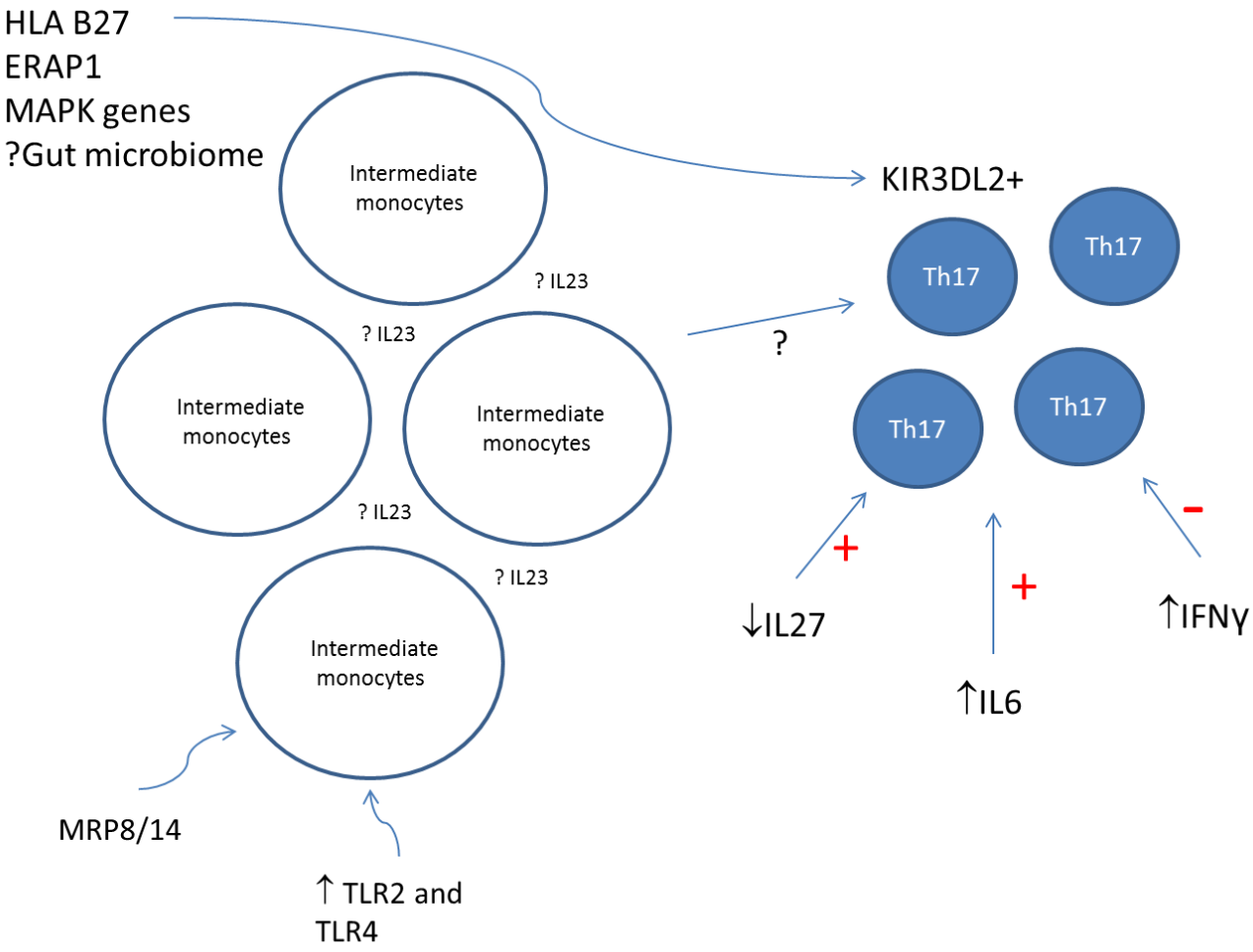


Figure 1.5 Diagram representing the existing knowledge on the pathogenesis of ERA

1.3 Thesis hypothesis and aims

1.3.1 Overarching thesis hypothesis

The pathogenesis of ERA is similar to that of adult SpA, demonstrated by IL23/IL17 pathway and intracellular stress responses from monocyte-derived macrophages (MDMs) from patients with ERA compared to adult patients with SpA.

1.3.2 Thesis aims

- To develop a bioassay for differentiating MDMs in vitro, to activate MDMs to produce pro-inflammatory cytokines and to induce the UPR

- To measure the production of IL23 and other pro-inflammatory cytokines from activated MDMs from patients with ERA compared to healthy controls, patients with adult SpA and an age-matched disease control group

- To identify any clinical features associated with differences in cytokine production from MDMs from patients with ERA

- To investigate the UPR in MDMs from patients with ERA, adult SpA and healthy controls and the effect of UPR induction on cytokine production in the in vitro bioassay

- To identify potential serum biomarkers in patients with ERA

- To undertake an initial investigation in to the potential effect of oestrogen on cytokine production from MDMs in the in vitro bioassay

CHAPTER 2: MATERIALS AND METHODS

Overview of Chapter 2

This chapter is divided into 3 parts. The first part (section 2.1) details the general materials and equipment used throughout this thesis and also specific materials and buffers used.

The second part (section 2.2) outlines the methods for the MDM activation bioassay developed for this project to allow the study of IL23 and other relevant cytokines and markers from MDMs. This includes PBMC and monocyte isolation, monocyte culture and macrophage activation by upregulation of HLA B, induction of TLR 4 signalling and induction of the UPR.

The final part of this chapter (sections 2.3-2.8) describes the methods for obtaining the outcome measures from the macrophage activation assay including the measurement of cytokines at the protein and RNA level, p38 MAPK signalling pathway activation and assessment of markers of the UPR. The methods for serum biomarker evaluation are also described.

2.1 Chemicals, general materials and equipment

2.1.1 Chemicals

All solid chemicals were dissolved in double-distilled (dd)H_2O, adjusted to the correct pH with 0.1M HCl or 0.1M NaOH and autoclaved or filter sterilised as required.

2.1.2 General materials and equipment

50 mL and 15 mL Falcon tubes (Cell-star, Greiner bio-one, Germany)

Micro-tubes 1.5 mL (390525, Biosigma, Italy)

Sepmate-50 tubes (15450, Stemcell, France)

Polystyrene round-bottom tubes (352054, BD bioscience, USA)

Cryotube vials (375418, Thermo Scientific, USA)

22µm filter (SLGP033RS, Millipore, USA)

Haemocytometer 0.0025mm2 (Marienfield, Germany)

Nanodrop spectrophotometer ND-1000 (Labtech International)

EasySep Magnet (18000, Stemcell, Canada)

Centrifuges: Sorvall Legend XTR (Thermo Scientific, USA)

1-14 K Refrigerated microfuge (Sigma, Germany)

Magnetic hand held plate washer (eBioscience, USA)

DNA Engine Opticon System (MJ Research, USA)

FACSVerse (BD Biosciences, USA)

Microplate reader (GENios, Tecan, Switzerland)

Bioplex 200 suspension array system (Bio-rad, USA)

2.1.3 Cell culture reagents

RPMI 1640 Glutamax (61870, Gibco, USA)

Heat inactivated foetal bovine serum (FBS) Performance Plus (10082, Gibco, USA)

Penicillin/Streptomycin (10000 units/mL/ 10µg/mL) (P4333, Sigma, USA)

Ficoll-Paque Plus (17144003, GE Healthcare, USA)

Tissue culture grade phosphate-buffered saline (PBS). Dulbecco's PBS (DPBS)/Modified without calcium or magnesium (SH30028, HyClone, GE Healthcare, USA)

EasySep Human CD14 Positive Selection Kit (18058, Stemcell, USA)

Hypure cell culture grade water – endotoxin free (SH30529, Hyclone, GE Healthcare USA)

Trypan blue (15250, Gibco, USA)

Dimethyl sulfoxide (DMSO) (D2650, Sigma, USA)

Macrophage colony stimulating factor (MCSF) (300-25, Peprotech, USA)

Granulocyte macrophage-colony stimulating factor (GMCSF) (130-093-862, Miltenyi Biotec, Germany)

Interferon gamma (IFNγ) (300-02, Peprotech, USA)

Lipopolysaccharide (LPS) (Salmonella Enterica) (L4641, Sigma, USA)

Tunicamycin (TM) (11089-65-9, Cayman Chemical, USA)

β-Oestradiol (E2758, Sigma, USA)

Phorbol 12-myristate 13-acetate (PMA) (SC3576, Santa Cruz Biotechnology, USA)

Anti-CD3 antibody (Clone UCHT1, MAB100, R&D Systems, USA)

Anti-CD28 antibody (Clone CD28.2, 555725, BD Biosciences, USA)

CellTiter 96 AQueous One Solution Cell Proliferation Assay (G3582, Promega, USA)

24 well plates (D7039, Nunclon, Sigma, USA)

RPMI 1640 Glutamax (Gibco) was supplemented with 1% penicillin/ streptomycin (P/S) (Sigma) and 10% heat inactivated FBS Performance Plus (Gibco).

A stock solution of ethylene diamine triacetic acid (EDTA) buffer 50 mL was prepared using PBS (tissue culture grade, GE Healthcare), 2% FBS (heat inactivated, performance plus, Gibco) and 1mM EDTA (adjusted to pH 7.4 and filter sterilised before use).

Adherent cells were removed from cell culture plates using a detachment buffer composed of 1 mM EDTA, 0.1% sodium azide in 50 mL PBS.

2.1.4 General buffers and solutions

PBS. A stock solution of 10x was prepared by dissolving 20 tablets (18912, Gibco, USA) in 1 L of ddH_2O. This was autoclaved and diluted 1:10 prior to use.

PBS/0.1% Tween. 1 mL 'Tween 20' (P1379, Sigma, USA) was added to 1 L of PBS.

2.1.5 Specific buffers and solutions

2.1.5.1 Protein extraction and quantification

Cell lysates were extracted using 50 µL of lysis buffer composed of the following: 50mM Tris-HCl pH 7.4, 150mM NaCl, 5mM EDTA pH 8, 1mM EGTA pH 8, 1% NP-40, 0.1% Sodium dodecyl sulphate (SDS), 0.5% Na-Deoxycholate, 1mM Sodium orthovanadate

A complete mini protease inhibitor cocktail tablet (11836153001, Roche, Germany) and Phostop Easypack tablet (04906845001, Roche, Germany) were added to 10 mL of the lysis buffer and aliquots of 500 µL were stored at -20°C and thawed only once.

Protein quantification was undertaken using a Bicinchoninic acid (BCA) assay (23225, Thermo Scientific, USA)

2.1.5.2. Western blotting equipment and reagents

3-(N-morpholino)propanesulfonic acid (MOPS) SDS running buffer (20x) (B000102, Invitrogen, USA)

Bolt (Novex) transfer buffer (20x) (BT00061, Life technologies, USA)

Bolt 4-12% Bis-Tris Plus 15 well Gels (NW04125, Thermo Fisher Scientific, UK)

Rainbow marker (RPN800E, Amersham, GE Healthcare, UK)

NuPage LDS Sample buffer 4x (NP0007, Thermo Fisher Scientific, UK)

NuPage sample reducing agent 10x (NP0009, Thermo Fisher Scientific, UK)

Hybond nitrocellulose blotting membrane (0.45µm) (RPN303D, Amersham, GE Healthcare, UK)

Skimmed milk powder (70166, Sigma, USA)

Primary Antibodies:

Rabbit anti-phospho-p38 MAPK antibody 1:1000 (9211, Cell signalling, USA)

Rabbit anti-P38 MAPK antibody 1:1000 (9212, Cell signalling, USA)

Secondary Antibody

Goat anti-rabbit IgG horse radish peroxidase (HRP) (SC-2004, Santa Cruz Biotechnology, USA)

Enhanced chemiluminescent (ECL) prime Western blotting detection reagent (RPN2232, Amersham, GE Healthcare, UK)

2.1.5.3 RNA extraction, quantification, detection and characterisation

TRIzol reagent (15596018, Life Technologies, USA)

Glycogen (AM9510, Invitrogen, USA)

PCR tubes 0.2 mL (A1402-3700, Star Labs, USA)

RNAase OUT (786-71, G-biosciences, USA)

PCR multiplate 96 well (MLL9651, Bio-rad, UK)

Optical flat 8-cap strips (S0803, Bio-rad, USA)

Quantitect reverse transcription kit (205313, Qiagen, USA)

SYBR Green Jumpstart Taq ReadyMix (S4438, Sigma, USA)

Quantitect primer assay (GAPDH) (QT01192646, Qiagen, USA)

Primers for quantitative polymerase chain reaction (qPCR) were stored in aliquots dissolved in Tris-EDTA (TE) buffer. A stock solution of 50 mL was prepared by adding 1M Tris pH 8 and 0.5M EDTA pH 8 to 49.6 mL diethylpyrocarbonate (DEPC) treated water.

2.1.5.4 DNA extraction, quantification, detection and characterisation

Purelink Genomic DNA mini kit (K1820-01, Invitrogen, USA)

Taq DNA Polymerase kit (201203, Qiagen, USA)

Agarose (A2576, Sigma, USA)

Tris Acetate-EDTA buffer (T6025, Sigma, USA)

Gel green (BT41005, Biotium, USA)

Blue/orange loading dye (G1881, Promega, USA)

TrackIt 50bp DNA ladder (10488043, Invitrogen, USA)

2.2. Cell culture

2.2.1 PBMC isolation

15 mL of whole blood was collected in sodium-heparin tubes (367878, BD, USA) and then diluted 1:2 with RPMI (supplemented with 1% P/S). 15 mL of Ficoll-Paque Plus was added to a sepmate-50 tube and the diluted blood was layered on top. The tube was centrifuged at 1200 g for 10 minutes. The top layer was transferred to a new Falcon tube, topped up to 50 mL with RPMI and centrifuged at 1800 rpm for 10 minutes. The supernatant was discarded and the cells were re-suspended in 25 mL RPMI (supplemented with 10% FBS). The tube was spun at 1800 rpm for 10 minutes and this wash was repeated once more. The cells were counted using a haemocytometer with an aliquot of 10 µL cell suspension and 10 µL trypan blue. The following formula was used to estimate cell number:

Average number of cells from 2 large squares	X	2 (dilution factor)	X	Volume of cell suspension	=	Total number of cells x 10^4

2.2.2 Monocyte isolation and differentiation

PBMCs were re-suspended in EDTA buffer (PBS, 2% FBS and 1mM EDTA) at a concentration of 1x10^8 per mL in a round-bottom polystyrene 5 mL tube. 100 µL per mL of EasySep positive selection cocktail was added and the cell suspension was incubated at room temperature for 15 minutes. 50 µL per mL of pre-mixed EasySep magnetic nanoparticles were added and the suspension was incubated at room temperature for a further 10 minutes. The cell suspension was then made up to a volume of 2.5 mL with EDTA buffer and placed inside the EasySep magnet for 5 minutes. The supernatant was then poured off and 2.5 mL EDTA buffer added to the tube which was then placed back inside the magnet for 5 minutes, after which the supernatant was poured off. This process was repeated once more and then 2 mL of RPMI (supplemented with 10% FBS) was added to the tube. Positively selected cells were then counted (by the method described above) and cultured at 0.5 x 10^6 per mL in 24 well plates (500 µL per well). MCSF 100 ng/mL or GMCSF 50 ng/mL was added to each well. Cells were cultured for 7 days (37°C, 5% CO_2).

2.2.3 Monocyte-derived macrophage activation assay

2.2.3.1 Upregulation of HLA B

On day 6 of the culture, cells were checked to ensure they had clearly differentiated in to MDMs and 4000 iu/mL of IFNγ was added to each well to upregulate HLA B.

2.2.3.2 Induction of TLR 4 signalling and the unfolded protein response

On day 7, cell culture media was removed and adherent cells were washed with PBS. 500 µL RPMI (supplemented with 1% P/S) (control cells) or RPMI (+1% P/S) containing LPS 50 ng/mL alone or LPS 50 ng/mL and/or TM 5µg/mL was added to each well for 4 or 24 hours (37°C, 5% CO_2). LPS was used to induce TLR 4 signalling and TM to induce the UPR. A summary of conditions can be found in table 2.1 below. Following this, cell culture supernatants were aliquoted and stored at -80°C until needed. Cell lysates and cells in TRIzol for RNA were also stored at -80°C until needed.

	Control	+LPS	+LPS +TM	+TM
Interferon γ 4000iu/mL	+	+	+	+
Lipopolysaccharide 50ng/mL	-	+	+	-
Tunicamycin 5µg/mL	-	-	+	+

Table 2.1. Summary of the conditions for the MDM activation assay

2.2.3.3 Oestrogen

To investigate the effect of adding oestrogen to MDMs stimulated under the above conditions, 10nM β-oestradiol (Sigma) was also added per well on day 7 in selected patients and healthy controls.

2.2.4 Flow cytometry for monocyte yield, purity and monocyte derived macrophage phenotype

2.2.4.1 Monocyte yield and purity

Monocytes and PBMCs were isolated as above, re-suspended in PBS at a concentration of 5 x 10^6/mL and added to a 96 well microplate (100 µL per well). The plate was spun at 500 g for 5 minutes and supernatants discarded. Cells were then re-suspended in 100 µL 10% goat serum (Invitrogen) and incubated for 15 minutes on ice. 100 µL of PBS was added to each well and the plate was centrifuged again at 500 g for 5 minutes, after which the supernatants were discarded.

For the live/dead wells, ethanol was added to 50 µL cells (PBMCs and isolated monocytes) and the plate was centrifuged at 500 g for 5 minutes. Cells were re-suspended in 100 µL PBS

and 100 µL of live cells were added. 100 µL of this mix was added to 2 wells of the microplate. To stain the cells, antibodies were added under the following conditions:

All cells (PBMCs and isolated monocytes) (2 wells): all stains: Live/ dead, HLA DR (BV711), CD14 (FITC), CD86 (PB)

Unstained (2 wells)

Isolated monocytes (2 wells): all stains

Isotype control: PB + Live/ dead, HLA DR (BV711), CD14 (FITC)

Isotype control: FITC + Live/ dead, HLA DR (BV711), CD86 (PB)

Isotype control: BV711 + Live/ dead, CD14 (FITC), CD86 (PB)

Following the addition of the antibodies, the plate was incubated for 30 minutes on ice in the dark. 100 µL of PBS was added then the plate was centrifuged at 500 g for 5 minutes and supernatants discarded. 50 µL of cell fixation and permeabilisation buffer was added to each well and the plate was incubated on ice for 30 minutes. Next the plate was re-spun at 500 g for 5 minutes and the supernatant discarded. Cells were re-suspended in 250 µL of PBS and transferred to FACS tubes. For compensation, beads were used as single controls for BV711, FITC and PB. Samples were run on a FACSVerse (BD biosciences) and analysed using FloJo software (TreeStar).

2.2.4.2 Monocyte derived macrophage phenotype

A further experiment was carried out to assess MDM phenotype after 6 days differentiation. Cells were kept on ice and washed with 1 mL ice cold PBS twice. Detachment buffer (500 µL) was then added to each well and pipetted up and down gently for 15 minutes to detach the cells from the plate without disrupting the cell surface. Next the cells were spun at 1500 g for 5 minutes at 4°C and re-suspended in 100 µL PBS as per the methods above under the following conditions:

All cells (2 wells): all stains: Live/dead, CD14 (FITC) and CD163 (PE)

Unstained (2 wells)

Isotype control: FITC + Live/ dead and CD163 (PE)

Isotype control: PE + live /dead and CD14 (FITC)

Once again, beads were used as single controls for compensation and the number of cells positive for CD14 and CD163 was measured on a FACSVerse and results analysed using FloJo

software. A summary of antibodies used in all flow cytometry experiments can be found in table 2.2 below.

Antibody	Fluorochrome	Concentration	Company
CD14	FITC	1:100	325604, BioLegend, USA
CD86	PB (pacific blue)	1:100	305423 BioLegend, USA
HLA DR	BV711	1:200	307643 BioLegend, USA
CD163	PE	1:10	333606, BioLegend, USA
Live/ dead	APC-Cy7		L34971, Invitrogen, USA
Isotype controls were used at the same concentration as the corresponding antibody			

Table 2.2. Antibodies used in flow cytometry experiments

A further evaluation of MDM phenotype was undertaken using qPCR, the methods for which are described below.

2.2.5 Cell viability assay

To ensure that the stimulation conditions of the MDM activation assay did not adversely affect cell numbers, a cell viability assay was performed. 100 µL of CellTiter 96 AQueous One Solution reagent (Promega) was pipetted into each well (still containing media) of MDMs following stimulation for 4 or 24 hours under the 4 conditions described above (table 2.1). The plate was then incubated for 1 hour (37°C, 5% CO_2) when a colour change was seen. Next, 100 µL of supernatant was transferred to a 96 well plate in triplicate for each of the conditions in the MDM activation assay and read on a plate reader at 490nm absorbance.

2.2.6 Synovial fluid mononuclear cell isolation

SF was collected in sterile tubes containing 35 µL of preservative free heparin (monoparin 1000iu/mL) and then diluted 1:1 with RPMI (supplemented with 1% P/S and 1µl heparin per mL). Hyaluronidase (Sigma H4272) was added to a final concentration of 10 units/mL and the SF was then incubated for 30-40 minutes at 37 °C. The SF was then layered on to 15 mL of Ficoll-Paque Plus and centrifuged at 400 g for 10 minutes with no brake. The top layer was transferred to a new Falcon tube, topped up to 50 mL with RPMI and washed twice and counted as per the method for PBMCs. The SFMCs were frozen in sterile medium (90 % FCS plus 10% DMSO) until required.

2.2.7 Positive control for cytokine and RNA detection, quantification and characterisation

In order to test inter-plate variability for Luminex and enzyme-linked immunosorbent assay (ELISA) experiments and to check the reliability of primers for PCR, a positive control was created with cells expressing the cytokines of interest for the MDM activation assay.

A solution of anti-CD3 and anti-CD28 (at 1 µg/mL each) was made in 2 mL of PBS. 1 mL each was added to 2 wells of a 6 well plate which was then incubated for 3 hours (37°C, 5% CO_2) after which time the PBS was removed. PBMCs isolated as above were re-suspended at a concentration of $2x10^6$/mL in RPMI (supplemented with 10% FBS) and 1mL was added to each well pre-coated with anti-CD3 and anti-CD28 plus 2 extra wells. LPS (at 1 µg/mL) and PMA (at 7 µg/mL) were added to these wells and the plate was incubated for 48 hours (37°C, 5% CO_2). Cell culture supernatants were pooled then aliquoted and stored at -80°C. Cells were pooled and stored in TRIzol at -80°C.

2.3. Cytokine detection and quantification

2.3.1 ELISAs for the detection of IL23 and TNFα

IL23 and TNFα cytokine levels were measured using Duoset ELISA kits (R&D Systems) as per manufacturer's instructions. Plates were coated with 100 µL capture antibody per well and incubated overnight at room temperature. Next, each well was washed with 400 µL wash buffer (x3) and blocked with 300 µL reagent diluent for 1 hour. After a further wash, 100 µL of standard or diluted cell culture supernatants were added in duplicate to the plate and incubated at room temperature for 2 hours. Following optimisation experiments, supernatants were diluted 1:100 for the TNFα ELISA and 1:25 for the IL23 ELISA. Next, the plate was washed again and 100 µL of detection antibody was added to the plate which was again incubated for 2 hours at room temperature. The plate was then washed and 100 µL of streptavidin-HRP was added. After 20 minutes and a further wash, 100 µL of substrate solution was added for 20 minutes followed by 50 µL of stop solution. Optical density was read immediately at 450nm and cytokine concentration (pg/mL) was determined by comparison with the standard curve. After analysis, samples with results outside the range of detection for the assay were repeated with adjustment in the dilution factor as necessary.

2.3.2 Proteome profile array

Proteome profiler (human cytokine array panel A) (R&D) was used as per manufacturer's instructions to measure 36 cytokines and chemokines in cell culture supernatants from MDMs to determine which cytokines and chemokines to study further via luminex.

Reagents and samples were prepared as per the protocol. Each membrane was blocked for 1 hour in a 4-well multi-dish on a rocking platform shaker at room temperature. 15 µL of detection antibody cocktail was added to the prepared samples and also incubated for 1 hour at room temperature. Blocking buffer was then aspirated from the 4-well multi-dish and the sample/ antibody mixture was added. The membranes were then incubated overnight on a rocking platform at 4°C. Next, the membranes were washed (3 x 10 minutes) and then placed back in to the 4-well multi-dish with Streptavidin-HRP. The membranes were incubated at room temperature for 30 minutes on a rocking platform shaker. After a

further wash, the membranes were placed on a plastic sheet and 1 mL of Chemi Reagent Mix was pipetted on to each membrane and spread evenly to all the corners. After 1 minute, the membranes were dried, covered with a plastic sheet and placed in an autoradiography film cassette. The membranes were then exposed to x-ray film for multiple exposure times from 30 seconds to 10 minutes.

The positive signals seen on developed film were identified using the template provided. The pixel density of each spot was analysed using QuantityOne software (Bio-rad, USA) and the average density of each pair of duplicate spots representing each cytokine was calculated and subtracted from the background signal. One blot was used for each condition of the MDM activation assay, making a total of 4 blots per patient / healthy control. To enable comparison between blots, an adjustment was made using the reference spots, using the control (untreated) condition as baseline. To enable comparison between the patients and healthy control, a further adjustment was made, using the average of the reference spots for all the conditions, using the healthy control as baseline. Cytokine level was expressed as arbitrary units of pixel density.

2.3.3 Luminex assay for the detection of other relevant cytokines and chemokines

Magnetic luminex screening assay (R&D) kits were used to measure levels of IL1, IL6, IFNγ, IL12p70, IL27, GMCSF, CCL4 and CCL5 (initially also IL17, IL2 and IL10) as per manufacturer's instructions. All reagents and standards were prepared according to the protocol. Microparticles were diluted; re-suspended and 50 µL was added to each well of the microplate. Next, 50 µL of standard or sample (cell culture supernatant from MDMs diluted 1:2) was added. The plate was sealed and incubated at room temperature for 2 hours on a horizontal plate shaker (800 rpm). The plate was then washed using a magnetic hand-held plate washer and 50 µL of Biotin antibody cocktail was added to each well. The plate was sealed again and incubated for 1 hour at room temperature on the horizontal plate shaker as above. Following a further wash, 50 µL of diluted Streptavidin-PE was added to each well; the plate was sealed and returned to the plate shaker for 30 minutes. Next, the plate was washed once more and the microparticles re-suspended in 100 µL of wash buffer. The plate was read on a Bio-plex 200 analyser (Bio-rad) with the following settings: 50 events/bead, minimum events: 0, flow rate: fast, sample size 50 µL, doublet discriminator gates: 8000-16500 and mean fluorescence intensity (MFI) was collected. Cytokine concentration (pg/mL) was determined by comparison with the standard curve.

2.4 Protein extraction, quantification and characterisation to analyse p38 MAPK phosphorylation

2.4.1 Protein extraction

Cell lysates from MDMs were extracted by adding 50 µL of ice cold lysis buffer to each well of the 24 well tissue culture plate which was then placed on ice. After 10 minutes, the cells

were scraped from the plate and placed in to micro-tubes on ice. Lysates were frozen at -80°C until needed. Cell lysates were thawed on ice and spun at 13 000 g for 5 minutes at 4°C so that the cell debris formed a pellet. The supernatant was then transferred to a clean micro-tube ready for use.

2.4.2. Determination of protein concentration

Determination of protein concentration was performed by the BCA assay. Protein standards were prepared by diluting stock BSA at 2mg/mL to give eight standard concentrations from 2000 µg/mL to 25 µg/mL. Working reagent was prepared by adding 50 parts of BCA reagent A to 1 part BCA reagent B. 5 µL of either standard albumin or sample was added to wells in a 96 well plate followed by 95 µL of working reagent (ratio 1:20). The plate was incubated for 30 minutes at 37°C and then read at 560nm on a microplate reader. Protein concentrations were determined by comparison with the albumin standard curve.

2.4.3. Western blotting

Samples were prepared by adding sample buffer (Thermo Fisher Scientific), sample reducing agent (Thermo Fisher Scientific) and the appropriate volume of ddH_2O to bring the protein concentration to 5 µg before boiling for 5 minutes. A protein marker (Amersham) was loaded, followed by the samples on to a 4-12% Bis-Tris 15 well gel (Thermo Fisher Scientific) which was electrophoresed at 165 volts in running buffer (Invitrogen) until the loading dye reached the bottom.

2.4.4. Transfer of protein to nitrocellulose

Sponges, filter paper and nitrocellulose blotting membrane (Amersham) were all pre-soaked in transfer buffer (prepared by diluting 20x Bolt Transfer buffer (Life technologies) and adding 20% methanol). The gel containing separated proteins was placed on filter paper on 2 layers of sponge and covered with membrane (cut to size) and a further 2 layers of filter paper and sponge. The proteins were then transferred to the membrane at 10 volts for 1 hour.

2.4.5. Immunodetection of proteins on western blots

To block non-specific protein binding, the membrane was incubated with skimmed milk (4%) for 1 hour on a rocking platform at room temperature. The membrane was then placed in primary antibody (1:1000 in skimmed milk 4%) and incubated overnight on a roller at 4°C. The membrane was then washed (3 x 10 minutes in PBS/0.1% Tween), placed in secondary antibody conjugated to HRP (1:1000 in skimmed milk 4%) and incubated for 1 hour at room temperature on a roller. Next the membrane was washed (as above) and excess PBST removed by blotting with a tissue. The membrane was placed on a plastic sheet and ECL (Amersham) was pipetted on, ensuring that the whole membrane was covered. After 5 minutes the membrane was dried, covered with a plastic sheet and placed in an autoradiography film cassette. The membrane was then exposed to x-ray film for multiple exposure times from 10 seconds to 2 minutes.

Once the phosphorylated protein expression had been detected, the membrane was washed in 0.2M sodium hydroxide (2 x 5 minutes) to remove the antibodies to the phosphorylated protein. The membrane was then re-blocked and then re-probed for the total protein as per the protocol above except that the incubation with primary antibody was for 1 hour at room temperature instead of overnight.

The pixel density of the protein bands was analysed using QuantityOne software (Bio-rad, USA) and phosphorylated protein expressed as a ratio of total protein. For each patient or healthy control the ratio of phosphorylated protein for each condition of the MDM activation assay was then calculated as a fold change of the control (untreated cells).

2.5 RNA extraction, detection, quantification and characterisation

2.5.1 RNA extraction

RNA was extracted from MDMs for each patient/ healthy control for each of the four conditions of the MDM activation assay as described above (table 2.1). First, 400 µL of TRIzol reagent (Life technologies) was added to each well of the 24 well plate and incubated for 5 minutes at room temperature. The cells in TRIzol were then transferred in to micro-tubes and stored at -80°C until needed. RNA was also extracted from SFMCs which were thawed quickly in a water bath and centrifuged at 1800 rpm for 10 minutes to form a pellet. The supernatant was discarded and then cells were transferred to micro-tubes and washed in RPMI (supplemented with 10% P/S) and centrifuged again. 400 µL TRIzol was added to the SFMCs which were stored at -80°C until needed or used straight away.

Samples were thawed quickly in a water bath, and then left at room temperature for 5 minutes. In a fume hood, 80 µL of chloroform was added to each tube, which were then inverted and mixed for 15 seconds and incubated at room temperature for 3 minutes. Samples were then centrifuged at 12 000 g for 15 minutes at 4°C causing the sample to separate in to a clear aqueous phase (containing RNA), an interphase and a phenol layer at the bottom. The aqueous phase was removed in to a clean micro-tube and 1 µL of glycogen and 200 µL of isopropanol was added. The tubes were inverted and mixed then incubated at room temperature for 10 minutes. Next, the samples were centrifuged at 12 000 g for 10 minutes at 4°C so that pellets of RNA were visible. The supernatant was removed and 400 µL of 75% ethanol was added to each tube and mixed. The sample was centrifuged again at 7500 g for 5 minutes at 4°C and the supernatant discarded. A master mix containing 50 µL DEPC H_2O, 5 µL 3M sodium acetate (pH 5.2) and 0.5 µL glycogen for each sample was made and 55.5 µL was added to each tube, along with 150 µL of ethanol. The tubes were mixed, then placed at -80°C for 1 hour after which they were centrifuged at 12 000 g for 30 minutes at 4°C. The supernatant was discarded once again and another ethanol wash was performed by adding 200 µL of 75% ethanol and centrifuging at 7500 g for 5 minutes at 4°C. The ethanol was then discarded and the tubes were allowed to air dry for 15 minutes. The RNA

was then dissolved in 20 µL of DEPC H_2O and concentration determined using a Nanodrop spectrophotometer.

2.5.2 cDNA synthesis

To transcribe the RNA in to complementary DNA (cDNA), a Quantiscript reverse transcription kit (Qiagen) was used. RNA and all components of the kit were kept on ice. RNA at a concentration of 1 µg was added to the appropriate volume of DEPC H_2O to bring the volume to 12 µL in a sterile micro-tube. 2 µL of gDNA wipeout buffer to eliminate genomic DNA contamination was added to each sample which were then heated to 42°C for 2 minutes using the GeneAmp PCR System 9700 (Applied Biosystems).

For the second stage, a master mix containing 4 µL of Quantiscript reverse transcriptase (RT) buffer, 1 µL of RT primer mix and 1 µL of RT enzyme per sample was prepared. 6 µL of the master mix was added to each sample bringing the total volume to 20 µL. The sample was then heated to 42°C for 15 minutes and the reaction was inactivated by heating at 95°C for 3 minutes. cDNA was then stored at -20°C until needed.

2.5.3 Quantitative polymerase chain reaction (qPCR)

To detect and quantify gene expression, qPCR was performed as follows: all reagents and cDNA were thawed on ice protected from direct light. cDNA was diluted 1:5 with DEPC H_2O (to ensure more accurate pipetting by using a larger volume), vortexed and spun. Primers were diluted in TE buffer to a working concentration of 10mM. A master mix consisting of: 12.5 µL SYBR green master mix, 1 µL each of forward (Fwd) and reverse (Rev) primer and 5.5 µL nuclease-free water was prepared and kept on ice until needed. 5 µL of cDNA was added to each well of a PCR microplate. 20 µL of master mix was added to each well. The plate was covered and spun to force the contents to the bottom of each well.

Samples were run on a DNA Engine Opticon System (MJ Research). A temperature gradient curve from 55-72°C was performed to determine the optimal annealing temperature for each primer. The following conditions were used: initial denaturing at 95°C for 10 minutes, 40 two-step cycles of 95°C for 15 seconds and 60 seconds at the annealing temperature which varied depending on the primer used (see table 2.3 below) after which a melt curve was performed from 65°C to 95°C, read every 0.3 seconds and held for 1 second between reads.

The cycle threshold (Ct) of the gene in question was normalised against a housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or in the case of spiced XBP1, unspliced XBP1 to give the ΔCt. ΔΔCt was calculated by subtracting ΔCt for the control (untreated) samples from ΔCt for the other conditions. $\Delta\Delta Ct^2$ was then calculated to give a fold change in the expression of the relevant gene compared to the control sample.

Gene	Forward primer	Reverse Primer	Anealing temperature (°C)
XBP1 spliced	CTGAGTCCGCAGCAGGTG	CAGAATCCATGGGGAGATGTT	60
XBP1 unspliced	CACTCAGACTACGTGCACCTCT	CAGAATCCATGGGGAGATGTT	60
CHOP	CTAGTGCCAATGATGTGA	ATATACAAGCTGAGACCT T	58
BiP	GTGGTGACCCGTCTGTG	TTGGTTGCTTGGCGTTGG	65
TNFα	CTTCTCCTTCCTGATCGTGG	GCTGGTTATCTCTCAGCTCCA	58
IL1	AAATACCTGTGGCCTTGGGC	TTTGGGATCTACACTCTCCAGCT	60
IL6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	58
IL17	AATCTCCACCGCAATGAGGA	ACGTTCCCATCAGCGTTGA	60
HLA B	GTCCACCGTCCCCATCG	ACGCAGCCTGAGAGTAGC	60
IIL12/23p40	CGGTCATCTGCCGCAAA	TGCCCATTCGCTCCAAGA	60
IL12p35	CTCCTGGACCACCTCAGTTTG	GGTGAAGGCATGGGAACATT	60
IL23p19	TGCAAAGGATCCACCAGGGTCTGA	TGAGTGCCATCCTTGAGCTGCTGC	65
STAT1	TGCTTGGATCAGCTGCAGAA	CCGAACTTGCTGCAGACTCT	60
CD163	GAGCAGCACATGGGAGATTG	TTGCACGAGGACAGTGTTTG	60
CD206	AATGAACGTGTGTGGATCGC	ATCAGCAGCCCAGTTAGTGT	60
CD80	CCATCCTGGGCCATTACCTT	CTCTGCATCTTGGGGCAAAG	60
CD200R	CCATCGTGGGATTCATTTGGT	GGCTGCATTTCATCCTCCTC	60
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA	60

Table 2.3 Primer sequences for RNA qPCR

2.6 DNA extraction, quantification, detection and characterisation for HLA B27 status

2.6.1 DNA extraction and quantification

PBMCs were thawed in a water bath, diluted in RPMI (supplemented with 1% P/S) and counted. Cells were then spun at 250 g for 5 minutes and the pelleted cells were re-suspended in PBS at a concentration of 5 x 10^6 in 200 µL. DNA was extracted using a PureLink genomic DNA kit (Invitrogen, USA). To obtain cell lysates, 20 µL of proteinase K was added to the sample followed by 20 µL of RNAse A. The sample was then mixed and briefly vortexed before being incubated at room temperature for 2 minutes. Next, 200 µL of PureLink genomic lysis buffer was added and the sample was mixed well and vortexed to obtain a homogeneous solution. The sample was then heated for 10 minutes in a water bath at 55°C to promote protein digestion. Following this, 200 µL 100% ethanol was added to each sample which was again mixed well and vortexed to obtain a homogeneous solution.

DNA was extracted using spin columns. Cell lysates in lysis buffer and ethanol were added to the column inside a collection tube and spun at 10 000 g for 1 minute. The collection tube was then discarded and replaced with a clean tube and 500 µL of wash buffer was added. The column was spun again at 10 000 g for 1 minute and the collection tube discarded and replaced. A further wash was performed with 500 µL of wash buffer and the column was spun at 14 000 g for 3 minutes. The collection tube was again discarded and replaced with a 1.5 mL microtube. DNA was eluted from the column by adding 25 µL of elution buffer, incubating at room temperature for 1 minute and then spinning the column at 14 000 g for 1 minute.

DNA concentration was quantified using a Nanodrop spectrophotometer.

2.6.2 DNA detection and characterisation

DNA was amplified using a Taq DNA polymerase kit (Qiagen). DNA and all components of the kit were thawed and kept on ice. Primers for HLA B27 and β-globin were diluted in TE buffer to a working concentration of 10mM (sequences are shown below in table 2.4). β-globin was used as an amplification control. DNA (150ng) was diluted with the appropriate volume of RNAse free H_2O in a sterile microtube. A master mix containing 5 µL of 10x PCR buffer, 1 µL of deoxyribonucleotide triphosphates (dNTPs), 2.5 µL each of HLA B27 and β-globin forward and reverse primers and 0.25 µL Taq polymerase for each sample was added to the DNA and H_2O making a total reaction volume of 50 µL. Samples were run on a GeneAmp PCR System 9700 (Applied Biosystems) using the following programme: initial denaturation for 3 minutes at 95°C followed by 3-step cycling consisting of denaturation for 35 seconds at 95°C, annealing for 45 seconds at 57°C and an extension of 55 seconds at 72° for 30 cycles and final extension of 8 minutes at 72°C. DNA was stored at -20°C until needed.

Gene	Forward primer	Reverse primer
HLA B27	GGGTCTCACACCCTCCAGAAT	CGGCGGTCCAGGAGCT
β-globin	CAACTTCATCCACGTTCACC	GAAGAGCCAAGGACAGGTAC

Table 2.4 Primer sequences for DNA PCR

To confirm the presence or absence of PCR product, gel electrophoresis was performed through a 2% agarose gel. To make the gel, 2 g of agarose (Sigma) was dissolved in 100 mL of Tris-Acetate EDTA (TAE) buffer and heated in a microwave for 2 minutes. Gel green (10 µL) was then added to the agarose which was poured in to a casting tray with a 15 well comb in situ, covered in foil and left to set at 4°C. Once set, the gel was placed in to the electrophoresis tank and covered with TAE buffer. Samples were prepared by mixing 20 µL of amplified DNA with 4 µL loading buffer (6x) (Promega) and were loaded on to the gel with a DNA marker (Invitrogen) and a positive control for HLA B27. The gel was electrophoresed at 160V for 30 minutes and DNA bands were visualised using a light box.

2.7 Biomarker detection

2.7.1 CRP ELISA

For those samples in the MDM activation assay where a CRP had not been performed in the hospital laboratory (for example healthy volunteer samples), CRP was measured to ensure no co-existing infection or condition was present which might influence the assay results. CRP was measured by DuoSet kit (R&D). Following optimisation experiments to calibrate results with the hospital laboratory, serum was diluted 1:10000 for the ELISA which was carried out as per the methods above for measuring levels of TNFα and IL23.

2.7.2 Dkk1 and MMP3 ELISAs

Quantikine ELISA kits (R&D) were used to measure Dkk1 and MMP3. Serum samples were diluted 1:8 for Dkk1 and 1:10 for MMP3. First, 100 µL of assay diluent was added to each well of the plate, followed by 100 µL of standard or diluted sample in duplicate. The plate was then incubated for 2 hours at room temperature on a horizontal plate shaker at 500 rpm. Next, the plate was washed (4 x 400 µL per well) and Dkk1 or MMP3 conjugate (200 µL) was added to each well. The plate was then incubated again for 2 hours at room temperate on a plate shaker. After a further wash, 200 µL of substrate solution was added and the plate was incubated for 30 minutes in the dark on the bench. The reaction was then stopped with 50 µL of stop solution and the plate was read immediately using a microplate reader at 450 nm wavelength.

2.7.3 Calprotectin (MRP 8/14) ELISA

To ensure reliability of the MRP 8/14 assay, serum samples were analysed by Professor Johannes Roth's laboratory at the University of Munster using a validated ELISA protocol previously used to analyse samples from patients with JIA (Frosch, Strey et al. 2000).

2.8 Statistical Analysis

Optimisation experiments were performed at least three times, independently and results shown as median with interquartile range. Outcome data was checked for normality by plotting histograms. In general, data was found not to be normally distributed and therefore non-parametric tests were used for analysis: Mann-Whitney U test for direct comparison between two groups and Kruskal-Wallis for comparing several groups. Statistical analysis was performed using SPSS (IBM Corp., Armonk, NY, USA) and GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

CHAPTER 3: ACTIVATION OF MONOCYTE-DERIVED MACROPHAGES WITH LIPOPOLYSACCHARIDE AND INDUCTION OF THE UNFOLDED PROTEIN RESPONSE LEADS TO ENHANCED PHOSPHORYLATION OF P38 MAP KINASE AND PRODUCTION OF PRO-INFLAMMATORY CYTOKINES

Overview of Chapter 3

This chapter describes the process undertaken to optimise an assay for differentiating MDMs and inducing the production of pro-inflammatory cytokines and the UPR within these cells.

The first part of this chapter focuses on experiments that analyse monocyte purity and yield and also to define the optimal method for MDM differentiation by analysing macrophage phenotype.

The second part of the chapter details the optimisation experiments undertaken to induce pro-inflammatory cytokine production with LPS and the UPR with TM.

Finally, having defined the protocol for the MDM activation assay, preliminary experiments are described demonstrating enhanced phosphorylation of p38 MAPK in a subgroup of patients and detailing the final outputs to be measured from the assay in future experiments. A summary of the demographics and clinical features of the patients and healthy controls recruited for this project is included.

Hypothesis for chapter 3

Enhanced p38 MAPK phosphorylation and pro-inflammatory cytokine production from MDMs will occur with LPS stimulation and UPR induction.

3.1 Differentiation of monocyte-derived macrophages

3.1.1 Introduction

MDMs were chosen as the cell of interest in this thesis because, along with T cells, macrophages are the most frequent cell found at sites of early active sacroiliitis in adult SpA (Bollow, Fischer et al. 2000). It is recognised that macrophages exhibit great heterogeneity depending on their environment and may exhibit an inflammatory or suppressor phenotype. These often coexist and are dependent on the activating and inhibitory signals of the surrounding tissue (Martinez and Gordon 2014). Tissue macrophages are difficult to obtain from patients, difficult to isolate from tissue and do not proliferate in culture, therefore MDMs are a useful surrogate (Eligini, Crisci et al. 2013). MCSF and GMCSF both promote the development of MDMs in vitro but induce different phenotypic populations (Lacey, Achuthan et al. 2012) with MCSF promoting a suppressor phenotype and GMCSF, a more inflammatory phenotype. In view of this, preliminary experiments were carried out to first ensure high yield and purity of isolated monocytes and then to decide on the most appropriate growth factor for MDM differentiation.

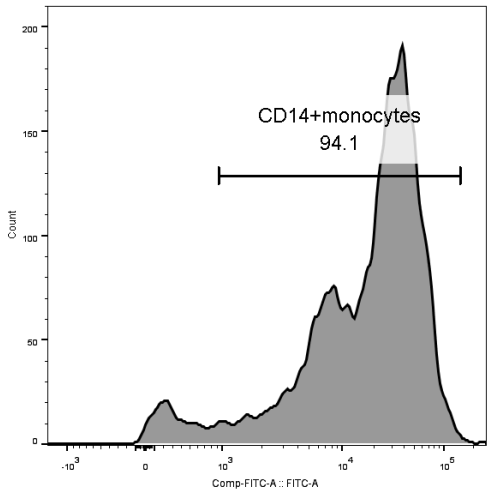
The study of other cells types, such as dendritic cells, which also play a major role in the pathogenesis of SpA, was considered. However, for the purposes of this thesis, experiments were focussed on one cell type. The study of dendritic cells is planned for future work.

3.1.2 Monocyte purity and yield

Firstly, an experiment was carried out to assess purity and yield of isolated monocytes via the methods described in section 2.2.4. From the same healthy volunteer, PBMCs and monocytes isolated as described in the methods (sections 2.2.1 and 2.2.2), were stained for CD14, CD16, CD86 and HLA DR.

Monocyte purity (defined as CD14 positive cells) using positive selection to isolate monocytes was 89.3%. The yield of total monocytes (in comparison to number of monocytes found in whole PBMCs) was 94.1% (figure 3.1 A). In addition, isolated monocytes were 58.8% HLA DR positive and 18.7% CD 86 positive suggesting some activation of the cells using this method of isolation. The majority of isolated monocytes were CD16 negative (90.8%).

A



B

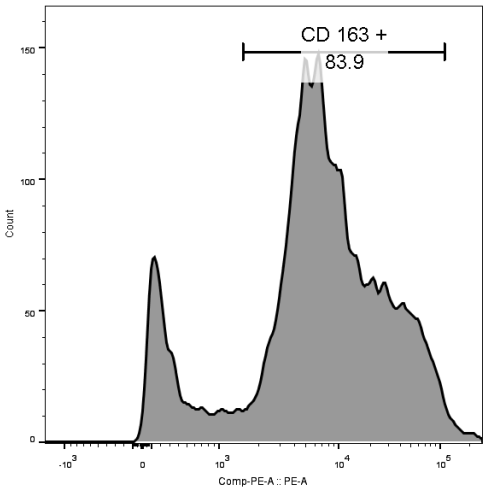


Figure 3.1. Flow cytometry on monocytes isolated with CD14 positive selection kit and differentiated monocyte-derived macrophages. Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) using a commercial CD14 positive selection kit ((Miltenyi Biotec, Germany.) Flow cytometry demonstrated 94.1% monocyte purity as determined by CD14+ marker positivity (A). Monocyte derived macrophages were generated in vitro by incubating isolated monocytes with macrophage colony stimulating factor (M-CSF) for 6 days. Flow cytometry demonstrated yields of 83.9% CD163+, a highly specific macrophage marker.

3.1.3 Monocyte isolation via negative selection

In view of the evidence that isolation of monocytes by the method described in section 2.2.4 using positive selection induced some cell activation, attempts were made to culture monocytes after negative selection. The yield using negative selection was significantly lower and unfortunately, those negatively selected cells did not culture well. The majority of the cells were dead on day 4. After three attempts to culture cells from negatively selected monocytes, it was decided to revert to positive selection.

3.1.4 Differentiation of MDMs with MCSF and GMCSF: GMCSF produces constitutively activated cells

Experiments were then carried out to determine the growth factor to be used to differentiate MDMs. Isolated monocytes were differentiated with either GMCSF (50ng/mL) or MCSF (100ng/mL) for 6 days. These doses were chosen following a review of the literature with regard to macrophage differentiation and from protocols previously published (Hashimoto, Yamada et al. 1997, Mia, Warnecke et al. 2014). Differentiated cells were then stimulated with LPS to determine how well each growth factor performed within the assay.

Results showed that GMCSF produced MDMs which were constitutively activated without consistent increase in p38 MAPK phosphorylation when stimulated with LPS. MCSF

produced MDMs that showed a stepwise enhancement of p38 MAPK phosphorylation with increasing doses of LPS (figure 3.2 A and B). In addition, TNFα levels, measured from the cell culture supernatants were low at baseline (median 30.52 pg/mL, IQR 14.22-61.18 pg/mL) and increased with LPS stimulation in MCSF- differentiated cells (for LPS 10 ng/mL: median 101.40 pg/mL, IQR 79.86-120.5 pg/mL, LPS 50 ng/mL: median 84.74 pg/mL, IQR 77.41-118.0 pg/mL). In contrast, GMCSF differentiated cells produced higher levels of TNFα at baseline (median 54.43 pg/mL, IQR 31.43-77.42 pg/mL) with minimal increase with LPS stimulation (for LPS 10 ng/mL: median 76.03 pg/mL, IQR 69.48-82.58 pg/mL, LPS 50 ng/mL: median 79.57 pg/mL, IQR 77.10-82.03 pg/mL, figure 3.2 C). Therefore, to ensure maximal differences were seen between the control (unstimulated) MDMs and those treated with LPS in this assay, monocyte differentiation with MCSF was chosen as the preferred cytokine to differentiate monocyte to macrophages.

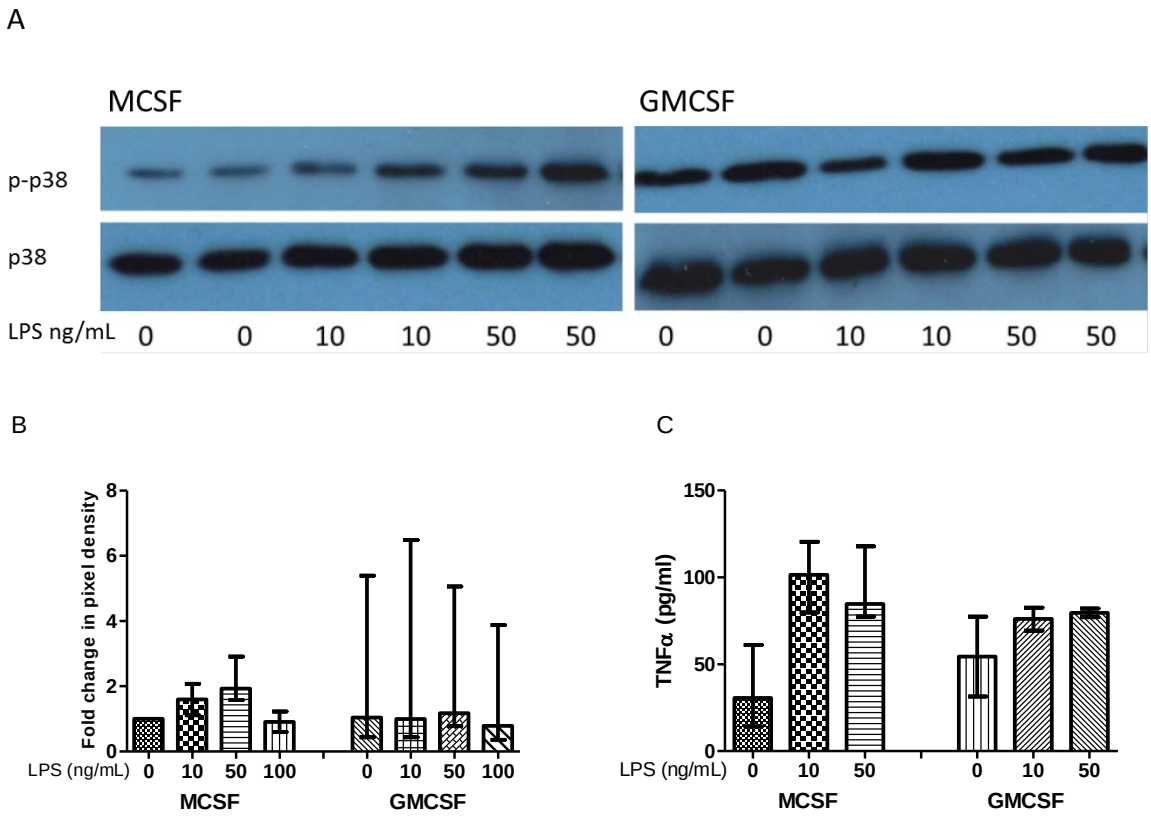


Figure 3.2. Phosphorylation of p38 MAPK and TNFα production in MDMs differentiated with MCSF, but not GMCSF is enhanced with LPS stimulation. A. Isolated monocytes were differentiated for 6 days with either MCSF (100 ng/mL) or GMCSF (50 ng/mL) and stimulated with LPS for 4 hours. p38 MAPK phosphorylation was measured from cell lysates using Western blotting. B. Graph shows median ± interquartile range of fold change in pixel density from Western blots from 4 independent experiments. C. Levels of TNFα were measured by ELISA. Graph shows median TNFα (pg/mL) ± interquartile range from 3 independent experiments.

3.1.5 MDM phenotype

Next, an experiment was carried out to define the phenotype of MDMs produced by MCSF differentiation in the knowledge that MCSF may induce a suppressor phenotype in contrast to the inflammatory phenotype seen with GMCSF. Markers of these phenotypes were chosen after a review of the literature (Jaguin, Houlbert et al. 2013, Chavez-Galan, Olleros et al. 2015). Results of qPCR showed higher gene expression of inflammatory phenotype markers (STAT1 and CD80) on GMCSF- differentiated cells compared to MCSF-differentiated cells but only after LPS stimulation (figure 3.3). Interestingly, when MCSF-differentiated MDMs were stimulated with LPS, a stepwise enhancement of these markers was seen with increasing LPS dose. Of the suppressor phenotype markers chosen, only CD163 was enhanced on MCSF-differentiated MDMs; and no difference was seen in CD200R or CD206 expression between MCSF-differentiated and GMCSF-differentiated MDMs. This experiment was only carried out on one sample as future experiments would involve the addition of IFNγ and LPS, thus skewing the MDMs towards an inflammatory phenotype.

Flow cytometry was subsequently used to identify surface markers on the MCSF-differentiated MDMs. As higher CD163 gene expression was observed from MCSF-differentiated MDMs with no difference seen in the other suppressive markers tested, expression CD163 was tested using flow cytometry along with CD14 and CD16. Of the CD14 positive MCSF-differentiated MDMs, 83.9% were also CD163 positive (figure 3.1 B). Interestingly, the proportion of CD16 positive cells had increased to 62.7% compared to 9.2% of freshly isolated monocytes. These results confirmed that the isolated monocytes had undergone differentiation and the markers seen were as expected surface markers of MCSF-differentiated MDMs (Boyette, Macedo et al. 2017).

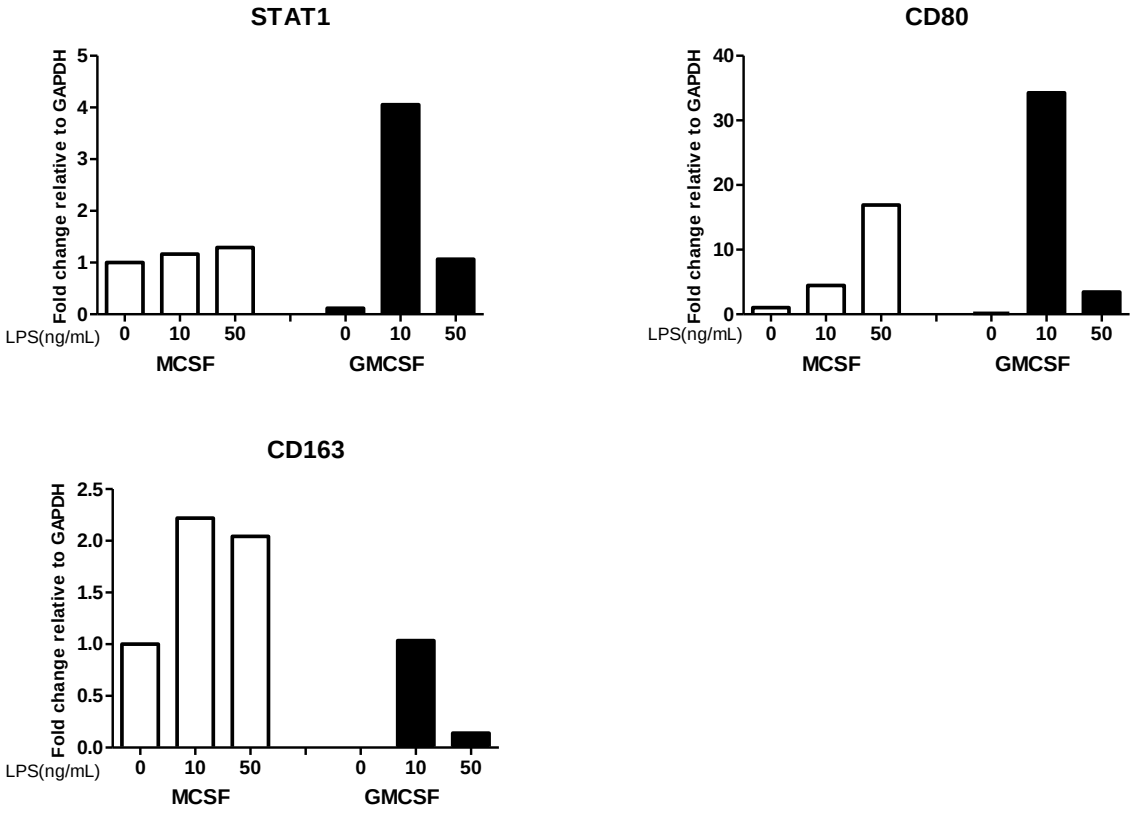


Figure 3.3. STAT1 and CD80 are increased on GMCSF-differentiated MDMs but only after LPS stimulation. CD163 is enhanced on MCSF-differentiated MDMs. Isolated monocytes were cultured for 6 days with either GMCSF or MCSF and stimulated with LPS for 4 hours. RNA was then extracted for analysis by qPCR (n=1).

3.2 Induction of pro-inflammatory pathways and the unfolded protein response.

3.2.1 Introduction

Once the method of differentiating MDMs was decided, further optimisation experiments were carried out to determine the factors to be used for MDM activation. Preliminary experiments indicated that MCSF-differentiated MDMs did not produce IL23 with LPS stimulation alone and this was consistent with previously published papers (Verreck, de Boer et al. 2004). As this was one of the main outcomes of this thesis, it was decided to add IFNγ to the assay on day 6 to induce a more inflammatory phenotype and thus the production of IL23 by MDMs. In addition, it was hypothesised that IFNγ would upregulate HLA B and therefore promote the UPR as seen in animal models (Turner, Delay et al. 2007).

LPS had been used in preliminary experiments to activate the MDMs and thus induce an inflammatory response. LPS has been shown to induce the production of IL23 in both animal and human MDMs (Colbert, DeLay et al. 2010, Zeng, Lindstrom et al. 2011) and was therefore chosen to activate the MDMs in this assay. As an inducer of TLR 4, LPS was thought to be particularly appropriate for the activation of MDMs in this thesis in view of the evidence of upregulation of TLR 4 found in patients with ERA (Myles and Aggarwal 2011). It was also felt to be relevant given that it is a component of gram negative bacteria and the gut microbiome, associated with increased gut permeability, appears to play a role in the pathogenesis of SpA (Martinez-Gonzalez, Cantero-Hinojosa et al. 1994). Other methods of activating MDMs were considered including the use of TNFα but as the measurement of this was a key output of the assay, it was decided that LPS would be the better option.

Phosphorylation of p38 MAPK from cell lysates and levels of TNFα from cell culture supernatants was used as evidence of the induced inflammatory response in these optimisation experiments. Other outputs were trialled during optimisation experiments including the measurement of other components of the pathways downstream of TLR 4 activation. ERK and phosphorylated ERK were measured by Western blot but proved less reliable than p38 MAPK. Measurement of NFκB nuclear translocation was also considered but as this involved separation of the cytoplasmic and nuclear fractions of the cell lysates, it was more complex than the measurement of p38 MAPK. In addition, p38 MAPK appears to be relevant to the pathogenesis of SpA in view of the evidence from genetic studies (Lamot, Borovecki et al. 2014, Ding, Guan et al. 2018, Wang, Han et al. 2018) and that the p38 pathway may not function properly in the presence of HLA B27 heavy chains (Sahlberg, Penttinen et al. 2007).

Next, to induce the UPR, TM was added to the assay. TM causes protein misfolding by blocking N-linked glycosylation of proteins in the ER. XBP1 spicing was used as a measure of

induction of the UPR using qPCR in these optimisation experiments. XBP1 is a transcription factor and, on induction of the UPR, a spliced form of XBP1 is released.

Finally, to ensure no deleterious effects were seen from these agents on cultured MDMs, experiments to test cell viability were carried out as well as further experiments to check the production of pro-inflammatory cytokines and phosphorylation of p38 MAPK.

3.2.2 IFNγ induces IL23 production and upregulation of HLA B in MDMs

Optimisation experiments were first carried out to determine the dose of IFNγ to be used in the assay. MDMs were incubated with a range of doses of IFNγ (1000-8000 iu/mL) for 24 hours prior to stimulation with LPS (50 ng/mL) for a further 24 hours. IL23 levels in the supernatant, detected by ELISA, were enhanced by all doses of IFNγ with a dose of 4000 iu/mL resulting in the highest level (median 54070 pg/mL, IQR 39010-65620 pg/mL) and lower levels seen with 8000 iu/mL (median 42830 pg/mL, IQR 26260-56320 pg/mL) suggesting that this dose was having a deleterious effect on the cells (figure 3.4A). IL23 was only detected when both IFNγ and LPS were added to the assay and only after 24 hours incubation. HLA B upregulation was measured by qPCR and was enhanced by all doses of IFNγ but most consistently at 4000 and 8000 iu/mL (median fold change 16.80 and 15.89 respectively with LPS stimulation) (figure 3.4B). Following these experiments it was decided that a dose of 4000 iu/mL IFNγ would be used in the assay.

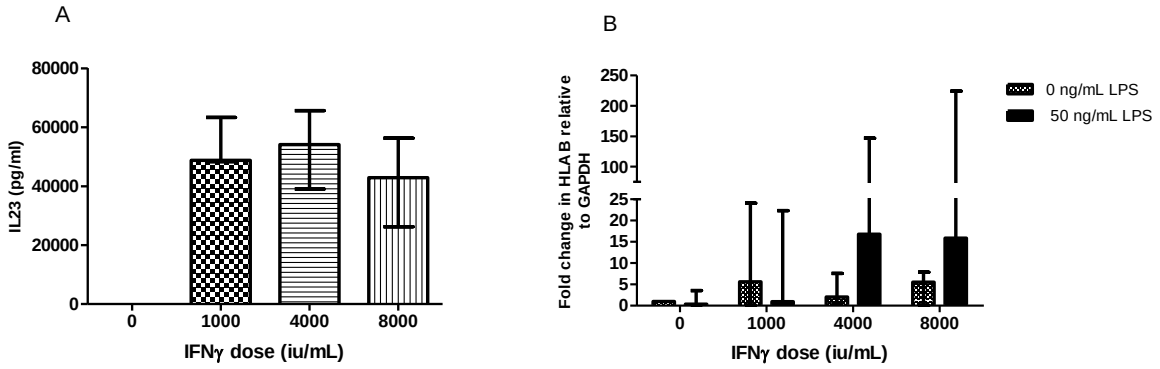


Figure 3.4 IL23 production and HLA B upregulation is enhanced by IFNγ. Isolated monocytes were cultured for 6 days with MCSF. On day 6 IFNγ was added for 24 hours after which media was changed and cells were washed prior to stimulation with LPS 50 ng/mL. IL23 from cell culture supernatants was measured by ELISA and HLA B from RNA by qPCR. Results shown are for 3 independent experiments (graphs show median + interquartile range).

To confirm that IFNγ was inducing a more inflammatory phenotype, MDM surface markers were tested on cells with and without the addition of IFNγ (4000 iu/mL). Results confirmed that STAT1 and CD80 (inflammatory surface markers) were enhanced by the addition of IFNγ, especially in the presence of LPS (figure 3.5 A and B) and that CD163 was reduced as

expected (figure 3.5C). Once again, although CD200R and CD206 were tested, no difference was seen in these markers between IFNγ-treated and untreated MDMs. Levels of TNFα in the cell culture supernatants were significantly higher in the IFNγ-treated cells (median 15021 pg/mL, IQR 11219-20599 pg/mL vs 41552 pg/mL, IQR 29095-48954 pg/mL, p=0.002, figure 3.5D).

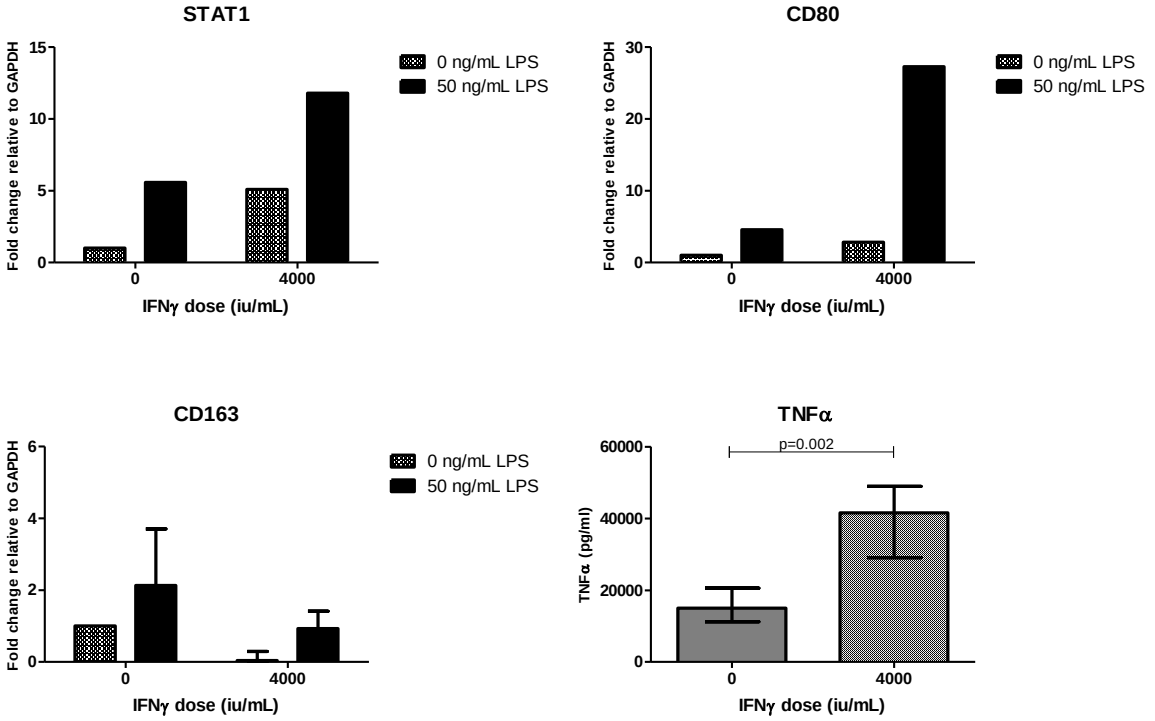


Figure 3.5. MDMs treated with IFNγ exhibit an inflammatory cell phenotype. MCSF-differentiated MDMs were incubated with IFNγ 4000 iu/mL for 24 hours prior to stimulation with LPS 50 ng/mL. STAT1, CD80 and CD163 were measured using qPCR (n=1 for STAT1 and CD80, n=4 for CD163). TNFα was measured in cell culture supernatants (n=6). Statistical analysis by Mann-Whitney U test.

3.2.3 LPS stimulation of MDMs results in enhanced phosphorylation of p38 MAPK and pro-inflammatory cytokine production

MDMs differentiated with MCSF for 6 days and stimulated with IFNγ (4000 iu/mL) for 24 hours, were incubated with a range of LPS doses (0.1 ng/mL- 100 ng/mL) for 4 hours. Enhanced phosphorylation of p38 MAPK was seen with 10 ng/mL (median fold change 1.59) and was further enhanced at a dose of 50 ng/mL of LPS (median fold change 1.93) (figure 3.2 and 3.6A). The level of TNFα in the cell culture supernatants was measured by ELISA. The highest concentrations were seen with 1, 10 and 50 ng/mL LPS (median 35315 pg/mL, 37558 pg/mL and 44758 pg/mL respectively) (figure 3.6B). A dose of 100 ng/mL LPS appeared to reduce phosphorylation of p38 (median fold change 0.91) and resulted in lower levels of TNFα (median 27526 pg/mL), indicating that this higher dose may be detrimental to the cells.

Following these experiments, a dose of 50 ng/mL LPS was chosen for the assay.

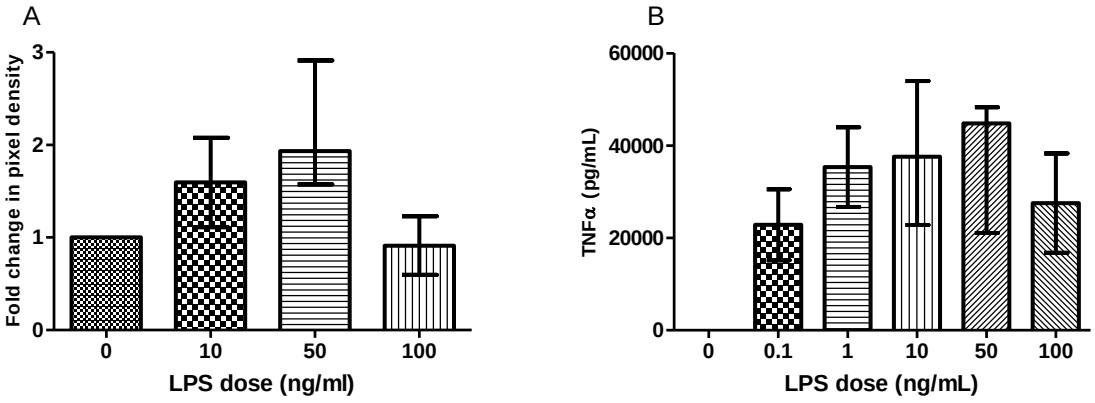


Figure 3.6. LPS stimulation results in enhanced phosphorylation of p38 MAPK and TNFα production by MDMs. MDMs were treated with LPS 0.1 ng/mL-100 ng/mL. p38 MAPK phosphorylation was measured in cell lysates by Western blotting (A). The concentration of TNFα was measured in cell culture supernatants by ELISA (B). Graphs show median (+ interquartile range) of 4 independent experiments.

3.2.4 LPS stimulation increases TNFα production over time

To determine the optimum time for measuring cytokine levels in cell culture supernatants from the MDM assay, a time course experiment was carried out with supernatants collected at 1, 4 and 24 hours following LPS stimulation. It was noted previously that IL23 was only detectable after 24 hours incubation with LPS. TNFα however, was present in the cell culture supernatants even after 1 hour (figure 3.7) and increased in concentration over time (median concentration: 1919 pg/mL at 1 hour, 18300 pg/mL at 4 hours and 73655 pg/mL at 24 hours). It was also noted that after 24 hours, TNFα was present in the cell culture supernatants even without LPS stimulation (median 2019 pg/mL). Following these experiments, it was decided to test supernatants at 24 hours for the majority of cytokines but also to collect after 4 hours incubation with LPS to measure TNFα levels.

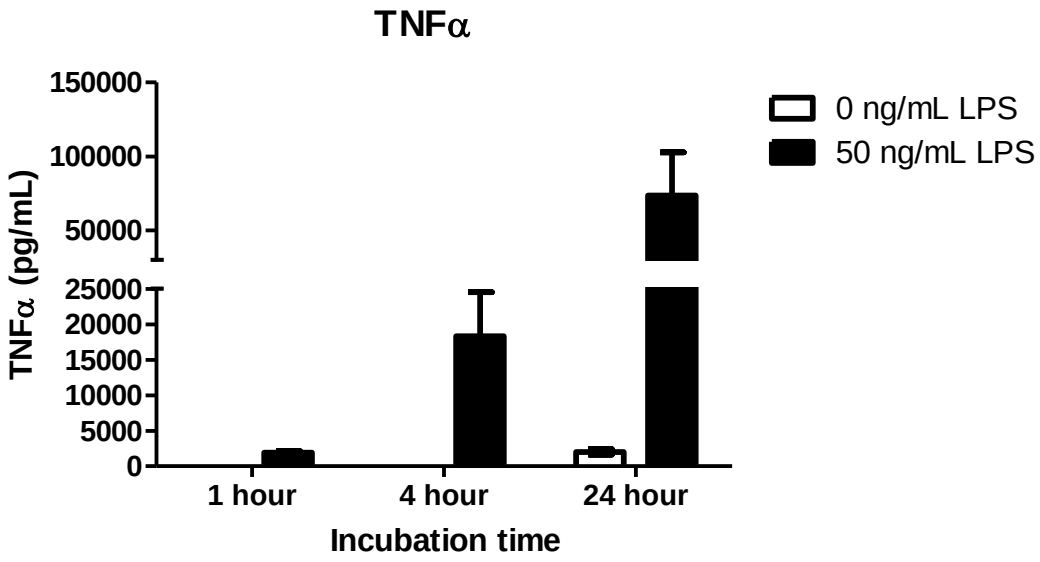


Figure 3.7. TNFα production by MDMs increases over time following LPS stimulation. MDMs were treated with LPS (50ng/mL) and cultured for 1, 4 and 24 hours. TNFα was measured in cell culture supernatants by ELISA. Graph shows median (+ interquartile range) of 3 independent experiments.

3.2.5 TM induces the unfolded protein response

MDMs, differentiated with MCSF and incubated with IFNγ for 24 hours, were treated with doses of TM (1-10 μg/mL) with and without LPS (50 ng/mL) for 4 and 24 hours. To test whether the UPR was being induced, qPCR for XBP1 splicing was performed. Results were expressed relative to unspliced XBP1.

Enhanced XBP1 splicing was seen with all doses of TM, independent of LPS dose. Without LPS, doses of 5 and 10 μg/mL appeared to induce XBP1 splicing the most (median fold change 8.24 and 8.13 respectively) and with LPS doses of 1 and 5 μg/mL were most effective (median fold change 9.79 and 9.94 respectively). LPS alone did not induce XBP1 splicing (figure 3.8 A). A dose of 5 μg/mL was therefore chosen for the assay. When incubated for 24 hours instead of 4 hours with TM (5 μg/mL), further enhancement of XBP1 splicing was seen (median fold change 10.48 vs 32.37 without LPS and 11.88 vs 42.81 with LPS) (figure 3.8 B).

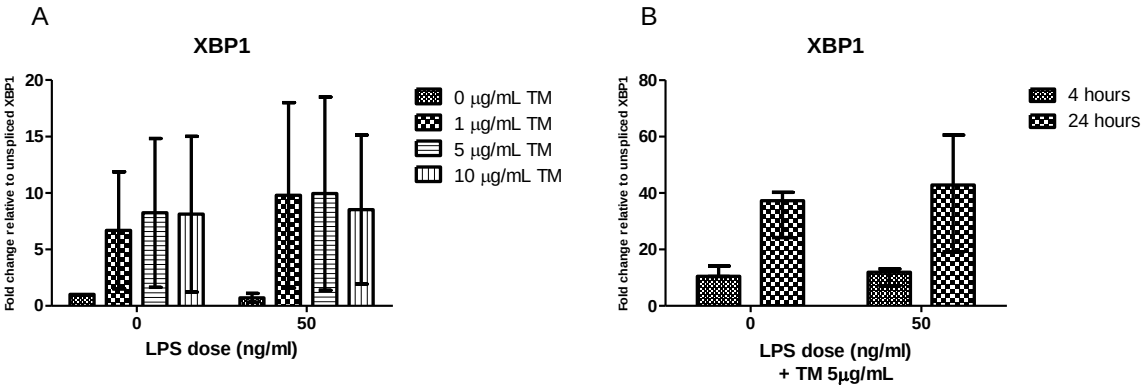


Figure 3.8. TM enhances XBP1 expression. A. MDMs were treated with doses of TM between 1 and 10 μg/mL with and without LPS (50ng/mL) for 4 hours. XBP1 splicing was measured by qPCR and expressed as a fold change of an unstimulated sample relative to unspliced XBP1. B. MDMs were treated with 5 μg/mL TM, with and without LPS (50ng/mL), for 4 and 24 hours. Graphs show median (+ interquartile range) of 3 independent experiments.

3.2.6 TM enhances phosphorylation of p38 MAPK but reduces the expression of TNFα and IL23

Further experiments were performed to ensure the addition of TM was not detrimental to p38 MAPK phosphorylation and to determine the effect on TNFα and IL23 production. In fact, the addition of TM (doses 1 and 5 μg/mL) seemed to further enhance p38 MAPK phosphorylation over and above that of LPS alone (figure 3.9 A) although no significant difference was seen across 3 independent experiments in p38 MAPK phosphorylation between stimulation with LPS alone, LPS combined with TM and TM alone (figure 3.9 B). In the presence of LPS and TM, TNFα (at 4 and 24 hours) and IL23 levels in the supernatant were lower compared to levels from MDMs incubated with LPS alone (TNFα 4 hours incubation: median 13720 pg/mL vs 30838 pg/mL, TNFα 24 hours incubation: median 73410 pg/mL vs 82776 pg/mL, IL23: median 15780 pg/mL vs 54070 pg/mL). This was unexpected as it was hypothesised that TM would also enhance pro-inflammatory cytokine production. It was therefore decided to also measure these cytokines at the RNA level in case blocking N-glycosylation was impairing their production at the protein level.

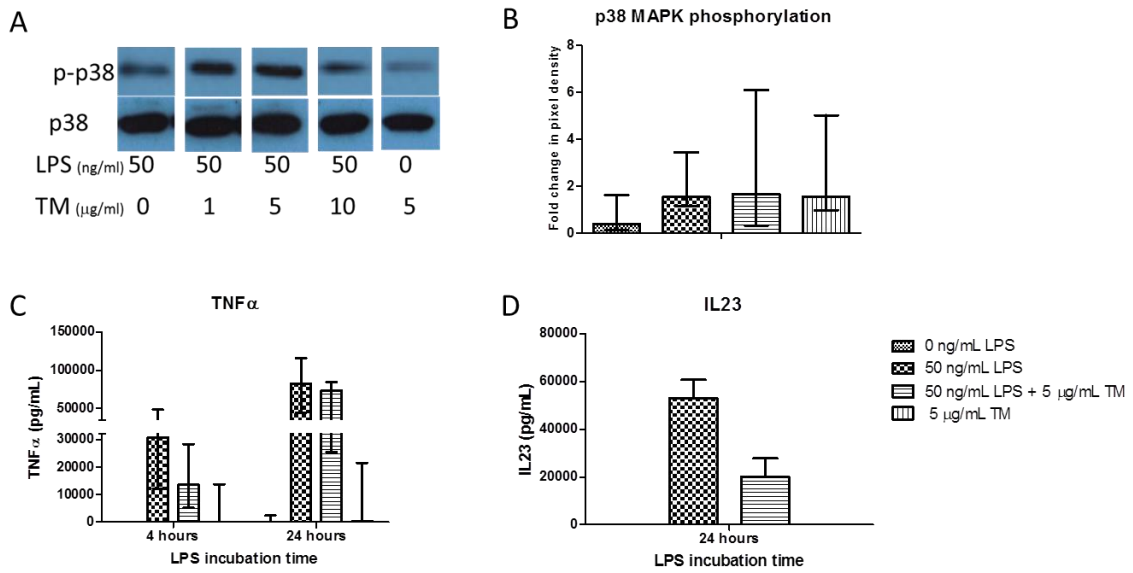


Figure 3.9. Induction of the UPR with TM does not adversely affect p38 MAPK phosphorylation but lowers production of TNFα and IL23. MDMs differentiated as above were treated with TM (5 μg/mL) with and without LPS (50 ng/mL) for 4 and 24 hours. p38 MAPK phosphorylation was measured by Western blotting and cytokines were measured by ELISA. A. Representative Western blot of 1 experiment. B, C and D show median (+ interquartile range) of 3 independent experiments.

3.2.7 Cell viability is maintained across all conditions of the MDM assay

Finally, experiments were carried out to ensure that the addition of IFNγ, LPS and TM was not toxic to the MDMs. This was done with a cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) in combination with phenazine ethosulfate (PES) which are reduced by viable cells to produce a formazan product which can be read on a microplate reader at 490nm. The quantity of formazan product is directly proportional to the number of living cells in culture. A negative control (made by incubating cells for 2 hours with 1mM H_2O_2) was used to eliminate background and cell viability was calculated relative to a control (unstimulated sample).

Cell viability at 4 hours ranged from 81.5% to 116.1% (median value) across all conditions with no significant difference between them (figure 3.10 A). At 24 hours, cell viability ranged from 66.6 to 105.5% (median value), with the lowest figure being with cells treated with TM alone (figure 3.10 B). There was no significant difference between any of conditions.

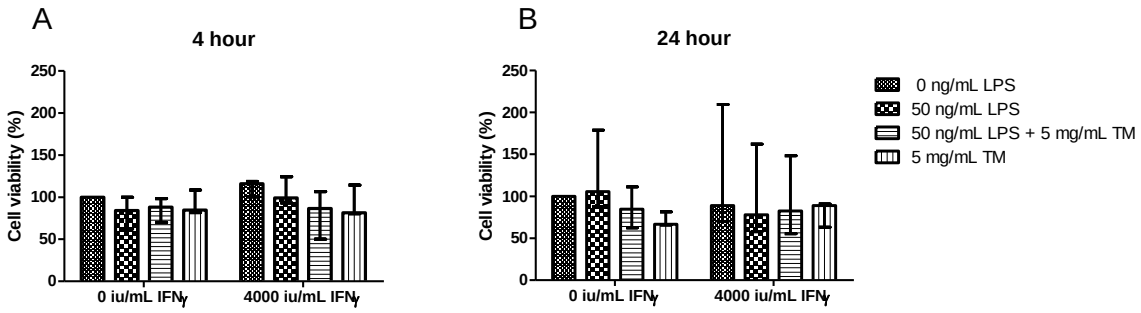


Figure 3.10. Cell viability is not significantly altered by the addition of IFNγ, LPS and TM. MDMs, differentiated for 6 days with MCSF, were incubated with and without IFNγ, LPS and TM for 4 and 24 hours. Cell viability was measured using an MTS assay (Promega). Graphs show median cell viability (%) (+ interquartile range) for 3 independent experiments.

3.3 Patients and healthy volunteers

3.3.1 Introduction

After optimisation of the MDM assay, patients and healthy volunteers were recruited from the following groups: ERA, polyarticular JIA (rheumatoid factor negative), AS, adolescent healthy volunteers and adult healthy volunteers. Each gave a 15 mL blood sample from which monocytes were isolated then differentiated in to MDMs as per the protocol described in chapter 2 (section 2.2).

Patients with ERA and polyarticular JIA aged 13-19 years were recruited from the adolescent rheumatology clinic at University College London Hospital (UCLH) over a period of 3 years (2014-2017) following local ethics committee approval (REC 11/LO/0330). Adult patients with AS were recruited from the SpA clinic. Each patient gave informed consent to participate in the study and in the case of patients aged less than 16 years, gave assent with parental consent. Blood samples were taken at the same time as other blood tests for routine clinical care. Each patient was given a code and all stored samples were anonymised. Clinical data was collected as part of routine clinical care and recorded in an anonymised database.

Adolescent healthy volunteers were recruited from a variety of sources including science days run by the Centre for Adolescent Rheumatology at UCL, healthy patients attending for urology or dental surgery (not for dental caries), friends of clinic patients and young people attending the lab for other reasons. Staff and students at UCL and UCLH were recruited as adult healthy volunteers. All healthy volunteers gave informed consent under the same ethics as above.

The primary aim was to compare cytokine production from MDMs from patients with ERA with a group of age-matched healthy volunteers. Patients with polyarticular JIA were recruited as a disease control group. It was also important to compare the results to adult patients with AS. This adult group required a group of adult healthy volunteers for comparison.

3.3.2 Demographics and clinical features of patients and healthy controls

The numbers of patients and healthy controls in the final analysis for cytokine production from MDMs is shown in table 3.1. Some were excluded from the final analysis (ERA=2, adolescent healthy control=1, adult healthy control=1, polyarticular JIA=1) on the basis that their MDMs on day 6 did not appear fully differentiated and when the supernatants from these cells were analysed, the level of pro-inflammatory cytokine production following LPS stimulation was much lower than expected.

Patients with ERA were mostly male (83.8%) and adolescent healthy controls were age and gender matched as far as possible. Despite attempts to recruit males in the disease control group, patients with polyarticular JIA were mostly female (86.7%). This group were also much more likely to have active peripheral arthritis (73.3%) compared to patients with ERA

(21.6%). However, active hip arthritis and enthesitis were more frequent in the ERA group (45.9% vs 26.9% and 62.2% vs 13.3% respectively) reflecting the known differences in JIA disease phenotypes (Fisher and Sen 2012). Around 62% of patients with ERA had definite axial involvement. Treatment with TNF inhibitors and NSAIDs was similar across all the disease groups but use of DMARDS was much less frequent in the adult AS group (7.1%) compared to both the ERA and polyarticular JIA groups (both 73%) as expected. There were difficulties in data collection for the adult AS group, especially as disease activity scores measured in the adult clinic included BASDAI and BASFI and thus differed from those measured in the adolescent clinic where the JIA core outcome variables were the most frequent disease activity score collected. A disease activity score for juvenile SpA, the Juvenile Spondyloarthritis Disease Activity (JSpADA) index (Weiss, Colbert et al. 2014) which has some overlap with disease activity scores for adult SpA, was considered for patients with ERA. However, some aspects of the score were not consistently collected in the clinic and therefore it was not possible to give every patient a score.

HLA B27 was tested as described in section 3.5 below in healthy controls and patients with polyarticular JIA where it had not been tested during routine clinical care. CRP was also measured by ELISA in healthy controls (as described in section 2.6.1) to enhance the confidence that no condition was present which would alter the result of the MDM assay.

	ERA	Adolescent healthy controls	AS	Adult healthy controls	Polyarticular JIA
Number in final analysis	37	21	14	21	15
Age (median)	16 yrs 4 mths	16 yrs 10 mths	35 yrs 6 mths	29 yrs 2 mths	16 yrs 11 mths
Male (%)	83.8	76.2	57.1	61.9	13.3
HLA B27 + (%)	67.6	0	*	0	0
CRP (median)	0.9	0.15	0.6	*	1.0
Disease duration (median)	3 yrs 10 mths	-	*	-	4 yrs 5 mths
Axial arthritis (%)	62.2**	0	100	0	0**
Active peripheral arthritis (%)	21.6	0	7.1	0	73.3
Hip arthritis (%)	45.9	0	0	0	26.9
Active enthesitis (%)	62.2	0	21.4*	0	13.3
Pain VAS (median)	3*	-	*	-	2.71*
Physician VAS (median)	1	-	-	-	1
(C)HAQ (median)	0*	-	-	-	0.25*
Swollen joint count (median)	0	-	-	-	0
Restricted joint count (median)	0.5	-	-	-	2
Active joint count (median)	0	-	-	-	0
Recent steroid treatment (%) * **	10.8	-	0	-	33.3
Current NSAID (%)	51.4	-	50	-	60
Current DMARD (%)	73	-	7.1	-	73.3
Current TNF inhibitor (%)	40.5	-	42.9	-	40
Extra-articular/ overlap manifestations (%) ** **	18.9	-	*	-	0
Chronic pain (%)	8.1	-	*	-	13.3

Table 3.1 Patients and healthy volunteers included in final analysis for cytokine production from supernatants from MDM assay. Each patient and healthy volunteer donated a 15 mL blood sample from which MDMs were differentiated and subsequently stimulated with LPS. Basic demographics were collected at the time of sample collection and clinical data for patients was collected retrospectively from clinic letters (*=clinical data missing, **=some patients also had 'indeterminate scan results with either mild facet joint and interspinous ligament inflammation or subtle changes at the sacroiliac joints not diagnostic of sacroiliitis (ERA 18.9%, polyarticular JIA 6.7%), ***=recent steroid treatment included oral, intra-articular or intravenous, ****=extra-articular/ overlap manifestations included acute anterior uveitis, inflammatory bowel disease and chronic recurrent multifocal osteomyelitis (CRMO).

3.4 Phosphorylation of p38 MAPK in MDMs stimulated with LPS +/- TM derived from patients with ERA compared to healthy controls

3.4.1. Introduction

Having finalised the methods for the MDM assay, initial experiments were carried out to study phosphorylation of p38 MAPK in patients with ERA compared to healthy controls. Phosphorylation of p38 MAPK results in transcription of pro-inflammatory cytokines and has been implicated in the pathogenesis of many inflammatory conditions (Cuenda and Rousseau 2007). In addition, there is evidence as discussed above, that the p38 MAPK pathway may be affected by the presence of HLA B27 (Sahlberg, Penttinen et al. 2007) therefore studying p38 MAPK phosphorylation was of interest in patients with ERA.

3.4.2. Phosphorylation of p38 MAPK in stimulated MDMs is no different in patients with ERA compared to healthy controls

Analysis of p38 MAPK phosphorylation was carried out by Western blotting on cell lysates from MDMs cultured as above and stimulated with LPS with and without TM for 4 hours. Fold change in pixel density was calculated relative to unstimulated MDMs for each patient and healthy control. As expected enhanced phosphorylation of p38 MAPK was seen with both LPS stimulation alone and with LPS in combination with TM. No significant difference was seen between patients and healthy controls (figure 3.11 A). Interestingly, when patients were sub-divided in to those who were HLA B27 positive and negative, there appeared to be enhanced phosphorylation of p38 MAPK in those who were HLA B27 positive. However, the differences were not statistically significant (figure 3.11 B).

It was noted that 8 patients had significantly enhanced phosphorylation of p38 MAPK compared to other patients and healthy controls. Of these, 6 had enthesitis at the time their sample was taken, 5 of these 6 were HLA B27 positive. When results from patients with active enthesitis were compared to those without active enthesitis (figure 3.11 C) a significant difference was seen in p38 phosphorylation in MDMs stimulated with LPS (median fold change in pixel density 2.14 vs 1.26, p=0.03). No significant difference was seen between those with axial ERA compared to those who had peripheral arthritis only or with other clinical features.

Following these experiments, it was decided that it would be of interest to compare the results of subsequent experiments not only between patients and healthy controls but also for subgroups of patients with differing clinical phenotype.

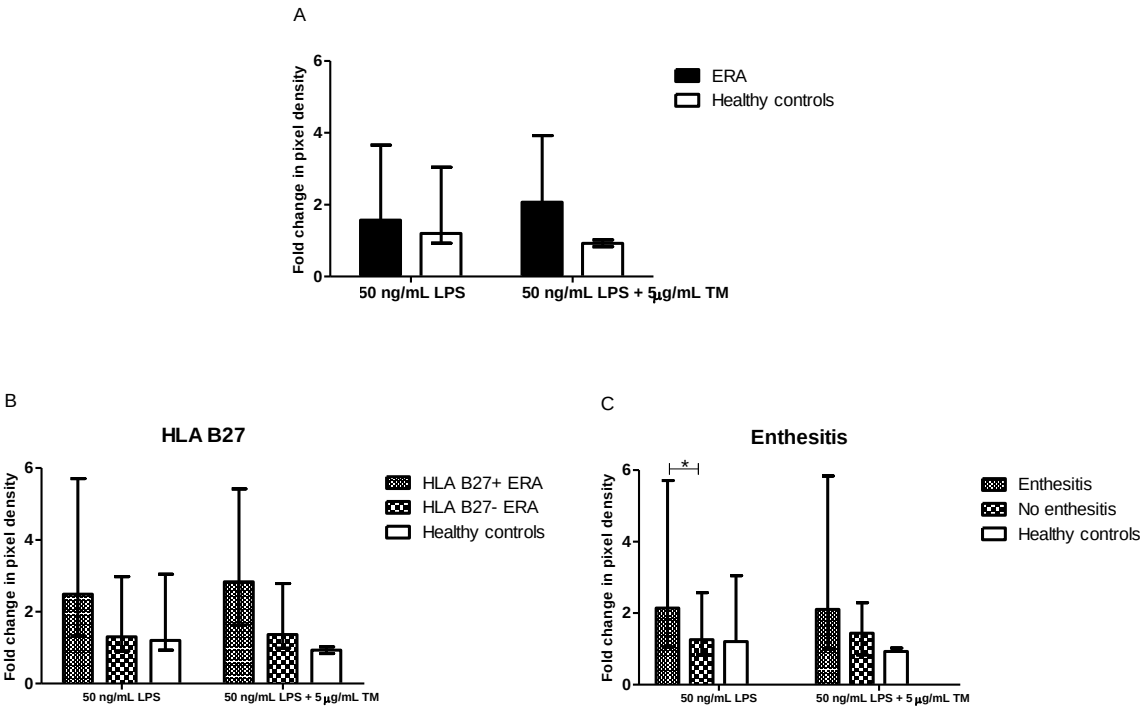


Figure 3.11. Enhanced phosphorylation of p38 occurs with active enthesitis. MDMs, differentiated as above, were stimulated with LPS (50 ng/mL) with and without TM (5 μg/mL) for 4 hours. Cell lysates were analysed by Western blotting and phosphorylated p38 was expressed as a ratio of total p38. Graphs show median fold change in pixel density (+interquartile range) compared to unstimulated MDMs for each patient and healthy control (ERA n=24 (15 HLA B27 positive, 14 with active enthesitis), adult healthy controls n=6). Statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (*$p<0.05$).

3.5 HLA B27 detection in healthy controls

3.5.1 Introduction

HLA B27 is strongly associated with SpA, including ERA, but is also present in some healthy individuals (Brewerton, Hart et al. 1973). In view of the above results suggesting HLA B27 may affect phosphorylation of p38 MAPK and therefore pro-inflammatory cytokine production (although the difference was not statistically significant) , it was important to establish HLA B27 status of all recruited patients and healthy controls. In the case of patients, this was already known from tests done as part of routine clinical care.

3.5.2 HLA B27 detection from DNA in healthy controls

Isolated DNA from PBMCs of healthy controls was amplified using Taq polymerase (Qiagen) and a known HLA B27-specific primer pair (E91/136) (Seipp, Erali et al. 2005) which amplifies a 135-bp fragment of the HLA B27 gene. B-globin (268-bp) was also amplified as an amplification control and DNA from 2 patients, one known to be HLA B27 positive and one HLA B27 negative, was used as reference for all samples tested (figure 3.12).

All healthy control samples tested negative for HLA B27 and therefore all were included in further analysis.

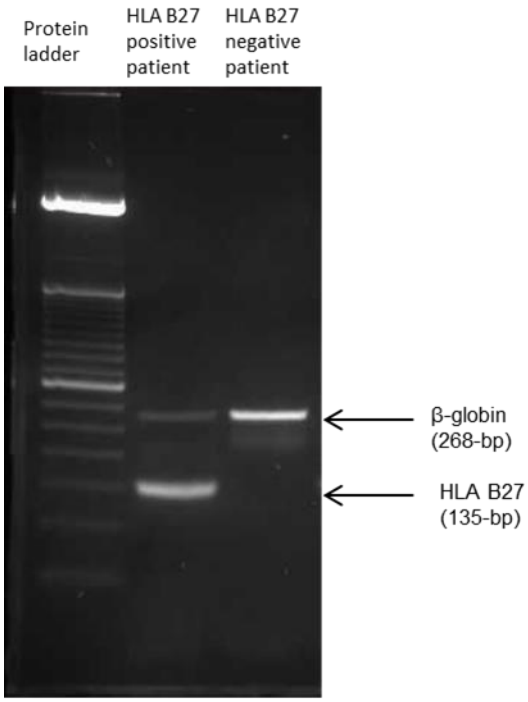


Figure 3.12. Confirmation of HLA B27 status using PCR. Isolated DNA (150ng) was amplified using Taq polymerase (Qiagen) and the E91/E136 primer pair in a total reaction volume of 50 µL. β-globin was also amplified as an amplification control. PCR products were fractionated on a 2% agarose gel with the addition of GelGreen (Biotium) and visualised using a light box.

3.6 Determination of final cytokine panel to be analysed for MDM assay

3.6.1 Introduction

Optimisation experiments for the MDM assay measured only TNFα and IL23 levels in cell culture supernatants from MDMs stimulated with LPS, with and without TM. Analysis of other cytokines was important in patients with ERA and was one of the main aims of this thesis. Therefore, preliminary experiments were carried out using a 9-plex Luminex assay measuring IL1, IL2, IL6, IL10, IL12, IL17, IFNγ in addition to TNFα and IL23 in samples from 5 patients and healthy controls. These experiments demonstrated that treatment of MDMs with LPS enhanced the production of IL1, IL6, IL10 and IL12 as well as IL23 although no significant differences were seen due to the small sample size. TNFα levels were too high to measure using this assay and would therefore be analysed on a separate ELISA to allow adequate dilution of the supernatants. IL2 levels were not enhanced by LPS or TM stimulation. To determine whether any other cytokines or chemokines would be of interest in this assay, assessment of a larger number of analytes was necessary.

3.6.2 Cytokine and chemokine detection using proteome profiler array

The proteome profiler array (R&D) is a membrane-based antibody assay detecting 36 cytokines and chemokines. Samples are mixed with a cocktail of biotinylated detection antibodies and incubated with the array membrane which is spotted with capture antibodies to the cytokines and chemokines (in duplicate). These are then visualised using ECL and the signal produced is proportional to the amount of analyte bound.

Supernatants collected after 24 hours incubation for each of the 4 conditions of the MDM assay (figure 2.1) were tested for 2 patients and 1 age-matched healthy control. The patient samples were from 2 patients with active ERA who were HLA B27 positive. An example of the 4 membranes acquired for each patient/ healthy control is shown in figure 3.13.

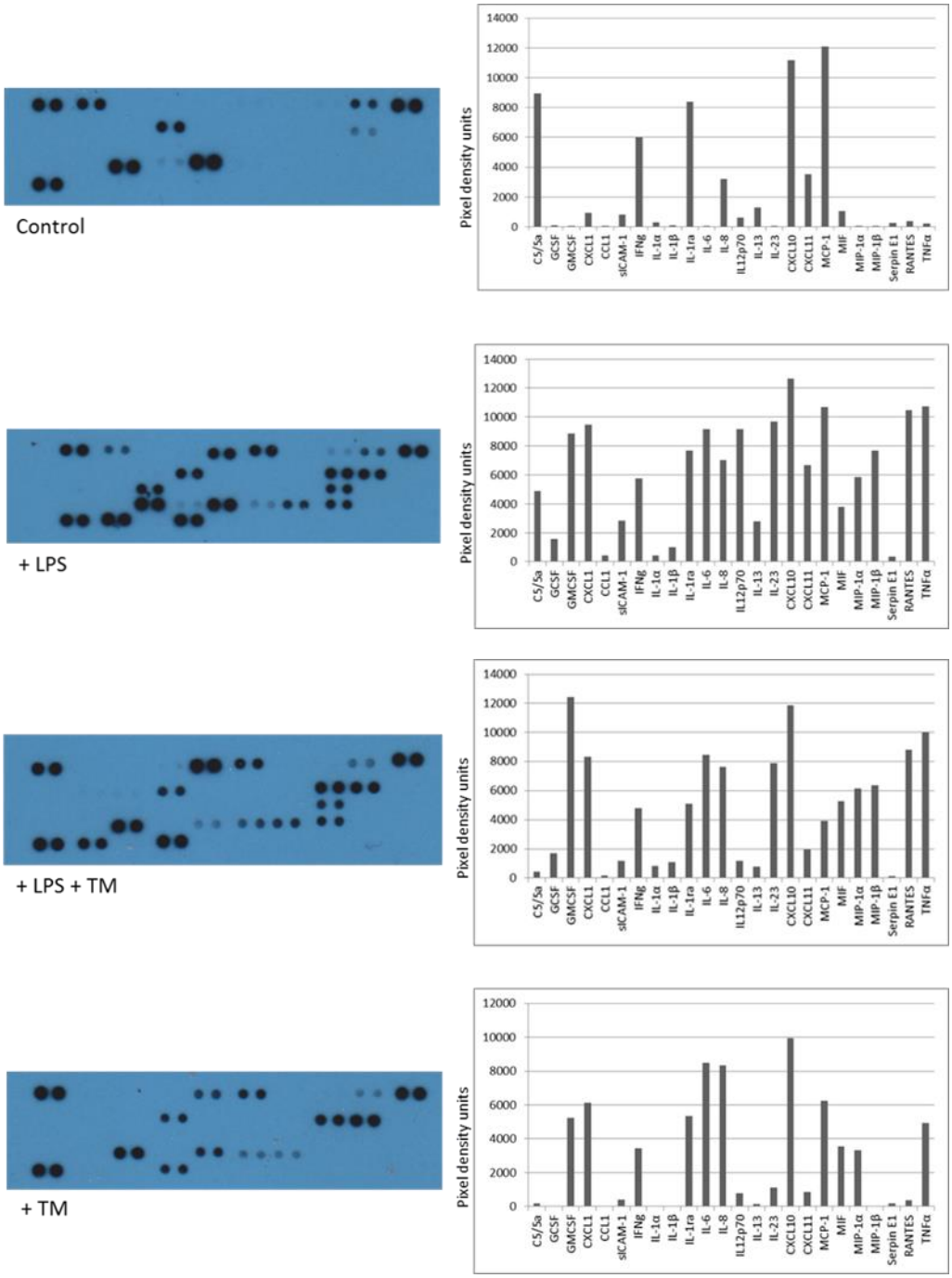


Figure 3.13. Example proteome profiler array from cell culture supernatants from MDMs from a patient with ERA. MDMs were cultured as above and stimulated with LPS and/ or TM. Each membrane shown represents a different condition (as labelled). Graphs show cytokine levels (in arbitrary pixel density units) for each blot calculated by taking the average pixel density for the pair of dots representing each cytokine on the membrane (quantified using QuantityOne software (Bio-rad, USA)) and subtracting this from the background signal. To enable comparison between blots, an adjustment was made using the reference spots, using the control (unstimulated) condition as baseline.

The results confirmed that LPS stimulation with and without TM (and indeed TM stimulation alone for some cytokines) induced the production of pro-inflammatory cytokines including TNFα, IL23, IL1β, IL6, and GMCSF (figure 3.13 and 3.14). IL12 production was only enhanced with LPS stimulation and not when TM was added to the assay which is consistent with previous literature demonstrating that IL12 is not induced by the UPR (Goodall, Wu et al. 2010). Analytes such as C-X-X motif chemokine (CXCL) 10, monocyte chemoattractant protein (MCP) 1 and IL1 receptor agonist (RA) were constitutively produced by MDMs and levels were not affected by LPS or TM stimulation. Some factors such as complement component 5a and Serpin E1 (also known as plasminogen activator inhibitor type 1) appeared to be down-regulated by stimulation with LPS and in particular TM. The following cytokines and chemokines were not detected in the supernatants from MDMs (either unstimulated or stimulated) in the profiler array: IL2, IL4, IL5, IL10, IL17, IL27, IL32 and CXCL12.

Although the sample size was small, potential differences were seen between the patients and the healthy control with TNFα, IL1β, GMCSF, IL12, macrophage inflammatory protein (MIP) 1β and Regulation on Activation, Normal T cell Expressed (RANTES) (figure 3.14). In particular, IL1β and IL12 were higher in supernatants from the patient samples (from LPS-stimulated MDMs with and without TM for IL1β and LPS-stimulated MDMs only for IL12). However, no definite conclusions could be drawn from these results because of the small numbers tested.

Following this assay, the final list of cytokines and chemokines to be analysed in all supernatants from patient and healthy control samples was determined: TNFα, IL23, IL1β, IL6, IL12, IFNγ, GMCSF, RANTES, and MIP1β. IL27 was added to this list as although no signal was seen in the proteome profiler array, it was of interest as a member of the IL23/IL12 family of cytokines. IL17, although produced by macrophages in vivo (Isailovic, Daigo et al. 2015), was not detected in the supernatants from MDMs by either luminex assay or proteome profiler array. As it was still a cytokine of interest, it was decided to explore other methods of detecting this.

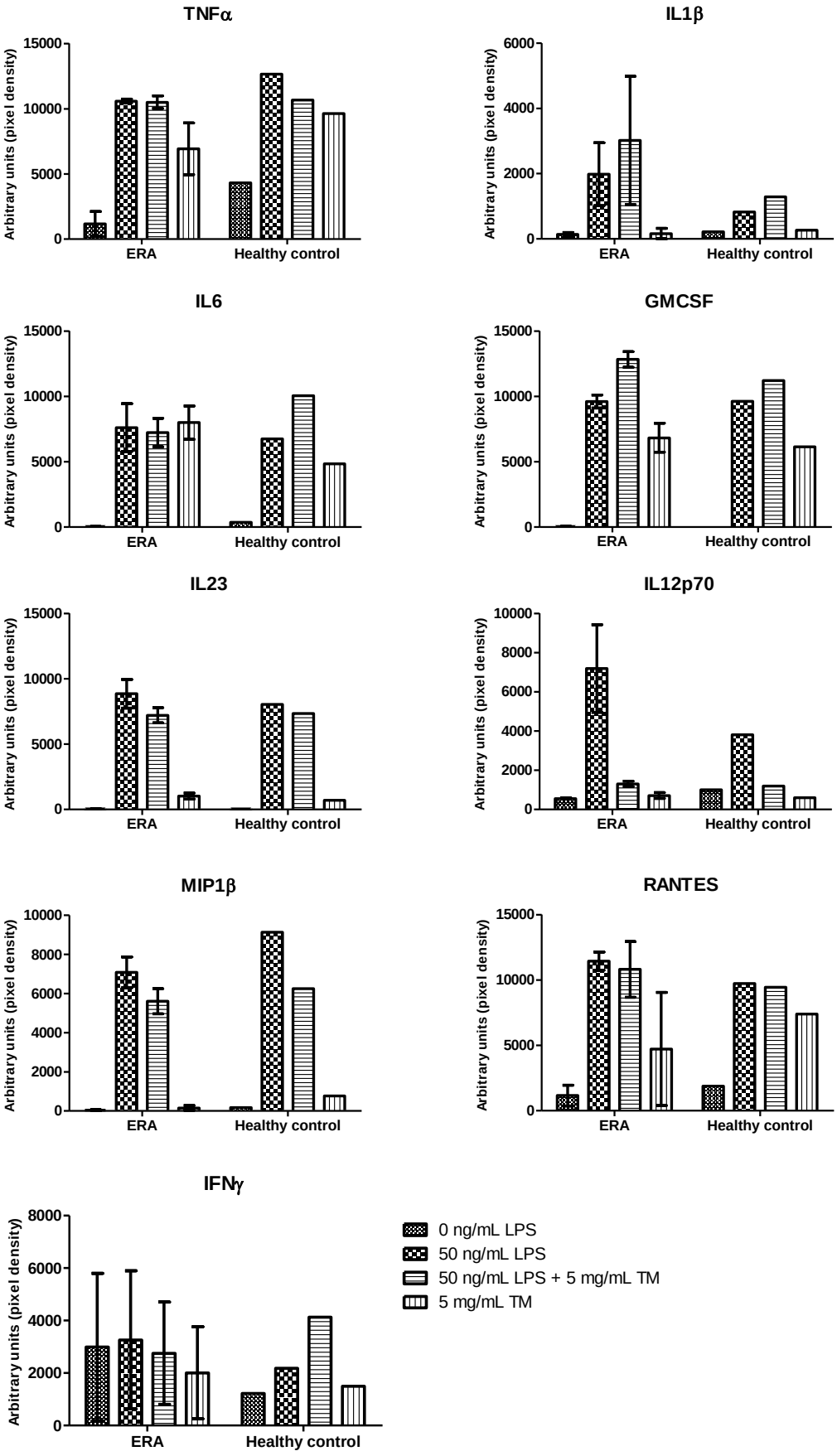

TNFα
IL1β
IL6
GMCSF
IL23
IL12p70
MIP1β
RANTES
IFNγ
Arbitrary units (pixel density)
ERA
Healthy control
0 ng/mL LPS
50 ng/mL LPS
50 ng/mL LPS + 5 mg/mL TM
5 mg/mL TM

Figure 3.14. LPS enhances the production of pro-inflammatory cytokines and chemokines as demonstrated by proteome profiler array. MDMs, cultured as above were stimulated with LPS and / or TM for 24 hours. Cell culture supernatants were collected and analysed by proteome profiler array. Graphs show median pixel density (+interquartile range), calculated as above (figure 3.13) and expressed as arbitrary pixel units for each condition of the MDM assay.

3.7 Discussion

Having made the decision to study MDMs in ERA, it was first important to establish a robust method for isolating monocytes, differentiating MDMs and inducing the production of IL23, other pro-inflammatory cytokines and also the UPR. In view of the fact that macrophages may acquire an inflammatory or suppressor phenotype depending on the growth factors present both in vitro and in vivo (Ribechini, Hutchinson et al. 2017), experiments were carried out to determine the optimal growth factor for differentiating MDMs in this assay to allow for differences to be seen between unstimulated and LPS-stimulated cells. MCSF-differentiated MDMs are often used as a model for tissue macrophages (Verreck, de Boer et al. 2004, Lacey, Achuthan et al. 2012). GMCSF, although used in the differentiation of dendritic cells, also effectively differentiates monocytes into macrophages (Robbins, Walzer et al. 2008). In this assay, when GMCSF and MCSF-differentiated MDMs were compared, it was found that GMCSF- differentiated MDMs were constitutively activated with enhanced phosphorylation of p38 MAPK and TNFα production even without LPS stimulation compared to MCSF-differentiated cells. MCSF-differentiated MDMs, however, had a lower basal expression of TNFα in the cell culture supernatants and exhibited low phosphorylation of p38 MAPK which enhanced with LPS stimulation. This is consistent with evidence that GMCSF produces a more inflammatory phenotype of macrophage resulting in the production of pro-inflammatory cytokines, while MCSF induces a more suppressor phenotype (Jaguin, Houlbert et al. 2013). It also reflects the different roles of MCSF and GMCSF in vivo, with MCSF continually produced and a key growth factor in maintaining macrophage homeostasis; in contrast to GMCSF, which is normally present at a low basal level and is released as part of the inflammatory response, resulting in an increase in inflammatory-phenotype macrophages (Ushach and Zlotnik 2016). For the purposes of this assay, in order to assess the relative thresholds of activation following LPS stimulation using phosphorylation of p38 MAPK and pro-inflammatory cytokine production, MCSF was chosen as the growth factor to be used.

In view of the evidence described above, it was hypothesised that the MDMs differentiated with MCSF in this assay would be of a suppressor phenotype. Preliminary experiments were undertaken to investigate this using markers thought to be associated with either a suppressor or inflammatory macrophage phenotype. There is an extensive list of these potential markers but no definitive marker profile that reliably differentiates these two phenotypes (Ribechini, Hutchinson et al. 2017). This is likely to be because of the significant overlap between these phenotypes with macrophage function exhibiting a spectrum from suppressor to inflammatory phenotype. This results in a range of marker expression rather than two distinct populations (Martinez and Gordon 2014). MDM phenotype may also switch over time in culture from an inflammatory phenotype initially to a suppressor phenotype by around day 7 which is likely to affect the expression of inflammatory and suppressor markers (Jones, Chang et al. 2007). Two potential inflammatory markers were chosen: STAT1 has been shown to be crucial for macrophage polarisation to an

inflammatory phenotype (Shuai, Stark et al. 1993); CD80 is a co-stimulatory molecule also expressed at high levels on macrophages with an inflammatory phenotype (Chavez-Galan, Olleros et al. 2015). Two markers which have been associated with suppressor-phenotype macrophages were also chosen: CD206 or macrophage mannose receptor (Roszer 2015) and CD200R (Koning, van Eijk et al. 2010) which are both transmembrane glycoproteins. An additional marker, CD163, was also tested. CD163 is a marker of macrophage differentiation along with CD16 (Ambarus, Krausz et al. 2012) but has also been suggested as a marker of suppressor phenotype macrophages although not in isolation (Barros, Hauck et al. 2013). In the context of this assay, the inflammatory phenotype markers were upregulated on GMCSF-differentiated cells compared to MCSF-differentiated MDMs. However, no difference was seen in the suppressor-phenotype markers except CD163 where expression was enhanced in the MCSF-differentiated MDMs. Interestingly, despite CD206 being designated as a prototypical marker of suppressor-type macrophages (Gordon and Pluddemann 2017), a study by Jaguin et al. did not find any difference in expression between inflammatory and suppressor phenotypes (Jaguin, Houlbert et al. 2013) which may explain this result.

Unsurprisingly, stimulation with LPS, a potent inducer of inflammatory phenotype macrophages (Guha and Mackman 2001), resulted in increased expression of STAT1 and CD80 in both MCSF and GMCSF-differentiated MDMs. LPS, a component of gram negative bacteria, induces pro-inflammatory activation of macrophages through binding with its receptor, TLR4, causing the phosphorylation of STAT1 (Toshchakov, Jones et al. 2002). Despite LPS inducing an inflammatory phenotype in MCSF-differentiated MDMs in this assay resulting in enhanced phosphorylation of p38 and TNFα production, preliminary experiments indicated that these MDMs did not produce IL23. This was consistent with other studies of MCSF-differentiated MDMs (Verreck, de Boer et al. 2004). Therefore, further enhancement of the inflammatory phenotype was necessary with IFNγ. IFNγ is commonly used in vitro to produce inflammatory phenotype macrophages (Murray, Allen et al. 2014) and has long been recognised as an activator of macrophages in vivo in response to microbial pathogens (Nathan, Murray et al. 1983). With the addition of IFNγ to MCSF-differentiated MDMs in this assay, further upregulation of the inflammatory macrophage phenotype markers, STAT1 and CD80 was seen. In addition, there was increased production of TNFα in cell culture supernatants and IL23 was also detected in the supernatant. Interestingly, CD163 appeared to be down-regulated by the addition of IFNγ.

In addition to studying the pro-inflammatory response of MDMs from patients with ERA, it was also of interest to study the UPR, in view of its potential role in the pathogenesis of SpA and therefore ERA. It was postulated that the use of IFNγ in this assay would assist in the induction of the UPR by upregulating HLA B (Turner, Delay et al. 2007). The UPR was not visualised after stimulation of MDMs with LPS alone, even in the presence of IFNγ, therefore it was necessary to add another agent to induce this. TM has been widely used as a pharmacological inducer of the UPR through its ability to block N-linked glycosylation and

therefore impair protein folding (Takatsuki and Tamura 1971). It has also been used to study the effect of UPR induction on cytokine production (Goodall, Wu et al. 2010). In this assay, TM was found to successfully upregulate HLA B and to induce the UPR, shown by splicing of XBP1. However, contrary to expectations and previous studies describing an increase in IL23 with TM following LPS stimulation (Goodall, Wu et al. 2010), the production of TNFα and IL23 was not enhanced by TM in this assay. In fact there was a reduction compared to levels from MDMs stimulated with LPS alone. It was thought that this may have been due to an adverse effect of TM on cell survival but this was not found to be the case with no significant difference seen in cell viability with the addition of TM to the assay at either 4 or 24 hours. Another possibility was that by blocking N-glycosylation, TM was affecting the formation and/ or stability of TNFα and IL23. The reduction in TNFα production with the addition of TM to LPS-stimulated cells was more marked after 4 hours incubation compared to 24 hours. The study by Goodall et al measured IL23 in the cell culture supernatants after 48 hours incubation and perhaps after this time, the adverse effect of TM on cytokine production was attenuated. It was decided to assess this further after testing more samples and also analyse these cytokines at both the protein and RNA level to avoid any adverse post-translational effects of TM on cytokine production.

Having established the protocol for the MDM assay, initial experiments were undertaken to analyse the induction of the pro-inflammatory response with LPS stimulation using phosphorylation of p38 MAPK as the output. Although these experiments did not demonstrate clear differences between patients and healthy controls over all, two groups of patients emerged that would be of potential interest for future experiments. Firstly, patients who were HLA B27 positive appeared to exhibit enhanced phosphorylation of p38 compared to those who were HLA B27 negative and healthy controls although this was not statistically significant. This was interesting in view of previous work by Sahlberg et al suggesting that the p38 pathway may not function properly in the presence of HLA B27 heavy chains (Sahlberg, Penttinen et al. 2007). The same group also demonstrated that the phosphorylation of STAT1 was enhanced in cells expressing HLA B27, thus predisposing these cells to a more inflammatory phenotype and suggesting a possible mechanism for MDMs from HLA B27 positive patients to exhibit an enhanced inflammatory profile (Ruuska, Sahlberg et al. 2012). The second group of patients exhibiting increased phosphorylation of p38 MAPK was those with active enthesitis. This was again interesting as there is some evidence from animal models that p38 MAPK is involved in chondrogenesis and ankylosing enthesitis (Greenblatt, Shim et al. 2010, Braem, Luyten et al. 2012). Approximately 70% of patients with active enthesitis were also HLA B27 positive and so this did not fully explain the result. It was thought that because those patients with active enthesitis had active disease, their MDMs may simply exhibit an enhanced inflammatory response thus explaining the enhanced phosphorylation of p38 MAPK. However, there did not appear to be other clinical features that were associated with enhanced phosphorylation of p38 MAPK

and CRP did not correlate with this. It was therefore decided that this group of patients with enthesitis would be of interest to study in future experiments.

In view of the p38 MAPK results, it was decided that a panel of pro-inflammatory cytokines should be studied in the cell culture supernatants from MDMs from patients with ERA stimulated with LPS. The proteome profiler allowed the analysis of a large number of cytokine and chemokines, thus complementing the preliminary luminex data and allowing a final list of analytes to be selected that were upregulated by stimulation with LPS with or without TM. The majority of these were prototypical innate inflammatory cytokines already implicated in the pathogenesis of adult SpA (Smith 2015) but not previously studied in MDMs in ERA. The IL23 family of cytokines were of particular interest because of their importance in the aetiology of SpA (Gaston, Goodall et al. 2011). Some cytokines, in particular IL17, were more difficult to detect in cell culture supernatants and it was therefore decided to analyse the production of these cytokines using RNA in addition. This was also necessary because of the potential effect of TM on cytokine production.

In summary this chapter describes the process of determining the optimal method for differentiating MDMs and the subsequent optimisation of the MDM activation assay for the experiments planned for this thesis. Despite this optimisation, it is acknowledged that as an in vitro method, this is an artificial system and because of the necessary additions to the MDM assay and the time taken to differentiate MDMs, the results need to be interpreted with caution. In vitro-based assays are widely used for the study of macrophages but significant variation is noted depending on many factors including the time points for measuring outputs and activation methods for the assay (Chamberlain, Holt-Casper et al. 2015). Studies have also demonstrated differences in cytokine production depending on the biomaterial utilised in culture and thus it is likely that MDMs undergo phenotypic changes with in vitro culture that would not occur in vivo (Jones, Chang et al. 2007). However, in view of the difficulty obtaining and culturing tissue macrophages, this in vitro system was a useful way of comparing innate immune responses between patients with ERA and healthy volunteers in addition to investigating the production of IL23 and other pro-inflammatory cytokine in MDMs from these groups. The experiments described in this chapter began to establish the important outputs for subsequent experiments presented in this thesis, and also began to identify subgroups of patients of interest for future study.

CHAPTER 4: IL23 AND RELATED CYTOKINES IN MONOCYTE-DERIVED MACROPHAGES FROM PATIENTS WITH ERA

Overview of Chapter 4

This chapter focuses on the findings from the MDM assay for the IL23 family of cytokines and the potential effect of clinical features and treatments on this output.

The first section describes the results from LPS-stimulated MDMs for the gene expression of the IL23 subunits IL23p19 and IL12/23p40 RNA in patients with ERA compared to healthy controls. This section also includes data from SFMCs in HLA B27 positive patients with ERA compared to a HLA B27 negative disease control group.

Following this, IL23 production at the protein level from LPS-stimulated MDMs from patients with ERA compared to healthy controls is described. These results are compared to a disease control group of patients with polyarticular JIA and adult patients with AS. This part of the chapter also includes subgroup analyses of patients with different clinical features and treatment regimes.

Finally, results from the MDM assay for other cytokines within this family are described including IL12 and IL27 protein production from cell culture supernatants and expression of IL12p35 RNA.

Hypothesis for Chapter 4

The gene expression of IL23p19 and production of IL23 from MDM from patients with ERA will be similar to that of adult patients with AS and higher than healthy controls.

4.1 IL23 gene expression from LPS-stimulated MDMs form patients with ERA and healthy controls

4.1.1 Introduction

IL23 was a key cytokine to study in ERA because of the central role of the IL23/17 axis in the pathogenesis of AS (Jethwa and Bowness 2016). IL23 is produced by activated antigen presenting cells such as macrophages and dendritic cells and has been implicated in maintaining chronic inflammation by promoting the expansion and survival of pathogenic Th17 cells and thus the expression of IL17A and F, IL22, IL21 and GMCSF (McGeachy, Chen et al. 2009, Lubberts 2015). IL23 is also key in the pathogenesis of enthesitis (Sherlock, Joyce-Shaikh et al. 2012). It is composed of a p19 subunit, unique to IL23 and a p40 subunit which is shared with IL12 (Oppmann, Lesley et al. 2000). The active form of IL23 consists of both the p19 and p40 subunit. The expression of the p19 subunit is tightly regulated and this controls the secretion of the active heterodimeric form of IL23 (Croxford, Mair et al. 2012).

Initial experiments were carried out to analyse gene expression by measuring the expression of both the IL23p19 and IL12/23p40 subunits by qPCR of RNA following stimulation of MDMs with LPS from a group of patients with ERA and age-matched healthy controls. These were preliminary experiments to identify potential differences in IL23 gene expression between MDMs from patients with ERA and healthy controls and therefore smaller numbers were tested from the final group of patients whose samples were analysed for cytokine expression at the protein level. This subgroup of patients had similar demographics and clinical characteristics to the larger final group.

4.1.2 IL23p19 gene expression correlates with IL12/23p40 gene expression in LPS-stimulated MDMs in patients with ERA

Following stimulation of MDMs with LPS for 24 hours, RNA was extracted and transcribed in to cDNA as described in section 2.5. qPCR was then performed and the results for IL23p19 and IL12/23p40 gene expression were normalised against GAPDH, after which a fold change was calculated relative to a control (unstimulated) sample for each patient and healthy control.

Firstly, analysis of IL23p19 and IL12/23p40 gene expression was undertaken to determine whether there was a correlation in the expression of these subunits from LPS-stimulated MDMs. Using Spearman's rank correlation coefficient, a significant correlation was seen in between expression of IL23p19 and IL12/23p40 from LPS-stimulated MDMs ($r=0.67$, $p=0.0002$, figure 4.1 A). However, no such correlation was seen in LPS-stimulated MDMs from healthy controls ($r=0.4$, $p=0.12$, figure 4.1 B).

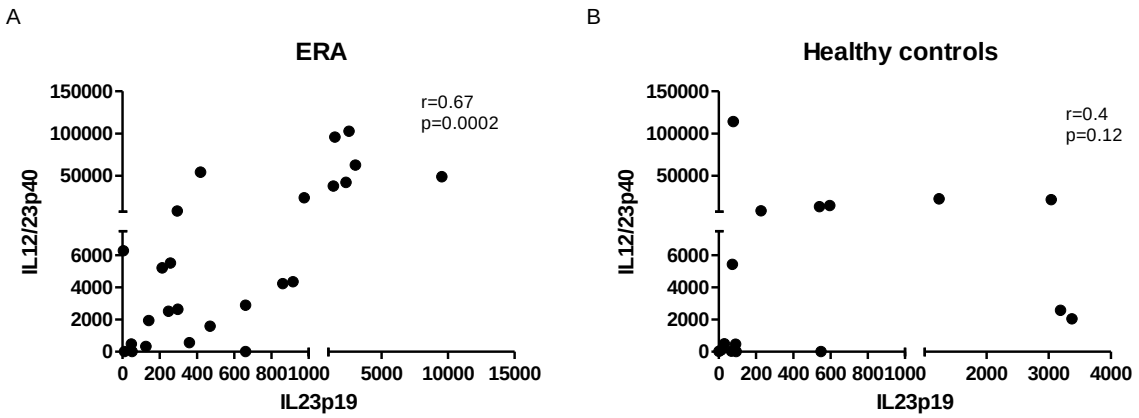


Figure 4.1 Correlation between IL23p19 and IL12/23p40 gene expression in LPS-stimulated MDMs from patients with ERA. RNA was isolated from MDMs which had been stimulated with LPS for 24 hours and transcribed in to cDNA. IL23p19 and IL12/23p40 genes were amplified using qPCR and normalised against a housekeeping gene (GAPDH). Fold change was calculated by comparing the expression of IL23p19 or IL12/23p40 from LPS-stimulated MDMs to the control (unstimulated) sample for each patient and healthy control. Graph A shows Spearman's rank correlation of IL23p19 expression vs IL12/23p40 expression for patients with ERA (n=25) and graph B for healthy controls (n=16).

4.1.3 Trend towards increased IL23p19 gene expression in patients with ERA compared to healthy controls

Next, the gene expression of IL23 p19 and IL12/23p40 was compared between LPS-stimulated MDMs from patients with ERA and age and gender-matched healthy controls. There was a trend towards higher gene expression of IL23p19 in LPS-stimulated MDMs from patients with ERA as compared to healthy controls (median fold change 445.0, IQR 137.1-1515 vs 91.0, IQR 58.91-560.6, p=0.06, figure 4.2A). There was no significant difference in expression of IL12/23p40 from LPS-stimulated MDMs from patients with ERA compared to healthy controls (median fold change 4360, IQR 1072-40125 vs 2311, IQR 46.77-14471, p=0.33), although the median was higher in patients with ERA but there was significant variability between sample results for patients and healthy controls (figure 4.2B).

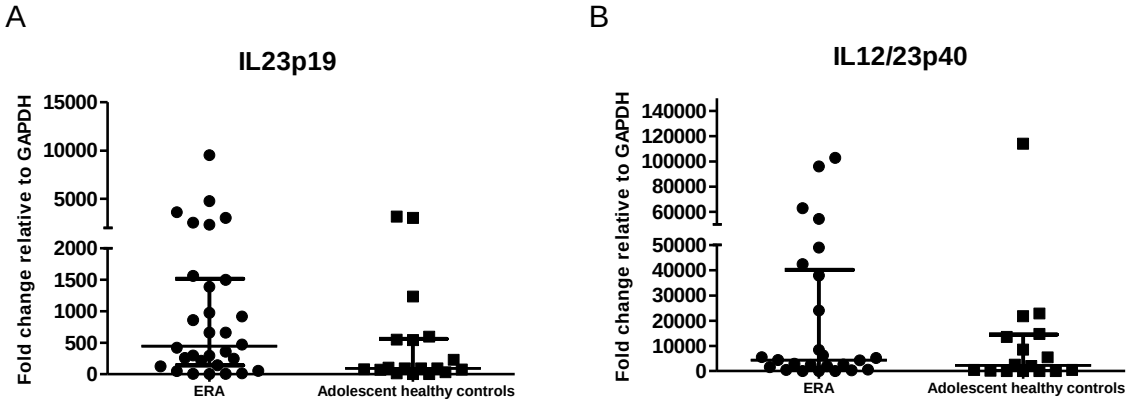


Figure 4.2 Trend towards higher IL23p19 gene expression in patients with ERA compared to healthy controls. RNA was isolated from MDMs stimulated with LPS for 24 hours and transcribed in to cDNA. IL23p19 and IL12/23p40 were amplified using qPCR and normalised against a housekeeping gene (GAPDH). Fold change was calculated by comparing the expression of IL23p19 or IL12/23p40 from LPS-stimulated MDMs to the control (unstimulated) sample for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann-Whitney U test (IL23p19: ERA n=30, adolescent healthy controls n=18, IL12/23p40: ERA n=25, adolescent healthy controls n=16).

4.1.4 IL23p19 and IL12/23p40 gene expression is not significantly affected by the presence of active enthesitis or HLA B27 status in LPS-stimulated MDMs

In view of the findings in section 3.3.2 showing enhanced phosphorylation of p38 MAPK in patients with enthesitis and a trend towards enhanced p38 MAPK phosphorylation in patients who were HLA B27 positive, analysis of both IL23p19 and IL12/23p40 in patients with these clinical features was undertaken.

No difference was found in IL23p19 expression (median fold change 444, IQR 159.2-1368 vs 638, IQR 5.93-1811) or IL12/23p40 expression (median fold change 4240, IQR 474.4-42348 vs 5327, IQR 2125-54136) from LPS-stimulated MDMs in patients with ERA with and without active enthesitis (n=20 vs n=10) or between these groups and healthy controls. Similarly, no significant difference in IL23p19 expression was observed between patients who were HLA B27 positive (median fold change 471.1, IQR 169.6-2060) compared to those who were HLA B27 negative (median 418.8, IQR 73.7- 946.0). However, there was a strong trend towards increased IL23p19 expression in patients who were HLA B27 positive compared to healthy controls, although this did not reach statistical significance (p=0.051, figure 4.3 A). In those patients where IL23p19 expression was very high (>10 times the median, n=4), all had axial ERA and were HLA B27 positive with either active axial or peripheral arthritis or enthesitis at the time of sample. There was no difference in IL12/23p40 expression from LPS-stimulated MDMs in patients with ERA who were HLA B27 positive compared to those who were HLA B27 negative (figure 4.3 B, table 4.2).

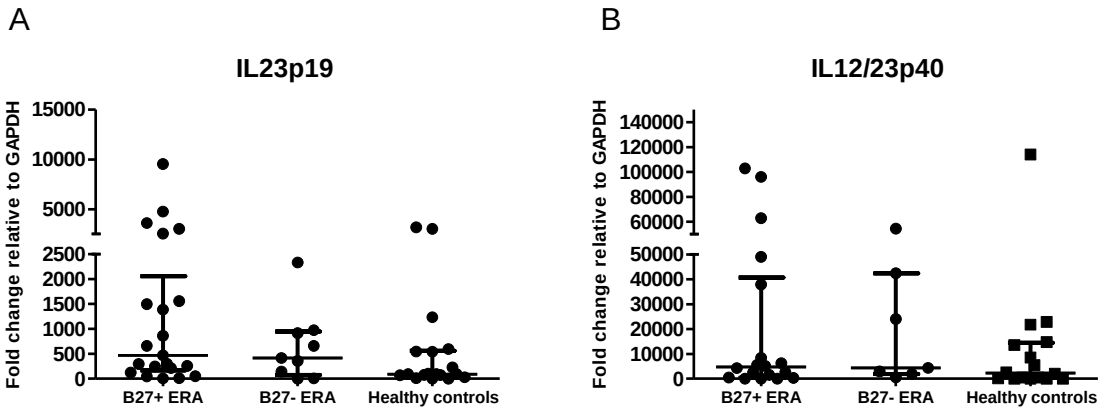


Figure 4.3 Trend towards higher IL23p19 expression in HLA B27 positive patients with ERA compared to healthy controls. RNA was isolated from MDMs stimulated with LPS for 24 hours and transcribed in to cDNA. IL23p19 and IL12/23p40 were amplified using qPCR and normalised against GAPDH. Fold change was calculated by comparing the expression of IL23p19 or IL12/23p40 from LPS-stimulated MDMs to the control (unstimulated) sample for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test groups (IL23p19: B27+ ERA n=21, B27- ERA n=9; IL12/23p40: B27+ ERA n=18, B27- ERA n=7, adolescent healthy controls n=18).

4.1.5 Disease activity and phenotype do not significantly affect gene expression of IL23p19 and IL12/23p40 in LPS-stimulated MDMs from patients with ERA

In view of the finding that the four patients with highest IL23p19 expression had axial arthritis and active disease at the time of sample, it was important to determine whether disease activity or other clinical features were associated with enhanced IL23p19 or IL12/23p40 expression. The JIA core variables collected allowed calculation of the Juvenile Arthritis Disease Activity Score (JADAS) (Consolaro, Ruperto et al. 2009) which calculates disease activity as inactive, low, medium or high based on physician's global assessment of disease activity, patient global assessment of well-being, ESR or CRP and active joint count. However, this score does not capture certain features associated with ERA well such as axial disease and enthesitis (Weiss, Colbert et al. 2014)and therefore these were assessed separately. Insufficient clinical information was available to calculate the JSpADA.

No significant differences were seen across the JADAS disease activity categories for either IL23p19 or IL12/23p40 expression (summary of results in table 4.2). However, this analysis was underpowered with small group sizes especially for the high and inactive disease groups and significant variability between patients (figure 4.4 A and B). Similarly, when comparing gene expression of both IL23p19 and IL12/23p40 in those with axial ERA to those with ERA and peripheral arthritis only or those with indeterminate ERA (who had some features of spinal or sacroiliac joint inflammation seen on MRI scan but insufficient to diagnose axial ERA), no significant difference was observed (table 4.2, figure 4.4 C and D). This analysis was also underpowered because of the small group sizes especially in those with peripheral ERA (n=5).

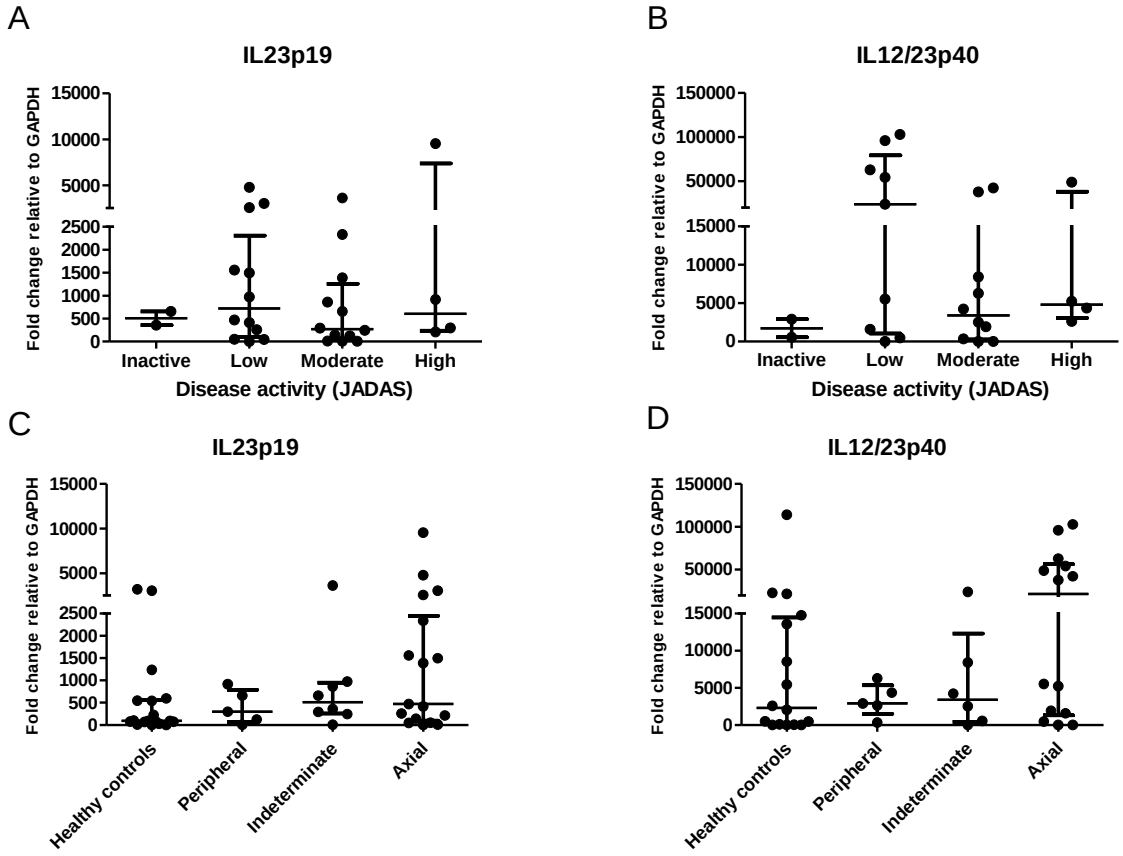


Figure 4.4 Disease activity and axial disease are not associated with significant differences in IL23p19 or IL12/23p40 gene expression in patients with ERA. RNA from LPS-stimulated MDMs was transcribed in to cDNA and amplified using qPCR. Fold change of IL23p19 and IL12/23p40 was calculated after normalisation with GAPDH, compared to a control sample for each patient/ healthy control. Box and whiskers plots show median with range (minimum to maximum), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test groups (Disease activity groups: IL23p19: inactive n=2, low n=12, moderate n=12, high n=4; IL12/23p40: inactive n=2, low n=9, moderate n=10, high n=4; IL23p19: peripheral ERA n=5, indeterminate ERA n=8, axial ERA n=17; IL12/23p40: peripheral ERA n=5, indeterminate ERA n=8, axial ERA n=17, adolescent healthy controls n=18 (IL23p19) and n=16 (IL12/23p40).

4.1.6 Gene expression of IL23p19 from LPS-stimulated MDMs is lower in patients with ERA and hip arthritis and higher in males with ERA compared to healthy controls

Interestingly, a trend towards lower IL23p19 gene expression was observed in those with hip arthritis (either active or historical) (median fold change 277.8, IQR 50.9-930.1 vs 861.1, IQR 270.7-2798) and gene expression in those without hip arthritis was higher than that of healthy controls (p=0.01, figure 4.5 A). Other clinical features such as active peripheral arthritis at the time of sample were not associated with enhanced IL23p19 or IL12/23p40 expression (table 4.2). In addition, there was no correlation with age, disease duration or CRP and IL23p19 or IL12/23p40 expression.

However, it was noted that IL23p19 expression was significantly higher in male patients with ERA compared male healthy controls (median fold change 661.7, IQR 133.2-1531 vs 90.51, IQR 40.98-386.8, p=0.04, figure 4.5 B). IL23p19 expression from LPS-stimulated MDMs was not significantly different in male patients with ERA compared to female patients, although the female group was small (n=5) (median fold change 298.2, IQR 134.5-1988). Similarly, there was no significant difference in IL12/23p40 gene expression from LPS-stimulated MDMs in male patients compared to female patients (median fold change 5221, IQR 1761-45666 vs 1604, IQR 142.6-4800) or healthy controls (median fold change 2048, IQR 30.7-14766).

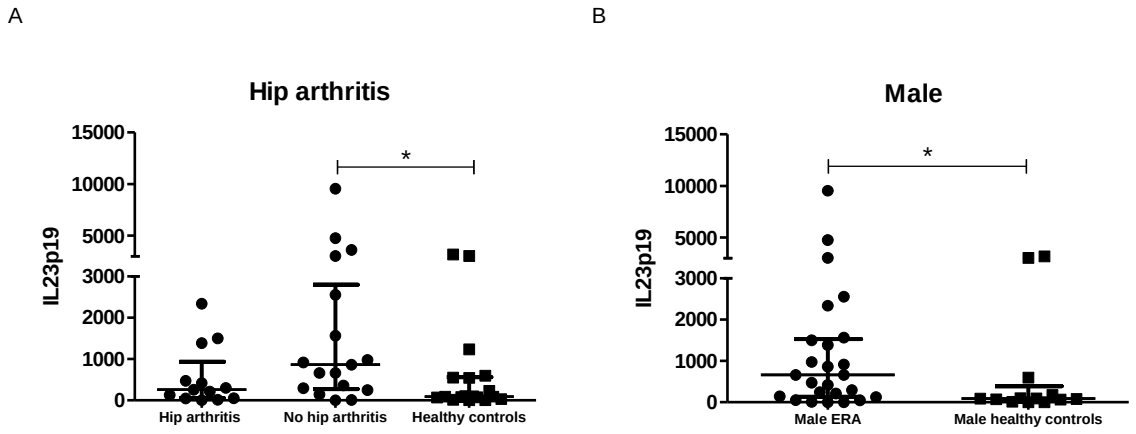


Figure 4.5 IL23p19 gene expression is lower in those with hip arthritis and higher in males with ERA compared to healthy controls. RNA from LPS-stimulated MDMs was transcribed in to cDNA and amplified using qPCR. Fold change of IL23p19 was calculated after normalisation with GAPDH, compared to a control sample for each patient/ healthy control. Box and whiskers plots show median with range (minimum to maximum), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test groups or Mann Whitney U test as appropriate (hip arthritis n=13, no hip arthritis n=17, adolescent healthy controls n=18, male patients with ERA n=25, male adolescent healthy controls n=13) (*p<0.05).

4.1.7 Enhanced IL23p19 gene expression in LPS-stimulated MDMs from patients with ERA on TNF inhibitors

To determine whether treatment had any effect on IL23p19 expression, fold change of IL23p19 and IL12/23p40 were analysed for patients receiving NSAIDs, DMARDs and TNF inhibitors. There was no difference seen in expression of IL23p19 or IL12/23 p40 in patients on NSAIDs compared to those not taking them (results summarised in table 4.2). In patients taking DMARDs (alone (n=11) and in combination with TNF inhibitors (n=11)), there was a trend towards higher IL23p19 and IL12/23p40 expression in those not taking DMARDs compared to those who were taking them (median fold change IL23p19: 1153, IQR 431.9-3473 vs 296.1, IQR 107.1-1106, p=0.09; median fold change IL12/23p40: 43443, IQR 3666-56479 vs 2896, IQR 474.7-8422, p=0.09) and a significant difference was noted between those not on DMARDs and healthy controls for both IL23p19 (p=0.04) and IL12/23p40 (p=0.04)(figure 4.6 A and B).

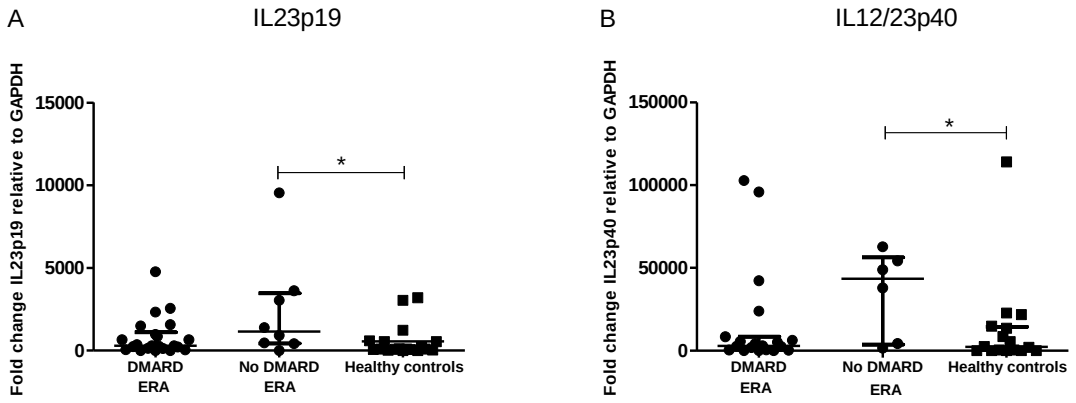


Figure 4.6 Trend towards enhanced IL23p19 and IL12/23p40 gene expression from LPS-stimulated MDMs in patients with ERA not on DMARDs. RNA was isolated from LPS-stimulated MDMs was transcribed in to cDNA. IL23p19 and IL12/23p40 were amplified using qPCR and normalised against GAPDH. Fold change was calculated by comparing the expression of IL23p19 or IL12/23p40 from LPS-stimulated MDMs to the control (unstimulated) sample for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test groups (*p<0.05) (IL23p19: DMARD treated ERA n=22, ERA not on DMARDs n=8 (7 were on either no treatment or NSAIDs only, 1 was on a TNF inhibitor only), adolescent healthy controls n=18; IL12/23p40: DMARD treated ERA n=19, ERA not on DMARDs n=6 (5 on no treatment or NSAIDs only and1 was on a TNF inhibitor only , adolescent healthy controls n=16).

Conversely, expression of IL23p19 was significantly higher in patients on TNF inhibitors compared to healthy controls (median fold change 861.1, IQR 303.4-2798, p=0.0073) and a trend was seen compared to patients not taking TNF inhibitors, although this did not reach statistical significance (median fold change 294.1, IQR 31.79-946.0, p=0.06) (figure 4.7). There was no significant difference in IL12/23p40 expression between those treated with TNF inhibitors and those not taking them or between those on TNF inhibitors and healthy controls (table 4.2).

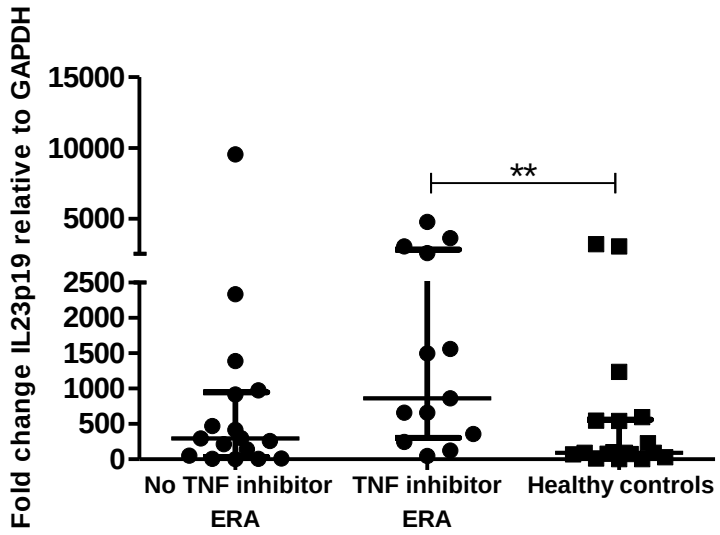


Figure 4.7 Trend towards enhanced IL23p19 gene expression in LPS-stimulated MDMs from patients with ERA treated with TNF inhibitors compared to those on other treatments. IL23p19 expression was analysed from RNA from MDMs using qPCR and normalised against GAPDH. Fold change was calculated relative to an unstimulated control for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test groups (**p<0.01) (TNF inhibitor treated ERA n=13, ERA not on TNF inhibitor n=17, adolescent healthy controls n=18).

4.1.8 IL23p19 and IL23p40 in SFMCs from patients with ERA and oligoarticular JIA

To determine whether the trends observed with IL23p19 and IL12/23p40 in LPS-stimulated MDMs from patients with ERA were mirrored in an ex vivo setting, IL23p19 and IL12/23p40 expression was measured from SFMCs using qPCR. SFMCs from HLA B27 positive patients with ERA were isolated from synovial fluid collected at the time of knee aspiration (as described in section 2.2.6). Ten samples from patients with ERA were identified, five of these also had MDMs cultured from PBMCs but the sample dates were different. It was decided, in the absence of a suitable non-inflammatory control group, to compare this group to a group of patients with persistent oligoarticular and extended oligoarticular JIA who were HLA B27 negative. As with the MDM assay, the proportion of male patients in the ERA group was higher (80% vs 30%). Five patients in with oligoarticular JIA and six with ERA were on DMARDs but only three patients were on TNF inhibition (two in the ERA group and one in the oligoarticular JIA group).

IL23p19 expression in SFMCs from HLA B27 positive patients with ERA was higher than expression from patients with oligoarticular JIA but this did not reach statistical significance (median fold change 13.5, IQR 5.77-24.01 vs 5.02, IQR 3.87-13.16, p=0.11, figure 4.8 A). Expression of IL12/23p40 was lower than IL23p19 and not significantly different between the two groups (median fold change 1.68, IQR 0.82-3.14 vs 2.69, IQR 1.21-4.27, figure 4.8 B). There was no difference in IL23p19 expression between male and female patients across both groups and numbers were too small to subdivide the ERA and oligoarticular JIA groups by gender or treatment. The patients all had active peripheral arthritis as they required joint aspiration and therefore it was not possible to differentiate them by disease activity. Only three of the patients with ERA had definite axial disease at the time the sample was taken and five patients had active enthesitis, although not at the site of joint aspiration. It was therefore not possible to undertake further subgroup analysis on these samples but it would be of interest to expand the numbers for future experiments.

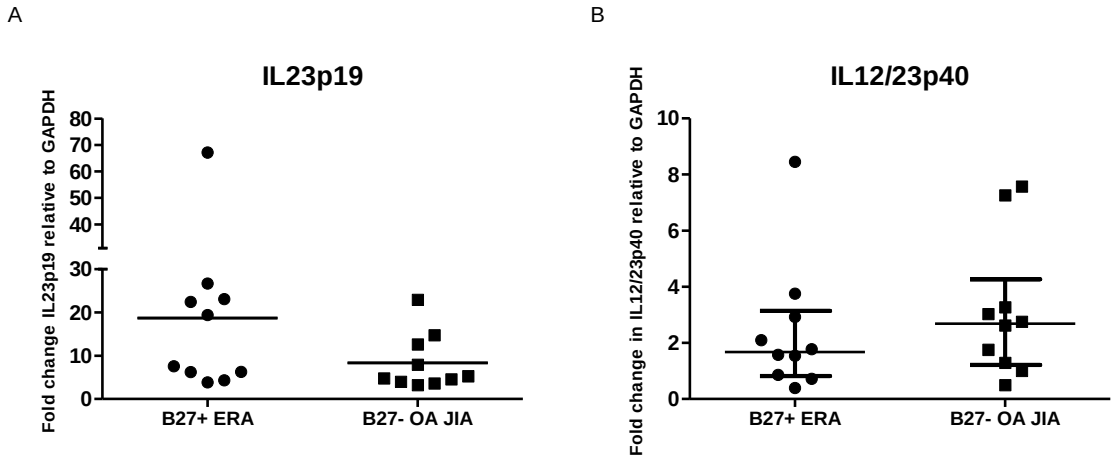


Figure 4.8 IL23p19 and IL12/23p40 gene expression are not significantly different in SFMCs in HLA B27 positive patients with ERA compared to HLA B27 negative patients with oligoarticular JIA. RNA was extracted from SFMCs and transcribed in to cDNA. IL23p19 and IL12/23p40 expression were analysed using qPCR and normalised against GAPDH. Fold change was calculated relative to a control value (the average ΔCt of the lowest ERA and oligoarticular JIA sample) in the absence of a non-inflammatory control sample. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann Whitney U test (HLA B27 positive ERA n=10, HLA B27 negative oligoarticular (OA) JIA n=10).

4.2 IL23 in cell culture supernatants from LPS-stimulated MDMs

4.2.1 Introduction

While gene expression of the individual subunits of IL23 gave an indication of IL23 expression in MDMs at a particular time point (in this case 24 hours after stimulation with LPS), it was important to verify that this translated in to secretion of the heterodimeric active form of IL23. Therefore, the next step was to analyse IL23 production from MDMs following LPS stimulation to determine whether the gene expression of IL23p19 and IL12/23p40 was mirrored by IL23 protein levels. These experiments were carried out on samples from the larger final group of patients with ERA and also in patients with polyarticular JIA, ankylosing spondylitis in addition to the adolescent and adult healthy controls.

4.2.2 IL23 is elevated in cell culture supernatants from MDMs from patients with ERA compared to healthy controls

The active form of IL23 was measured by ELISA in cell culture supernatants from the MDM assay. IL23 was not detectable in MDMs not stimulated with LPS. Following LPS stimulation for 24 hours, higher levels were found from MDMs from patients with ERA compared to healthy controls (median: 53580 pg/mL, IQR 35735-83945 pg/mL vs 32110 pg/mL, IQR 13745-48235 pg/mL, p=0.01) and also from patients with polyarticular JIA (median: 51340 pg/mL, IQR 36390-77830 pg/mL, p=0.04) compared to healthy controls. MDMs from patients with AS also produced higher levels of IL23 compared to adult healthy controls (median: 53835 pg/mL, IQR 35305-80648 pg/mL vs 34180 pg/mL, IQR 16628-49403 pg/mL, p=0.03) (figure 4.9). It was noted that 10 patients (6 with ERA, 3 with polyarticular JIA and 1 with AS) had very high levels of IL23 production (>100000 pg/mL) following LPS stimulation and therefore further analysis was undertaken to identify features associated with very high IL23 levels. Interestingly, only one of these patients with ERA also had very high levels of IL23p19 expression.

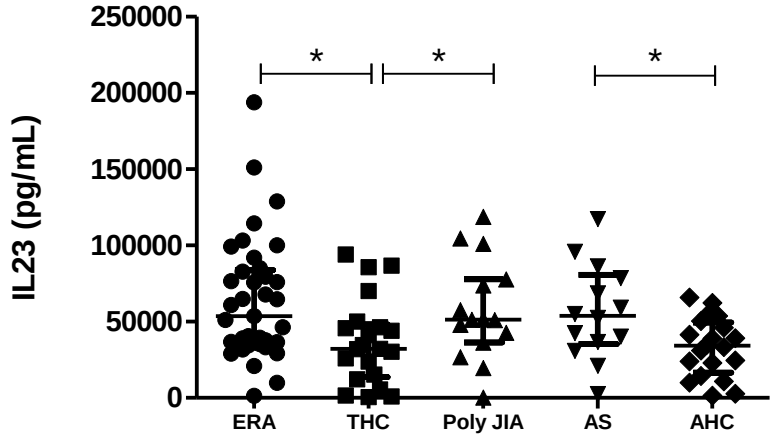


Figure 4.9 Patients with inflammatory arthritis produce higher levels of IL23 from LPS-stimulated MDMs compared to healthy controls. MDMs were differentiated as described in section 2.2 and stimulated with LPS for 24 hours. IL23 was measured in cell culture supernatants by ELISA. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (*$p<0.05$) (ERA n=37, THC=teenage healthy controls n=21, Poly JIA=polyarticular JIA, n=15, AS n=14, AHC=adult healthy controls n=21).

4.2.3 Higher IL23 production is associated with HLA B27 and enthesitis

In view of the findings in section 3.3.2 showing enhanced phosphorylation of p38 MAPK in patients with enthesitis and a trend towards enhanced p38 MAPK phosphorylation in patients who were HLA B27 positive, analysis of IL23 levels in patients having these features was undertaken first . In patients with ERA, LPS-stimulated MDMs from those who were HLA B27 positive expressed significantly higher levels of IL23 compared to healthy controls (median 64650 pg/mL, IQR 36400-99650 pg/mL vs 32110 pg/mL, IQR 13745-48235 pg/mL, p=0.0067) but not significantly more than those who were HLA B27 negative (45130 pg/mL, IQR 32645-76413 pg/mL) (figure 4.10 A). LPS-stimulated MDMs from patients with ERA with active enthesitis at the time their sample was taken, also produced significantly higher levels of IL23 compared to healthy controls (median 75905 pg/mL, IQR 36728-99425 pg/mL, p=0.001) and those without active enthesitis (median 38425 pg/mL, IQR 29923-63725 pg/mL, p=0.014) (figure 4.10 B). Interestingly all 6 patients with ERA with very high IL23 levels from their LPS-stimulated MDMs (>100000 pg/mL) had active enthesitis and were HLA B27 positive.

In patients with AS, there was insufficient data recorded to compare those with and without enthesitis and HLA B27 results were not available in 7 patients (50%). Interestingly, in patients with polyarticular JIA who were all HLA B27 negative, those who's MDMs produced very high levels of IL23 following LPS stimulation (>100000 pg/mL, n=3) were documented as having enthesitis on the day their sample was taken.

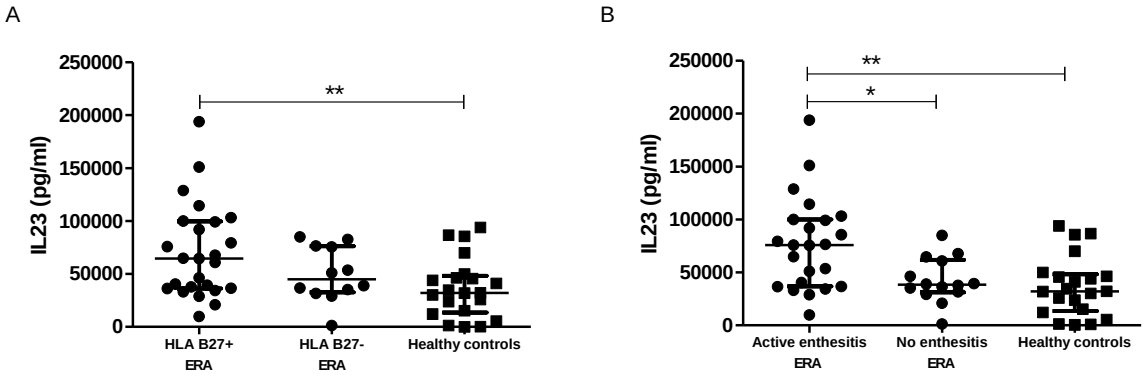


Figure 4.10 LPS-stimulated MDMs from patients with ERA who are HLA B27 positive and have active enthesitis produce more IL23 compared to healthy controls. MDMs were differentiated as described in section 2.2 and stimulated with LPS for 24 hours. IL23 was measured from cell culture supernatants by ELISA. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (HLA B27 positive ERA n=25, HLA B27 negative ERA n=12, ERA with active enthesitis n=23, ERA with no enthesitis n=14, adolescent healthy controls n=21 *p<0.05, **p<0.01).

4.2.4 Analysis of IL23 production and other clinical features including disease activity, arthritis and ERA subtype

Next, IL23 levels from LPS-stimulated MDMs were analysed in relation to disease activity and other clinical features in patients with ERA. As discussed above, insufficient data was collected to calculate the JSpADA index and therefore the JADAS was used as an indication of disease activity. Data on axial arthritis and enthesitis was analysed separately. There was no significant difference between the JADAS disease activity categories for patients with ERA (results summarised in table 4.3), although the groups with inactive disease and high disease activity were small (n=2 and n=6 respectively) making meaningful comparison difficult. A significant difference was seen between those with low disease activity (median 64650 pg/mL, IQR 35210-79380 pg/mL) and healthy controls (p=0.04) and also between those with moderate disease activity (median 52350 pg/mL, IQR 35523-94808 pg/mL) and healthy controls (p=0.03, figure 4.11 A).

With regard to ERA subtype, IL23 levels from LPS-stimulated MDMs from patients in both the axial ERA and indeterminate ERA (with some features of axial involvement but not sufficient to be diagnostic of sacroiliitis or inflammatory spinal disease) categories were higher than healthy controls (median 53580 pg/mL, IQR 35210-79380 pg/mL, p=0.03 and 75790 pg/mL, IQR 36225-110455 pg/mL, p=0.04 respectively, table 4.11 B). However, there was no significant difference between the ERA subtype groups or between those with peripheral ERA and healthy controls (table 4.3), although this group was small (n=5).

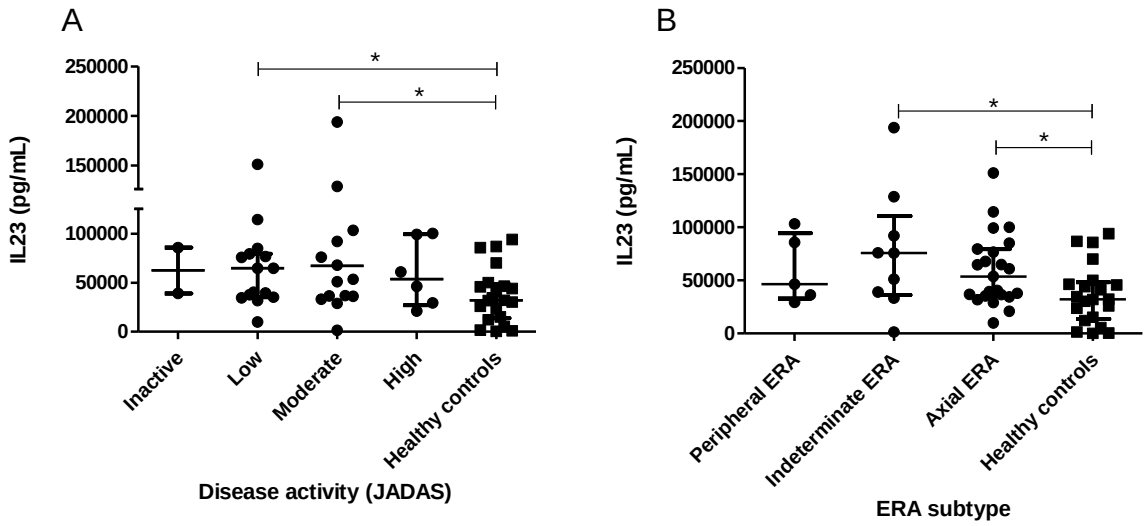


Figure 4.11 IL23 production from MDMs from patients with ERA is not significantly different between those with peripheral ERA and axial ERA or between disease activity categories of the JADAS. MDMs were differentiated as described in the protocol above (section 2.2) and stimulated with LPS for 24 hours. IL23 was measured in cell culture supernatants by ELISA. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (peripheral ERA n=5, indeterminate ERA n= 9, axial ERA n=23, teenage healthy controls n=21, inactive ERA n=2, low disease activity n=15, moderate disease activity n=14, high disease activity n=6, *p<0.05).

Hip arthritis, a common feature of ERA and often associated with a poor prognosis (Jadon, Ramanan et al. 2013), was analysed separately but there was no significant difference between those who had a history of hip arthritis and those who did not (table 4.3), although levels from those with a history of hip arthritis were higher than healthy controls (p=0.03) which was in contrast to the results for IL23p19 expression. There was also no significant difference seen between patients with ERA with active peripheral arthritis at the time of sample compared to those without (table 4.3) but again, levels from those with active peripheral arthritis at the time of sample were higher than healthy controls (p=0.01).

There were similar findings for the patients with polyarticular JIA for peripheral arthritis and hip arthritis although the numbers in this group were much smaller than the ERA group. The JADAS is a better marker for disease activity in polyarticular JIA and LPS-stimulated MDMs from those in the moderate disease activity group expressed significantly higher levels of IL23 compared to healthy controls (median 73850 pg/mL, IQR 51445-111800 pg/mL, p=0.0076), although numbers in each group were small (inactive n=3, low n=4, moderate n=5, high n=3) and there was no overall difference in IL23 levels between the groups.

Patients with AS had predominantly axial disease and only one also had active peripheral arthritis at the time of sample. It was therefore not possible to compare IL23 production between subgroups with differing clinical features for the AS group. In addition, disease activity scores (BASDAI and BASFI) were only available in half the patients and therefore patients with AS could not be categorised according to disease activity.

Importantly, there was no correlation in IL23 levels from LPS-stimulated MDMs with CRP in any of the patient or healthy control groups. There were a small number of patients with extra-articular manifestations associated with ERA such as acute anterior uveitis and inflammatory bowel disease (n=7) and LPS-stimulated MDMs from these patients did not secrete higher levels of IL23 compared to those without extra-articular manifestations. Interestingly, LPS-stimulated MDMs from male patients with ERA produced more IL23 compared to male healthy controls (median 64650 pg/mL, IQR 36260-92010 pg/mL vs 36525 pg/mL, IQR 7135-49148 pg/mL, p=0.01) and female patients with ERA, although the number in this group was small (n=6) and this did not reach statistical significance (median 37840 pg/mL, IQR 32238-50918 pg/mL, p=0.11). This was also the case for male patients with AS, where IL23 levels from LPS-stimulated MDMs were higher than male healthy controls (median 57135 pg/mL, IQR 43343-84463 pg/mL vs 33500 pg/mL, IQR 16920-40380 pg/mL, p=0.0066) and also female patients with AS (median 39700 pg/mL, IQR 16538-80800 pg/mL) although again this did not reach statistical significance as the numbers were small (n=6).

Finally, analyses were undertaken to determine whether there was any correlation between either disease duration or age and level of IL23 production from MDMs in patients with ERA, polyarticular JIA or AS. Using Spearman's rank correlation coefficient, no such correlations were found.

4.2.5 Treatment with TNF inhibitors is associated with higher levels of IL23 production from LPS-stimulated MDMs

To determine the effect of treatment on IL23 production from LPS-stimulated MDMs from patients with ERA, analysis was undertaken of IL23 levels of patients taking NSAIDS, DMARDs and TNF inhibitors. No difference was found between those taking NSAIDs (table 4.3). Similarly, there was no difference in IL23 levels between those taking DMARDs and those not taking DMARDs (table 4.3) although there was a significant difference between those taking DMARDs and healthy controls (p=0.0041). A small number of patients had also had recent treatment with steroids (n=4 in the ERA group, n=5 in the polyarticular JIA group) but this did not significantly affect levels of IL23 production from MDMs.

In contrast, IL23 expression from LPS-stimulated MDMs from those with ERA on TNF inhibitors was significantly higher than those not on TNF inhibitors (median 70353 pg/mL, IQR 39380-97608 pg/mL vs 36790 pg/mL, IQR 30210-76620 pg/mL, p=0.04) and compared to healthy controls (p=0.0049) (figure 4.12 A). This was the case for both TNF inhibitors (etanercept and adalimumab) used for treatment in this group of patients with ERA with no significant difference between them. It was also noted that those on combination treatment with both a TNF inhibitor and a DMARD had significantly higher IL23 expression compared to those taking only a DMARD (median 85000 pg/mL, IQR 45290-108800 pg/mL vs 41535 pg/mL, IQR 33830-67570 pg/mL, p=0.02).

Interestingly, LPS-stimulated MDMs from patients with AS on TNF inhibitors also produced increased levels of IL23 compared to those not on TNF inhibition (median 72810 pg/mL, IQR 51925-101365 pg/mL(n=6) vs 38500 pg/mL, IQR 23613-64613 pg/mL (n=8), p=0.03) and healthy controls (p=0.0026) although numbers were small (figure 4.12 B). However, there was no difference in IL23 levels in those with polyarticular JIA on TNF inhibitors compared to those not on TNF inhibition, although again the numbers were small (median 58295 pg/mL, IQR 27334-105625 pg/mL (n=6) vs 513340 pg/ml, IQR 37370-67685 pg/mL (n=9)).

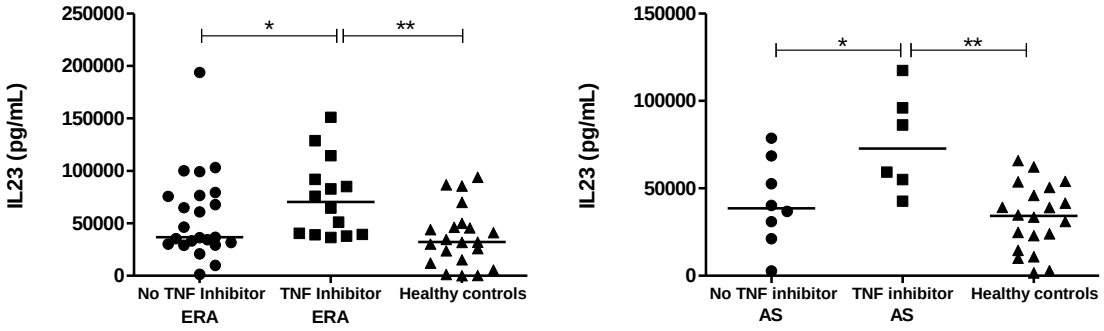


Figure 4.12 Treatment with TNF inhibitors is associated with higher IL23 production from MDMs from patients with ERA and AS compared to those not on TNF inhibition. MDMs were differentiated as above (section 2.2) and stimulated with LPS for 24 hours. IL23 was measured in cell culture supernatants by ELISA. Box and whisker plots show median (+minimum to maximum range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (TNF inhibitor treated ERA (n=14), ERA not treated with TNF inhibitor n=23, TNF inhibitor treated AS n=6, AS not treated with TNF inhibitor n=8, healthy controls were adolescent healthy controls for ERA (n=21) and adult healthy controls for AS (n=21), *$p<0.05$, **$p<0.01$).

4.3 Other cytokines in the IL23 family: IL12 and IL27

4.3.1 Introduction

The IL12/23 family of cytokines includes four heterodimeric cytokines linked by common subunits and receptors. IL23 and IL12 share the p40 subunit and are considered pro-inflammatory cytokines (Croxford, Kulig et al. 2014). One of the main functions of IL12 is the induction and differentiation of IFNγ-producing Th1 cells (Agarwal, Raghavan et al. 2009). IL27 and IL35 play a more immunoregulatory role but only IL27 is produced by antigen presenting cells whereas IL35 is produced by regulatory T and B cells (Vignali and Kuchroo 2012). IL27 has been implicated in the pathogenesis of SpA in genetic studies (Brown, Kenna et al. 2016) and with elevated levels in the serum of patients with AS (Lin, Lu et al. 2015). It exhibits an inhibitory effect by abrogating GMCSF production from T cells (Codarri, Gyulveszi et al. 2011) and by reducing induction of the inflammasome in dendritic cells (Mascanfroni, Yeste et al. 2013).

In view of the findings of elevated IL23 in cell culture supernatants from LPS-stimulated MDMs from patients with ERA compared to healthy controls, it was of interest to study other cytokines within this family. IL12 and IL27, the 2 other cytokines in this family known to be produced by macrophages were therefore analysed by Luminex assay and the results compared to the same group of age and gender-matched healthy controls.

4.3.2 IL27 secretion is significantly higher from MDMs from patients with ERA without LPS stimulation

Unlike IL23 and IL12, IL27 was detected in cell culture supernatants from MDMs without LPS stimulation. Following differentiation as described above in section 2.2 and the addition of IFNγ, these cells were washed and the media replaced. MDMs were then cultured for a further 24 hours but no additional stimuli were added. Levels of IL27 were significantly higher from MDMs from patients with ERA compared to healthy controls (median 393.0 pg/mL, IQR 312.3-482.6 pg/mL vs 315.9 pg/mL, IQR 207.3-368.9 pg/mL, p=0.0096) and patients with polyarticular JIA (median 315.2 pg/mL, IQR 271.9-357.1 pg/mL, p=0.01) (figure 4.13). The difference between patients with AS and adult healthy controls was not significant (median 347.9, IQR 259.1-447.2 pg/mL vs 312.3 pg/mL, IQR 199.3-327.5 pg/mL, p=0.1).

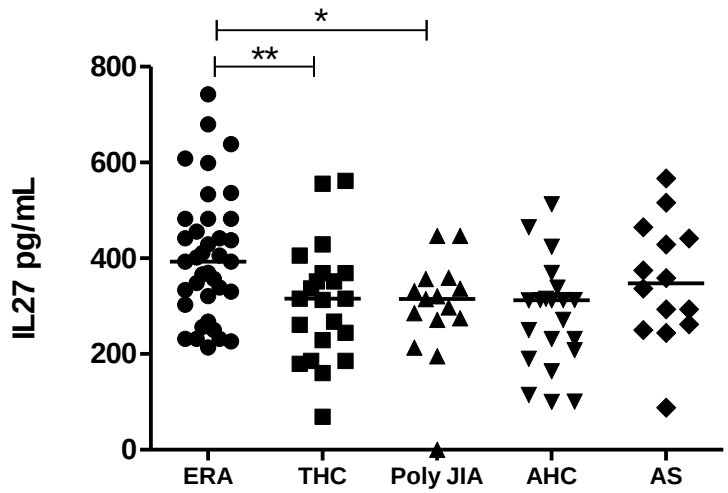


Figure 4.13 IL27 production from MDMs from patients with ERA is significantly higher than healthy controls and patients with polyarticular JIA. MDMs were cultured as per the protocol in section 2.2. The media was replaced and MDMs were cultured for a further 24 hours after which cell culture supernatants were collected and analysed by luminex assay. Box and whisker plots show median (+minimum to maximum range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA n=37, THC=teenage healthy controls n=21, polyarticular JIA n=15, AHC=adult healthy controls n=21, AS n=14, *$p<0.05$, **$p<0.01$).

4.3.3 IL27 production from MDMs without LPS stimulation: clinical features, disease activity and treatment

IL27 levels from MDMs without LPS stimulation were not significantly different in patients with active enthesitis compared to those without (median 404.5 pg/mL, IQR 333.8-482.6 pg/mL vs 357.1 pg/mL, IQR 258.5-442.3 pg/mL) although there was a significant difference between patients with enthesitis and healthy controls (p=0.0045). Similarly, there was no significant difference in IL27 levels from MDMs without LPS stimulation in patients who were HLA B27 positive compared to those who were HLA B27 negative (median 393.0 pg/mL, IQR 312.3-508.1 pg/mL vs 408.2 pg/mL, IQR 265.2-452.2 pg/mL).

In addition, no significant difference was seen between patients with axial ERA (median 357.1 pg/mL, IQR 256.2-455.7 pg/mL) compared to those with peripheral arthritis only (median 366.1 pg/mL, IQR 307.8-560.7 pg/mL), although some of the highest levels of IL27 were seen in the indeterminate category which was significantly different to healthy controls (median 437.8 pg/mL, IQR 381-603.7 pg/mL, p=0.004). High disease activity, measured according to JADAS (median 357.1, IQR 311.7-455.7 pg/mL, n=6), was not associated with increased IL27 levels and no significant difference was seen across the disease activity categories (moderate: median 421.7 pg/mL, IQR 263.2-615.8 pg/mL, n=14; low: median 357.1 pg/mL, IQR 303.3-455.7 pg/mL, n=15; inactive: median 405.5 pg/mL, IQR 369.7-441.4 pg/mL, n=2).

Treatment with TNF inhibition did not significantly affect levels of IL27 from MDMs without LPS stimulation from patients with ERA (median 402.0 pg/mL, IQR 337.8-608.2 pg/mL vs 379.5 pg/mL, IQR 254.7-462.5 pg/mL), although levels from patients on TNF inhibitors were significantly higher than healthy controls (p=0.0058). Treatment with DMARDs did not significantly affect IL27 production from MDMs without LPS treatment (median on treatment 402 pg/mL, IQR 303.3-482.6 pg/mL vs median off treatment 379.5 pg/mL, IQR 318.4-534.2 pg/mL).

4.3.4 Levels of IL12 and IL27 from LPS-stimulated MDMs are significantly higher from patients with ERA compared to healthy controls.

Next, analysis was undertaken of IL12 and IL27 from cell culture supernatants following LPS stimulation of MDMs for 24 hours. Both IL12 and IL27 were higher from MDMs from patients with ERA compared to healthy controls (IL12: median 3753 pg/mL, IQR 2055-7688 pg/mL vs 2733 pg/mL, IQR 1327-4309 pg/mL, p=0.037 and IL27: median 1986 pg/mL, IQR 1505-2268 pg/mL vs 1603 pg/mL, IQR 1121-1918 pg/mL, p=0.019). The median for polyarticular JIA for both IL12 and IL27 was higher than healthy controls (IL12: 4533 pg/mL, IQR 1683-6128 pg/mL and IL27: 1747 pg/mL, IQR 1356-2366 pg/mL) but this did not reach statistical significance because the range of results was wide. For IL12, LPS-stimulated MDMs from patients with AS also produced significantly higher levels compared to adult healthy controls (median 4134 pg/mL, IQR 2716-5794 pg/mL vs 2300 pg/mL, IQR 608.3-3579 pg/mL, p=0.01) but this was not the case for IL27, (figure 4.14 A and B).

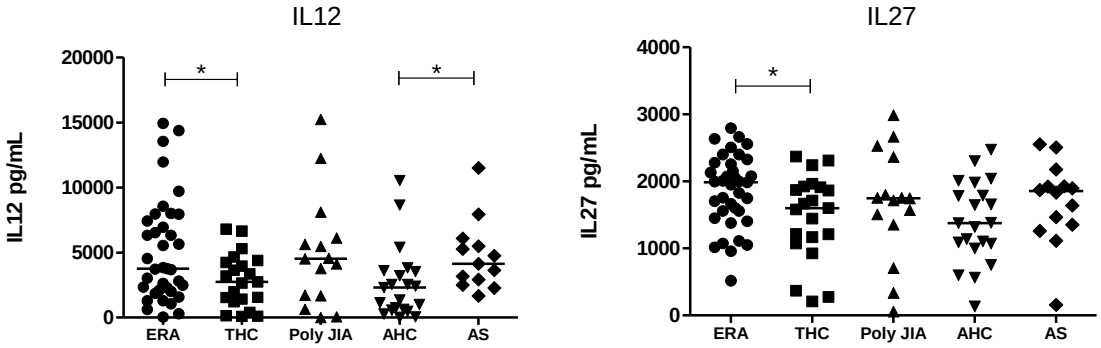


Figure 4.14 LPS-stimulated MDMs from patients with ERA secrete higher levels of IL12 and IL27 compared to healthy controls. MDMs were stimulated with LPS for 24 hours after which the cell culture supernatants were collected and analysed by Luminex assay. Box and whisker plots show median (+minimum to maximum range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA n=37, THC=teenage healthy controls n=21, polyarticular JIA n=15, AHC=adult healthy controls n=21, AS n=14, *$p<0.05$).

4.3.5 Effect of clinical features and disease activity on IL12 and IL27 production from LPS-stimulated MDMs from patients with ERA

Next analysis was undertaken to determine whether any clinical features or disease activity had an effect on IL12 or IL27 secretion from LPS-stimulated MDMs. As with IL23, IL12 and IL27 were produced at higher levels from patients with ERA who were HLA B27 positive compared to healthy controls (IL12: median 5646 pg/mL, IQR 2075-8289 pg/mL, p=0.017, IL27: median 1998 pg/mL, IQR 1574-2304 pg/mL, p=0.018). There was no significant difference for either cytokine between HLA B27 positive and negative patients (table 4.3, figure 4.15 A and B).

In patients with ERA and active enthesitis, IL27 production was significantly higher from LPS-stimulated MDMs compared to those with no enthesitis at the time of sample (median 2079 pg/mL, IQR 1666-2410 pg/mL vs 1632 pg/mL, IQR 1399-2000 pg/mL, p=0.04 and also healthy controls (p=0.0079, figure 4.15 D). IL12 was also higher from MDMs in those with active enthesitis compared to healthy controls (median 4543 pg/mL, IQR 2485-8013 pg/mL, p=0.018) and although secretion was higher in those with enthesitis compared to those without (median 3025 pg/mL, IQR 1287-5817 pg/mL), this did not reach statistical significance (figure 4.15 C).

In those with active peripheral arthritis at the time of sample, IL27 production from LPS-stimulated MDMs was significantly higher compared to those without active peripheral arthritis (median 2340 pg/mL, IQR 1823-2657 pg/mL vs 1828 pg/mL, IQR 1393-2105 pg/mL, p=0.015) and also healthy controls (p=0.0032, figure 4.15 F). However, no difference was seen in IL12 production between those with and without active peripheral arthritis (median 5988 pg/mL, IQR 2322-9141 pg/mL vs 3696 pg/mL, IQR 2055-7440 pg/mL) but again levels from those with active peripheral arthritis were higher than healthy controls (p=0.02, figure 4.15 E). A history of hip arthritis was not associated with higher levels of either IL12 or IL27 production from LPS-stimulated MDMs compared to those without a history of hip arthritis (IL12 median 3850 pg/mL, IQR 1957-7979 pg/mL vs 3739 pg/mL, IQR 2035-7156 pg/mL; IL27 median 1998 pg/mL, IQR 1559-2294 pg/mL vs 1792 pg/mL, IQR 1444-2274 pg/mL) but levels of IL27 were higher in those with hip arthritis compared to healthy controls (p=0.02, table 4.3).

No significant difference was seen in IL12 and IL27 levels between LPS-stimulated MDMs from patients with peripheral ERA compared to those with axial ERA or indeterminate ERA (table 4.3). Similarly, no significant difference was seen in IL12 or IL27 expression from LPS-stimulated MDMs between the JADAS disease activity categories for patients with ERA (table 4.3).

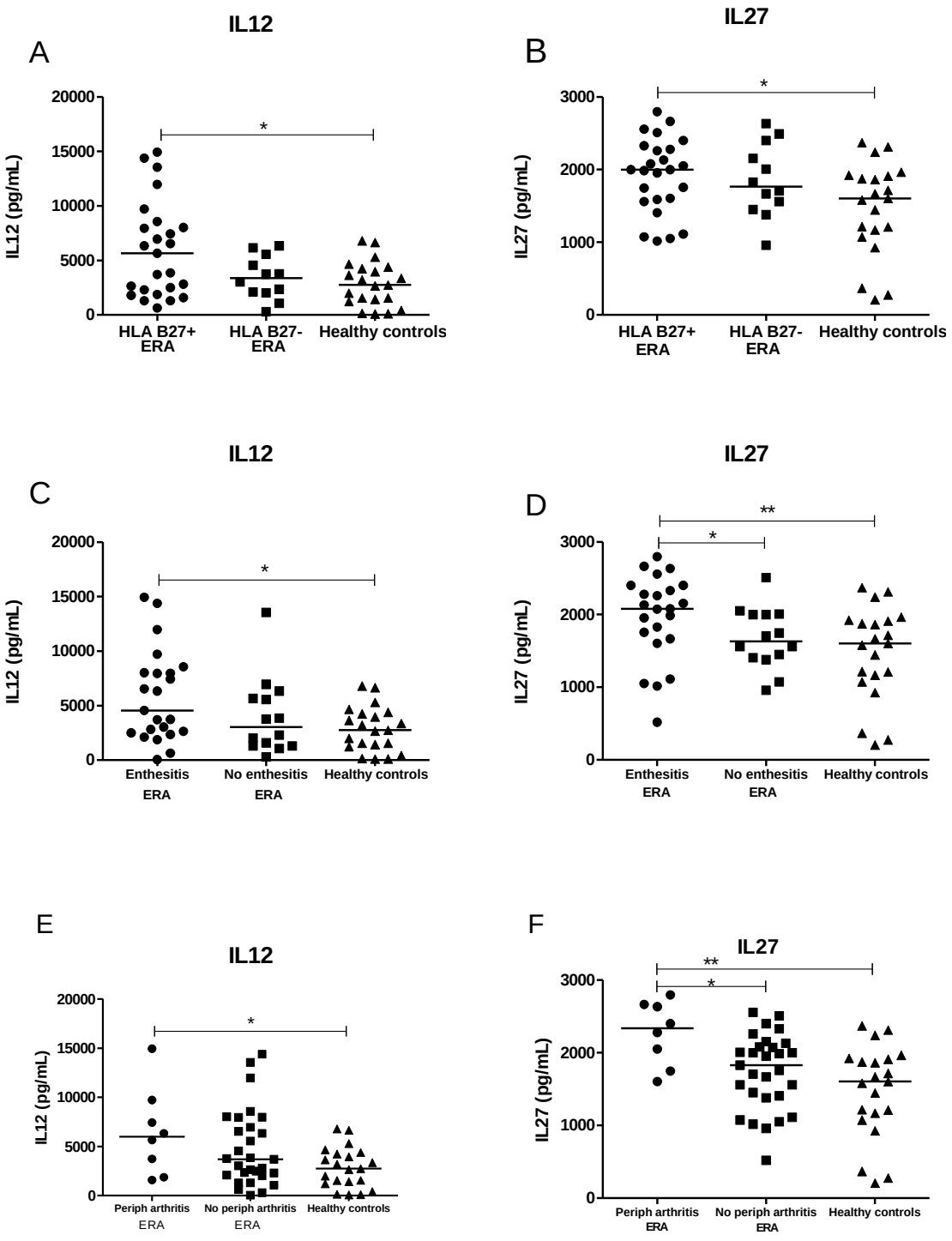


Figure 4.15 IL12 and IL27 production from LPS-stimulated MDMs in patients with ERA is higher in those with HLA B27, active enthesitis and peripheral arthritis compared to healthy controls. MDMs were differentiated as described in the protocol above (section 2.2) and stimulated with LPS for 24 hours. IL12 and IL27 were measured in cell culture supernatants by Luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (HLA B27+ ERA n=25, HLA B27- ERA n=12, ERA with active

enthesitis n=23, ERA without enthesitis n=14, ERA with peripheral arthritis n=8, ERA without peripheral arthritis n=29, adolescent healthy controls n=21.

No correlation was found between IL12 and IL27 production and CRP, age at sample date or disease duration from LPS-stimulated MDMs or from MDMs without LPS stimulation in the case of IL27. In addition, there was no significant difference between levels of IL12 and IL27 in males and females with ERA or in patients with and without extra-articular manifestations.

4.3.6 Treatment with TNF inhibitors and DMARDs does not significantly affect IL12 and IL27 levels from LPS-stimulated MDMs from patients with ERA

In view of the observation that IL23 levels from LPS-stimulated MDMs were significantly higher in patients with ERA on TNF inhibitors, it was of interest to determine whether treatment had any impact on production of IL12 and IL27 in patients with ERA. Levels were compared for patients on TNF inhibitors, DMARDS and NSAIDs to those not on them. Interestingly, there was no difference in levels of IL12 and IL27 from LPS-stimulated MDMs from patients with ERA on TNF inhibitors compared to those not on them (IL12 median 6330 pg/mL, IQR 2296-7954 pg/mL vs 3373 pg/mL, IQR 1784-6625 pg/mL and IL27 median 1828 pg/mL, IQR 1406-2330 pg/mL vs 1992 pg/mL, IQR 1532-2185 pg/mL) but there was a difference in IL12 levels between patients with ERA on TNF inhibitors and healthy controls ($p=0.03$). Similarly, no difference was seen between patients on DMARDs (with or without TNF inhibitors) compared to those not on them and this was also the case for patients on NSAIDs (table 4.3).

In addition, contrary to the IL23 findings for patients with AS on TNF inhibitors, there was no difference in IL12 or IL27 levels from LPS-stimulated MDMs for patients on and off TNF inhibitors (IL12 median 4658 pg/mL, IQR 2450-8827 pg/mL vs 3653 pg/mL, IQR 1983-5147 pg/mL; IL27 median 1854 pg/mL, IQR 1511-2072 pg/mL vs 1684 pg/mL, IQR 1286-2117 pg/mL). This was also the case for patients with polyarticular JIA on and off TNF inhibitors (IL12 median 5008 pg/mL, IQR 1276-7658 pg/mL vs 4123 pg/mL, IQR 1187- 6862 pg/mL; IL27 median 1738 pg/mL, IQR 1193-1983 pg/mL vs 1747 pg/mL, IQR 1031-2517 pg/mL).

4.3.7 IL12 and IL27 production from LPS-stimulated MDMs correlate with levels of IL23 production in patients with ERA

There were similarities and differences in expression of IL12 and IL27 from LPS-stimulated MDMs in patients with ERA compared to IL23. There were clear differences between patients and healthy controls for all these cytokines but the effect of treatment with TNF inhibitors differed with increased IL23 expression in patients with ERA and AS on treatment not mirrored by an increase of IL12 or IL27 expression. It was therefore of interest to investigate whether levels of IL12 and IL27 correlated well with IL23 production. Using Spearman's rank correlation, there was a significant positive correlation between both IL12 ($r=0.68$, $p<0.0001$) and IL27 ($r=0.62$, $p<0.0001$) levels and IL23 production from LPS-stimulated MDMs in patients with ERA (figure 4.16 A and B). There was also a correlation

between IL12 and IL27 production although this was less significant (r=0.52, p=0.001). In healthy controls, there was a strong correlation between IL23 levels and IL12 from LPS-stimulated MDMs (r=0.76, p<0.0001) and a less significant correlation between IL12 and IL27 (r=0.57, p=0.007) but no correlation between IL23 and IL27.

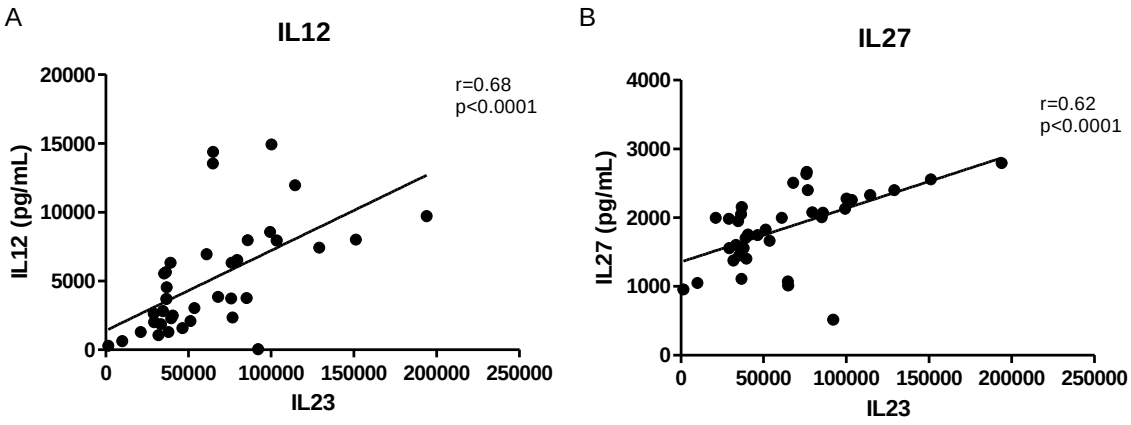


Figure 4.16 Levels of IL12 and IL27 from LPS-stimulated MDMs from patients with ERA correlate with IL23 production. MDMs were differentiated as above and stimulated with LPS for 24 hours. Cell culture supernatants were then collected and analysed by Luminex assay for IL12 and IL27. Graphs show Spearman's rank correlation between IL23 and IL12 (A) and IL27 (B) for patients with ERA (n=37).

4.4 Analysis of IL12p35 RNA from LPS-stimulated MDMs

4.4.1 Introduction

In view of the strong correlation between IL23 and IL12 in both patients with ERA and healthy controls, it was of interest to further analyse IL12 gene expression. IL12, like IL23, is a heterodimeric cytokine formed of the IL12/23p40 subunit which it shares with IL23 (see section 4.2) and IL12p35 which is specific to IL12. Transcription of IL12p35 is tightly regulated and this controls the secretion of IL12 (Croxford, Mair et al. 2012). Experiments were therefore undertaken to quantify RNA expression of IL12p35 using qPCR from MDMs following LPS stimulation for 24 hours from the same subgroup of patients and healthy controls used to analyse IL23p19 and IL12/23p40.

4.4.2 IL12p35 expression is not significantly higher in patients with ERA compared to healthy controls

Contrary to IL23p19, where a strong trend for enhanced expression was seen in patients with ERA compared to healthy controls, there was no difference in IL12p35 expression between patients with ERA and healthy controls (median fold change 141, IQR 69.07-288.0 vs 152.3, IQR 23.43-348.6, figure 4.17 A). Similarly, there was no difference in expression of IL12p35 from LPS-stimulated MDMs in patients who were HLA B27 positive compared to those that were HLA B27 negative (median fold change 141, IQR 69.07-288.0 vs 136.1, IQR 58.62-305.7, figure 4.17 B). Further analysis did not identify any other associations between IL12p35 gene expression and clinical features disease activity or treatment apart from a trend towards lower gene expression of IL12p35 from LPS-stimulated MDMs in those patients with hip arthritis, as seen for IL23p19 (p=0.06, table 4.2).

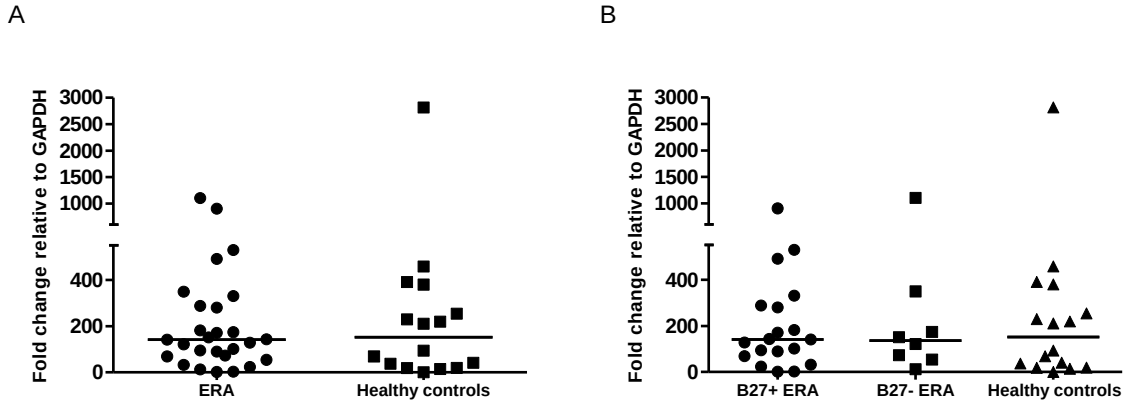


Figure 4.17 IL12p35 expression in LPS-stimulated MDMs is not significantly different between patients with ERA and healthy controls. IL23p35 expression was analysed from RNA from MDMs using qPCR and normalised against GAPDH. Fold change was calculated relative to an unstimulated control for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann-Whitney U test (ERA n=27 (B27 positive n=19, B27 negative n=8), healthy controls n=16)).

4.4.3 IL12p35 gene expression correlates with IL12/23p40 and IL23p19

To determine whether there was any correlation between the gene expression of IL12p35 and IL12/23p40 and also IL23p19 from LPS-stimulated MDMs in patients with ERA, Spearman's rank correlation was undertaken. There was a moderate correlation between both IL23p19 (r=0.57, p=0.002, figure 4.17 A) and IL12/23p40 (r=0.61, p=0.002, figure 4.17 C) and IL12p35. In healthy controls, the correlation in IL12p35 expression with IL12/23p40 expression from LPS-stimulated MDMs was not as strong (r=0.54, p=0.04, figure 4.17 D) and there was no correlation between IL12p35 and IL23p19 expression (r=0.41, p=0.12, figure 4.17 B).

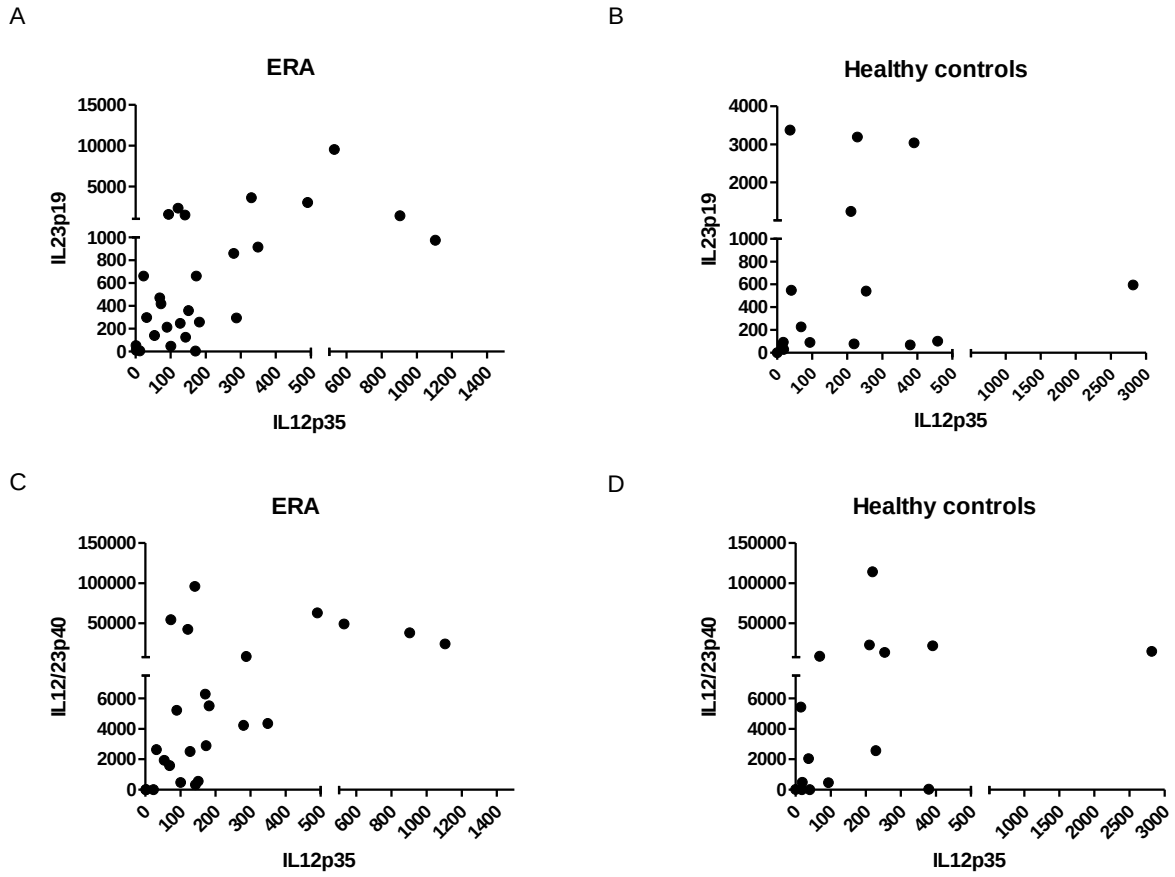


Figure 4.18 Correlation between IL12p35, IL23p19 and IL12/23p40 expression in LPS-stimulated MDMs from patients with ERA. RNA was isolated from MDMs which had been stimulated with LPS for 24 hours and transcribed in to cDNA. IL12p35, IL23p19 and IL12/23p40 genes were amplified using qPCR and normalised against a housekeeping gene (GAPDH). Fold change was calculated by comparing the expression of IL12p35, IL23p19 or IL12/23p40 from LPS-stimulated MDMs to the control (unstimulated) sample for each patient and healthy control. Graphs shows Spearman's rank correlation for patients with ERA (n=25, A and C) and for healthy controls (n=16, B and D).

4.5 Tables summarising the results from chapter 4

ERA Groups		IL23p19	IL12/23p40	IL12p35
Age		No correlation	No correlation	No correlation
Disease duration		No correlation	No correlation	No correlation
CRP		No correlation	No correlation	No correlation
HLA B27	Positive	471.1 (169.6-2060)	4731 (439.1-40673)	141 (69.07-288.0)
	Negative	418.8 (73.7- 946.0)	4360 (1938-42348)	136.1 (58.62-305.7)
Sex	Male	661.7 (133.2-1531) *	5221 (1761-45666)	134 (72.02-303.4)
	Female	298.2 (134.5-1988)	1604 (142.6-4800)	151.2 (16.88-256.6)
Active enthesitis	Yes	444 (159.2-1368)	4240 (474.4-42348)	134.4 (70.06-286.0)
	No	638 (5.93-1811)	5327 (2125-54136)	151.2 (31.78-349.7)
ERA subtype	Peripheral	298.2 (65.13-789.1)	2896 (1490-5327)	171.3 (87.4-261.7)
	Indeterminate	510.6 (259.0-946.9)	3381 (420.7-12314)	215.7 (49.33-320.1)
	Axial	471.1 (96.69-2446)	21710 (1307-56479)	97.74 (65.26-259.5)
Active peripheral arthritis	Yes	479.9 (259.0-2957)	6295 (2521-23988)	229.6 (55.83-480.3)
	No	445.0 (107.1-1515)	4300 (538.8-45348)	121.1 (69.07-182.3)
Hip arthritis	Yes	277.8 (50.9-930.1)	3934 (308.5-41236)	95.5 (41.10-142.5)
	No	861.1 (270.7-2798) *	4360 (2229-36485)	173.6 (94.35-349.7)
Disease activity (JADAS)	Low	723.3 (103.7-2308)	23988 (1030-79408)	101.1 (71.04-336.7)
	Moderate	270.7 (39.77-1257)	3381 (250.7-15792)	135.5 (30.78-286.0)
	High	607.3 (234.9-7385)	4790 (3075-38043)	219.8 (46.31-485.0)
TNF inhibitor	Yes	861.1 (303.4-2798) **	2709 (439.1-71137)	142 (99.43-200.3)
	No	294.1 (31.79-946.0)	5518 (1938-37902)	121 (42.8-340.3)
DMARD	Yes	296.1 (107.1-1106)	2896 (474.7-8422)	128 (58.82-173.6)
	No	1153 (431.9-3473) *	43443 (3666-56479) *	340.0 (70.06-520.3)
NSAID	Yes	661.7 (92.7-2127)	5369 (2173-44007)	172.5 (73.19-298.7)
	No	389.2 (137.1-1433)	2521 (560.5-37902)	101.1 (61.44-321.2)

Table 4.1 Summary of results for gene expression of IL23 p19, IL12/23p40 and IL12p35 from LPS-stimulated MDMs in patients with ERA. Gene expression was analysed from RNA from MDMs stimulated with LPS for 24 hours using qPCR and normalised against GAPDH. Fold change was calculated relative to an unstimulated control for each patient and healthy control. Table shows median fold change and IQR in brackets, statistical analysis by Mann-Whitney U test or Kruskal-Wallis test as appropriate. Key:

Trend towards difference between groups (p<0.1)	* Difference between group and healthy controls (p<0.05)
	** Difference between group and healthy controls (p<0.01)

ERA patient groups		IL23 (pg/mL)	IL12 (pg/mL)	IL27 (pg/mL)
Age		No correlation	No correlation	No correlation
Disease duration		No correlation	No correlation	No correlation
CRP		No correlation	No correlation	No correlation
HLA B27	Positive	64650 (36400-99650)**	5646 (2075-8289)*	1998 (1574-2304)*
	Negative	45130 (32645-76413)	3373 (2035-5291)	1767 (1478-2340)
Sex	Male	64650 (36260-92010)*	3850 (2095-7944)	
	Female	37840 (32238-50918)	3162 (1784-8342)	
Active enthesitis	Yes	75905 (36728-99425)**	4543 (2485-8013)*	2079 (1666-2410)**
	No	38425 (29923-63725)	3025 (1287-5817)	1632 (1399-2000)
ERA subtype	Peripheral	46280 (32780-94455)	5646 (1794-7949)	2052 (1653-2166)
	Indeterminate	75790 (36225-110455)*	3725 (1070-6881)	1828 (1281-2650)
	Axial	53580 (35210-79380)*	3753 (2340-8013)	1986 (1406-2154)
Active peripheral arthritis	Yes	75705 (38765-121700)*	5988 (2322-9141)*	2340 (1823-2657)**
	No	51120 (34860-82190)	3696 (IQR 2055-7440)	1828 (1393-2105)
Hip arthritis	Yes	60950 (33150-89290)*	3850 (1957-7979)	1998 (1559-2294)*
	No	45805 (36603-85533)	3739 (2035-7156)	1792 (1444-2274)
Disease activity (JADAS)	Low	64650 (35210-79380)*	3725 (2296-8013)	1756 (1379-2330)
	Moderate	52350 (35523-94808)*	3773 (2035-6605)	2019 (1480-2428)
	High	53615 (27233-99425)	4475 (1502-10157)	1998 (1700-2169)
TNF inhibitor	Yes	70353 (39380-97608)**	6330 (2296-7954)*	1828 (1406-2330)
	No	36790 (30210-76620)	3373 (1784-6625)	1992 (1532-2185)
DMARD	Yes	60950 (36790-92010)**	5540 (2216-7954)	1986 (1451-2258)
	No	38515 (27233-77310)	2412 (1712-7954)	1877 (1446-2309)
NSAID	Yes	53850 (31790-92010)	5540 (1854-8565)	1828 (1379-2132)
	No	53615 (37570-80785)	3739 (2329-6625)	2002 (1669-2348)

Table 4.2 Summary of results for IL23 family cytokine production from LPS-stimulated MDMs and clinical features in patients with ERA. Cytokine levels were measured in cell culture supernatants following incubation of MDMs with LPS for 24 hours using ELISA or Luminex assay. Table shows median cytokine level for each group with IQR in brackets, statistical analysis by Mann-Whitney U test or Kruskal-Wallis test as appropriate. Key:

Trend towards difference between groups (p<0.1)	* Difference between group and healthy controls (p<0.05)
Significant difference between groups (p<0.05)	** Difference between group and healthy controls (p<0.01)
Significant difference between groups (p<0.01)	

4.6 Discussion

The data in this chapter indicates that, in this assay, LPS-stimulated MDMs from patients with ERA produce more IL23, IL12 and IL27 compared to age-matched healthy controls. There is also a trend towards enhanced IL23p19 gene expression, especially in males. MDMs from patients with AS also produce more IL23 and IL12 compared to healthy controls and MDMs from patients with polyarticular JIA produce more IL23 compared to healthy controls.

This chapter starts by describing the patients and healthy controls recruited for this study. It was initially hoped to recruit patients with ERA in the early stages of disease (less than 3 years after diagnosis), with active arthritis and not treated with TNF inhibitors. However, this proved too restrictive and recruitment was slow, therefore all patients with ERA were subsequently included in the study. This, in fact, was useful as it allowed comparisons between patients treated with TNF inhibitors compared to those not on them and also demonstrated that in this assay that there was no correlation between disease duration and age and the production of IL23, IL12 and IL27 by LPS-stimulated MDMs.

Recruiting an age and gender-matched disease control group was problematic as ERA is the only subtype of JIA with a male predominance. Rheumatoid factor negative polyarticular JIA was chosen as it had a similar prevalence in the adolescent clinic and a similar prognosis to ERA (Guzman, Oen et al. 2015). It was hoped that a high proportion of male patients with polyarticular JIA could be recruited. However, this proved difficult and the number of females who gave samples was much higher than males because the proportion of female patients in this disease control group were higher. One male who was recruited had to be excluded as he was found to be HLA B27 positive on review of his case notes. The patients with polyarticular JIA tended to have more active peripheral arthritis but less hip arthritis than patients with ERA which was perhaps unsurprising given the differing phenotype of these JIA subtypes. The polyarticular JIA group was also more heterogeneous than hoped, three patients had enthesitis, one had indeterminate changes on MRI of the spine and sacroiliac joints and one went on to be diagnosed with psoriatic arthritis instead of polyarticular JIA. However, ERA is also a heterogeneous condition, with some patients experiencing only peripheral arthritis and enthesitis whilst others also develop axial disease. Those with indeterminate changes on MRI of the spine and SIJ, for example interspinous ligament inflammation, facet joint arthritis or mild sacroiliac joint enhancement, either progressed to axial disease or, in some cases, exhibited complete resolution of these changes and therefore were a challenging group to classify. This heterogeneity perhaps explains some of the variability in all outputs analysed in this chapter seen within these two groups. However, significant variability was also seen within the healthy control groups in IL23, IL12 and IL27 production, as well as RNA expression of IL23p19, IL12/23p40 and IL12p35 in the adolescent healthy controls. This is consistent with studies in the literature which report a wide variance in innate immune inflammatory responses between individuals in response to LPS determined by differences in gene expression (Wurfel, Park et

al. 2005). This perhaps explains the inherent variability seen in responses of MDMs to LPS stimulation in this assay.

The next challenge was clinical data collection. Whilst a standard set of core outcome variables was collected in the adolescent clinic, BASDAI and BASFI were more commonly collected in the adult SpA clinic. The outcome data collected was therefore not the same in the adolescent and adult clinics. In addition, the core outcome variables (and JADAS) which worked well for polyarticular JIA, did not capture features of disease specific to ERA and therefore information on enthesitis and axial arthritis had to be collected separately. For future studies, ensuring sufficient data is available to calculate the JSpADA and ASDAS for patients with ERA and AS respectively would improve reliability of the disease activity score collected. It would then be of interest to analyse outcomes such as IL23 in relation to these disease activity scores.

Given the key role of IL23 in the pathogenesis of SpA and in particular AS (Gaston, Goodall et al. 2011), IL23 was the first output to be analysed from the MDM assay. Initial experiments were carried out examining the gene expression of the separate subunits of IL23, IL23p19 and IL12/23p40 in RNA from MDMs from patients with ERA compared to healthy controls 24 hours after LPS stimulation. This time point was chosen because of the preliminary data discussed in chapter 3 demonstrating that IL23 was not detectable in the cell culture supernatants after 4 hours LPS stimulation but was present at 24 hours (section 3.2.4). Ideally, several time points would have been investigated to determine the optimal timing for quantification of IL23p19 and IL12/23p40 gene expression. However, the number of time points for output collection was limited by the relatively small amount of blood and therefore MDMs that could be collected for each patient and healthy control. The only other alternative was collection of RNA after 4 hours LPS stimulation and this was thought to be too early in view of the late production of IL23 in the cell culture supernatants. In addition, MDMs collected at 4 hours were used for detection of p38 MAPK.

Overall, there was a trend towards higher IL23p19 expression in patients with ERA, especially those who were HLA B27 positive, although the difference was not statistically significant except when male patients were compared to male healthy controls. The distinction between males and females was made because of the evidence of differences between males and females with SpA (Rusman, van Vollenhoven et al. 2018) and therefore it was of interest to investigate whether any differences were seen in the outputs from the MDM assay between males and females. It was, however, difficult to compare males and females as the number of females recruited to the study in the ERA group was small in line with the male predominance seen in ERA.

Contrary to phosphorylation of p38 MAPK, no difference was seen in either IL23p19 or IL12/23p40 expression with enthesitis. Apart from IL23p19 gene expression and hip arthritis, other clinical features and disease activity did not have an effect on IL23p19 or IL12/23p40 expression. This is perhaps because the study was underpowered to analyse these

subgroups. In particular, the groups for high and inactive disease activity were small and therefore meaningful comparison for these groups was difficult. Hip arthritis was analysed separately because of the evidence of greater need for hip arthroplasty in patients with juvenile onset AS compared to adult AS (Jadon, Shaddick et al. 2015). The trend towards lower IL23p19 gene expression in those with hip arthritis was unexpected and maybe have been a chance finding or perhaps explained by the fact that a lower percentage of those with hip arthritis were treated with TNF inhibitors (associated with high IL23p19 gene expression) compared to those without hip arthritis (23.1% vs 52.9%). In addition, a lower proportion of patients with hip arthritis were male compared to those without hip arthritis (76.9% vs 88.2%) and although this difference was not significant, it may have contributed.

In terms of treatment, the majority of patients were on treatment with either DMARDs alone or a combination of DMARDs and TNF inhibitors. No difference was seen between these groups but there was a trend towards higher IL23p19 and IL12/23p40 in patients who were not on DMARDS compared to those treated with them. This group of patients not on DMARDs included one patient on a TNF inhibitor only and the rest were patients on NSAIDs only or on no treatment. This result fitted with the hypothesis that IL23 gene expression would be higher in patients not on treatment and that treatment should reduce expression of pro-inflammatory cytokines such as IL23. Interestingly, and contrary to expectations, this was not the case with TNF inhibition. IL23p19 gene expression was significantly higher in patients on TNF inhibition compared healthy controls and there was a strong trend towards increased IL23p19 in LPS-stimulated MDMs from TNF inhibitor-treated patients compared to those not on them. This result was unexpected and was therefore analysed further in levels of IL23 secretion from cell culture supernatants.

As discussed in chapter 3, the MDM assay was an artificial system and it was of interest to see whether the results were mirrored in an ex vivo setting and specifically within the joint of patients with ERA. Analysis of IL23p19 and IL12/23p40 was undertaken in synovial fluid because of the availability of samples from HLA B27 positive patients with ERA and in the absence of tissue samples such as biopsy specimens from inflamed sacroiliac joint of patients with ERA. The difficulty with testing these samples was the lack of a control. In adult patients, SF from patients with osteoarthritis is often used but it was felt this would not be an appropriate control given the young age of the patients with ERA. Preliminary experiments were carried out using PBMCs from an age-matched healthy volunteer as a control but the results were significantly different between PBMCs and SF and therefore this was not an appropriate control to use. It was decided to compare the results of HLA B27 positive patients with ERA to IL23p19 and IL12/23p40 expression in SFMCs in group of HLA B27 negative patients with oligoarticular JIA (both persistent and extended groups were included). The average of the lowest value ΔC(t) from each group was then taken as the control value. Interestingly, despite the small sample size tested, a trend was seen towards higher IL23p19 expression in SFMCs from HLA B27 positive patients with ERA. Numbers were too small for subgroup analysis of factors such as clinical characteristics or effect of

treatment and it would be of interest to expand the sample size of these groups and also to compare with other JIA subtypes in the future.

Next, experiments were undertaken to analyse IL23 production from LPS-stimulated MDMs in cell culture supernatants. RNA expression of the separate subunits of IL23 gave an indication of IL23 production at a particular time point (in this case 24 hours after LPS stimulation). However, it was important to determine whether the trends seen in IL23p19 and IL12/23p40 expression were translated in to secretion of the active form of IL23. Interestingly, the results for IL23 secretion from LPS-stimulated MDMs demonstrated clearer differences between patients and healthy controls, with significantly higher levels seen in patients with ERA. This result was mirrored in patients with AS compared to a group of adult healthy controls. Zeng et al described similar findings in their study of LPS-stimulated MDMs from patients with AS (Zeng, Lindstrom et al. 2011). Levels were also significantly higher from LPS-stimulated MDMs from patients with polyarticular JIA compared to age-matched healthy controls. This supports the theory that IL23 may be important in the pathogenesis of inflammatory arthritis in general as demonstrated in animal models where mice deficient in IL23 were protected from developing collagen induced arthritis (Murphy, Langrish et al. 2003).

In patients with ERA, IL23 production from LPS-stimulated MDMs was higher in those who were HLA B27 positive and male compared to healthy controls. These two features have been associated with worse prognosis in patients with ERA, with male patients more likely to develop sacroiliitis and those patients who were HLA B27 positive (as well as those with sacroiliitis) less likely to achieve drug free remission after 8 years of follow up (Berntson, Nordal et al. 2013). MDMs from male patients with AS also produced significantly higher levels of IL23 compared to healthy controls. It was not possible to compare HLA B27 positive patients with AS to those who were HLA B27 negative because of a lack of clinical information available. The observation of higher IL23 production from LPS-stimulated MDMs in males was interesting in view of findings from previous studies indicating elevated levels of pro-inflammatory cytokines, in particular TNFα and IL17A and also increased numbers of Th17 cells in male patients with AS compared to female patients (Gracey, Yao et al. 2016). Elevated IL23 production from macrophages in male patients with AS may provide a mechanism for the increased numbers of Th17 cells observed. This may in turn partially explain the observation that male patients with AS have a worse prognosis in terms of radiographic progression compared to female patients (Rusman, van Vollenhoven et al. 2018). In adult SpA, HLA B27 is linked to early onset and increased severity of axial disease (Chung, Machado et al. 2011). However, the mechanism linking HLA B27 to elevated IL23 is unclear. A number of theories have been suggested in the literature including the link between gut dysbiosis, autophagy and IL23 production (Ciccia, Accardo-Palumbo et al. 2014, Wendling 2016) and also the fact that HLA B27 misfolds and forms aberrant homodimers which may result in induction of the UPR and the production of IL23.

LPS stimulated-MDMs from patients with enthesitis at the time of sample also produced higher levels of IL23 compared to those without active enthesitis and healthy controls. This was the case for patients with ERA and for the 3 patients with polyarticular JIA who were also documented as having enthesitis. Enthesitis is central to the pathogenesis of SpA (McGonagle, Benjamin et al. 2002) and evidence from animal models suggests that IL23 plays an essential role in the development of enthesitis by acting on a unique subset of entheseal resident T cells resulting in entheseal inflammation and new bone formation (Sherlock, Joyce-Shaikh et al. 2012). Thus the finding of elevated levels of IL23 from MDMs from patients with enthesitis was interesting; especially in light of the recent ECLIPSA study which demonstrated that blockade of IL12/23p40 in psoriatic arthritis resulted in significant improvement in enthesitis when compared to treatment with TNF inhibitors (Araujo, Englbrecht et al. 2019).

As with IL23p19 and IL12/23p40, no difference in IL23 production was seen across the JADAS disease activity groups in ERA, though MDMs from the groups with larger numbers (low and moderate) secreted significantly higher levels of IL23 compared to healthy controls. Similarly, there was no difference between those with peripheral ERA only, indeterminate and axial ERA but MDMs from those in the larger indeterminate and axial ERA groups produced significantly more IL23 compared to healthy controls. The levels of IL23 secretion in the indeterminate ERA group varied widely and it would be of interest to follow these patients to determine whether high IL23 from LPS-stimulated MDMs is predictive of progression to axial ERA. Those with active peripheral arthritis at the time of sample produced significantly higher levels of IL23 from their MDMs compared to healthy controls but not compared to those patients with ERA without active peripheral arthritis. This was also the case for those with a history of hip arthritis. Thus the results across these subgroups reflected the overall differences seen in IL23 expression from LPS-stimulated MDMs in patients with ERA compared to healthy controls but it was not possible to differentiate between them. As mentioned above, this may in part be due to the small numbers in some of the subgroups, such as peripheral ERA and the inactive and high disease activity groups, thus making the study underpowered for analysis of these subgroups. No correlation with CRP was observed but in adult patients with AS, CRP is known to be a poor predictor of disease activity (Claushuis, de Vries et al. 2015) and this is also the case in ERA.

Interestingly, the unexpected finding of elevated IL23p19 gene expression from LPS-stimulated MDMs in patients with ERA treated with TNF inhibitors was mirrored by significantly higher levels of IL23 secretion in cell culture supernatants from LPS-stimulated MDMs in patients on TNF inhibitors compared to those not on them. This was also the case for patients with AS but not for those with polyarticular JIA. MDMs from patients on DMARDs also produced higher levels of IL23 compared to healthy controls but after further analysis it was found that this was likely to be due to those patients on a combination of DMARD and TNF inhibitor producing significantly higher levels of IL23 compared to those on a DMARD alone. It was thought that the higher levels of IL23 secreted by LPS-stimulated

MDMs in these groups could be due to more severe disease in those on TNF inhibitors or higher rates of HLA B27 or enthesitis. However, further analysis revealed that this was not the case. Patients on TNF inhibitors had lower disease activity according to JADAS criteria, with no patients in the high disease activity group, most patients in the low or moderate activity groups (50% and 33.3%) and both inactive patients in the study on treatment with TNF inhibitors. Equally, there was no difference in the percentage of patients with enthesitis treated with TNF inhibitors with a third of patients in both the treated and untreated group having active enthesitis and no significant difference in gender between those on TNF inhibitors and those not on them (86.7% male vs 81.8% male). The proportion of patients who were HLA B27 positive was slightly higher in the group treated with TNF inhibitors (73.3% vs 63.6%). The proportion of patients with peripheral ERA only was no different between the TNF inhibitor treated group and those not on treatment. There was a slightly higher percentage of patients with ERA in the indeterminate group treated with TNF inhibitors (33.3% vs 22.2%) but a slightly higher proportion of axial ERA in the group not on TNF inhibitors (61.1% vs 50%). Of those with axial ERA on TNF inhibitors, it was interesting to note that 75% still had evidence of some inflammation on their most recent sacroiliac joint and spine MRI scan, albeit this was usually mild. There were 2 patients in the study with inflammatory sacroiliac joint MRI changes described as severe but these were both in the group not yet treated with TNF inhibitors. Therefore, the theory that high IL23 levels from LPS-stimulated MDMs of those on TNF inhibitors was related to severe disease or active disease did not appear to be the case.

Another explanation was that by blocking the TNF inflammatory pathway, the IL23 pathway was paradoxically upregulated. There is some evidence for this from previous studies. Notley et al found increased numbers of pathological Th17 cells in an animal model of inflammatory arthritis following treatment with TNF inhibition. They observed a decrease in expression of IL12/23p40 with the administration of TNFα and suggested that the reason for the expansion of Th17 cells was due to a paradoxical increase in IL12/23p40 and thus IL23 following TNF blockade (Notley, Inglis et al. 2008). An earlier study also demonstrated reduced expression of IL12/23p40 in a dose-dependent fashion in response to TNFα administration which led to a reduction in IL23 production by macrophages and dendritic cells in response to stimulation with LPS and IFNγ (Zakharova and Ziegler 2005) thus supporting the theory that TNF blockade results in a paradoxical increase in IL23. This is also supported by the increased incidence or exacerbation of conditions where IL23 is strongly implicated in pathogenesis, such as psoriasis, Crohn's disease and multiple sclerosis seen in patients treated with TNF inhibitors (Chowers, Sturm et al. 2010). It may also explain why patients on TNF inhibitors with both ERA and AS often have persistent inflammation of their sacroiliac joints seen on MRI and bony progression despite treatment with TNF inhibitors (van der Heijde, Ramiro et al. 2017). Interestingly, the effect of TNF inhibition on IL23p19 expression was not studied in the work described above and this appeared to be increased in the context of the MDM assay in this project as opposed to IL12/23p40 in patients on TNF

inhibitors. This is perhaps because the expression of IL12/23p40 was only measured at one time point, 24 hours after LPS stimulation, which may have been too late to see any difference in the expression of this IL23 subunit. It would be of interest to investigate this further and measure the effect of both TNFα administration and TNF inhibition on both IL23p19 and IL21/23p40 expression in the MDM assay.

Following the experiments to investigate IL23 in LPS-stimulated MDMs, it was important to investigate other members of this cytokine family. Experiments were undertaken to quantify the production of IL12 and IL27 from MDMs from patients with ERA, AS and polyarticular JIA compared to healthy controls. In LPS-stimulated MDMs, there was a strong correlation between the production of both IL12 and IL27 and IL23 especially in patients with ERA but there were also some differences in expression of these cytokines. IL27 was the only cytokine detected at baseline in MDMs without LPS stimulation, with significantly higher expression in patients with ERA compared to healthy controls and, interestingly in contrast to IL23, also compared to patients with polyarticular JIA. Similar to IL23, IL27 expression from unstimulated MDMs was higher in patients with ERA and enthesitis compared to healthy controls and also in patients on TNF inhibitors and healthy controls but no significant difference was seen with HLA B27. In LPS-stimulated MDMs, some results were similar to IL23 expression, including higher IL27 and IL12 production in patients with ERA compared to healthy controls and also, for IL12, patients with AS and adult healthy controls. As with IL23, higher levels of IL27 and IL12 were associated with the presence of enthesitis and HLA B27 and for IL27, the presence of active peripheral arthritis. However, in contrast to IL23 expression, LPS-stimulated MDMs from those patients treated with TNF inhibitors did not produce higher levels of IL27 compared to those not on treatment. IL12 levels were significantly higher from patients treated with TNF inhibitors compared to healthy controls but not compared to those patients not treated with TNF inhibitors. This supports the theory that TNF inhibition may upregulate the expression of IL12/23p40, the subunit of IL23 shared with IL12, although this was not seen in this assay at the time point measured. The expression of the other subunit of IL12, IL12p35, was no different between patients and healthy controls and was not affected by any clinical features or treatment.

The results of these experiments indicate a potential role for the IL23 family of cytokines in the pathogenesis of ERA, particularly in a subgroup of patients with active enthesitis and perhaps in those who are male, HLA B27 positive and with active peripheral arthritis. This is consistent with the wealth of evidence that IL23 is strongly implicated in the pathogenesis of SpA (Lubberts 2015) and in particular that IL23 is a key cytokine in the development of enthesitis (Watad, Cuthbert et al. 2018). Increased expression of IL23 and to a lesser extent IL12, from LPS-stimulated MDMs in patients treated with TNF inhibitors in this assay suggests that this pathway is upregulated by TNF blockade and perhaps explains why inflammation seen at the sacroiliac joints on MRI does not fully resolve and bony progression still occurs despite TNF inhibitors in this group of patients. This supports the concept that it may be beneficial to target both of these pathways in patients with SpA

(Poddubnyy and Sieper 2017). Given that IL23 appears to be a promising therapeutic target in inflammatory arthritis and initial trials of the IL12/23p40 blocker, ustekinumab, were effective in controlling spinal symptoms in AS (Poddubnyy, Hermann et al. 2014), it is perhaps surprising that recent clinical trials of IL12/23p40 blockade (Tahir 2018) and IL23p19 blockade (Baeten, Ostergaard et al. 2018) in adult patients with established AS have not demonstrated significant clinical improvements and not met their primary endpoints. The reason for this is unclear but it is possible that there may be an early window of opportunity for the use of IL23 blockade in the initial phase of disease when enthesitis is the predominant clinical feature and that once structural changes are established IL23 inhibition is no longer or less effective. This is supported by studies in animal models demonstrating that IL23p19 inhibition is effective if given after the initial arthritic stimulus but before arthritis is established (Cornelissen, Asmawidjaja et al. 2013).This is particularly pertinent in patients with ERA where there is often a long relapsing and remitting course of peripheral arthritis and enthesitis before the onset of sacroiliitis which may provide this window of opportunity when IL23 blockade may be effective.

The limitations of these experiments include the limitations of the MDM assay as discussed above (section 3.6) and also the need to increase numbers of patients in the groups related to clinical features such as disease activity and peripheral and axial ERA to help to determine whether these features significantly affect expression of this group of cytokines. It would also be of interest to study longitudinal samples to analyse IL23 expression form LPS-stimulated MDMs for patients pre- and post-TNF inhibition to determine whether upregulation is truly seen with TNF blockade. In view of the recent studies suggesting that IL23 inhibition may not be effective in the treatment of AS, the next chapter describes the analysis of other pro-inflammatory cytokines that may be involved in the pathogenesis of ERA.

CHAPTER 5: ANALYSIS OF OTHER KEY CYTOKINES FROM MONOCYTE-DERIVED MACROPHAGES IN PATIENTS WITH ERA

Overview of chapter 5

This chapter expands on the results of the MDM assay described in chapter 4 and comprises data on the production of other pro-inflammatory cytokines from MDMs and also their gene expression in patients and healthy controls.

The first section will focus on TNFα, IL1β and IL6 gene expression and protein production from LPS-stimulated MDMs and includes subgroup analysis for patients with ERA with differing clinical features and treatment. Next, the production of GMCSF is discussed and the results for the chemokines CCL4 and CCL5 are described. The next section will describe IFNγ secretion from MDMs and also IFNγ-regulated gene expression from SFMCs in HLA B27 positive patients with ERA compared to a group of HLA B27 negative patients with oligoarticular JIA. Finally, correlations between the cytokines analysed in this chapter and the cytokines from the IL23 family are described.

Hypothesis for Chapter 5

The gene expression and production of pro-inflammatory cytokines from MDMs from patients with ERA will be similar to adult patients with AS and higher than healthy controls

5.1 TNFα, IL1β and IL6 gene expression from LPS-stimulated MDMs

5.1.1 Introduction

Following the analysis of cytokines within the IL23 family, it was important to measure other pro-inflammatory cytokines of potential relevance to the pathogenesis of ERA. Cytokines of interest had been identified in optimisation experiments using the proteome profiler array (section 3.5.2). In common with IL23, IL12 and IL27, TNFα, IL1β and IL6 have all been implicated in the pathogenesis of adult SpA by genetic association, expression in animal models and in in vitro and in vivo human studies (Hreggvidsdottir, Noordenbos et al. 2014). In addition, inhibition of TNFα is a highly effective treatment for AS, resulting in a reduction in inflammation and symptomatic improvement, although without consistent inhibition of new bone formation (van der Heijde, Ramiro et al. 2017). Activated macrophages are potent producers of TNFα, IL1β and IL6 (Arango Duque and Descoteaux 2014) and therefore it was of interest to analyse the production of these from LPS-stimulated MDMs from patients with ERA.

As with IL23, initial experiments were carried out analysing gene expression from RNA isolated from LPS-stimulated MDMs from a group of patients with ERA and age-matched healthy controls. As these were preliminary experiments, smaller numbers were tested compared to the final group of patients analysed for cytokine expression at the protein level. This subgroup of patients was the same as those analysed for IL23p19, IL12/23p40 and IL12p35 gene expression and had similar demographics and clinical characteristics to the larger final group used for analysis of cytokine secretion.

5.1.2 Gene expression of TNFα, IL1β and IL6 is not significantly different from LPS-stimulated MDMs in patients with ERA compared to healthy controls

Following stimulation of MDMs with LPS for 24 hours, RNA was extracted and transcribed in to cDNA as described in section 2.5. qPCR was then performed and the results for TNFα, IL1β and IL6 expression were normalised against GAPDH, after which a fold change was calculated relative to a control (unstimulated) sample for each patient and healthy control.

Although the medians tended to be higher in patients with ERA, no significant difference was seen in the expression of TNFα, IL1β or IL6 from LPS-stimulated MDMs between patients with ERA and healthy controls (TNFα: median fold change 5.152, IQR 1.633-8.856 vs 2.142, IQR 0.933-13.93; IL1β median fold change 111.4, IQR 52.2-641.1 vs 75.06, IQR 2.362-292.0; IL6 median fold change 1463, IQR 490.2-2327 vs 1314, IQR 308.7-2353; figure 5.1). The results for IL1β in particular exhibited significant variability especially in patients with ERA.

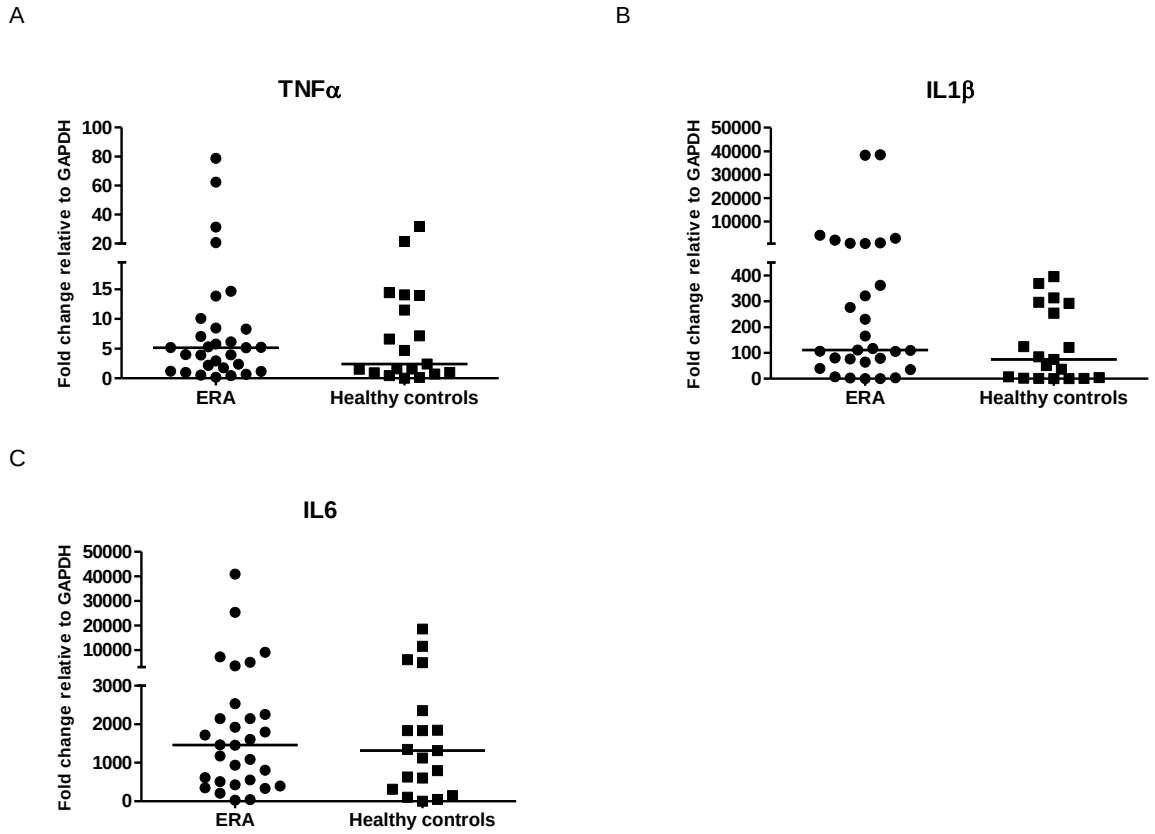


Figure 5.1 Gene expression of TNFα, IL1β and IL6 is no different in LPS-stimulated MDMs from patients with ERA compared to healthy controls. RNA was isolated from MDMs stimulated with LPS for 24 hours and transcribed in to cDNA. TNFα, IL1β and IL6 were amplified using qPCR and normalised against a housekeeping gene (GAPDH). Fold change was calculated by comparing the expression of TNFα, IL1β or IL6 from LPS-stimulated MDMs to the control (unstimulated) sample for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann-Whitney U test (ERA n=30, teenage healthy control n=18).

5.1.3 Associations of enthesitis, HLA B27 haplotype and male sex with TNFα, IL1β and IL6 gene expression in LPS-stimulated MDMs

In view of the findings in chapter 4 demonstrating increased levels of IL23 from LPS-stimulated MDMs in patients with ERA and active enthesitis, as well as in those who were HLA B27 positive and in male patients, analysis of TNFα, IL1β and IL6 gene expression was first undertaken in patients with these clinical features.

However, as with IL12/23p40 and IL12p35, no significant difference was seen in TNFα, IL1β or IL6 gene expression from LPS-stimulated MDMs in patients who were HLA B27 positive compared to those who were HLA B27 negative (results summarised in table 5.1). Interestingly, TNFα expression was higher from LPS-stimulated MDMs from patients with ERA without active enthesitis at the time of sample compared to those with active enthesitis (median fold change 9.169, IQR 4.85-16.14 vs 3.433, IQR 1.035-6.055, p=0.01, figure 5.2 A). There was no significant difference in IL1β or IL6 gene expression from LPS-stimulated MDMs between those with and without active enthesitis (table 5.1, figure 5.2 B and C). No significant difference was seen between LPS-stimulated MDMs from male and female patients with ERA in the gene expression of TNFα, IL1β and IL6 (table 5.1). However, as discussed previously, the number in the female patient group was small (n=6) compared to the male patient group (n=24).

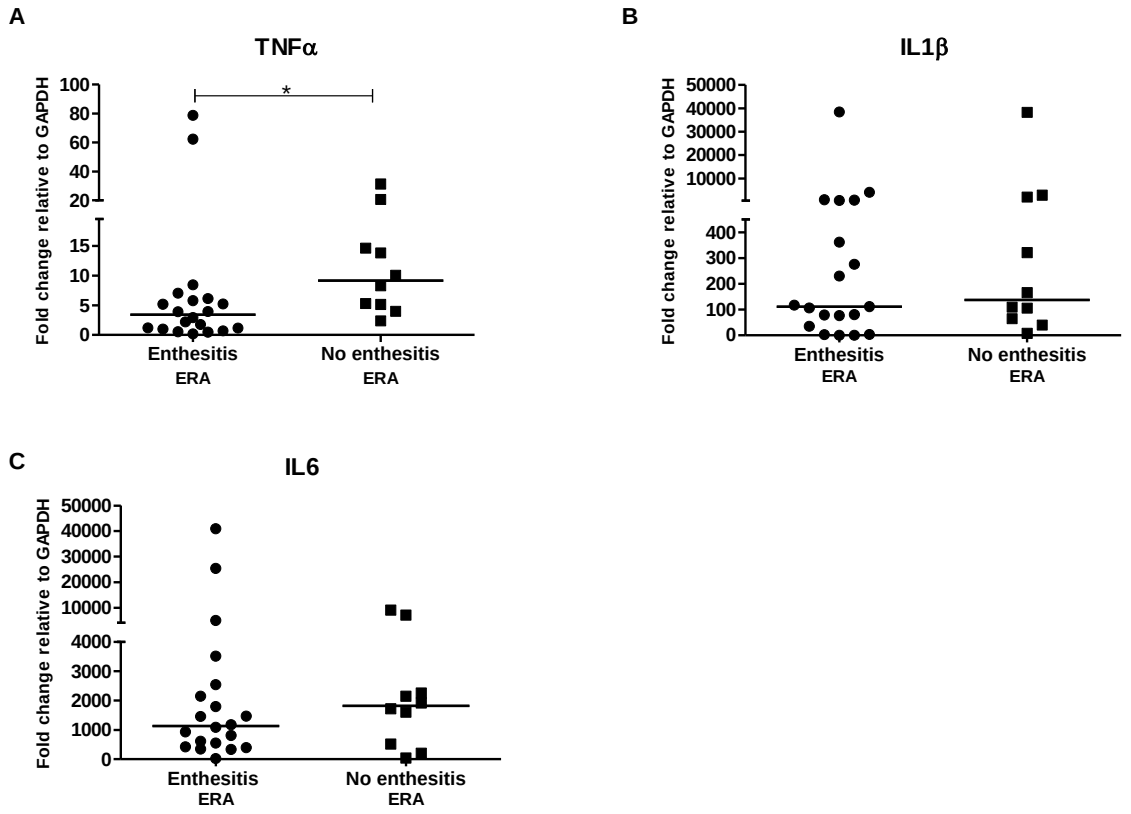


Figure 5.2 Gene expression of TNFα in LPS-stimulated MDMs is higher in patients with ERA without active enthesitis compared to those with enthesitis. RNA was isolated from MDMs stimulated with LPS for 24 hours and transcribed in to cDNA. TNFα, IL1β and IL6 were amplified using qPCR and normalised against GAPDH. Fold change was calculated by comparing the expression of the gene of interest from LPS-stimulated MDMs to the control (unstimulated) sample for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann Whitney U test (ERA with enthesitis n=20, ERA without enthesitis n=10).

5.1.4 Effect of disease activity, other clinical features and treatment on gene expression of TNFα, IL1β and IL6

Next, it was important to determine whether disease activity or other clinical features such as active peripheral arthritis had an effect on the gene expression of TNFα, IL1β and IL6 from LPS-stimulated MDMs. No significant differences were seen across the JADAS disease activity groups for TNFα, IL1β and IL6 or between those groups and healthy controls (table 5.1). The group with inactive disease by JADAS criteria was very small (n=2) and therefore meaningful comparison with the other groups and healthy controls was not possible.

When comparing those with peripheral ERA only to those with indeterminate and axial ERA, there was a trend towards higher median fold change for the gene expression of all three

cytokines in those with axial disease although no significant differences were found between the groups and some of the groups were small (TNFα: median fold change peripheral ERA 4.000, IQR 3.931-114.23, indeterminate ERA 4.712, IQR 0.7041-9.656, axial ERA 5.169, IQR 1.479-7.215; IL1β: peripheral ERA 76.64, IQR 24.00-299.0, indeterminate ERA 106.9, IQR 64.45-117.0, axial ERA 362.0, IQR 57.42-2445; IL6: peripheral ERA 809, IQR 429.6-1932, indeterminate ERA 1590, IQR 753.9-2061, axial ERA 1468, IQR 410.4-6095, figure 5.3, table 5.1). However, IL1β gene expression from LPS-stimulated MDMs in those with axial ERA was significantly higher than healthy controls (p=0.026, figure 5.3 B).

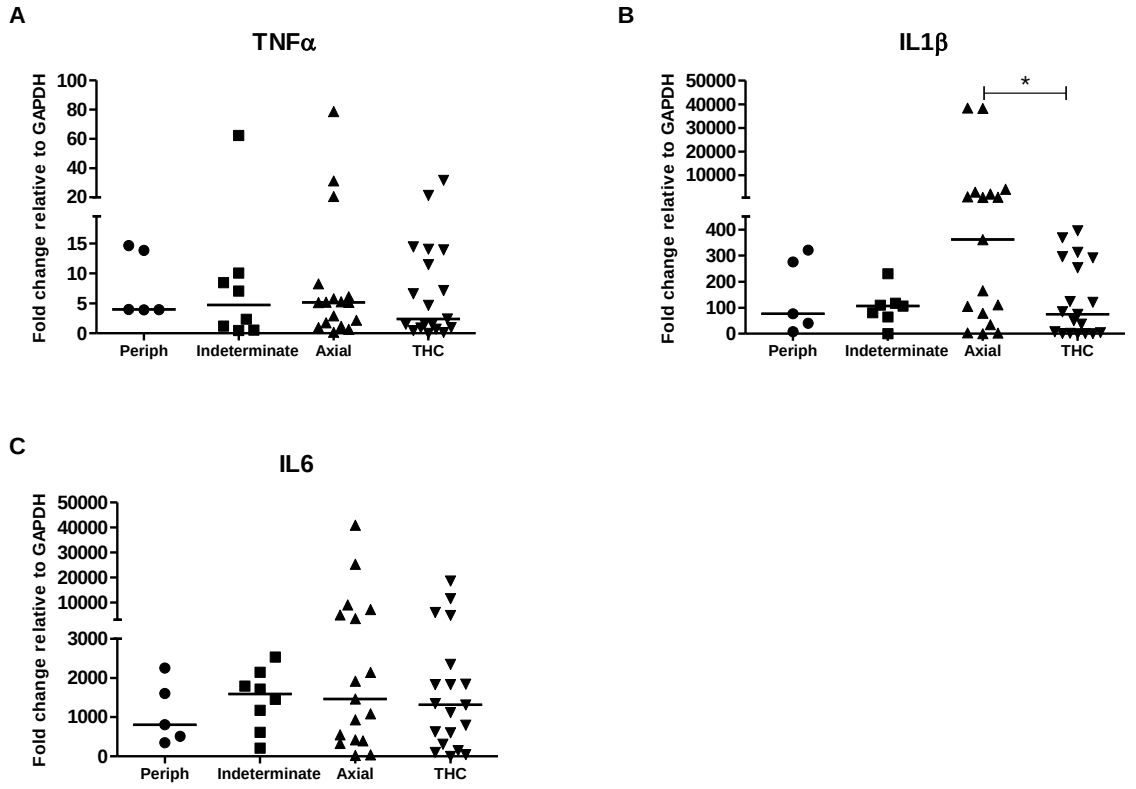


Figure 5.3 Gene expression of IL1β is significantly higher from LPS-stimulated MDMs in those with axial ERA compared to healthy controls. TNFα, IL1β and IL6 expression were analysed from RNA from LPS-stimulated MDMs using qPCR and normalised against GAPDH. Fold change was calculated relative to an unstimulated control for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann-Whitney U test (peripheral ERA n=5, indeterminate ERA n=8, axial ERA n=7, THC = teenage healthy control n=18, *p<0.05).

Other clinical features such as active peripheral arthritis, hip arthritis and extra-articular manifestations did not affect TNFα or IL1β gene expression. However, as seen with IL23p19, the gene expression of IL6 was higher in those patients with ERA without a history of hip arthritis compared to those with hip arthritis (p=0.04). There was no correlation between the gene expression of any of the three cytokines with CRP, age or disease duration (table 5.1).

Similarly, treatment with DMARDs did not significantly affect the expression of TNFα and IL1β from LPS-stimulated MDMs (table 5.1). For IL6, there was a strong trend towards higher gene expression from LPS-stimulated MDMs for those not on DMARDs compared to those on treatment but this did not reach statistical significance (2619, IQR 1284-20812 vs 1013, IQR 405.3-2150, p=0.052). Contrary to the findings for IL23p19, treatment with TNF inhibitors did not result in higher median fold change in gene expression of TNFα, IL1β or IL6 compared to those not on treatment (table 5.1).

5.2 TNFα, IL1β and IL6 secretion from LPS-stimulated MDMs

5.2.1 Introduction

Next, it was important to measure levels of TNFα, IL1β and IL6 in the cell culture supernatants from LPS-stimulated MDMs to verify whether the gene expression of these cytokines translated in to protein production. As with IL23, IL12 and IL27, these experiments were carried out on samples from the larger final group of patients with ERA and also in patients with polyarticular JIA and AS, in addition to the adolescent and adult healthy controls (table 3.1). Previously (section 3.2.4), it was observed that TNFα was secreted early from LPS-stimulated MDMs in comparison to other cytokines such as IL23. It was therefore decided to measure this cytokine in the cell culture supernatants after 4 hours incubation with LPS instead of 24 hours as for the other cytokines. TNFα levels were measured by ELISA. IL1β and IL6 were measured by Luminex assay in cell culture supernatants from MDMs after 24 hours stimulation with LPS.

5.2.2 TNFα secretion is elevated in cell culture supernatants from MDMs from patients with ERA compared to healthy controls

Levels of TNFα from LPS-stimulated MDMs in patients with ERA were found to be higher than levels from age-matched healthy controls (median 52445 pg/mL, IQR 28483-67190 pg/mL vs 29090 pg/mL, IQR 14845-47785 pg/mL, p=0.011, figure 5.4 A). Levels were also higher in patients with polyarticular JIA (median 41220 pg/mL, IQR 18680-54590 pg/mL) and AS compared to healthy controls (median 36510 pg/mL, IQR 24745-48570 pg/mL vs 25010 pg/mL, IQR 16455-39740 pg/mL) but these results did not reach statistical significance.

There was a trend towards higher IL1β levels from LPS-stimulated MDMs in patients with ERA compared to healthy controls (median 802.2 pg/mL, IQR 339.6-1318 pg/mL vs 458.6

pg/mL, IQR 190.9-768.6 pg/mL, p=0.075, figure 5.4 B) and significantly higher levels in patients with AS compared to adult healthy controls (median 689.4 pg/mL, IQR 424.3-1057 pg/mL vs 347.5, IQR 147.4-475.3 pg/mL, p=0.0036). There was no significant difference in IL1β expression from LPS-stimulated MDMs in patients with polyarticular JIA compared to controls (median 496.7 pg/mL, IQR 209.7-1150 pg/mL).

For IL6, higher levels were seen from LPS-stimulated MDMs from patients with AS compared to adult healthy controls (median 26342 pg/mL, IQR 21294-28006 pg/mL vs 20913 pg/mL, IQR 14045-23588 pg/mL, p=0.021). However, no significant difference was seen between patients with ERA and polyarticular JIA compared to age-matched healthy controls (median 19892 pg/mL, IQR 18289-27100 pg/mL vs 23214 pg/mL, IQR 18207-28004 pg/mL vs 24597 pg/mL, IQR 17147-26290 pg/mL, figure 5.4 C).

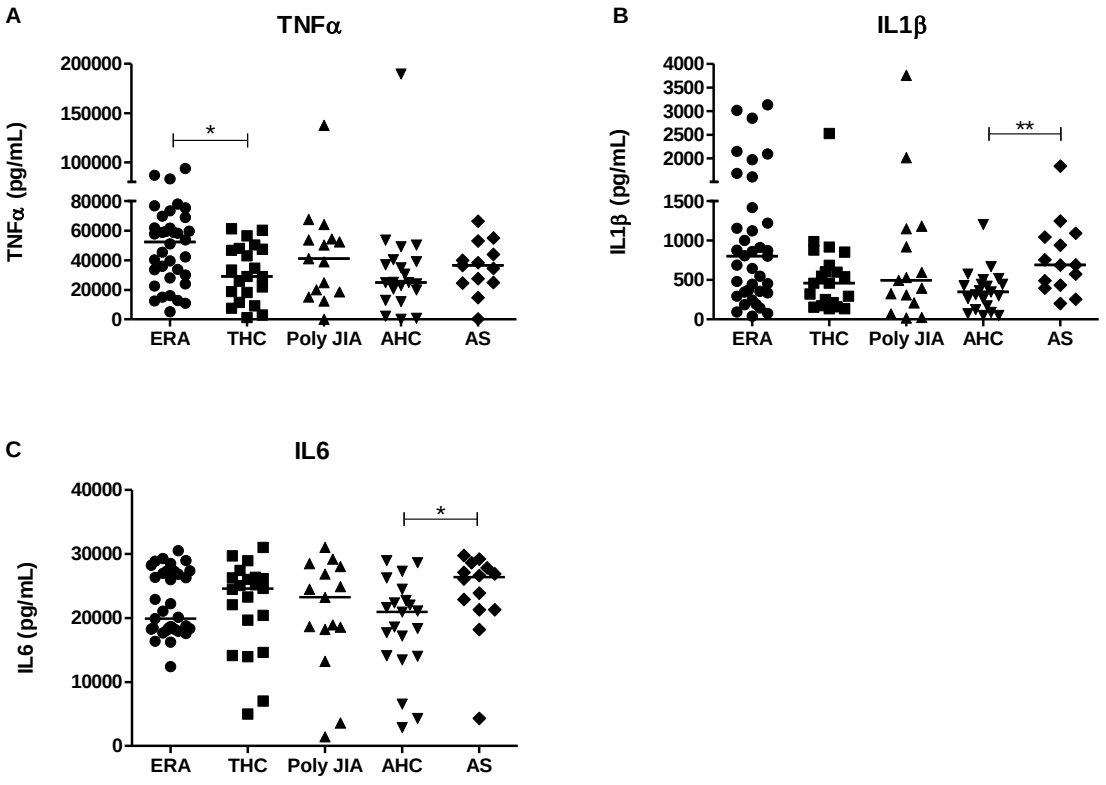


Figure 5.4 TNFα secretion from LPS-stimulated MDMs is higher in patients with ERA compared to healthy controls. MDMs, differentiated as described in section 2.2, were stimulated with LPS for 4 or 24 hours. TNFα was measured in cell culture supernatants by ELISA. IL1β and IL6 were measured by luminex assay. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (*p<0.05) (ERA n=37, THC=teenage healthy controls n=21, Poly JIA=polyarticular JIA, n=15, AS n=14, AHC=adult healthy controls n=21).

5.2.3 HLA B27, enthesitis and male sex are associated with higher TNFα and IL1β expression from LPS-stimulated MDMs in patients with ERA

In patients with ERA, LPS-stimulated MDMs from those who were HLA B27 positive expressed significantly higher levels of TNFα (median 49645 pg/mL, IQR 30948-72540 pg/mL, p=0.013, figure 5.5 A) and IL1β (median 805.7 pg/mL, IQR 362.1-1318 pg/mL, p=0.04, figure 5.5 B) compared to healthy controls. However, no significant difference was seen between patients who were HLA B27 positive compared to those who were HLA B27 negative for these cytokines (TNFα: median 54445 pg/mL, IQR 22905-59695 pg/mL; IL1β 745 pg/mL, IQR 304.3-1758 pg/mL). For IL6, no difference was seen between those who were HLA B27 positive (median 19892 pg/mL, IQR 18245-27790 pg/mL) and healthy controls or those patients with ERA who were HLA B27 negative (median 20557 pg/mL, IQR 18429-25207 pg/mL, figure 5.5 C).

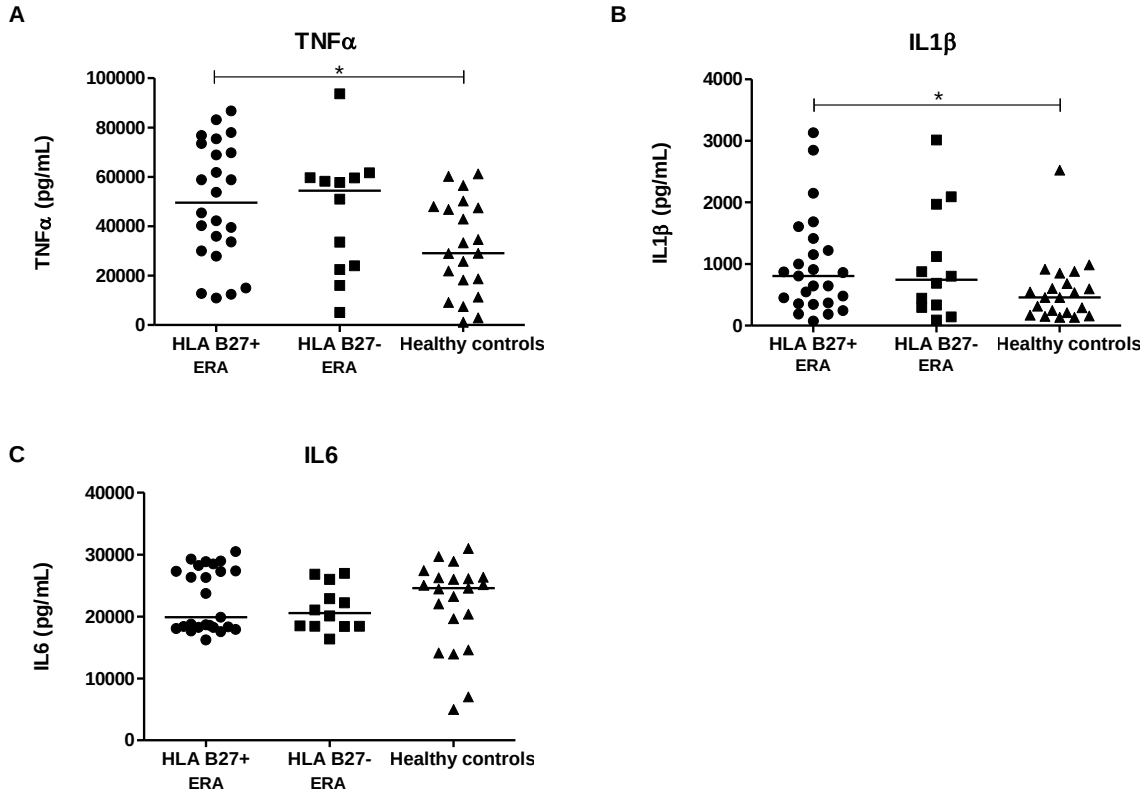


Figure 5.5 Levels of TNFα and IL1β secretion are higher in HLA B27 positive patients with ERA compared to healthy controls. MDMs were differentiated as described in section 2.2 and stimulated with LPS for 4 or 24 hours. TNFα, IL1β and IL6 were measured from cell culture supernatants by ELISA or luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (HLA B27 positive ERA=25, HLA B27 negative ERA =12, adolescent healthy controls n=21, *p<0.05).

Next, analysis was undertaken for patients with and without active enthesitis at the time of sample collection. Interestingly, the results for TNFα and IL1β mirrored those of IL23 with significantly higher levels produced by LPS-stimulated MDMs in patients with ERA with active enthesitis compared to those without active enthesitis (TNFα: median 59660 pg/mL, IQR 35373-75795 pg/mL vs 36640 pg/mL, IQR 20925-54938 pg/mL, p=0.017, figure 5.6 A; IL1β median 871.2 pg/mL, IQR 480.7-1608 pg/mL vs 345.3 pg/mL, IQR 188.9-935.0 pg/mL, p=0.027, figure 5.6 B) and healthy controls (p=0.0014 and p=0.0098 respectively). However, those patients with ERA who had the highest level of TNFα and IL1β production from LPS-stimulated MDMs were not the same as those noted to have very high levels of IL23 and also enthesitis (section 4.3.3), although 3 out of the 6 patients were the same. Similarly, for patients with polyarticular JIA, those with the highest levels of TNFα and IL1β were not the same as those who exhibited very high levels of IL23 expression and enthesitis (section 4.2.3).

Once again, no differences were seen in IL6 production from LPS-stimulated MDMs between those patients with ERA with and without active enthesitis (median 21041 pg/mL, IQR 18382-28221 pg/mL vs 18540 pg/mL, IQR 17861-23650 pg/mL) or compared to healthy controls (figure 5.6 C).

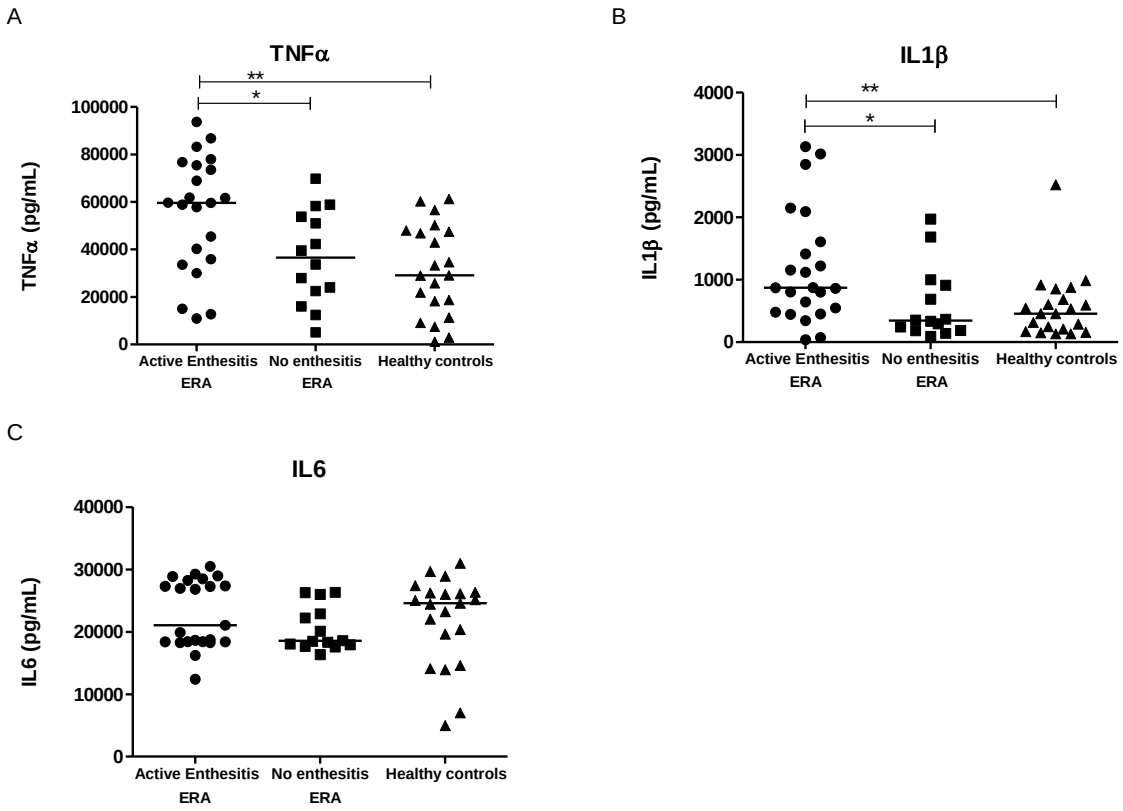


Figure 5.6 Levels of TNFα and IL1β secretion are higher in patients with ERA with active enthesitis compared to those without active enthesitis and healthy controls. MDMs from patients with ERA and age-matched healthy controls were stimulated with LPS for 4 or 24 hours and cytokines were detected in the cell culture supernatants by ELISA or luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA with active enthesitis n=23, ERA with no enthesitis n=14, adolescent healthy controls n=21, *p<0.05, **p<0.01).

In view of the finding that IL23 production was higher from LPS-stimulated MDMs from male patients with ERA compared to male healthy controls, the production of TNFα, IL1β and IL6 was compared between male and female patients with ERA and healthy controls. As noted before the female group was much smaller than the male group, however, a strong trend was seen towards higher production of TNFα and IL1β from LPS-stimulated MDMs in male patients with ERA compared to female patients (TNFα: median 58065 pg/mL, IQR 35373-69173 pg/mL vs 23050 pg/mL, IQR 14455-46093 pg/mL, p=0.09, figure 5.7 A; IL1β median 871.2 pg/mL, IQR 356.3-1608 vs 356.4 pg/mL, IQR 239.3-612.3 pg/mL, p=0.06, figure 5.7 B). As with IL23, a significant difference was seen between male patients with ERA and male healthy controls (TNFα: p=0.0077, IL1β: p= 0.047). For IL6 production from LPS-stimulated MDMs, no difference was observed between male and female patients with ERA (median 20072 pg/mL, IQR 18377-27264 pg/mL vs 19108 pg/mL, IQR 17745-23882 pg/mL) or compared to healthy controls (figure 5.7 C).

In patients with AS, higher levels of TNFα and IL1β were also observed in male patients compared to female patients (TNFα: median 39200 pg/mL, IQR 29880-50840 pg/mL vs 24950 pg/mL, IQR 7606-50535 pg/mL; IL1β 847.9 pg/mL, IQR 468.1-1082 pg/mL vs 531.5 pg/mL, IQR 389.6-830.4 pg/mL) but the numbers in each group were small (male AS n=8, female AS n=6) and this did not reach statistical significance.

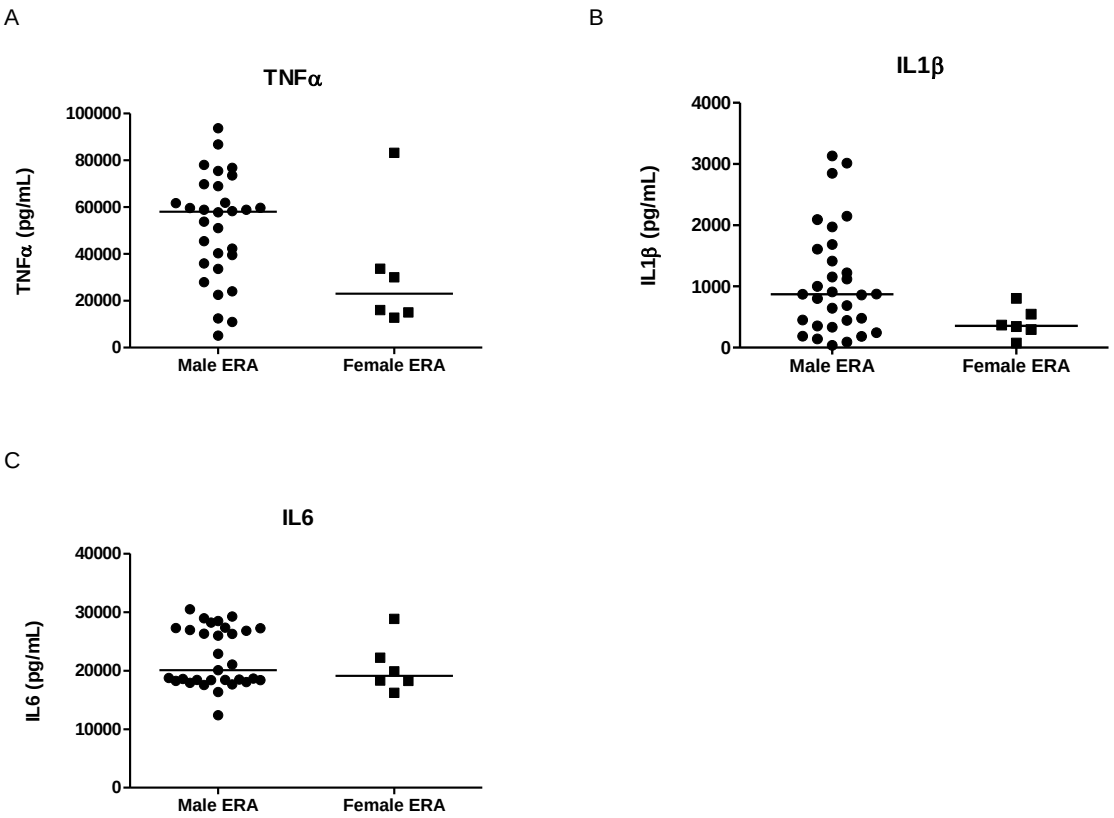


Figure 5.7 Trend towards higher TNFα and IL1β expression from LPS-stimulated MDMs from male patients with ERA compared to female patients. MDMs were stimulated with LPS for 4 or 24 hours. TNFα, IL1β and IL6 were measured from cell culture supernatants by ELISA or luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Mann-Whitney U test (male ERA n=31, female ERA n=6).

5.2.4 Disease activity, other clinical features and production of TNFα, IL1β and IL6 from LPS-stimulated MDMs

It was next important to analyse whether any of the other clinical associations seen with IL23 were also observed with TNFα, IL1β and IL6. As discussed in chapter 4 (section 4.2.5), the JADAS was used as an indication of disease activity but does not capture certain features associated with ERA well, therefore axial disease and enthesitis were analysed separately.

No significant difference was observed in patients with ERA between the JADAS disease activity groups for TNFα, IL1β or IL6 production from LPS-stimulated MDMs (figure 5.8, results summarised in table 5.2). The group with inactive disease was very small (n=2) and therefore it was difficult to make meaningful comparisons for this group. The group with high disease activity was also small (n=6). For TNFα, LPS-stimulated MDMs from those with moderate disease activity secreted higher levels of TNFα compared to healthy controls (p=0.0029, figure 5.8 A). For IL1β and IL6, there was a trend towards increased production in the moderate and high disease activity groups (table 5.2, figure 5.8 B and C) but this did not reach statistical significance. A trend towards higher production of IL1β from LPS-stimulated MDMs was observed between those with high disease activity compared to controls (p=0.058) but for the other groups there was no significant difference compared to healthy controls.

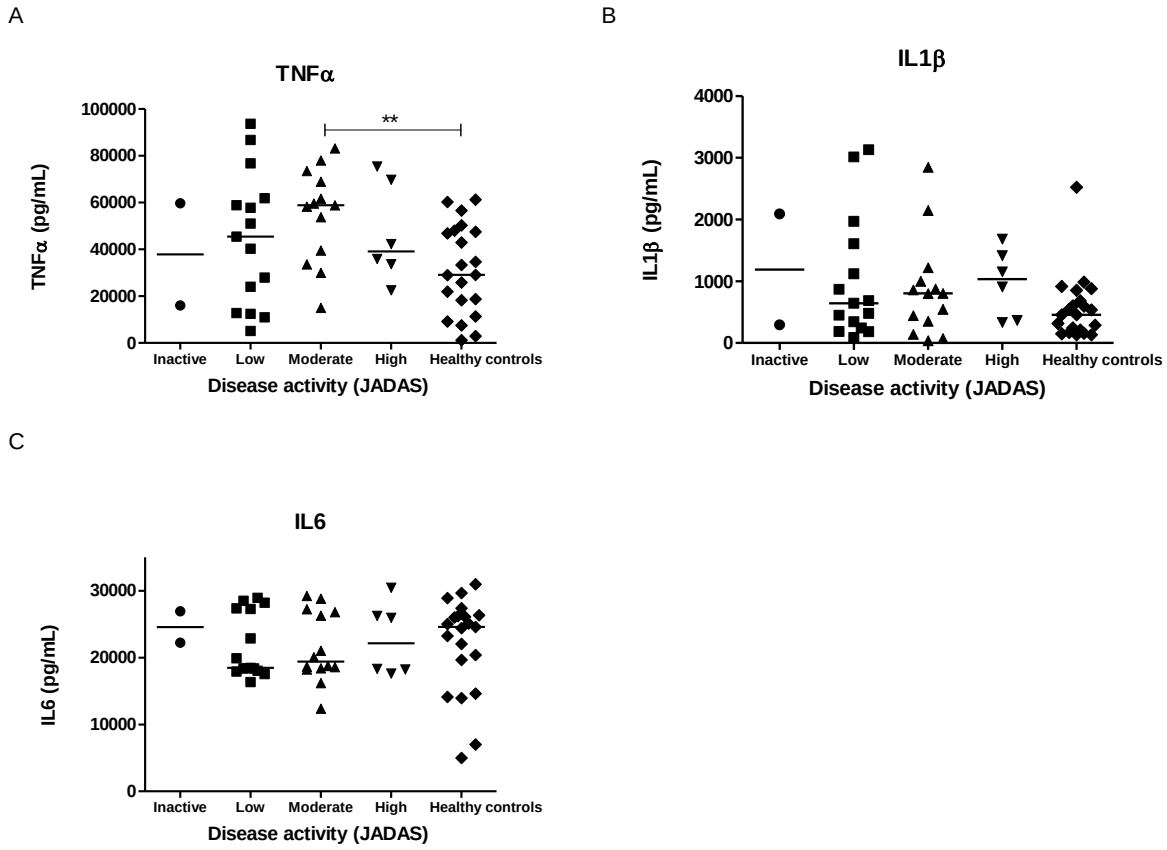


Figure 5.8 TNFα production from LPS-stimulated MDMs is higher in patients with ERA with moderate disease activity compared to healthy controls. MDMs were differentiated as described in the protocol above (section 2.2) and stimulated with LPS for 4 or 24 hours. TNFα, IL1β and IL6 were measured in cell culture supernatants by ELISA or luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (inactive ERA n=2, low disease activity n=15, moderate disease activity n=14, high disease activity n=6, adolescent healthy controls n=21, **p<0.01).

When comparing ERA subtypes, no difference in TNFα, IL1β or IL6 production from LPS-stimulated MDMs was seen between those patients with peripheral ERA and those with indeterminate or axial disease (table 5.2). For TNFα, as with IL23, the highest levels were seen in those with indeterminate ERA which were significantly higher than healthy controls (p=0.005) and there was a trend towards higher levels in those with axial ERA compared to healthy controls (p=0.054). As noted before the group with ERA with peripheral arthritis only was small (n=5).

Similarly, for IL1β, higher levels were observed from LPS-stimulated MDMs in those with indeterminate ERA and axial ERA compared to those with peripheral ERA but the difference was not statistically significant (table 5.2). There was a trend towards higher IL1β production in those with axial ERA compared to healthy controls (p=0.091, figure 5.9 B).

For IL6, there was no significant difference between those with indeterminate and axial ERA compared to those with peripheral ERA (table 5.2) and healthy controls (figure 5.9 C).

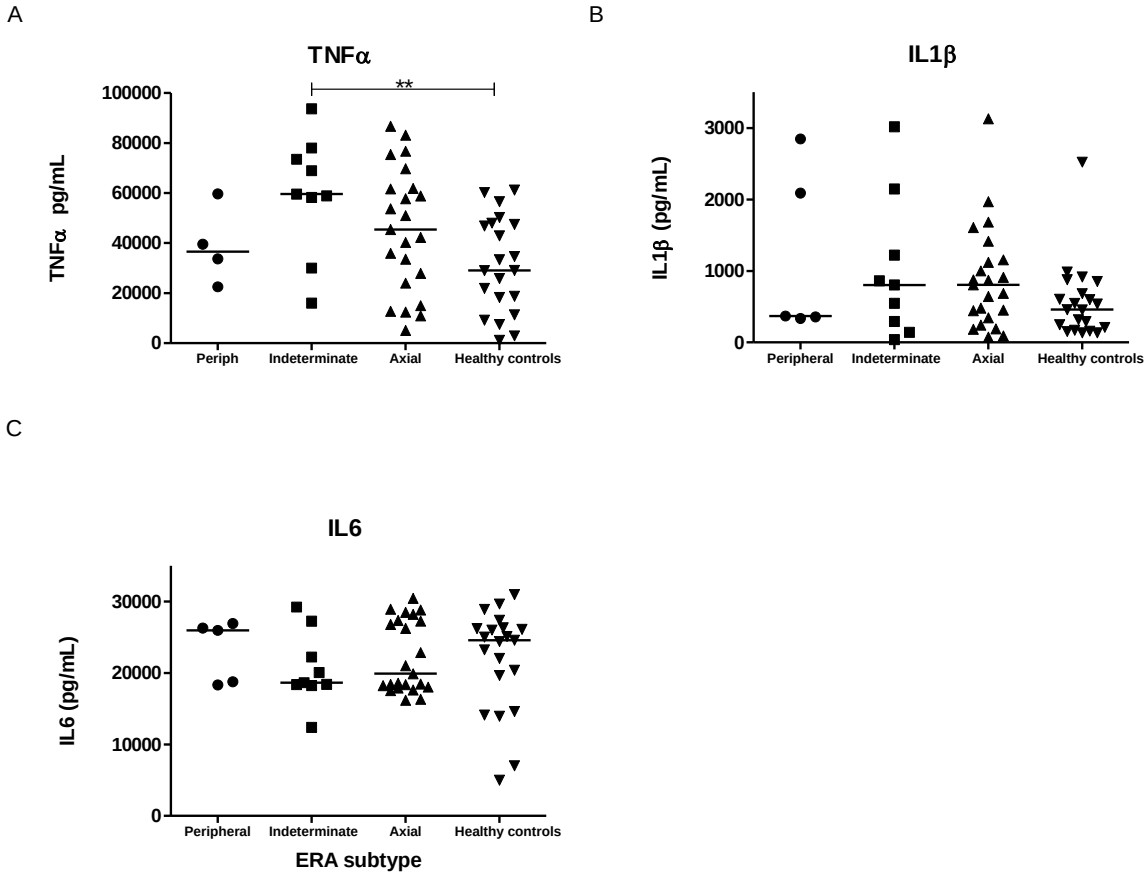


Figure 5.9 TNFα production from LPS-stimulated MDMs is higher in patients with the indeterminate subtype of ERA compared to healthy controls. MDMs were stimulated with LPS for 4 or 24 hours. TNFα, IL1β and IL6 were measured in cell culture supernatants by ELISA or luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (peripheral ERA n=5, indeterminate ERA n= 9, axial ERA n=23, adolescent healthy controls n=21, **p<0.01).

There was a trend towards higher production of TNFα from LPS-stimulated MDMs from patients with ERA and active peripheral arthritis at the time of sample compared to those without active peripheral arthritis (median 71235 pg/mL, IQR 35185-93720 pg/mL vs 48280 pg/mL, IQR 22905-59695 pg/mL (p=0.083). However, for IL1β and IL6 no differences were observed (table 5.2). Compared to healthy controls, TNFα and IL1β production from LPS-stimulated MDMs from those with active peripheral arthritis was significantly higher (TNFα: p=0.0058; IL1β p=0.029). Hip arthritis was again analysed separately but no significant differences were seen between those patients with ERA with and without hip arthritis for any of the 3 cytokines (table 5.2) although levels of IL1β from LPS-stimulated MDMs from patients with ERA with active hip arthritis were significantly higher than healthy controls (p=0.024).

As with IL23, there was no correlation found between TNFα, IL1β or IL6 production from LPS-stimulated MDMs in patients with ERA and CRP level at the time of sample, age or disease duration. Extra-articular manifestations were present in 7 patients with ERA and in this group and TNFα production from LPS-stimulated MDMs was significantly higher in this group compared to healthy controls (median 58910 pg/mL, IQR 40320-75450 pg/mL, p=0.029) but not significantly different to those patients not having extra-articular manifestations of disease (median 45480 pg/mL, IQR 25985-65410 pg/mL). Production of IL1β and IL6 from LPS-stimulated MDMs was no different in patients with ERA with and without extra-articular manifestations.

In the other patient groups (AS and polyarticular JIA), as discussed in chapter 4 (section 4.2.4), it was difficult to analyse TNFα, IL1β and IL6 production from LPS-stimulated MDMs in relation to clinical features or disease activity because of the small numbers in each subgroup (for example JADAS category in polyarticular JIA) or lack of available clinical information (for example BASDAI and BASFI in patients with AS). Subgroup analysis was therefore not undertaken for these groups.

5.2.5 Treatment with TNF inhibitors and DMARDs does not significantly affect TNFα, IL1β and IL6 levels from LPS-stimulated MDMs from patients with ERA

In view of the observation that IL23 levels from LPS-stimulated MDMs were significantly higher in patients with ERA on TNF inhibitors, it was of interest to determine whether TNFα, IL1β and IL6 were affected by treatment in patients with ERA. Levels were compared for patients on TNF inhibitors, DMARDS and NSAIDs to those not on them. In contrast to IL23, there was no difference in levels of TNFα, IL1β and IL6 from LPS-stimulated MDMs from patients with ERA on TNF inhibitors compared to those not on them (table 5.2). However, TNFα production from LPS-stimulated MDMs from patients on TNF inhibitors was higher than healthy controls (p=0.021).

Similarly, no difference was observed between patients on DMARDs (with or without TNF inhibitors) compared to those not on them (table 5.2). Again, for TNFα, higher levels were

seen from LPS-stimulated MDMs in patients with ERA on DMARDs compared to healthy controls (p=0.011). This was perhaps due to higher levels of TNFα seen in those patients also taking a TNF inhibitor combined with a DMARD (n=12) compared to those on a DMARD alone (n=14) (median 59250 pg/mL, IQR 33748-70588 pg/mL vs 37755 pg/mL, IQR 21198-71543 pg/mL) and levels of TNFα production in those on combined treatment were significantly higher than healthy controls (p=0.0083).

Treatment with NSAIDs did not appear to significantly affect the production of TNFα, IL1β or IL6 (table 5.2). A small number of patients (n=4) had been recently treated with corticosteroids and this did not appear to affect the production of TNFα, IL1 and IL6 from LPS-stimulated MDMs in patients with ERA.

Similarly, and contrary to the IL23 findings for patients with AS on TNF inhibitors, there was no difference in TNFα, IL1β and IL6 expression from LPS-stimulated MDMs in those treated with TNF inhibitors (n=6) compared to those not treated with them (n=8) (TNFα: median 33525 pg/mL, IQR 24458-46813 pg/mL vs 38360 pg/mL, IQR 24540-53110 pg/mL; IL1β: 993.1 pg/mL, IQR 626.9-1280 pg/mL vs 531.5 pg/mL, IQR 291.0-737.7 pg/mL; IL6 26855 pg/mL, IQR 22453-28291 pg/mL vs 24481 pg/mL, 21289-28175 pg/mL) although there was a trend towards higher IL1β production (p=0.08) which was significantly higher than adult healthy controls (p=0.0029). In patients with polyarticular JIA, no difference was observed in TNFα, IL1β and IL6 production from LPS-stimulated MDMs between patients treated with TNF inhibitors (n=6) compared to those not taking them (n=9) (TNFα: median 29525 pg/mL, IQR 9330-47833 pg/mL vs 52590 pg/mL, IQR 21755-59405 pg/mL; IL1β: 402.8 pg/mL, IQR 174.5-743.4 vs 530.5 pg/mL, IQR 174.5-1584 pg/mL; IL6 18611 pg/mL, IQR 14559-25357 pg/mL vs 24897 pg/mL, IQR 16071-28822 pg/mL) but the subgroup sizes were small.

5.2.6 TNFα and IL1β production from LPS-stimulated MDMs correlate with IL23 production in patients with ERA

In chapter 4 (section 4.4.7), correlations were noted between the production of IL23 from LPS-stimulated MDMs and levels of IL12 and IL27 in patients with ERA as well as healthy controls. It was therefore of interest to determine whether levels of TNFα, IL1β and IL6 production from LPS-stimulated MDMs correlated with IL23. TNFα production correlated with levels of IL23 (r=0.44, p=0.0074, figure 5.10 A) and IL1β levels strongly correlated with IL23 (r=0.57, p=0.0002, figure 5.10 B). However, IL6 production did not correlate with IL23 (figure 5.10 C). Interestingly, for adolescent healthy controls and patients with polyarticular JIA, no correlations were found between IL23 and TNFα, IL1β or IL6. In patients with AS, only a borderline correlation between IL23 and IL1β was seen (r=0.5, p=0.049).

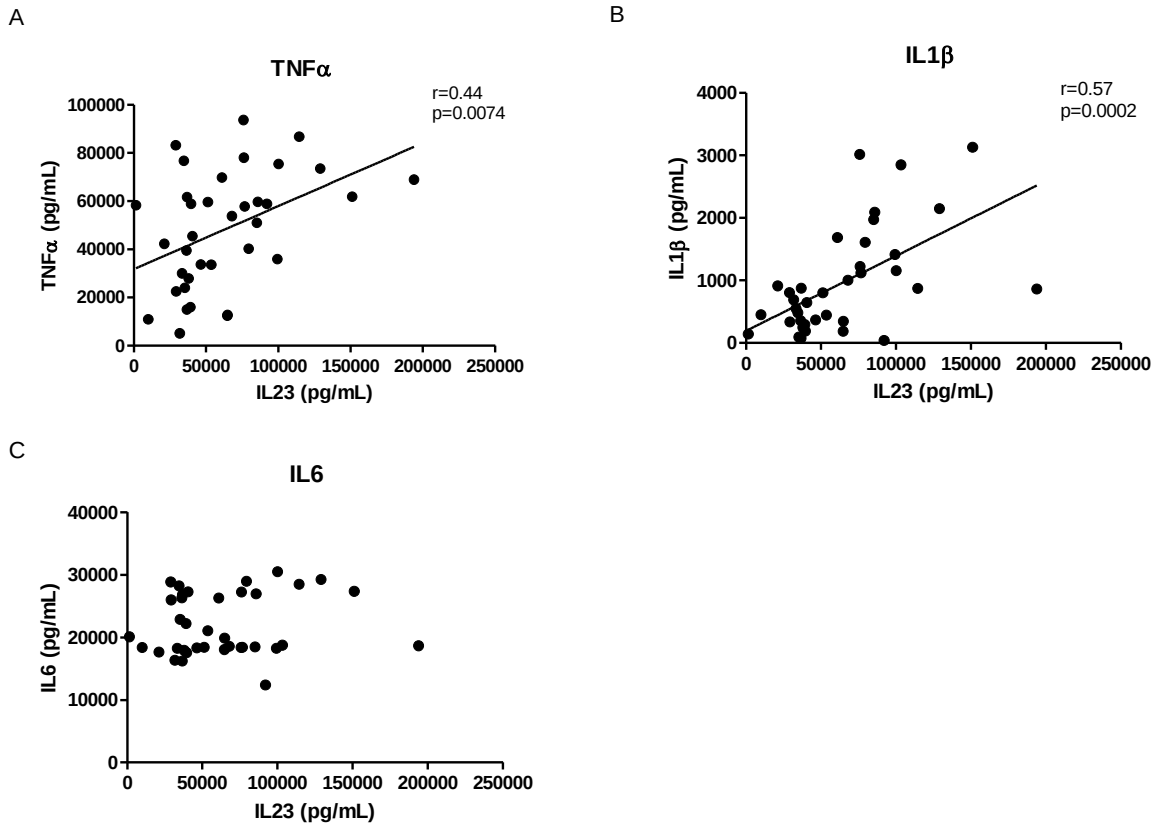


Figure 5.10 TNFα and IL1β levels from LPS-stimulated MDMs from patients with ERA correlate with IL23 production. MDMs were differentiated and stimulated with LPS for 4 or 24 hours. Cell culture supernatants were then collected and analysed by ELISA or luminex assay for TNFα, IL1β and IL6. Graphs show Spearman's rank correlation between IL23 and TNFα (A), IL1β (B) and IL6 (C) for patients with ERA (n=37).

5.3 Detecting IL17 from LPS-stimulated MDMs

5.3.1 Introduction

As discussed in section 3.5.2, IL17 production was not detected by either Luminex assay or proteome profiler array in cell culture supernatants from MDMs stimulated with LPS for 24 hours. As a cytokine of particular interest, being heavily implicated in the pathogenesis of SpA (Smith 2016), it was decided to explore whether IL17 gene expression could be detected in LPS stimulated MDMs using qPCR. In a small number of samples (ERA n=22, adolescent healthy controls n=12), RNA was isolated from MDMs following stimulation with LPS for 24 hours.

5.3.2 Gene expression of IL17 is not significantly different between patients with ERA and healthy controls

There was no significant difference observed between IL17 expression from RNA isolated from LPS-stimulated MDMs from patients with ERA compared to healthy controls (median fold change 3.909, IQR 0.343-18.35 vs 1.642, IQR 0.144-10.00, figure 5.11 A), although the results were highly variable in both groups and around a third of patients and healthy controls exhibiting no expression at all (ERA 36.3%, healthy controls 33.3%). Contrary to the findings for IL23p19 gene expression, there was no difference in IL17 expression from LPS-stimulated MDMs between HLA B27 positive and negative patients with ERA (median fold change 10.80, IQR 1.677-33.25 vs 1.602, IQR 0.053-14.72, figure 5.11 B). However, similar to IL23p19, there was a trend towards higher IL17 gene expression in patients with active enthesitis at the time of sample compared to those with no active enthesitis (median fold change 11.16, IQR 0.901-29.24 vs 0.582, IQR 0.051-5.776, p=0.091, figure 5.11 C).

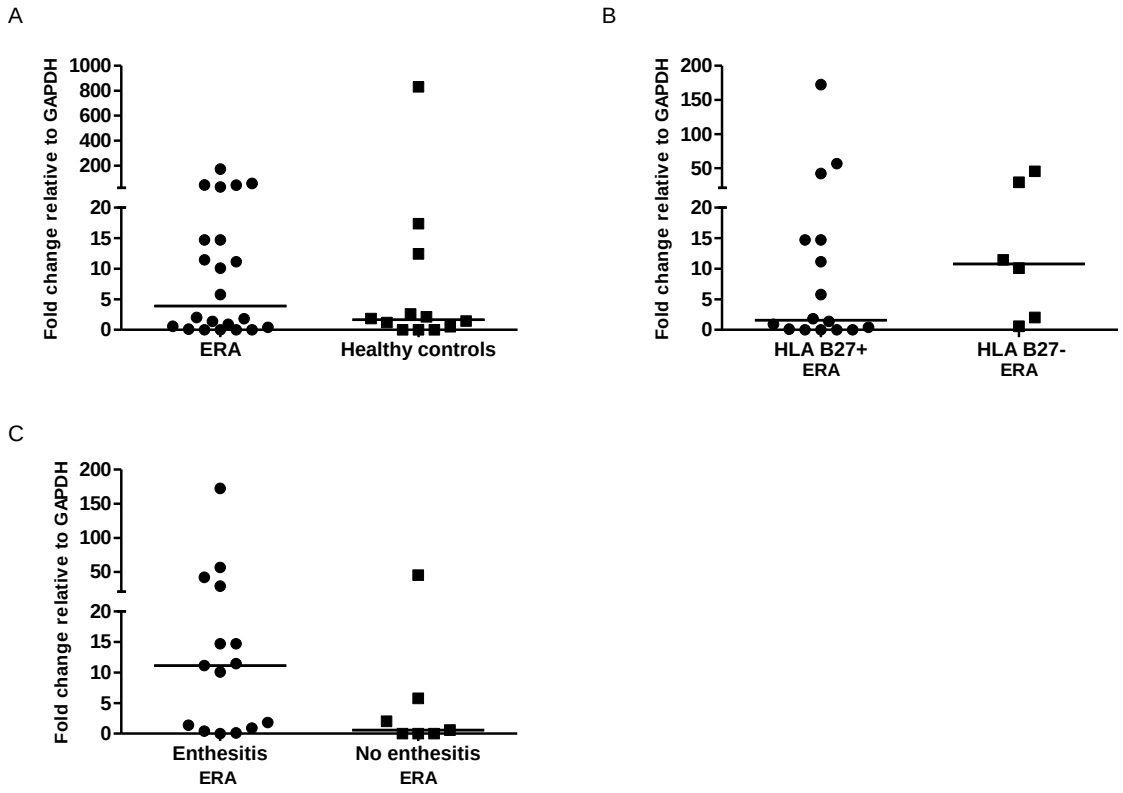


Figure 5.11 IL17 gene expression is no different between patients with ERA and healthy controls. IL17 gene expression was analysed from RNA from LPS-stimulated MDMs using qPCR and normalised against GAPDH. Fold change was calculated relative to an unstimulated control for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann-Whitney U test (ERA n=22, adolescent healthy controls n=12, HLA B27 positive ERA n=16, HLA B27 negative ERA n=6, ERA with active enthesitis n=15, ERA without active enthesitis n=7).

In view of the importance of IL17 in the pathogenesis of SpA, further attempts were made to determine whether gene expression of IL17 translated in to IL17 protein production. Attempts were made to detect IL17 at an earlier time point following LPS stimulation (4 hours) but no IL17 was detected in cell culture supernatants by ELISA. IL17 gene expression was also tested in MDMs from a small number of patients (n=15) and healthy controls (n=5) after 4 hours LPS stimulation but the fold change in expression was generally lower than after 24 hours stimulation (median fold change ERA: 1.548, IQR 0.025-17.27; healthy controls, 0.145, IQR 0.007-10.78), although again the results were highly variable with no fold increase observed in 47% of patients with ERA and 67% of healthy controls. It was concluded that in this assay, LPS-stimulated MDMs did not express significant levels of IL17 or alternatively that the stability of IL17 at the time points tested made it difficult to detect.

5.4 GMCSF production in the cell culture supernatants from MDMs from patients with ERA, polyarticular JIA, AS and adolescent and adult healthy controls

5.4.1 Introduction

The next cytokine to be analysed from the cell culture supernatants from MDMs from patients and healthy controls was GMCSF. This cytokine had been identified in the proteome profiler array (section 3.6.2) as a cytokine of interest with potential differences seen in expression between patients with ERA and the healthy control. In addition to being a haemopoietic growth factor (Metcalf 2008), GMCSF plays a central role in regulating innate immunity (Cornish, Campbell et al. 2009) and has recently been implicated in the pathogenesis of SpA (Al-Mossawi, Chen et al. 2017) with increased numbers of GMCSF producing Th17 cells found in patients with SpA compared to healthy controls and patients with rheumatoid arthritis.

5.4.2 GMCSF production is no different from MDMs without LPS stimulation in patients with ERA compared to healthy controls

As with IL27, GMCSF was detected in cell culture supernatants from MDMs without LPS stimulation. Following differentiation as described above in section 2.2 and the addition of IFNγ, MDMs were washed and the media replaced. MDMs were then cultured for a further 24 hours but no additional stimuli were added. GMCSF was measured by Luminex assay in the cell culture supernatants. Levels were no different in patients with ERA compared to adolescent healthy controls (median 121.3 pg/mL, IQR 96.6-194.0 pg/mL vs 157.1 pg/mL, IQR 124.2-203.3 pg/mL, figure 5.12). In addition, there was no significant difference between GMCSF production from MDMs without LPS stimulation from patients with AS and adult healthy controls (median 186.1 pg/mL, IQR 147.1-210.1 pg/mL vs 150.5 pg/mL, IQR 117.9-191.4 pg/mL) or between patients with polyarticular JIA and adolescent healthy controls (median 145.3 pg/mL, IQR 77.62-170.6 pg/mL).

In view of the findings for IL23 and other cytokines demonstrating significant differences between patients with ERA who were HLA B27 positive, male and had active enthesitis at the time of sample compared to healthy controls, these features were analysed but no differences were seen in GMCSF production from MDMs without LPS stimulation (table 5.3).

Further analysis was undertaken to determine whether any other clinical features or treatment were associated with higher levels of GMCSF from MDMs without LPS stimulation but no such differences were found (table 5.3).

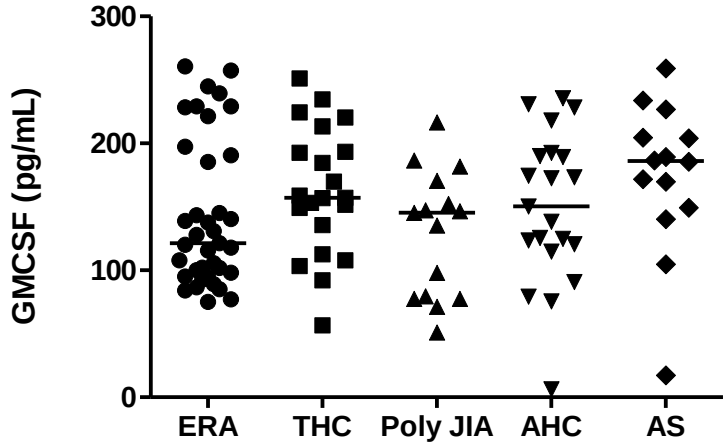


Figure 5.12 Levels of GMCSF production from MDMs without LPS stimulation are not significantly different between patients and healthy controls. MDMs were cultured as per the protocol in section 2.2. The media was replaced and MDMs were cultured for a further 24 hours after which cell culture supernatants were collected and analysed by luminex assay. Box and whisker plots show median (+minimum to maximum range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA n=37, THC=teenage healthy controls n=21, polyarticular JIA n=15, AHC=adult healthy controls n=21, AS n=14).

5.4.3 GMCSF levels are higher from LPS-stimulated MDMs from patients with ERA compared to healthy controls

Next, analysis of GMCSF production in cell culture supernatants from MDMs following LPS stimulation was undertaken. As seen with IL23, levels of GMCSF from LPS-stimulated MDMs were higher from patients with ERA compared to adolescent healthy controls (median 1853 pg/mL, IQR 1125-3061 pg/mL vs 1175 pg/mL, IQR 607.0-1779 pg/mL, p=0.010, figure 5.13). This was also the case for patients with AS compared to adult healthy controls (median 1578 pg/mL, IQR 992.7-2703 pg/mL vs 1044 pg/mL, IQR 433.9-1510 pg/mL, p=0.038). There was no significant difference in levels from LPS-stimulated MDMs in those with polyarticular JIA (median 1535 pg/mL, IQR 727.1-2267 pg/mL) compared with adolescent healthy controls.

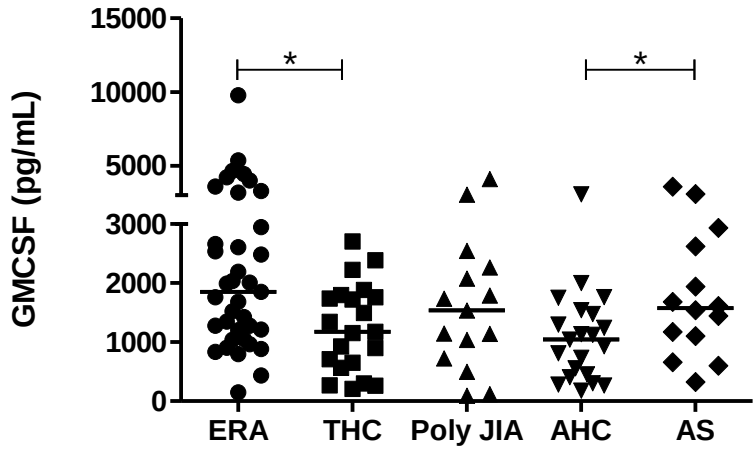


Figure 5.13 LPS-stimulated MDMs from patients with ERA and AS secrete higher levels of GMCSF compared to healthy controls. MDMs were stimulated with LPS for 24 hours after which the cell culture supernatants were collected and GMCSF was measured by luminex assay. Box and whisker plots show median (+minimum to maximum range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA n=37, THC=teenage healthy controls n=21, polyarticular JIA n=15, AHC=adult healthy controls n=21, AS n=14, *p<0.05).

5.4.4 GMCSF production from LPS-stimulated MDMs is higher in male patients with ERA and in those with active enthesitis compared to healthy controls

Next analysis was undertaken to determine whether any clinical features or disease activity had an effect on GMCSF secretion from LPS-stimulated MDMs. There was no difference in GMCSF levels between HLA B27 positive patients compared to those who were HLA B27 negative (median 1685 pg/mL, IQR 1046-3122 pg/mL vs 2115 pg/mL, IQR 1228-3048 pg/mL, figure 5.14 A), although in both groups levels were significantly higher than healthy controls (p=0.034 and 0.019 respectively) reflecting the higher levels from patients with ERA in general. Levels of GMCSF production were significantly higher from LPS-stimulated MDMs in male patients with ERA compared to female patients (median 2038 pg/mL, IQR 1278-3298 pg/mL vs 1088 pg/mL, IQR 826.0-1571 pg/mL, p=0.030, figure 5.14 B) and compared to male adolescent healthy controls (p=0.0052). In patients with ERA with active enthesitis at the time of sample, there was a trend towards higher GMCSF production from LPS-stimulated MDMs compared to those without active enthesitis (median 2191 pg/mL, IQR 1278-3298 pg/mL vs 1312 pg/mL, IQR 897.5-2265 pg/mL, p=0.077, figure 5.14 C) and significantly higher production compared to healthy controls (p=0.0026).

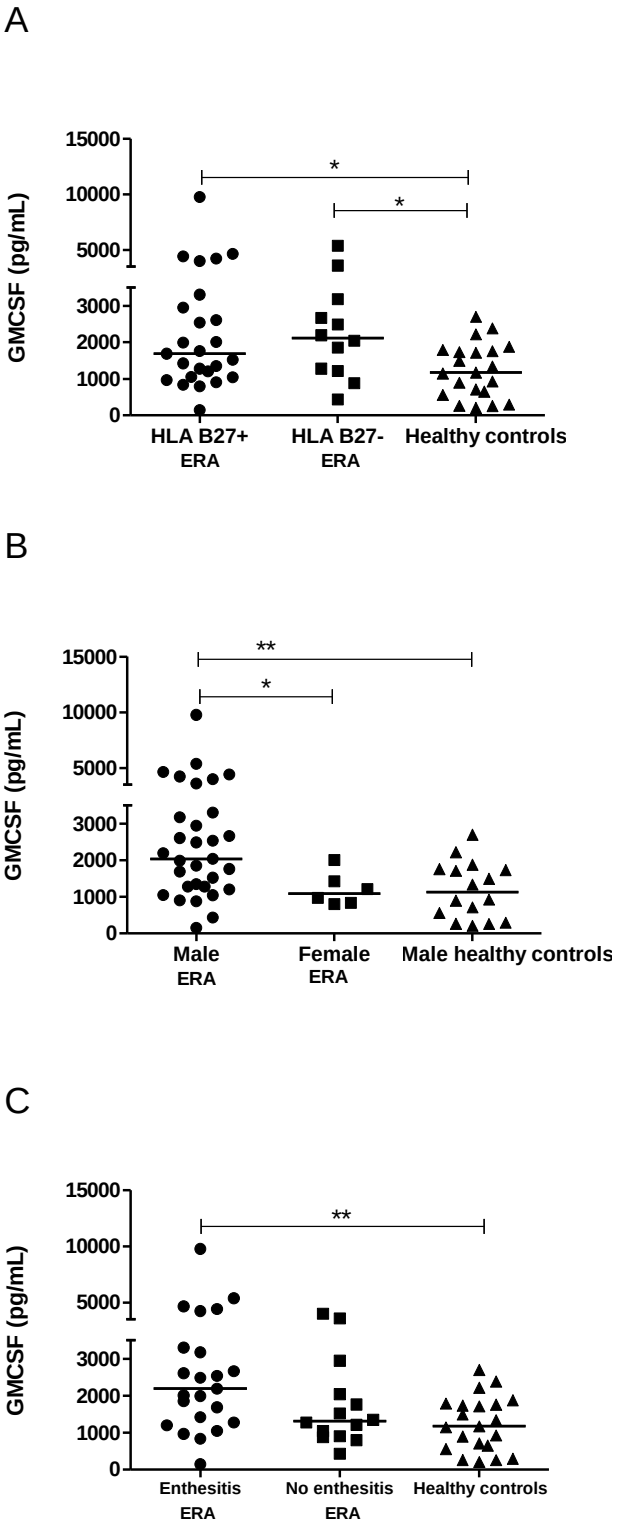


Figure 5.14 GMCSF production from LPS-stimulated MDMs is higher in patients with ERA compared to healthy controls, especially in those who are male and those with active enthesitis. MDMs were differentiated as previously described (section 2.2) and stimulated with LPS for 24 hours. GMCSF was measured in cell culture supernatants by Luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by

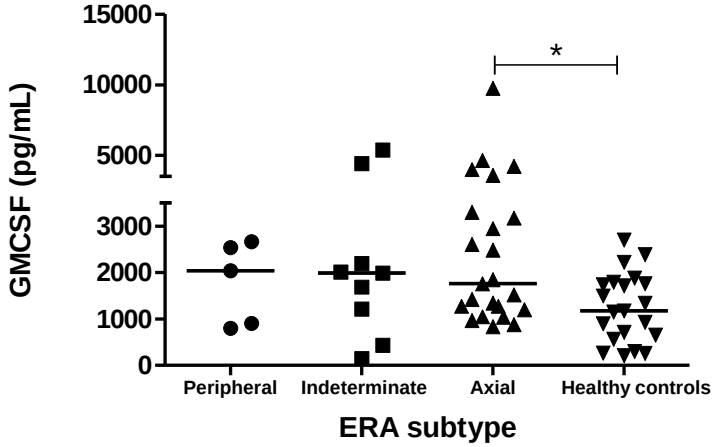
Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (HLA B27+ ERA n=25, HLA B27- ERA n=12, male ERA n=31, female ERA n=6, male healthy controls n=16, ERA with active enthesitis n=23, ERA without enthesitis n=14, adolescent healthy controls n=21, *p<0.05, **p<0.01).

Next, comparison was made between those patients with axial ERA and those with peripheral and indeterminate ERA. No significant difference was seen in GMCSF levels from LPS-stimulated MDMs between patients with axial ERA compared to those with peripheral arthritis only or indeterminate ERA, although levels from those with axial ERA were significantly higher than healthy controls (p=0.010, figure 5.15 A). In addition, there was no difference between those with active peripheral arthritis at the time of sample compared to those without (table 5.2). Similarly, no difference was observed between those with hip arthritis compared to those without (table 5.2), although levels of GMCSF from LPS-stimulated MDMs from both those with active peripheral arthritis and hip arthritis were higher than healthy controls (p=0.043 and 0.015 respectively).

There was a trend towards higher GMCSF production from LPS-stimulated MDMs in those with higher disease activity by JADAS criteria but there was no significant difference between the groups A trend was also seen towards higher GMCSF production compared to healthy controls in the moderate and high disease activity categories (p=0.089 and 0.058 respectively) and levels in the low disease activity group were significantly higher than healthy controls (p=0.037, figure 5.15 B).

As with other cytokines, no correlation was seen between GMCSF production from LPS-stimulated MDMs and CRP, disease duration or age of the patient or healthy controls. In addition, GMCSF production from LPS-stimulated MDMs in patients who had extra-articular manifestations of ERA such as anterior uveitis or inflammatory bowel disease was not significantly higher than those without extra-articular manifestations (median 2191 pg/mL, IQR 1049-3592 pg/mL vs 1807 pg/mL, IQR 1162-3004 pg/mL).

A



B

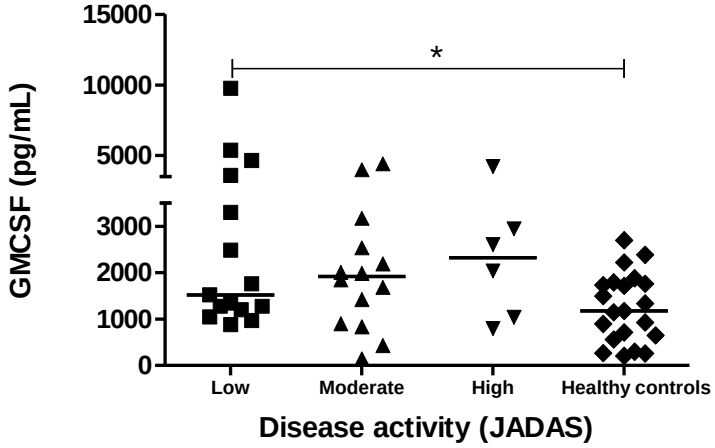


Figure 5.15 GMCSF production from LPS-stimulated MDMs is higher in patients with axial ERA compared to healthy controls. MDMs were differentiated and stimulated with LPS for 24 hours. GMCSF was measured in cell culture supernatants by luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (peripheral ERA n=5, indeterminate ERA n= 9, axial ERA n=23, adolescent healthy controls n=21, low disease activity n=15, moderate disease activity n=14, high disease activity n=6, *$p<0.05$).

5.4.5 Treatment with TNF inhibitors is not associated with higher GMCSF production from LPS-stimulated MDMs

Contrary to the findings with IL23 (section 4.3.5), there was no significant difference in GMCSF production from LPS-stimulated MDMs in those patients with ERA treated with TNF inhibitors compared to those not on them, although GMCSF levels from both treated and untreated groups were significantly higher than healthy controls (p=0.022 and 0.033 respectively). In addition there were no significant differences in GMCSF production from LPS-stimulated MDMs between patients on treatment with DMARDs compared to those on treatment and patients treated with NSAIDs compared to those not on them (table 5.2).

5.4.6 GMCSF production from LPS-stimulated MDMs correlates with IL23 production

Finally, analysis was undertaken to determine whether levels of GMCSF from LPS-stimulated MDMs correlated with IL23 production. A strong correlation was observed in patients with ERA (r=0.61, p<0.0001, figure 5.16). Correlations were also observed for adolescent healthy controls (r=0.58, p=0.0054), adult healthy controls (r=0.50, p=0.020)and patients with polyarticular JIA (r=0.55, p=0.032) and AS (r=0.57, p=0.032).

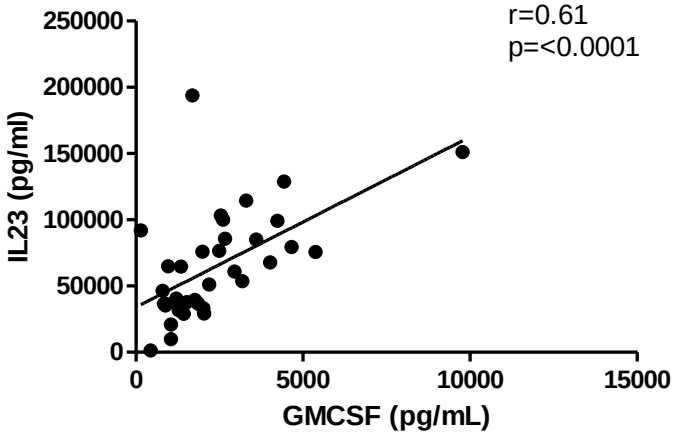


Figure 5.16 Levels of GMCSF from LPS-stimulated MDMs from patients with ERA correlate with IL23 production. MDMs were differentiated as above and stimulated with LPS for 24 hours. Cell culture supernatants were then collected and analysed by luminex assay for GMCSF. Graphs show Spearman's rank correlation between IL23 and GMCSF for patients with ERA (n=37).

5.5 Analysis of CCL4 and CCL5 in the cell culture supernatants from MDMs from patients with ERA, polyarticular JIA, AS and adolescent and adult healthy controls

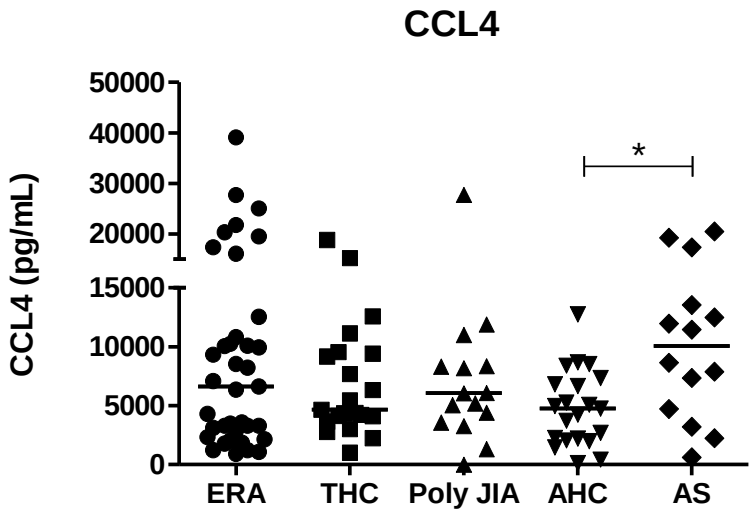
5.5.1 Introduction

Next, analysis was undertaken of two chemokines identified by the proteome profiler array (section 3.6.2) as exhibiting differences in expression between the patients with ERA and healthy controls. CCL4 (MIP 1β) and CCL5 (RANTES) are pro-inflammatory chemokines known to be secreted by inflammatory-phenotype macrophages (Mantovani, Sica et al. 2004) which exert chemotactic activity towards T cells, monocytes and NK cells (Szekanecz, Vegvari et al. 2010).

5.5.2 CCL4 and CCL5 production in cell culture supernatants from MDMs without LPS stimulation is no different in patients with ERA compared to healthy controls

As with GMCSF and IL27, both CCL4 and CCL5 were detected in cell culture supernatants from MDMs without LPS stimulation. No significant difference was observed in levels of CCL4 or CCL5 between patients with ERA or patients with polyarticular JIA and age-matched healthy controls (CCL4: ERA median 6630 pg/mL, IQR 2602-11700 pg/mL, polyarticular JIA median 6056 pg/mL, IQR 3568-8367 pg/mL, healthy controls median 4653 pg/mL, IQR 3886-9479 pg/mL; CCL5: ERA median 709.2 pg/mL, IQR 241.8-1399 pg/mL, polyarticular JIA 562.7 pg/mL, IQR 318.0-887.4 pg/mL, healthy controls median 661.3 pg/mL, IQR 302.8-1078 pg/mL, figure 5.17). In patients with AS, MDMs without LPS stimulation produced more CCL4 compared to adult healthy controls (median 10071 pg/mL, IQR 4374-14510 pg/mL vs 4771 pg/mL, IQR 2155-7095 pg/mL, p=0.011, figure 5.17 A) and there was also a trend towards higher CCL5 expression (median 714.7 pg/mL, IQR 465.9-1459 pg/mL vs 461.9 pg/mL, IQR 223.1-712.5 pg/mL, p=0.064, figure 5.17 B).

A



B

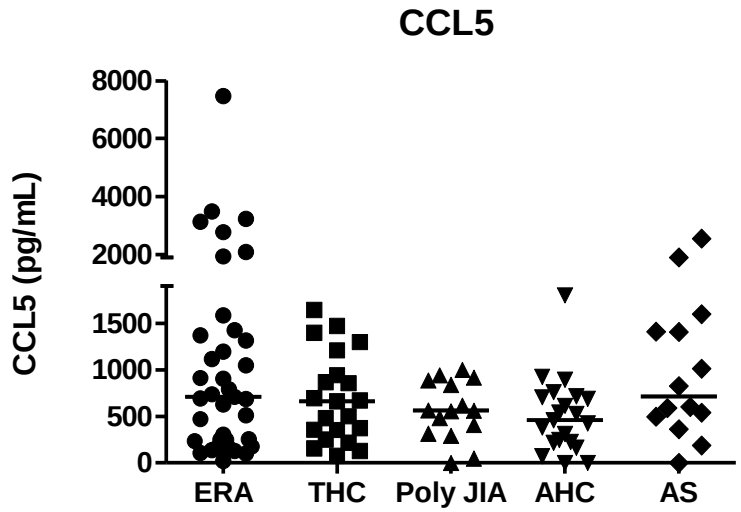


Figure 5.17 CCL4 production from MDMs without LPS stimulation is higher in patients with AS compared to adult healthy controls. MDMs were cultured as per the protocol in section 2.2. The media was replaced and MDMs were cultured for a further 24 hours after which cell culture supernatants were collected and analysed by Luminex assay. Box and whisker plots show median (+minimum to maximum range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA n=37, THC=teenage healthy controls n=21, polyarticular JIA n=15, AHC=adult healthy controls n=21, AS n=14, *$p<0.05$).

5.5.3 CCL4 and CCL5 production from MDMs without LPS stimulation is not affected by clinical features, disease activity or treatment in patients with ERA

CCL4 and CCL5 production was analysed in subgroups of patients with ERA with different clinical features but no significant differences were found between male and female patients, HLA B27 positive and negative patients, patients with active enthesitis compared to those without or between these groups and healthy controls for either cytokine (table 5.3).

Other clinical features such as active peripheral arthritis and axial ERA did not significantly affect CCL4 or CCL5 production from MDMs without LPS stimulation. There was no difference across the disease activity or treatment groups either (table 5.3) or between these groups and healthy controls.

CCL4 and CCL5 production from MDMs without LPS stimulation did not correlate with CRP, age, disease duration of patients with ERA or with IL23 production from LPS-stimulated MDMs. However, there was a strong correlation between CCL4 and CCL5 production ($r=0.83$, $p<0.0001$, figure 5.18).

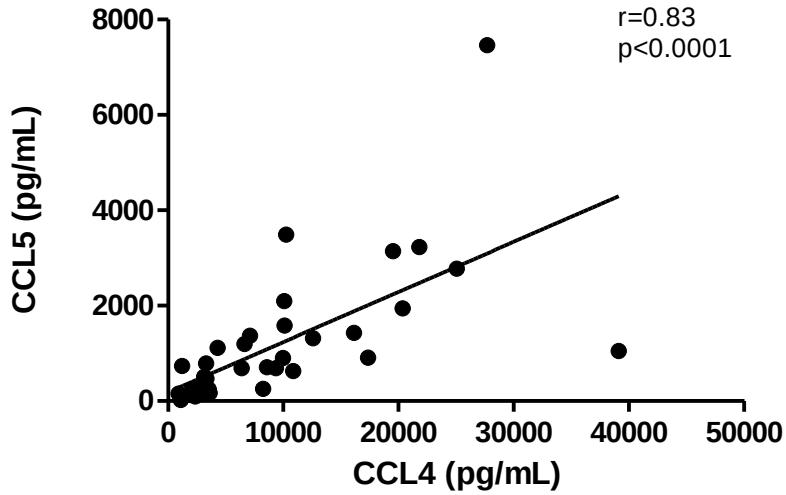


Figure 5.18 Levels of CCL4 from MDMs without LPS stimulation in patients with ERA correlate with CCL5 production. MDMs were differentiated and stimulated with LPS for 24 hours. Cell culture supernatants were then collected and analysed by Luminex assay for CCL4 and CCL5. Graphs show Spearman's rank correlation between CCL4 and CCL5 for patients with ERA (n=37).

5.5.4 Levels of CCL5 in cell culture supernatants from LPS-stimulated MDMs are higher in patients with ERA compared to healthy controls

Next, analysis of CCL4 and CCL5 was undertaken in cell culture supernatants from LPS-stimulated MDMs. CCL5 levels were significantly higher from patients with ERA compared to healthy controls (median 42177 pg/mL, IQR 30313-53937 pg/mL vs 29626 pg/mL, IQR 20664-44991 pg/mL, p=0.014, figure 5.19 B). No significant difference was observed in CCL5 production between patients with polyarticular JIA and healthy controls (median 34846 pg/mL, IQR 23209-56412 pg/mL) or between patients with AS and adult healthy controls (median 35305 pg/mL, IQR 25378-52576 pg/mL vs 31130 pg/mL, IQR 14921-44512 pg/mL). In addition, no significant difference was noted in CCL4 production from LPS-stimulated MDMs between patients with ERA or polyarticular JIA and healthy controls (ERA: median 107792 pg/mL, IQR 87620-135595 pg/mL, polyarticular JIA: median 110027 pg/mL, IQR 80296-157238 pg/mL vs 133707 pg/mL, IQR 71120-149874 pg/mL, figure 5.19 A). However, there was a trend towards higher production of CCL4 from LPS-stimulated MDMs in patients with AS compared to adult healthy controls (median 125782 pg/mL, IQR 90836-199393 pg/mL vs 82247 pg/mL, IQR 61853-117226 pg/mL, p=0.061).

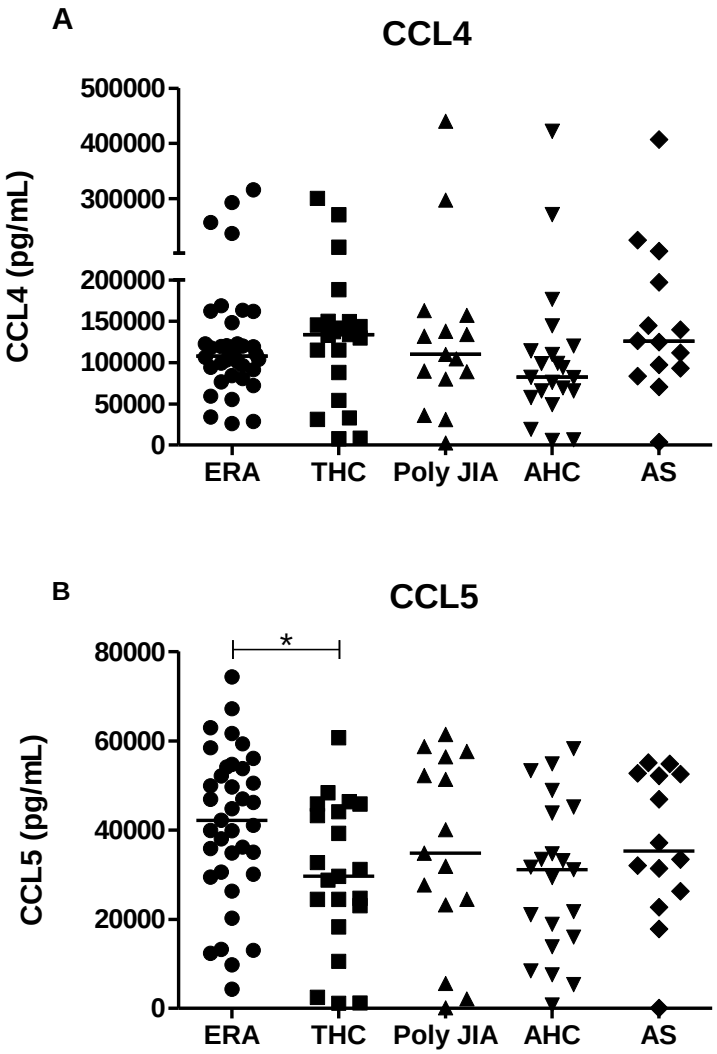


Figure 5.19 CCL5 production from LPS-stimulated MDMs is higher from patients with ERA compared to healthy controls. MDMs were stimulated with LPS for 24 hours after which the cell culture supernatants were collected and CCL4 and CCL5 were measured by Luminex assay. Box and whisker plots show median (+minimum to maximum range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA n=37, THC=teenage healthy controls n=21, polyarticular JIA n=15, AHC=adult healthy controls n=21, AS n=14, *p<0.05).

5.5.5 Effect of clinical features, disease activity and treatment on CCL4 and CCL5 production from LPS-stimulated MDMs from patients with ERA

There was no difference in CCL4 and CCL5 production from LPS-stimulated MDMs in patients with ERA who were HLA B27 positive compared to those who were HLA B27 negative or between male patients with ERA compared to female patients (table 5.2).

CCL5 production was significantly higher from LPS-stimulated MDMs in patients with ERA with active enthesitis compared to healthy controls (median 46592 pg/mL, IQR 30072-58407 pg/mL, p=0.034) but not compared to patients with ERA without enthesitis at the time of sample (median 355996 pg/mL, IQR 30278-47782 pg/mL, figure 5.20 B). There was no difference in CCL4 production in patients with ERA and enthesitis compared to those without or compared to healthy controls (figure 5.20 A, table 5.2). There was no significant difference between the peripheral, indeterminate and axial ERA subtypes for either CCL4 or CCL5 production from LPS-stimulated MDMs although CCL5 production for those within the peripheral ERA category was higher than healthy controls (p=0.041, table 5.2, figure 5.20 D). Similarly, in those patients with ERA with active peripheral arthritis at the time of sample, CCL5 production from LPS-stimulated MDMs was significantly higher than healthy controls (median 52161 pg/mL, IQR 40246-58472 pg/mL, p=0.016) but not compared to patients with ERA without active peripheral arthritis (median 39912 pg/mL, IQR 29761-51298 pg/mL). There was no significant difference in CCL4 production in patients with ERA with and without active peripheral arthritis or compared to healthy controls (table 5.2). In addition, there was no difference between the disease activity groups by JADAS criteria for either CCL4 or CCL5 or between these groups and healthy controls (table 5.2, figure 5.20 E and F). The inactive disease activity category was not included in the analysis because of its small size (n=2).

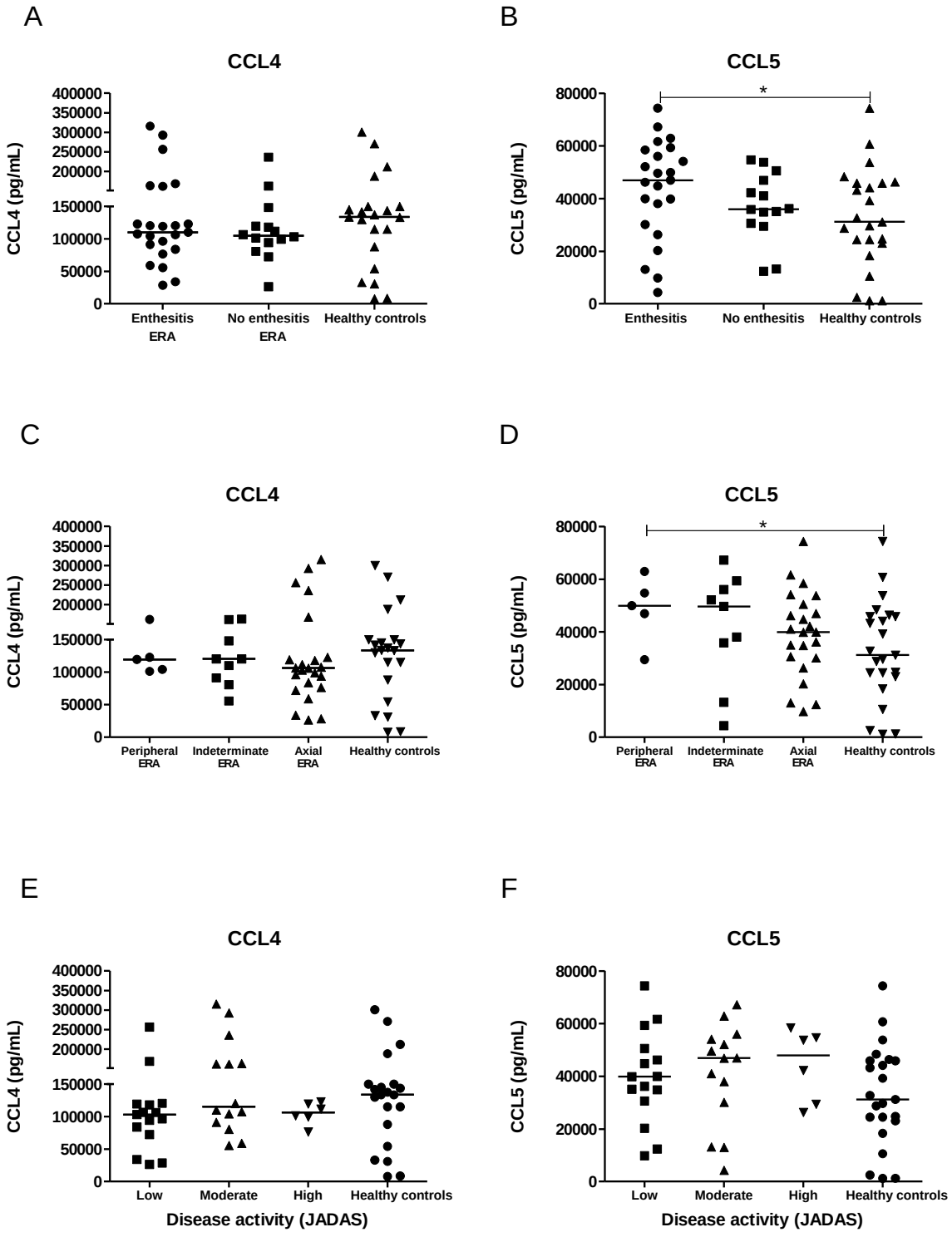


Figure 5.20 CCL5 production from LPS-stimulated MDMs is higher in patients with active enthesitis and in those with the peripheral ERA subtype compared to healthy controls. MDMs were differentiated as described in the protocol above (section 2.2) and stimulated with LPS for 24 hours. CCL4 and CCL5 were measured in cell culture supernatants by Luminex assay. Box and whisker plots show median and minimum to maximum range,

statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA with active enthesitis n=23, ERA without enthesitis n=14, peripheral ERA n=5, indeterminate ERA n= 9, axial ERA n=23, adolescent healthy controls n=21, low disease activity n=15, moderate disease activity n=14, high disease activity n=6, *p<0.05).

Treatment with NSAIDs, DMARDs and TNF inhibitors did not significantly affect CCL4 or CCL5 production from LPS-stimulated MDMs in patients with ERA (table 5.2).

As with CCL4 and CCL5 production from MDMs without LPS stimulation, there was a strong correlation between CCL4 and CCL5 production from LPS-stimulated MDMs for patients with ERA (r=0.43, p=0.0086, figure 5.21). This was also the case for patients with polyarticular JIA (r=0.66, p=0.0073) and for adolescent healthy controls (r=0.61, p=0.0031). The correlation between CCL4 and CCL5 production was less strong in adult healthy controls (r=0.48, p=0.029) and was not observed in patients with AS. There was no correlation with IL23 levels from LPS-stimulated MDMs for any of the patient or healthy controls groups but interestingly there was a correlation with levels of TNFα and IL1β production for both CCL4 (r=0.45, p=0.0064 and r=0.44, p=0.0065) and CCL5 (r=0.51, p=0.0016 and r=0.61, p<0.0001) in patients with ERA. A full analysis of correlations of cytokines and chemokines in this chapter can be found in figure 5.28.

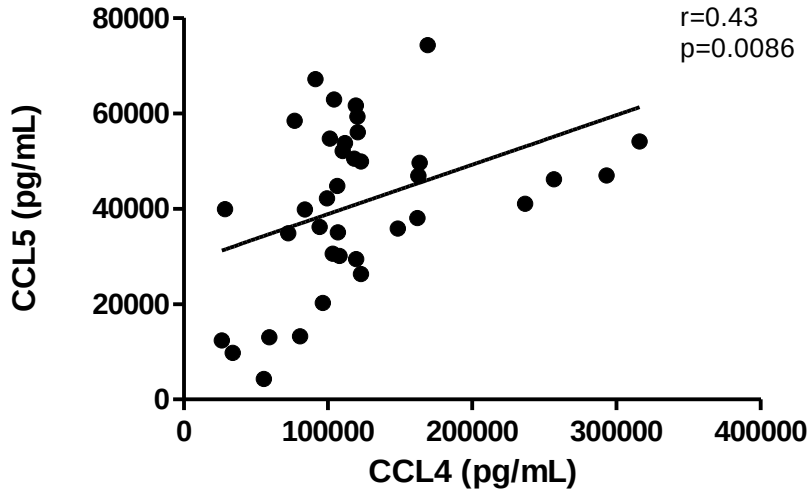


Figure 5.21 Levels of CCL4 from LPS-stimulated MDMs from patients with ERA correlate with CCL5 production. MDMs were differentiated and stimulated with LPS for 24 hours. Cell culture supernatants were then collected and analysed by Luminex assay for CCL4 and CCL5. Graphs show Spearman's rank correlation between CCL4 and CCL5 for patients with ERA (n=37).

5.6 Analysis of IFNγ from MDMs in patients compared to healthy controls

5.6.1 Introduction

The next cytokine analysed in the cell culture supernatants from MDMs was IFNγ. Although this cytokine was used in the assay to induce a more inflammatory MDM phenotype and thus the production of IL23 (section 3.2.2), supernatants containing IFNγ were removed on day 7 and replaced by fresh media with or without LPS. IFNγ production was of interest to study from MDMs in patients with ERA because of the evidence from macrophages in adult patients with AS of dysregulation of IFNγ gene expression or a 'reverse IFNγ signature' (Smith, Barnes et al. 2008, Fert, Cagnard et al. 2014). IFNγ is known to suppress the inflammatory response and attenuate the differentiation and expansion of Th17 cells (Lee, Lee et al. 2013).

5.6.2 IFNγ production from MDMs without LPS stimulation is lower in patients with ERA compared to healthy controls and patients with polyarticular JIA

First, IFNγ production was analysed in cell culture supernatants without LPS stimulation. Following differentiation as described above (section 2.2) and the addition of IFNγ for 24 hours, MDMs were washed and the media replaced. MDMs were then cultured for a further 24 hours but no additional stimuli were added. IFNγ production by MDMs was measured by Luminex assay in the cell culture supernatants. Levels of IFNγ were significantly lower form MDMs from patients with ERA compared to adolescent healthy controls and patients with polyarticular JIA (median 3796 pg/mL, IQR 2755-6849 pg/mL vs 6638 pg/mL, IQR 3989-9388 pg/mL, p=0.0021 and 5902 pg/mL, IQR 4445-8529 pg/mL, p=0.025, figure 5.22). IFNγ levels were also lower from MDMs without LPS stimulation in patients with AS compared to adult healthy controls but the difference was not statistically significant (median 3452 pg/mL, IQR 2826-5318 pg/mL vs 4610 pg/mL, IQR 3680-6097 pg/mL).

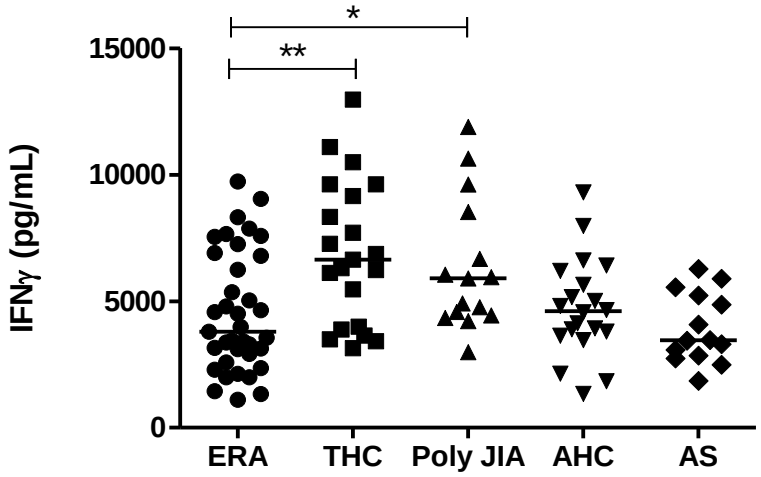


Figure 5.22 IFNγ production is significantly lower from MDMs without LPS stimulation in patients with ERA compared to healthy controls and patients with polyarticular JIA. MDMs were cultured as per the protocol in section 2.2. The media was replaced and MDMs were cultured for a further 24 hours after which cell culture supernatants were collected and analysed by Luminex assay. Box and whisker plots show median (+minimum to maximum range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA n=37, THC=teenage healthy controls n=21, polyarticular JIA n=15, AHC=adult healthy controls n=21, AS n=14, *p<0.05, **p<0.01).

5.6.3 Effect of clinical features and disease activity on IFNγ production from MDMs without LPS stimulation

There were no significant differences between patients who were HLA B27 positive and negative or between male and female patients in the production of IFNγ from MDMs without LPS stimulation (table 5.3). However, MDMs from HLA B27 positive and negative patients and male patients produced significantly lower IFNγ levels compared to healthy controls (HLA B27 positive: p=0.0055, HLA B27 negative: p=0.034, male ERA: p=0.0014).

In patients with active enthesitis at the time of sample compared to those with no enthesitis, no difference was observed in IFNγ levels (table 5.3), although levels were significantly lower than healthy controls in both groups (p=0.0039 and p=0.025). There was a trend towards higher IFNγ expression from MDMs without LPS stimulation in patients with axial ERA compared to those with peripheral ERA and to a lesser extent indeterminate ERA (table 5.3, figure 5.23 A) but the difference between the groups was not statistically significant. However, IFNγ levels from both the axial and peripheral ERA groups were significantly lower than healthy controls (peripheral ERA: p=0.0034, indeterminate ERA: p=0.057, axial ERA: p=0.017). This was also the case for those with active peripheral arthritis at the time of sample and those without active peripheral arthritis compared to healthy controls (p=0.023 and p=0.0049 respectively) although there was no significant difference between these groups (table 5.3). Similarly, there was no difference in IFNγ production from MDMs without LPS stimulation between those with and without hip arthritis (table 5.3) but again in both groups IFNγ expression was significantly lower than healthy controls (p=0.015 and p=0.0055 respectively).

Disease activity did not significantly affect IFNγ production from MDMs without LPS stimulation in patients with ERA (table 5.3) although levels of IFNγ for all the groups were significantly lower than healthy controls (low: p=0.021, moderate: p=0.018, high: p=0.029, figure 5.23 B).

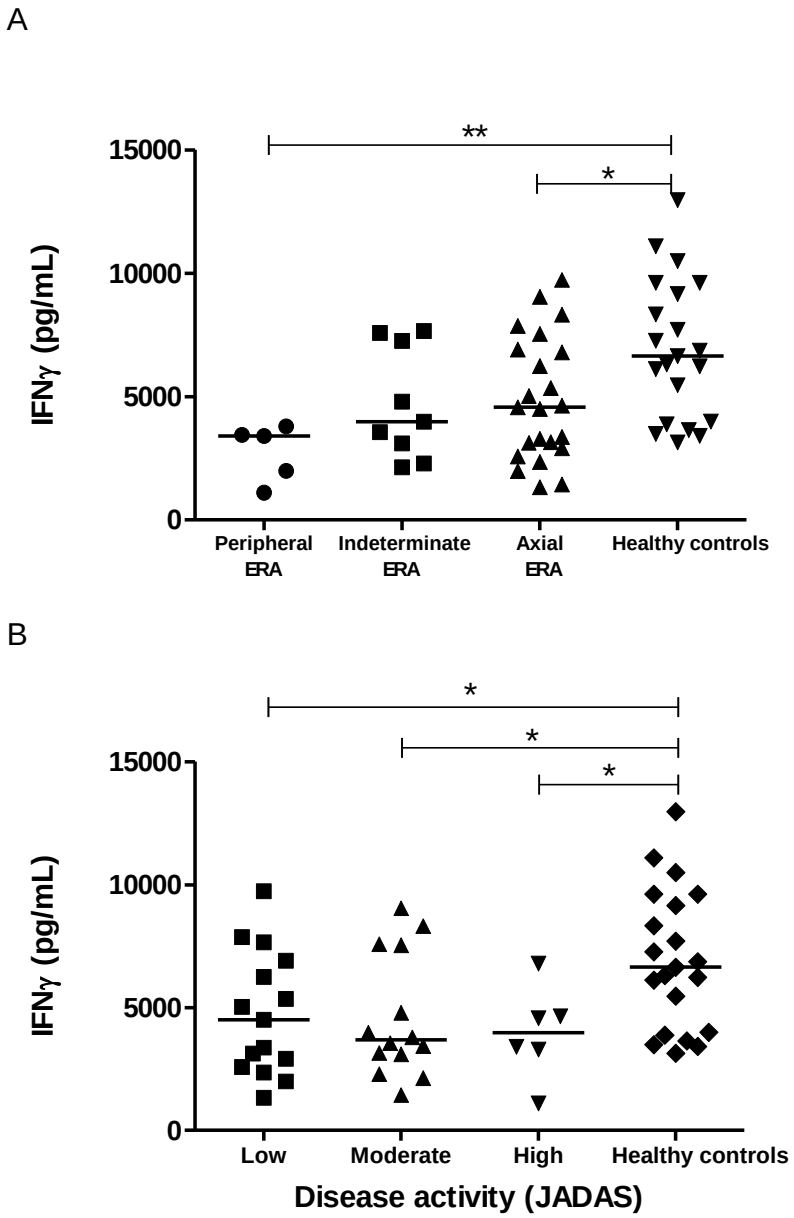


Figure 5.23 IFNγ production from MDMs without LPS stimulation is no different between ERA subtypes and disease activity groups. MDMs were cultured as per the protocol in section 2.2. The media was replaced and MDMs were cultured for a further 24 hours after which cell culture supernatants were collected and analysed by Luminex assay. Box and whisker plots show median (+minimum to maximum range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (peripheral ERA n=5, indeterminate ERA n= 9, axial ERA n=23, adolescent healthy controls n=21, low disease activity n=15, moderate disease activity n=14, high disease activity n=6, *p<0.05, **p<0.01).

Treatment with NSAIDs, DMARDs and TNF inhibitors did not affect IFNγ production from MDMs without LPS stimulation in patients with ERA but levels from all the patient groups were lower than healthy controls (NSAID treated: p=0.0041, no NSAID: p=0.019, DMARD

treated: p=0.011, no DMARD: p=0.005, TNF inhibitor-treated: p=0.014, no TNF inhibitor: p=0.0063, table 5.3).

In patients with extra-articular manifestations of ERA, no significant difference was observed compared to those without extra-articular manifestations (median 3297 pg/mL, IQR 2133-5037 pg/mL vs 4150 pg/mL, IQR 2978-7329 pg/mL) but in both groups, levels were lower than healthy controls (p=0.0058 and p=0.0085). No correlation was noted between IFNγ levels from MDMs without LPS stimulation and CRP, age or disease duration for patients with ERA and for the other patient and healthy control groups. In addition no correlation was found between IFNγ levels and IL23 production from LPS-stimulated MDMs.

5.6.4 IFNγ production from LPS-stimulated MDMs is significantly lower than healthy controls and patients with polyarticular JIA

IFNγ levels were then analysed from MDMs stimulated with LPS in patients with ERA, polyarticular JIA and AS compared to adolescent and adult healthy controls. As observed in MDMs without LPS stimulation, levels of IFNγ in the cell culture supernatants were significantly lower in patients with ERA compared to healthy controls (median 8836 pg/mL, IQR 7240-11255 pg/mL vs 11693 pg/mL, IQR 9481-13435 pg/mL, p=0.0076) and compared to patients with polyarticular JIA (median 11584 pg/mL, IQR 9149-14984 pg/mL, p=0.015, figure 5.24). There was no significant difference in IFNγ production from LPS-stimulated MDMs in patients with AS compared to adult healthy controls (median 9485 pg/mL, IQR 6391-11770 pg/mL vs 9253 pg/mL, IQR 7897-10684 pg/mL).

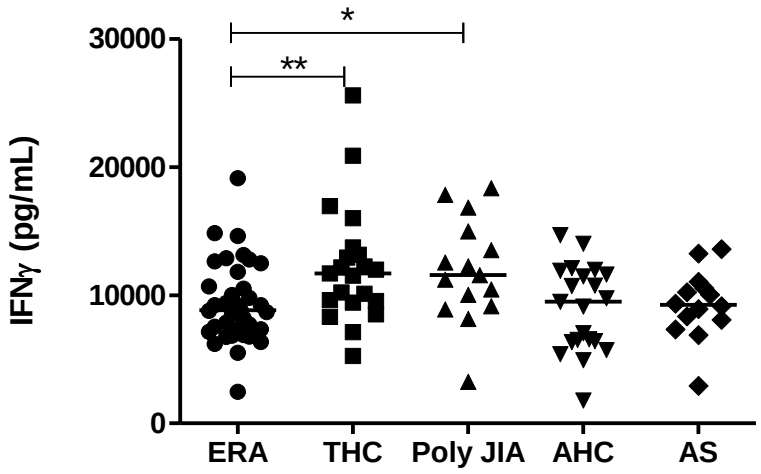


Figure 5.24 LPS-stimulated MDMs from patients with ERA produce less IFNγ than MDMs from healthy controls and patients with polyarticular JIA. MDMs were stimulated with LPS for 24 hours after which the cell culture supernatants were collected and analysed by Luminex assay. Box and whisker plots show median (+minimum to maximum range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA n=37, THC=teenage healthy controls n=21, polyarticular JIA n=15, AHC=adult healthy controls n=21, AS n=14, *p<0.05, **p<0.01).

5.6.5 Production of IFNγ from LPS-stimulated MDMs in patients with ERA is lower across the subgroups of clinical features, disease activity and treatment compared to healthy controls

The results from this section are summarised in table 5.2. As seen in MDMs without LPS stimulation, there was no difference in IFNγ production between patients who were HLA B27 positive compared to those who were HLA B27 negative (figure 5.25 A) but IFNγ levels from LPS-stimulated MDMs from patients who were HLA B27 positive were significantly lower than healthy controls (p=0.015). Similarly, there was no difference in IFNγ levels between male patients with ERA compared to female patients (figure 5.25 B) but levels from LPS-stimulated MDMs in male patients were significantly lower than healthy controls (p=0.0077).

In patients with active enthesitis, there was no difference in IFNγ production compared to those without enthesitis at the time of sample (figure 5.25 C). However, levels of IFNγ from LPS-stimulated MDMs from patients with active enthesitis were significantly lower than healthy controls (p=0.0060). There was a trend towards higher IFNγ production in LPS-stimulated MDMs from patients with axial ERA compared to peripheral ERA (median 9218 pg/mL, IQR 8210-12783 pg/mL vs 7350 pg/mL, IQR 7012-9100 pg/mL, p=0.092) and indeterminate ERA (median 7708 pg/mL, IQR 5934-9616 pg/mL, p=0.064, figure 5.25 D). Levels of IFNγ were significantly lower from those in the peripheral and indeterminate ERA categories compared to healthy controls (p=0.019 and p=0.006 respectively). No difference was observed in IFNγ production from LPS-stimulated MDMs between those with active peripheral arthritis at the time of sample compared to those with no peripheral arthritis although levels from both groups were significantly lower than healthy controls (p=0.014 and p=0.025 respectively), reflecting the lower levels in patients with ERA overall. Similarly, no difference was observed between those with and without a history of hip arthritis but levels from those without hip arthritis were lower than healthy controls (p=0.0028).

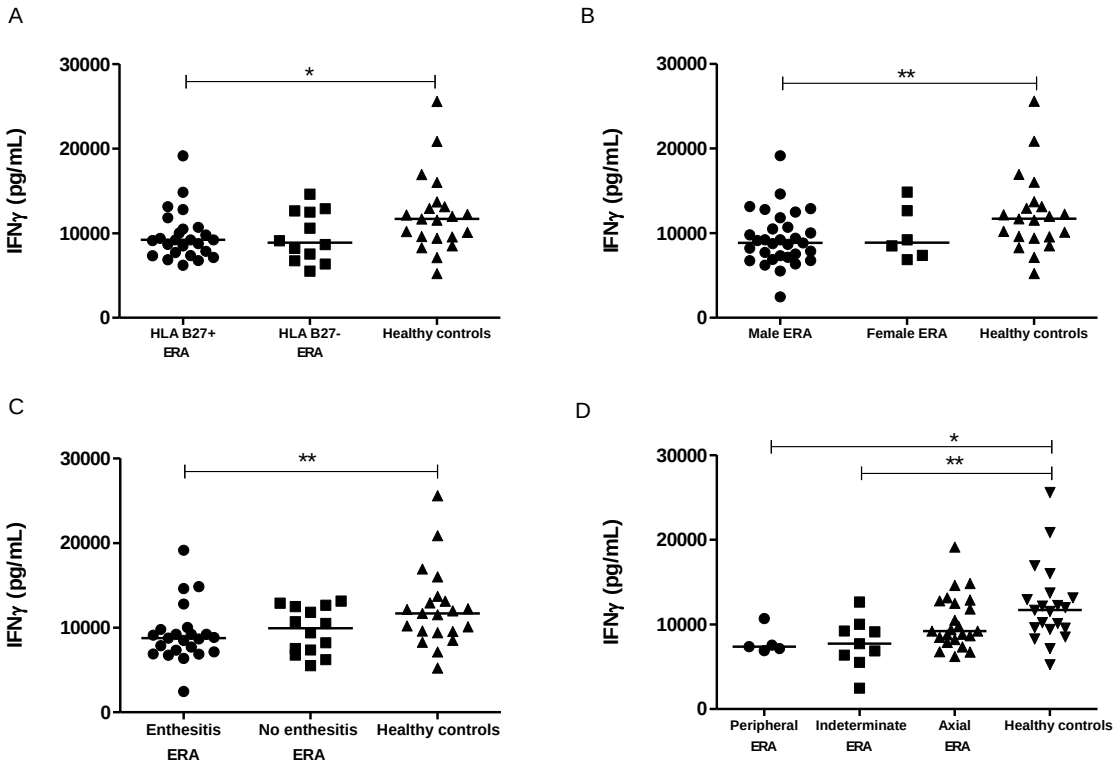


Figure 5.25 IFNγ production from LPS-stimulated MDMs is lower in HLA B27 positive patients, male patients, patients with active enthesitis and those in the peripheral and indeterminate categories of ERA compared to healthy controls. MDMs were differentiated as described in the protocol above (section 2.2) and stimulated with LPS for 24 hours. IFNγ was measured in cell culture supernatants by Luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (HLA B27+ ERA n=25, HLA B27- ERA n=12, male ERA n=31, female ERA n=6,ERA with active enthesitis n=23, ERA without enthesitis n=14, peripheral ERA n=5, indeterminate ERA n= 9, axial ERA n=23, adolescent healthy controls n=21,*p<0.05, **p<0.01).

Disease activity according to JADAS criteria did not affect levels of IFNγ from MDMs stimulated with LPS (table 5.2) and only levels from those in the moderate category were significantly lower than healthy controls (p=0.0067). There was no difference in IFNγ expression form LPS-stimulated MDMs in those patients with ERA with and without extra-articular manifestation of disease (median 8497 pg/mL, IQR 6353-9790 pg/mL vs 9166 pg/mL, IQR 7289-12022 pg/mL). In addition, treatment did not significantly affect levels of IFNγ from LPS-stimulated MDMs, with no difference seen between patients taking and not taking TNF inhibitors, DMARDs and NSAIDs (table 5.2). Levels of IFNγ from all of these groups were significantly lower than healthy controls, reflecting the lower levels observed in patients with ERA overall compared to healthy controls.

No correlation was observed between levels of IFNγ from LPS-stimulated MDMs and CRP, age or disease duration for patients with ERA, polyarticular JIA and AS. In addition, no correlation was seen with levels of IL23 production from LPS-stimulated MDMs in any of the patient or healthy control categories.

5.6.6 Expression of IFNγ regulated genes in SFMCs from patients with ERA and oligoarticular JIA

The findings of low IFNγ at the protein level for both LPS-stimulated MDMs and MDMs without LPS stimulation in patients with ERA compared to healthy controls was consistent with the 'reverse IFNγ signature' previously reported in patients with AS (Smith, Barnes et al. 2008, Fert, Cagnard et al. 2014). It was therefore of interest to determine whether these results would be mirrored in gene expression of IFNγ or its regulated genes. However, Smith el al determined that the addition of IFNγ to their assay normalised IFNγ gene dysregulation (Smith, Barnes et al. 2008) and therefore analysis of gene expression of IFNγ or IFNγ regulated genes from the MDMs in the assay studied in this project (where IFNγ was added) was unlikely to be helpful. Instead, the expression of a panel of 4 IFNγ regulated genes was analysed in the same SFMCs used to measure IL23p19 and IL12/23p40 gene expression (section 4.2.7). These were from 10 HLA B27 positive patients with ERA and, in the absence of a non-inflammatory control group, patients with oligoarticular JIA who were HLA B27 negative. SFMCs were isolated from synovial fluid collected at the time of knee aspiration (as described in section 2.2.6) and gene expression was measured by qPCR and normalised against GAPDH.

Interestingly, the expression of 2 of the genes tested IP10 (CXCL10) and MIG (CXCL9) was lower in patients with ERA compared to those with oligoarticular JIA (IP10: median fold change 574.0, IQR 36.98-1172 vs 1135, IQR 11.9-1956; MIG: median fold change 550.7, IQR 203.0-806.2 vs 800.6, IQR 210.1-2265, figure 5.26 A and C) but, perhaps because of the small numbers tested and wide variation in results, this did not reach statistical significance. In contrast, there was no difference in the expression of IRF1 and STAT1 between patients with ERA and oligoarticular JIA (IRF1: median fold change 8.140, IQR 6.000-11.67 vs 7.135, IQR 5.756-11.43; STAT1: median fold change 2.042, IQR 1.693-3.681 vs 2.567, IQR 2.071-

3.837, figure 5.26 B and D). It would be of interest to expand the numbers of samples tested in future experiments.

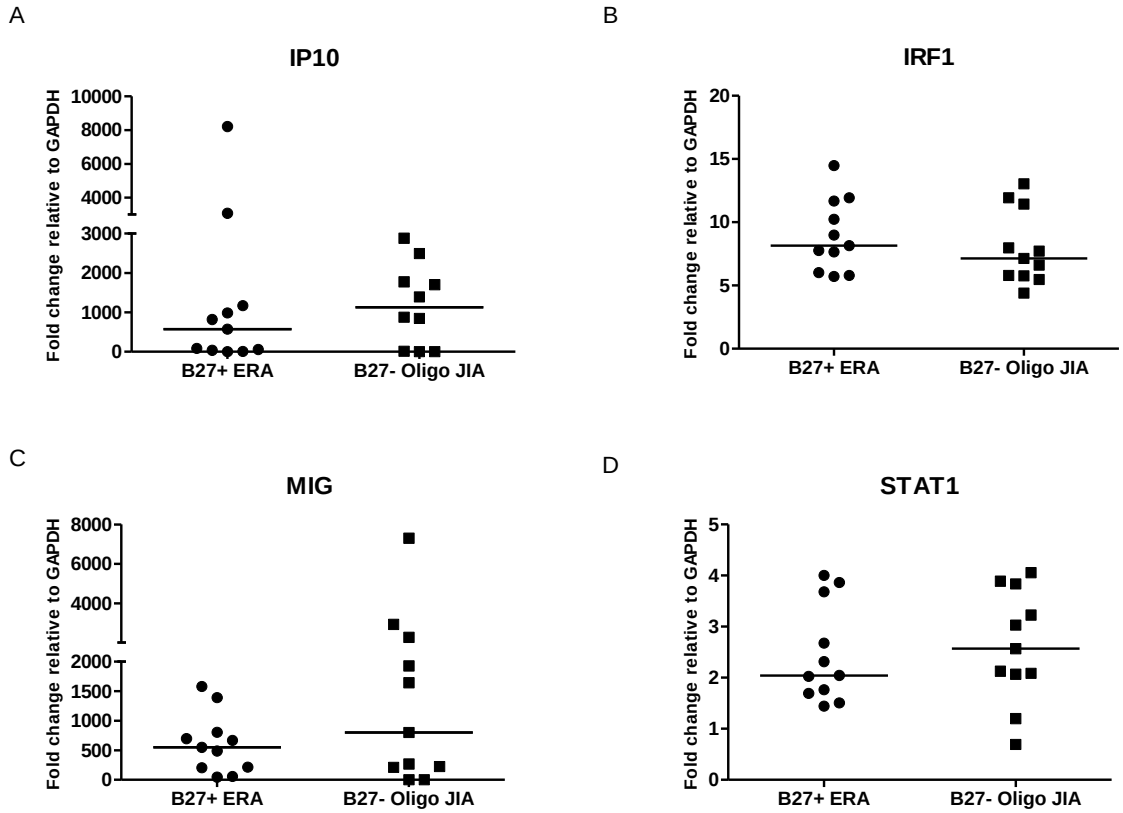


Figure 5.26 Expression of IP10, IRF1, MIG and STA1 are not significantly different in SFMCs in HLA B27 positive patients with ERA compared to HLA B27 negative patients with oligoarticular JIA. RNA was extracted from SFMCs and transcribed in to cDNA. Gene expression was analysed using qPCR and normalised against GAPDH. Fold change was calculated relative to a control value (the average ΔCt of the lowest ERA and oligoarticular JIA sample) in the absence of a non-inflammatory control sample. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann Whitney U Test (HLA B27 positive ERA n=10, HLA B27 negative oligoarticular JIA n=10).

5.7 Summary of results in chapter 5 for cytokine and chemokine production in cell culture supernatants from MDMs with and without LPS stimulation and cytokine gene expression

5.7.1 Introduction

In view of the multiple analyses undertaken for this chapter, tables were constructed summarising the results and highlighting significant differences in the gene expression and production of cytokines and chemokines from MDMs associated with clinical features, disease activity and treatment in patients with ERA. The majority of the differences represented higher production of the cytokine or chemokine in patients compared to controls but for IFNγ, the differences seen represented lower levels compared to healthy controls. In addition, summary heat maps for the correlations observed between the production of cytokines and chemokines from LPS-stimulated MDMs in patients with ERA, polyarticular JIA and AS, as well as adolescent and adult healthy controls are included (figure 5.28).

5.7.2 Tables summarising the results from chapter 5 including cytokine gene expression and cytokine and chemokine production from MDMs with and without LPS stimulation

ERA Groups		TNFα gene expression	IL1β gene expression	IL6 gene expression
Age		No correlation	No correlation	No correlation
Disease duration		No correlation	No correlation	No correlation
CRP		No correlation	No correlation	No correlation
HLA B27	Positive (n=21)	5.134 (1.092-8.369)	111.9 (14.92-1704)	1468 (468.3-3737)
	Negative (n=9)	5.278 (2.070-12.34)	111.4 (84.93-422.2)	935.8 (379.5-1759)
Sex	Male (n=24)	5.169 (1.488-11.14)	114.2 (36.62-677.6)	1722 (488.6-3028)
	Female (n=6)	4.00 (1.443-8.559)	109.9 (79.9-19377)	1090 (359.2-1322)
Active enthesitis	Yes (n=20)	3.433 (1.035-6.055)	111.4 (35.51-568.1)	1133 (456.6-2442)
	No (n=10)	9.169 (4.85-16.14)	137.7 (58.32-2339)	1823 (435.6-3488)
ERA subtype	Peripheral (n=5)	4.000 (3.931-114.23)	76.64 (24.00-299.0)	809 (429.6-1932)
	Indeterminate (n=8)	4.712 (0.704-9.656)	106.9 (64.45-117.0)	1590 (753.9-2061)
	Axial (n=7)	5.169 (1.479-7.215)	362.0 (57.42-2445)*	1468 (410.4-6095)
Active peripheral arthritis	Yes (n=8)	5.531 (1.635-12.49)	106.9 (8.057-321.8)	1973 (1247-2468)
	No (n=22)	5.152 (1.629-8.726)	114.2 (58.32-739.2)	1013 (384.0-2492)
Hip arthritis	Yes (n=13)	4.000 (1.084-5.527)	105.9 (19.68-465.1)	512.0 (340.2-2493)
	No (n=17)	7.062 (2.070-17.65)	114.2 (68.45-1704)	1795 (992.6-2398)
Disease activity (JADAS)	Low (n=12)	5.527 (5.143-28.68)	296.4 (22.73-1704)	1860 (348.9-4635)
	Moderate (n=12)	2.070 (0.808-7.977)	106.9 (64.45-165.4)	1463 (693.8-2230)
	High (n=4)	3.474 (0.873-11.97)	180.9 (36.62-311)	1059 (446.5-31081)
TNF inhibitor	Yes (n=12)	5.658 (3.924-18.02)	196.6 (84.2-1704)	1691 (413.7-4391)
	No (n=18)	3.474 (1.132-7.367)	105.4 (37.73-444.9)	1322 (490.2-2177)
DMARD	Yes (n=22)	6.419 (2.509-13.04)	122.9 (46.07-677.6)	1013 (405.3-2150)
	No (n=8)	4.567 (1.193-8.856)	111.4 (56.07-588.3)	2619 (1284-20812)
NSAID	Yes (n=16)	3.433 (0.808-6.616)	106.9 (39.95-814.6)	1317 (662.1-2150)
	No (n=14)	5.962 (3.549-15.38)	138.4 (49.13-604.6)	1759 (384.0-4433)

Table 5.1 Summary of results for gene expression of TNFα, IL1β and IL6 from LPS-stimulated MDMs in patients with ERA. Gene expression was analysed from RNA from MDMs stimulated with LPS for 24 hours using qPCR and normalised against GAPDH. Fold change was calculated relative to an unstimulated control for each patient and healthy control. Table shows median fold change and IQR in brackets, statistical analysis by Mann-Whitney U test or Kruskal-Wallis test as appropriate. Key:

Trend towards difference between groups (p<0.1)	* Difference between group and healthy controls (p<0.05)
Significant difference between groups (p<0.05)	

ERA patient groups		TNFα (pg/mL)	IL1β (pg/mL)	IL6 (pg/mL)	GMCSF (pg/mL)	CCL4 (pg/mL)	CCL5 (pg/mL)	IFNγ (pg/mL)
Age		No correlation	No correlation	No correlation	No correlation	No correlation	No correlation	No correlation
Disease duration		No correlation	No correlation	No correlation	No correlation	No correlation	No correlation	No correlation
CRP		No correlation	No correlation	No correlation	No correlation	No correlation	No correlation	No correlation
HLA B27	Positive (n=25)	49645 (30948-72540)*	805.7 (362.1-1318)*	19892 (18245-27790)	1685 (1046-3122) *	106369 (87620-162191)	44775 (35599-54394)	9210 (7529-10594) *
	Negative (n=12)	54445 (22905-59695)	745 (304.3-1758)	20557 (18429-25207)	2115 (1228-3048) *	118652 (104295-122149)	41405 (30193-51686)	8895 (6941-12596)
Sex	Male (n=31)	58065 (35373-69173) **	871.2 (356.3-1608) *	20072 (18377-27264)	2038 (1278-3298) **	107792 (91269-122840)	42177 (30554-52074)	8836 (7142-10691) **
	Female (n=6)	23050 (14455-46093)	356.4 (239.3-612.3)	19108 (17745-23882)	1088 (826.0-1571)	110870 (52879-190261)	44974 (12212-55039)	8858 (7226-13184)
Active enthesitis	Yes (n=23)	59660 (35373-75795)**	871.2 (480.7-1608)**	21041 (18382-28221)	2191 (1278-3298) **	110068 (83971-161890)	46592 (30072-58407) *	9942 (7204-12524) **
	No (n=14)	36640 (20925-54938)	345.3 (188.9-935.0)	18540 (17861-23650)	1312 (897.5-2265)	104932 (90733-126745)	355996 (30278-47782)	8764 (7142-9790)
ERA subtype	Peripheral (n=5)	36640 (25338-54685)	367.9 (345.3-2469)	25985 (18545-26619)	2038 (848.9-2602)	119541 (102651-142621)	49931 (38158-58724) *	7350 (7012-9100) *
	Indeterminate (n=9)	59590 (44170-75775) **	802.3 (218.3-1684)	18652 (18317-24747)	1990 (823.0-3310)	120350 (85934-155120)	49623 (24549-57667)	7708 (5934-9616) **
	Axial (n=23)	45480 (2400-61850)	805.7 (344.9-1157)	19892 (18047-27358)	1761 (1202-3298) *	106369 (76698-122840)	39912 (30072-50523)	9218 (8210-12783)
Active peripheral arthritis	Yes (n=8)	71235 (35185-93720) **	1009 (412.8-1916) *	22477 (18377-28746)	2000 (1098-3973) *	121682 (106000-162342)	52161 (40246-58472) *	8979 (7440-9819) *
	No (n=29)	48280 (22905-59695)	688.2 (269.0-1269)	19892 (18150-26881)	1761 (1125-3061)	106369 (78649-121145)	39912 (29761-51298)	8764 (7018-12560) *
Hip arthritis	Yes (n=17)	41300 (26403-67798)	871.2 (448.2-1512) *	19812 (18352-27790)	2486 (1046-3656) *	104085 (86555-144101)	44775 (32682-56553)	9217 (7601-12838)
	No (n=20)	58590 (28483-67163)	595.6, (203.4-1204)	19362 (18099-26908)	1807 (1204-2503)	118791 (85796-141972)	38935 (27070-50375)	8800 (6788-9863) **
Disease activity (JADAS)	Low (n=15)	45480 (12790-61850)	643.4 (243.7-1608)	18482 (18047-27358)	1521 (1202-3592) *	103130 (72364-119261)	39830 (30554-50523)	9122 (7338-12783)
	Moderate (n=14)	58910 (36580-71235) **	804.0 (302.8-1056)	19419 (18388-26936)	1921 (886.1-2699)	115296 (88601-181669)	46910 (25864-54582)	8583 (6728-10189) **
	High (n=6)	39210 (30933-71198)	1035 (359.5-1483)	22154 (18100-27326)	2322 (980.2-3268)	106457 (93576-120366)	47980 (29916-56645)	9027 (7482-11986)
TNF inhibitor	Yes (n=15)	58885 (24998-64763) *	802.0 (190.0-2091)	18766 (17933-27279)	1990 (1211-3298) *	106734 (59183-148350)	39830 (35065-49931)	8764 (6767-12488) *

	No (n=22)	41300 (28515-69173)	746.9 (365.0-1131)	19982 (18364-26433)	1769 (1023-3004) *	109745 (95125-132745)	45821 (29916-56645)	8979 (7482-10973) *
DMARD	Yes (n=27)	58885 (26978-70710) *	802.3 (344.9-1685)	19892 (18323-26936)	1761 (1211-3176)	107792 (91269-161890)	46868 (34811-54090)	9210 (7338-12488) *
	No (n=10)	43880 (28150-57938)	778.1 (268.3-1131)	19335 (18103-27699)	2024 (990.6-2959)	112815 (83128-121103)	40444 (23018-47596)	8303 (6826-9868) *
NSAID	Yes (n=19)	35960 (12790-61850)	688.2 (344.9-1157)	19892 (18252-26936)	1685 (1042-2606)	107792 (76698-122840)	42177 (26277-52074)	8836 (6893-10691) *
	No (n=18)	57810 (37025-71640)	873.5 (281.6-1756)	19419 (18364-27514)	2169 (1209-3698)	109216 (91576-151735)	42916 (36065-53844)	8943 (7289-11986) *

Table 5.2 Summary of results for cytokine production from LPS-stimulated MDMs and clinical features in patients with ERA. Cytokine levels were measured in cell culture supernatants following incubation of MDMs with LPS for 4 or 24 hours using ELISA or Luminex assay. Table shows median cytokine level for each group with IQR in brackets, statistical analysis by Mann-Whitney U test or Kruskal-Wallis test as appropriate. Key:

Trend towards difference between groups (p<0.1)	*	Difference between group and healthy controls (p<0.05)
Significant difference between groups (p<0.05)	**	Difference between group and healthy controls (p<0.01)

ERA patient groups		GMCSF (pg/mL)	CCL4 (pg/mL)	CCL5 (pg/mL)	IFNγ (pg/mL)
Age		No correlation	No correlation	No correlation	No correlation
Disease duration		No correlation	No correlation	No correlation	No correlation
CRP		No correlation	No correlation	No correlation	No correlation
HLA B27	Positive (n=25)	120.0 (97.25-188.1)	4298 (2244-10074)	739 (213.3-1839)	4505 (3015-6261) **
	Negative (n=12)	119.6 (95.86-182.7)	11700 (2981-19288)	1256 (508.9-1814)	3480 (2448-7559) *
Sex	Male (n=31)	118.7 (98.08-143.3)	6630 (2868-10834)	739.0 (251.7-1371)	3796 (3108-6243) **
	Female (n=6)	203.4 (86.23-233.1)	6470 (1980-22182)	431.3 (144.8-3323)	4776 (1362-8673)
Active enthesitis	Yes (n=23)	130.9 (100.0-221.4)	6630 (3300-17341)	692.7 (251.7-2094)	3438 (2359-6904) **
	No (n=14)	116.8 (91.75-140.5)	5346 (1712-17341)	724.1 (161.7-1385)	4150 (3312-6910) *
ERA subtype	Peripheral (n=5)	115.7 (95.52-184.1)	6630 (2342-16038)	1196 (959.6-2228)	3400 (1548-3617) **
	Indeterminate (n=9)	118.7 (92.4-178.6)	10057 (4751-15590)	692.7 (569.6-2663)	3985 (2702-7419)
	Axial (n=23)	130.9 (95.12-190.7)	3510 (2153-10090)	709.2 (177.9-1116)	4566 (2923-6904) *
Active peripheral arthritis	Yes (n=8)	111.8 (87.35-133.6)	7302 (3244-9881)	598.9 (191.1-1743)	3546 (2499-6885) *
	No (n=29)	128.0 (99.05-194.0)	6630 (2244-14340)	793.1 (241.8-1399)	3985 (2755-6849) **
Hip arthritis	Yes (n=17)	120.0 (97.4-188.0)	3590 (2602-10031)	709.2 (209.3-1244)	4566 (2177-6849) *
	No (n=20)	120.0 (95.86-182.7)	8791 (2339-15227)	715.8 (239.7-1814)	3678 (3113-7003) **
Disease activity (JADAS)	Low (n=15)	117.9 (94.27-143.4)	3305 (2153-16114)	469.1 (177.9-1371)	4504 (2587-6904) *
	Moderate (n=14)	117.2 (99.53-192.3)	9661 (2004-13003)	689.9 (216-2355)	3678 (2905-7554) *
	High (n=6)	128.8 (88.19-140.5)	8397 (4121-10709)	912.8 (233.5-1383)	3983 (2748-5178) *
TNF inhibitor	Yes (n=15)	118.7 (98.08-228.45)	10057 (3300-17341)	912.1 (628.5-2094)	3985 (3108-6904) *
	No (n=22)	120.7 (93.01-155.2)	3944 (2290-10001)	489.9 (218.5-1166)	3687 (2343-6982) **
DMARD	Yes (n=27)	117.9 (95.12-197.3) pg/mL vs	6373 (2336-16114)	793.1 (169.1-1585)	4504 (3108-6904) *
	No (n=10)	134.3 (100.2-156.6)	8397 (3017-10620)	580.9 (247.5-1008)	3349 (2530-5311) **
NSAID	Yes (n=19)	118.7 (98.08-228.4)	7103 (2336-10248)	709.2 (254.7-1316)	3985 (2296-5037) **
	No (n=18)	120.7 (94.91-155.2)	4981 (3013-19719)	740.1 (220.5-1675)	3499 (2839-7329) *

Table 5.3 Summary of results for cytokine production from MDMs without LPS stimulation and clinical features in patients with ERA. Cytokine levels were measured in cell culture supernatants following incubation of MDMs for 4 or 24 hours without the addition of LPS using ELISA or Luminex assay. Table shows median cytokine level for each group with IQR in brackets, statistical analysis by Mann-Whitney U test or Kruskal-Wallis test as appropriate.

Key:

*	Difference between group and healthy controls (p<0.05)
**	Difference between group and healthy controls (p<0.01)

5.7.3 Significant differences in production of chemokines and cytokines from MDMs in patients with ERA compared to healthy controls

A heat map was constructed to highlight the differences in cytokine and chemokine production from LPS-stimulated MDMs between ERA subgroups analysed in chapters 4 and 5 and healthy controls. Similar patterns of association were noted between many cytokines including IL23, IL12, IL27, TNFα, IL1β, GMCSF and IFNγ. IL6 and CCL4 were the exceptions with no clear difference in expression compared to healthy controls or association with any clinical features in ERA.

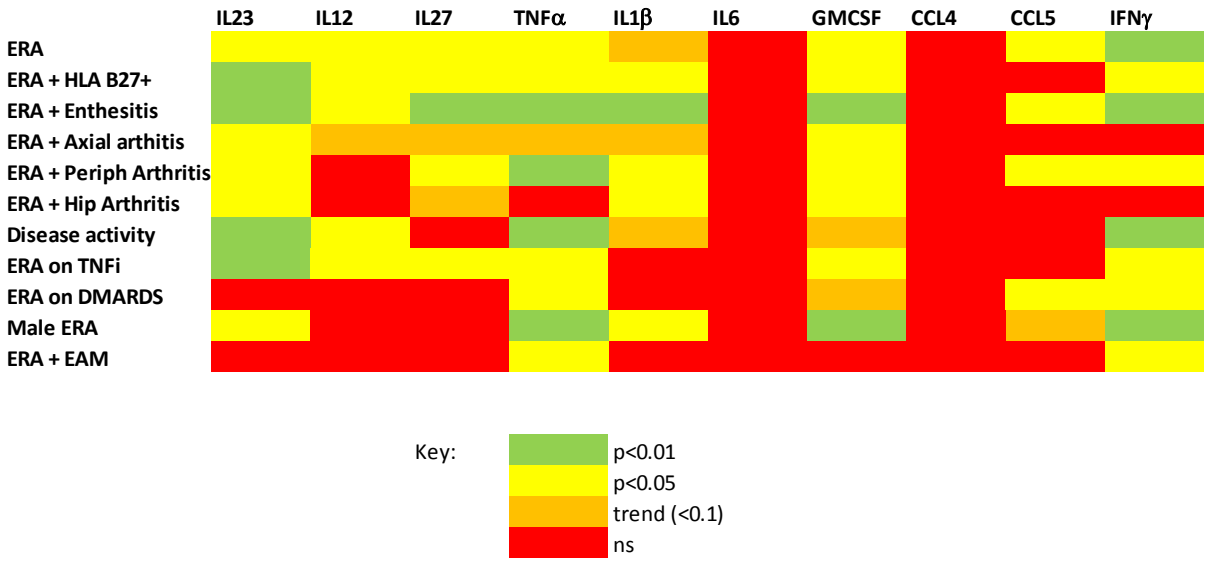


Figure 5.27 Summary of results for chapters 4 and 5. Heat map shows results for LPS-stimulated MDMs from patients with ERA compared to healthy controls highlighting where significant differences were found (TNFi=TNF inhibitors, EAM=extra-articular manifestations, patients with ERA n=37).

5.7.4 Correlations between cytokines and chemokines analysed in cell culture supernatants from LPS-stimulated MDMs

Heat maps were also constructed to summarise the correlations between cytokine and chemokine production from LPS-stimulated MDMs across the patient and healthy control group. One heat map was made for each group as the patterns of correlations were not the same. It was interesting to note relatively few correlations in the AS group compared to the ERA group and this is likely to be due to the smaller size of the AS group making the threshold for significant correlations more difficult to reach.

For the ERA and adolescent healthy control groups IFNγ levels did not correlate with any other cytokine. Similarly for the AS group, IFNγ only correlated with IL1β, IL6 and GMCSF whereas more correlations were observed for IFNγ in the polyarticular JIA and adult healthy control group. CCL4 and TNFα did not correlate with any other cytokines in the adult healthy control group.

ERA

	IL23	IL12	IL27	TNFα	IL1β	IL6	GMCSF	CCL4	CCL5	IFNγ
IL23		r=0.68	r=0.62	r=0.44	r=0.57	r=0.23	r=0.61	r=0.01	r=0.24	r=0.03
IL12	r=0.68		r=0.52	r=0.26	r=0.43	r=0.49	r=0.46	r=0.09	r=0.13	r=0.29
IL27	r=0.62	r=0.52		r=0.67	r=0.83	r=0.47	r=0.69	r=0.53	r=0.65	r=0.26
TNFα	r=0.44	r=0.26	r=0.67		r=0.57	r=0.52	r=0.43	r=0.45	r=0.51	r=0.06
IL1β	r=0.57	r=0.43	r=0.83	r=0.57		r=0.4	r=0.8	r=0.44	r=0.61	r=0.23
IL6	r=0.23	r=0.49	r=0.47	r=0.52	r=0.4		r=0.34	r=0.54	r=0.15	r=0.12
GMCSF	r=0.61	r=0.46	r=0.69	r=0.43	r=0.8	r=0.34		r=0.38	r=0.40	r=0.11
CCL4	r=0.01	r=0.09	r=0.53	r=0.45	r=0.44	r=0.54	r=0.38		r=0.43	r=0.21
CCL5	r=0.24	r=0.13	r=0.65	r=0.51	r=0.61	r=0.15	r=0.40	r=0.43		r=0.04
IFNγ	r=0.03	r=0.29	r=0.26	r=0.06	r=0.23	r=0.12	r=0.11	r=0.21	r=0.04	

Adolescent healthy controls

	IL23	IL12	IL27	TNFα	IL1β	IL6	GMCSF	CCL4	CCL5	IFNγ
IL23		r=0.76	r=0.24	r=0.15	r=0.21	r=0.41	r=0.58	r=0.17	r=0.36	r=0.01
IL12	r=0.76		r=0.57	r=0.58	r=0.31	r=0.70	r=0.50	r=0.40	r=0.42	r=0.19
IL27	r=0.24	r=0.57		r=0.75	r=0.58	r=0.85	r=0.54	r=0.57	r=0.75	r=0.23
TNFα	r=0.15	r=0.58	r=0.75		r=0.40	r=0.72	r=0.26	r=0.56	r=0.57	r=0.13
IL1β	r=0.21	r=0.31	r=0.58	r=0.40		r=0.76	r=0.63	r=0.43	r=0.36	r=0.12
IL6	r=0.41	r=0.70	r=0.85	r=0.72	r=0.76		r=0.65	r=0.50	r=0.53	r=0.16
GMCSF	r=0.58	r=0.50	r=0.54	r=0.26	r=0.63	r=0.65		r=0.28	r=0.42	r=0.03
CCL4	r=0.17	r=0.40	r=0.57	r=0.56	r=0.43	r=0.50	r=0.28		r=0.61	r=0.02
CCL5	r=0.36	r=0.42	r=0.75	r=0.57	r=0.36	r=0.53	r=0.42	r=0.61		r=-0.17
IFNγ	r=0.01	r=0.19	r=0.23	r=0.13	r=0.12	r=0.16	r=0.03	r=0.02	r=-0.17	

Polyarticular JIA

	IL23	IL12	IL27	TNFα	IL1β	IL6	GMCSF	CCL4	CCL5	IFNγ
IL23		r=0.74	r=0.56	r=0.02	r=0.30	r=0.33	r=0.55	r=0.31	r=0.33	r=0.57
IL12	r=0.74		r=0.56	r=0.55	r=0.62	r=0.49	r=0.71	r=0.32	r=0.49	r=0.57
IL27	r=0.56	r=0.56		r=0.40	r=0.70	r=0.65	r=0.82	r=0.82	r=0.77	r=0.54
TNFα	r=0.02	r=0.55	r=0.40		r=0.45	r=0.42	r=0.23	r=0.49	r=0.52	r=0.09
IL1β	r=0.30	r=0.62	r=0.70	r=0.45		r=0.78	r=0.73	r=0.77	r=0.58	r=0.28
IL6	r=0.33	r=0.49	r=0.65	r=0.42	r=0.78		r=0.76	r=0.82	r=0.46	r=0.63
GMCSF	r=0.55	r=0.71	r=0.82	r=0.23	r=0.73	r=0.76		r=0.66	r=0.61	r=0.71
CCL4	r=0.31	r=0.32	r=0.82	r=0.49	r=0.77	r=0.82	r=0.66		r=0.66	r=0.39
CCL5	r=0.33	r=0.49	r=0.77	r=0.52	r=0.58	r=0.46	r=0.61	r=0.66		r=0.22
IFNγ	r=0.57	r=0.57	r=0.54	r=0.09	r=0.28	r=0.63	r=0.71	r=0.39	r=0.22	

Adult healthy controls

	IL23	IL12	IL27	TNFα	IL1β	IL6	GMCSF	CCL4	CCL5	IFNγ
IL23		r=0.56	r=0.49	r=0.14	r=0.34	r=0.55	r=0.50	r=0.22	r=0.44	r=0.14
IL12	r=0.56		r=0.81	r=0.25	r=0.83	r=0.87	r=0.81	r=0.17	r=0.68	r=0.59
IL27	r=0.49	r=0.81		r=0.43	r=0.86	r=0.72	r=0.81	r=0.34	r=0.90	r=0.51
TNFα	r=0.14	r=0.25	r=0.43		r=0.27	r=0.32	r=0.09	r=0.42	r=0.38	r=-0.21
IL1β	r=0.34	r=0.83	r=0.86	r=0.27		r=0.86	r=0.75	r=0.39	r=0.73	r=0.56
IL6	r=0.55	r=0.87	r=0.72	r=0.32	r=0.86		r=0.67	r=0.22	r=0.75	r=0.46
GMCSF	r=0.50	r=0.81	r=0.81	r=0.09	r=0.75	r=0.67		r=0.25	r=0.72	r=0.42
CCL4	r=0.22	r=0.17	r=0.34	r=0.42	r=0.39	r=0.22	r=0.25		r=0.48	r=0.19
CCL5	r=0.44	r=0.68	r=0.90	r=0.38	r=0.73	r=0.75	r=0.72	r=0.48		r=0.56
IFNγ	r=0.14	r=0.59	r=0.51	r=-0.21	r=0.56	r=0.46	r=0.42	r=0.19	r=0.56	

AS

	IL23	IL12	IL27	TNFα	IL1β	IL6	GMCSF	CCL4	CCL5	IFNγ
IL23		r=0.63	r=0.34	r=0.05	r=0.53	r=0.23	r=0.57	r=0.19	r=0.33	r=0.38
IL12	r=0.63		r=0.49	r=0.22	r=0.45	r=0.54	r=0.67	r=0.46	r=0.27	r=0.41
IL27	r=0.34	r=0.49		r=0.47	r=0.51	r=0.65	r=0.45	r=0.77	r=0.85	r=0.16
TNFα	r=0.05	r=0.22	r=0.47		r=0.42	r=0.18	r=0.31	r=0.42	r=0.33	r=0.04
IL1β	r=0.53	r=0.45	r=0.51	r=0.42		r=0.45	r=0.69	r=0.48	r=0.41	r=0.59
IL6	r=0.23	r=0.54	r=0.65	r=0.18	r=0.45		r=0.26	r=0.78	r=0.38	r=0.59
GMCSF	r=0.57	r=0.67	r=0.45	r=0.31	r=0.69	r=0.26		r=0.44	r=0.27	r=0.57
CCL4	r=0.19	r=0.46	r=0.77	r=0.42	r=0.48	r=0.78	r=0.44		r=0.42	r=0.53
CCL5	r=0.33	r=0.27	r=0.85	r=0.33	r=0.41	r=0.38	r=0.27	r=0.42		r=-0.08
IFNγ	r=0.38	r=0.41	r=0.16	r=0.04	r=0.59	r=0.59	r=0.57	r=0.53	r=-0.08	

Key:
- p<0.001
- p<0.01
- p<0.05
- trend (<0.1)
- not significant

Figure 5.28 Correlations between cytokines and chemokines from LPS-stimulated MDMs. Heat map shows a summary of correlations for each patient and healthy control group.

5.8 Discussion

Following the associations observed in chapter 4 for the expression of IL23 and other cytokines within the IL23 family, it was important to establish whether these associations were also observed with other cytokines and chemokines produced by MDMs analysed in this assay. The choice of cytokines and chemokines analysed was based on those implicated in the pathogenesis of SpA in addition to those identified as of potential interest in the preliminary experiments described in chapter 3, such as the proteome profiler array (section 3.5.2).

The first group of cytokines to be analysed was TNFα, IL1β and IL6. Macrophages are the main source of these pro-inflammatory cytokines (Arango Duque and Descoteaux 2014) all of which have been implicated in the pathogenesis of SpA (Hreggvidsdottir, Noordenbos et al. 2014). Treatment with TNF inhibitors is an effective treatment for SpA (van der Heijde, Ramiro et al. 2017) and also ERA (Constantin, Foeldvari et al. 2016), thus implicating TNFα as a key cytokine in both conditions. TNF inhibition results in reduced synovial inflammatory cell infiltrate in patients with SpA (Baeten, Kruithof et al. 2001). In addition, polymorphisms in TNFα signalling pathways have been identified by GWAS in patients with AS (Hreggvidsdottir, Noordenbos et al. 2014). Similarly, for IL1β, GWAS studies have highlighted significant genetic associations with the IL1 pathway in patients with AS (Monnet, Kadi et al. 2012) and genetic polymorphisms have also been identified in patients with ERA (Aggarwal, Srivastava et al. 2012). However, blockade of the IL1 receptor has not proved to be an effective treatment for patients with SpA (Haibel, Rudwaleit et al. 2005). There is also evidence for the involvement of IL6 in the pathogenesis of SpA through GWAS studies identifying polymorphisms in the IL6 receptor (Cortes, Hadler et al. 2013) and through the observation of elevated serum levels in patients with AS (Pedersen, Sorensen et al. 2011) as well as the finding of IL6 in the sacroiliac joints of patients with AS (Francois, Neure et al. 2006). However, despite this, the blockade of the IL6 receptor using tocilizumab in patients with AS has not proved effective (Sieper 2016).

As with IL23, initial experiments were carried out to analyse gene expression of TNFα, IL1β and IL6 from MDMs stimulated with LPS for 24 hours in the same subgroup of patients with ERA and adolescent healthy controls used to analyse IL23p19 and IL12/23p40. As discussed in chapter 4 (section 4.7) it would have been ideal to collect and analyse gene expression at more than one time point, especially for cytokines such as TNFα which were secreted early from MDMs in the context of this assay. However, the number of time points was limited by the relatively small amount of blood collected and therefore number of MDMs available after culture. Other assays, such as p38 MAPK detection, were collected using MDMs at the earlier time point of 4 hours post LPS stimulation and therefore RNA collection was often only possible at the later time point of 24 hours after LPS stimulation.

Although the median gene expression for TNFα, IL1β and IL6 tended to be higher for patients with ERA, no significant difference was observed between patients and healthy

controls. This was in keeping with the findings for IL12/23p40 but may also have been because the study was underpowered to see differences between these groups. In addition, no association with HLA B27, male sex and disease activity was observed. Contrary to the findings for IL23 in cell culture supernatants, LPS-stimulated MDMs from patients with ERA and active enthesitis at the time of sample had significantly lower gene expression of TNFα compared to those without enthesits. This may have been a chance finding as it was not mirrored by the findings for TNFα production from LPS-stimulated MDMs in cell culture supernatants (discussed below) although these were analysed after 4 hours LPS stimulation instead of 24 hours as for gene expression. The finding was interesting because IL23 production in the cell culture supernatants was only detectable after 24 hours LPS stimulation and was significantly higher in patients with enthesitis. This suggests a possible inverse relationship between TNFα gene expression and IL23 production from LPS-stimulated MDMs. This is supported by the theory discussed in section 4.7 that inhibition of, or lower levels of TNFα result in increased IL23 production (Notley, Inglis et al. 2008) (Zakharova and Ziegler 2005) (Mayordomo, Silva et al. 2018) and thus enhanced Th17 response. To confirm this, in future experiments, it would be of interest to measure TNFα secretion in the cell culture supernatants from MDMs after 24 hours stimulation with LPS to determine whether differences seen between patients and healthy controls and associations seen with clinical features such as enthesitis are reversed for TNFα when IL23 is present at high levels. It would also be of interest to analyse the effect of IL23 inhibition on TNFα levels in the assay. If there was a true paradoxical relationship, it could be hypothesised that the use of IL23 inhibitors in patients may result in increased TNFα expression and thus may explain the lack of efficacy seen so far in clinical trials for patients with AS.

The presence of axial arthritis and thus a more severe clinical phenotype of ERA resulted in a trend towards increased gene expression of TNFα and IL1β but only the difference in IL1β expression was significantly higher in those with axial ERA compared to healthy controls and the numbers in each of the ERA subgroups were small. Treatment with DMARDs and TNF inhibitors did not result in significant differences in the gene expression of TNFα, IL1β or IL6 from LPS-stimulated MDMs in patients with ERA.

The results for the production of TNFα, IL1β and IL6 in cell culture supernatants from LPS-stimulated MDMs were analysed from the larger final group of patients and healthy controls as detailed in table 3.1, perhaps making it more likely to see differences between the groups. Also, as mentioned above, because of data from preliminary experiments described in section 3.2.4, TNFα production was measured separately at the earlier time point of 4 hours after LPS stimulation. As with IL23, levels of TNFα production from LPS-stimulated MDMs were higher in patients with ERA compared to adolescent healthy controls but no significant difference was observed in IL1β or IL6 production. In general, however, levels for all 3 cytokines tended to be higher in all the groups with inflammatory arthritis and levels of

IL1β and IL6 were significantly higher in patients with AS compared to adult healthy controls.

In patients with ERA, as with IL23 production from the cell culture supernatants from LPS-stimulated MDMs, HLA B27 and active enthesitis were associated with significantly higher levels of TNFα and IL1β production compared to healthy controls. Similarly, LPS-stimulated MDMs from male patients with ERA secreted significantly higher levels of TNFα and IL1β compared to healthy controls which was also in keeping with previous studies demonstrating higher levels of pro-inflammatory cytokines in male patients with AS compared to female patients (Gracey, Yao et al. 2016) (Huang, Tso et al. 2012). Interestingly, there was no association noted for IL6 with any of these clinical features.

Disease activity and ERA subtype did not significantly affect TNFα, IL1β and IL6 production from LPS-stimulated MDMs in patients with ERA but the subgroups were perhaps too small to see any differences between them.

In contrast with the results for IL23, TNFα, IL1β and IL6 levels from LPS-stimulated MDMs were not significantly affected by treatment with TNF inhibitors and DMARDs although, in keeping with IL12, TNFα expression in those treated with TNF inhibitors was higher than healthy controls. As discussed above, this was for TNFα measured at 4 hours post LPS stimulation prior to significant levels of IL23 production by LPS-stimulated MDMs. It would be of interest in future experiments to measure TNFα production after 24 hours of LPS stimulation to see whether the upregulation of IL23 reversed this result.

Many of the associations with clinical features for TNFα and IL1β mirrored the results for IL23 production from LPS-stimulated MDMs and this was confirmed by the correlations noted between these cytokines in patients with ERA. No such correlation was found for IL6 and IL23 production, however, which was unsurprising given there were fewer similarities in the findings for IL6 production and the clinical features associated with the higher production of IL23.

IL17 was an important cytokine for analysis in this assay because of the evidence implicating it as a key cytokine in the pathogenesis of SpA (Smith 2016). Although Th17 cells are major producers of this cytokine, a wide variety of innate immune cells including innate lymphoid cells, NK cells, mast cells, macrophages, and neutrophils also produce IL17 (Isailovic, Daigo et al. 2015) and it was therefore of interest to determine the production of IL17 by MDMs in this assay. Despite the detection of IL17 gene expression within LPS-stimulated MDMs, albeit with a high degree of variation in the levels detected, this did not translate to the detection of IL17 at the protein level, either at 4 hours or 24 hours post LPS-stimulation. It was therefore concluded that the time points used may not have been correct for the detection of IL17 or the stability of IL17 was not sufficient to measure it in this assay.

The next cytokine to be analysed was GMCSF. GMCSF is strongly implicated in the pathogenesis of inflammatory arthritis (Wicks and Roberts 2016) and recent clinical trials have demonstrated efficacy for the use of GMCSF inhibitors in the treatment of rheumatoid arthritis (Burmester, McInnes et al. 2018). GMCSF is also implicated in the pathogenesis of SpA (Al-Mossawi, Chen et al. 2017) and JIA (Piper, Pesenacker et al. 2014) although both of these studies focus on T cell expression of GMCSF. However, macrophages are significant producers of GMCSF (Shiomi and Usui 2015) and therefore it was of interest to study GMCSF production from MDMs in this assay.

As with IL27, GMCSF was detected in the cell culture supernatants of MDMs after 24 hours culture without LPS stimulation. However, there was no difference in the levels of production between patents with ERA, polyarticular JIA or AS and healthy controls. No significant associations were noted with clinical features either including HLA B27, male sex, active enthesitis, disease activity, axial arthritis and treatment. Conversely, when MDMs were stimulated with LPS, levels were generally higher in patients with inflammatory arthritis, in particular ERA and AS compared to healthy controls, although the presence of HLA B27 did not have any effect on GMCSF production. As with IL23, and consistent with higher levels of pro-inflammatory cytokines in male patients with SpA (Rusman, van Vollenhoven et al. 2018), higher GMCSF production was noted from LPS-stimulated MDMs from male patients with ERA compared to females. Similarly, there was also a trend towards higher GMCSF production in patients with active enthesitis and active peripheral arthritis compared to those without, in those with axial ERA and in those with higher levels of disease activity. However, in contrast to the results for IL23, treatment with TNF inhibitors did not significantly affect levels of GMCSF production from LPS-stimulated MDMs. Despite this, the similarities with IL23 production resulted in a strong correlation between GMCSF and IL23 levels from LPS-stimulated MDMs. Interestingly, GMCSF was the only cytokine apart from IL12 to correlate with IL23 across all the patient and healthy control groups analysed. In addition, GMCSF levels correlated with IL12 and IL1β production in all the groups analysed. In view of the evidence from the literature that GMCSF promotes a pro-inflammatory macrophage phenotype (Ushach and Zlotnik 2016), it was hypothesised that the high levels observed from LPS-stimulated MDMs in this assay may drive the production of other pro-inflammatory cytokines, in particular IL23. Thus, it would be of interest to investigate the effect of GMCSF blockade in this assay on the production of IL23 and other pro-inflammatory cytokines. Extrapolating this further, clinical trials of GMCSF are already underway in adults with inflammatory arthritis including SpA, and therefore GMCSF inhibition may be a promising therapeutic option for patients with ERA.

The chemokines CCL4 and CCL5 were analysed next. Both have been identified by previous studies as important in the pathogenesis of rheumatoid arthritis (Agere, Akhtar et al. 2017) (Kuo, Huang et al. 2018) and, in particular CCL5, of potential significance in JIA (Pharoah, Varsani et al. 2006) and SpA (Chan, Filer et al. 2008). Both chemokines were chosen for analysis following the results of the proteome profiler array (section 3.6.2) which suggested

potential differences in expression of these chemokines between the patients with ERA tested and healthy control.

Both CCL4 and CCL5 were detected from MDMs without LPS stimulation following 24 hours culture. However, the only significant difference found between patients and healthy controls was for CCL4 production from MDMs from patients with AS compared to adult healthy controls. No other significant differences were observed for MDMs without LPS stimulation between patient and healthy control groups or for any of the clinical features analysed. Trends were seen towards lower CCL4 and CCL5 production in patients with axial ERA compared to peripheral ERA and higher CCL4 and CCL5 production with higher disease activity but the differences seen were not significant. In contrast, the results for LPS-stimulated MDMs demonstrated significantly higher CCL5 production in patients with ERA compared to adolescent healthy controls and also in those patients with ERA with active enthesitis and peripheral arthritis at the time of sample compared to healthy controls. Treatment with TNF inhibitors and DMARDs and other clinical features which were of interest in view of the findings for IL23 and other cytokines, such as HLA B27 and male sex, were not associated with higher levels of CCL4 or CCL5 production from LPS-stimulated MDMs. Unsurprisingly, no correlation was found between CCL4 and CCL5 production and IL23 production from LPS-stimulated MDMs. However, both CCL4 and CCL5 correlated more closely with IL27 production for each of the analysed patient and healthy control groups. Other correlations were noted especially in the adolescent age groups with pro-inflammatory cytokines such as TNFα, IL1β and IL6 (particularly with CCL4) and for the majority of groups CCL4 and CCL5 production correlated except for the AS patient group where the small group size may have reduced the likelihood of significant correlations being seen.

The final cytokine to be analysed in this chapter was IFNγ. This cytokine was of particular interest in patients with ERA because of the evidence that IFNγ gene dysregulation may play a role in the pathogenesis of SpA (Smith, Barnes et al. 2008) (Fert, Cagnard et al. 2014). In addition, IFNγ has been shown to inhibit the development of Th17 cells and thus the production of IL17 (Sarkar, Cooney et al. 2009) (Lee, Lee et al. 2013) and low dose treatment with IFNγ may be beneficial in patients with rheumatoid arthritis (Lemmel, Brackertz et al. 1988). The fact that it was necessary to add IFNγ to the assay to induce a pro-inflammatory MDM phenotype and thus IL23 was of concern when interpreting IFNγ production in the cell culture supernatants. However, as the supernatants containing the added IFNγ were removed following the initial 7 day culture and replaced with fresh media with or without LPS, it was decided to analyse IFNγ production from MDMs in the assay but to interpret the results with caution.

IFNγ was detected in cell culture supernatants from MDMs without LPS stimulation and was significantly lower in patients with ERA compared to healthy controls and patients with polyarticular JIA. In patients with AS, levels were also lower than adult healthy controls

although the difference was not significant. The pattern of IFNγ secretion was very similar for LPS-stimulated MDMs with significantly lower levels from patients with ERA compared to healthy controls and patients with polyarticular JIA. The difference between ERA and polyarticular JIA was interesting and perhaps provides one explanation as to why the IL23/IL17 axis is so important to the pathogenesis of SpA compared to other forms of inflammatory arthritis. It is possible that low levels of IFNγ production may result in reduced inhibition of pathogenic Th17 cell differentiation and survival therefore causing high levels of IL17 production. This is supported by previous studies demonstrating that IFNγ may inhibit the IL23-driven development of Th17 cells (Harrington, Hatton et al. 2005, Lee, Lee et al. 2013) and therefore low levels of IFNγ, as seen in this assay in patients with ERA, may contribute to the pathogenic role of IL23. It would be of interest to study this further in a larger group of patients with AS and also adult patients with rheumatoid arthritis to see whether similar differences are observed.

For both MDMs without LPS stimulation and LPS-stimulated MDMs, no difference in IFNγ secretion was observed between clinical features noted to be of significance for IL23 production such as HLA B27, male sex, active enthesitis and active peripheral arthritis at the time of sample. For MDMs without LPS-stimulation, the majority of the subgroups of patients with ERA analysed had lower levels of IFNγ compared to adolescent healthy controls and for LPS-stimulated MDMs, those patients who were HLA B27 positive, male and had active enthesitis exhibited lower levels of IFNγ compared to healthy controls. The lack of association with clinical features found to be of significance for the production of IL23 was surprising but perhaps suggests an independent mechanism influencing the production of IFNγ compared to IL23. This was supported by the lack of correlations observed between IFNγ production and the levels of other pro-inflammatory cytokines including IL23.

In both MDMs without LPS stimulation and LPS-stimulated MDMs, there was a trend towards higher levels of IFNγ in patients with axial ERA compared to those with peripheral ERA although the differences were not significant. This may have been a chance finding but may explain why IFNγ levels were not significantly lower in patients with AS, all of whom had axial disease, compared to healthy controls. This would also be of interest to study further in a larger group of patients.

A trend was seen towards higher levels of IFNγ from MDMs without LPS stimulation in patients with lower levels of disease activity although no significant differences were observed between the groups. This would be consistent with the theory of low IFNγ levels resulting in increased Th17 cell differentiation and thus IL17 production and therefore high disease activity and again it would be interesting to confirm this in a larger group of patients. Treatment with TNF inhibitors and DMARDs was not associated with significant difference in IFNγ production for either MDMs without LPS stimulation or LPS-stimulated MDMs.

The results for IFNγ production from both MDMs without LPS stimulation and LPS-stimulated MDMs were consistent with the results from the work by Smith et al who found a reverse IFNγ signature in macrophages from AS patients, with under-expression of genes upregulated by IFNγ and overexpression of genes down-regulated by IFNγ. The study also found lower expression of the IFNγ gene in macrophages from patients with AS but did not assess IFNγ production at the protein level (Smith, Barnes et al. 2008). The same study noted that by adding IFNγ to the assay, the IFNγ gene dysregulation was reversed. It was therefore decided not to analyse the expression of IFNγ regulated genes from the MDMs in this assay. However, it was of interest to try to validate the findings for IFNγ in the cell culture supernatants from MDMs and it was therefore decided to analyse the expression of a panel of 4 IFNγ-regulated genes in the SFMCs used previously for to measure the expression of IL23p19 and IL12/23p40 by qPCR. There was significant variation in the results and the lack of a non-inflammatory control group was problematic (as discussed in section 4.2.7). However, it was interesting to note that the expression of 2 of the genes chosen (IP10 and MIG) was lower in SFMCs from HLA B27 positive patients with ERA compared to HLA B27 negative patients with oligoarticular JIA although, perhaps because of the relatively small numbers tested and variability in the results, the difference between the groups was not significant. It would be of interest to undertake further analysis with a larger group of patients and increased number of IFNγ-regulated genes tested.

The limitations of the results in this chapter include the previously discussed fact that the MDM assay is an artificial in vitro system and therefore validating the results in an ex vivo setting would be beneficial to confirm their relevance to the pathogenesis of ERA. In addition, the group sizes for some of the subgroup analyses were small (including disease activity and ERA by peripheral, indeterminate and axial subtype) and thus the study was underpowered to see differences between these subgroups.

Overall, the results in this chapter demonstrate that the production of other pro-inflammatory cytokines and in particular TNFα, IL1β and GMCSF follow a similar pattern to IL23 in this assay, especially in relation to features such as HLA B27, male sex and active enthesitis. IL6 was one exception which is interesting in view of the fact that blocking IL6 is not an effective treatment for SpA (Sieper 2016). The other notable exception was IFNγ with production from both MDMs without LPS and following LPS stimulation exhibiting almost the inverse pattern of secretion compared to other pro-inflammatory cytokines. No correlations were observed however, leading to the conclusion that the production of IFNγ may be influenced by different mechanisms to other pro-inflammatory cytokines and which would be of interest to investigate in future work.

CHAPTER 6: THE UNFOLDED PROTEIN RESPONSE AND ITS EFFECT ON LEVELS OF PRO-INFLAMMATORY CYTOKINES FROM MONOCYTE DERIVED MACROPHAGES

Overview of chapter 6

The aim of this chapter was to study the induction of the UPR in patients with ERA compared to adolescent healthy controls and to analyse the effect of this on the gene expression and production of pro-inflammatory cytokines studied in previous chapters.

The first part of the chapter analyses levels of gene expression of three markers of the UPR (spliced XBP1, BiP and CHOP) in MDMs from patients with ERA and healthy controls and also SFMCs from HLA B27 positive patients with ERA compared to HLA B27 negative patients with oligoarticular JIA.

The next part of the chapter focusses on the effect of inducing the UPR in MDMs on the gene expression and production of cytokines within the IL23 family and other pro-inflammatory cytokines in patients with ERA compared to healthy controls.

In the final part of the chapter, correlations between markers of the UPR and pro-inflammatory cytokine production are discussed.

Hypothesis for Chapter 6

Markers of the UPR will be higher in MDMs from patients with ERA and AS compared to healthy controls and indeuction of the UPR will enhance cytokine gene expression and production.

6.1 Markers of the UPR in MDMs from patients with ERA compared to healthy controls

6.1.1 Introduction

In view of the findings in chapters 4 and 5 demonstrating higher levels of production of some pro-inflammatory cytokines including IL23, TNFα and GMCSF from MDMs from patients with ERA compared to adolescent healthy controls, the next step was to try to understand the mechanism behind this. In SpA, several theories exist to explain the increased production of pro-inflammatory cytokines from innate immune cells. The strong association of SpA and in particular AS with HLA B27 supports one theory suggesting that the misfolding of HLA B27 may result in accumulation of peptides within the ER and thus the induction of the UPR leading to the production of pro-inflammatory cytokines (Colbert, DeLay et al. 2010). Autophagy may also be induced as a result of protein misfolding and lead to the production of pro-inflammatory cytokines (Ciccia, Accardo-Palumbo et al. 2014). In addition, gut dysbiosis and expansion of gut-derived ILCs is increasingly recognised as key to the pathogenesis of SpA (Ciccia, Guggino et al. 2015) and again may result in increased expression of pro-inflammatory cytokines (Gilis, Mortier et al. 2018) as can the formation of HLA B27 free heavy chains at the cell surface (Wong-Baeza, Ridley et al. 2013).

Evidence for the involvement of the UPR in the pathogenesis of SpA is more convincing in animal models than human subjects (Smith 2018). However, in view of the strong association of ERA with HLA B27 and because of the increased expression noted in some cytokines including IL23 in LPS-stimulated MDMs from HLA B27 positive patients, it was decided to investigate the UPR and its effect on the production of pro-inflammatory cytokines in this assay.

The UPR modulates cytokine production via the activation of three pathways: IRE1α, PERK and ATF6α (Grootjans, Kaser et al. 2016). Three markers of the UPR were chosen for analysis in this assay. BiP, a chaperone protein, is bound to all 3 of the above proteins in their inactive state. Upon activation of the UPR, BiP binds to misfolded proteins, releasing and therefore activating IRE1α, PERK and ATF6α. XBP1 is a transcription factor spliced as a result of the activation of the IRE1α pathway resulting in the induction of pro-inflammatory signalling pathways (via MAPK and NFκB) and cytokine release. CHOP is upregulated via the PERK pathway and the translation of ATF4 and triggers apoptotic and autophagy pathways (Dufey, Sepulveda et al. 2014).

Analysis of XBP1 splicing, CHOP and BiP was carried out by measuring gene expression from RNA isolated from MDMs from a group of patients with ERA and age-matched healthy controls. This subgroup of patients was the same as those analysed for IL23p19, IL12/23p40 and IL12p35 gene expression and had similar demographics and clinical characteristics to the larger final group used for analysis of cytokine secretion (ERA n=30, healthy control

n=18). Analysis was first undertaken in MDMs without LPS and TM stimulation and then in MDMs following both LPS stimulation and induction of the UPR using TM.

6.1.2 Gene expression of XBP1 and CHOP is not significantly different in MDMs without LPS and TM stimulation in patients with ERA compared to healthy controls

Following culture of MDMs for 24 hours (without the addition of LPS or TM), RNA was extracted and transcribed in to cDNA as described in section 2.5. qPCR was then performed and the results for CHOP expression were normalised against GAPDH, after which a fold change was calculated relative to a control sample. The same control sample (from MDMs without LPS or TM stimulation) from a healthy control was used for all subsequent experiments described in this section. The results for spliced XBP1 were normalised against unspliced XBP1. Subgroup analysis was performed for clinical features and treatment but the group sizes were small and therefore the study was perhaps under-powered to see differences between these subgroups.

As noted in section 3.2.5, fold change in spliced XBP1 and CHOP was generally low without the addition of TM to the assay. No significant difference was found in gene expression of XBP1 or CHOP between MDMs from patients with ERA compared to age-matched healthy controls (XBP1: median fold change 0.9727, IQR 0.6830-1.357 vs 0.8706, IQR 0.5987-2.732; CHOP: median fold change 1.189, IQR 0.5587-2.799 vs 1.840, IQR 0.5510-4.959).

Subgroup analysis was undertaken to determine associations with clinical features and gene expression of spliced XBP1 and CHOP. However, no significant differences were noted between the subgroups analysed (table 6.1) although, as mentioned above, some of the subgroups were small.

6.1.3 Gene expression of BiP is higher in MDMs without LPS or TM stimulation from HLA B27 positive patients with ERA compared to healthy controls

The gene expression of BiP was also measured using qPCR (as described in section 2.5) following the culture of MDMs for 24 hours (without the addition of LPS or TM) and the results were normalised against GAPDH, after which a fold change was calculated relative to the control sample. The results for BiP demonstrated a different pattern in that there was a strong trend towards higher gene expression in MDMs without LPS and TM stimulation in patients with ERA compared to age-matched healthy controls (median fold change 2.182, IQR 0.7210-7.024 vs 0.8108, IQR 0.2462-3.353, p=0.058, figure 6.1 A). MDMs from patients who were HLA B27 positive exhibited higher BiP expression compared to healthy controls (median fold change 3.630, IQR 1.056-8.375, p=0.019) but not compared to patients who were HLA B27 negative (median fold change 1.338, IQR 0.1977-3.409, figure 6.1 B).

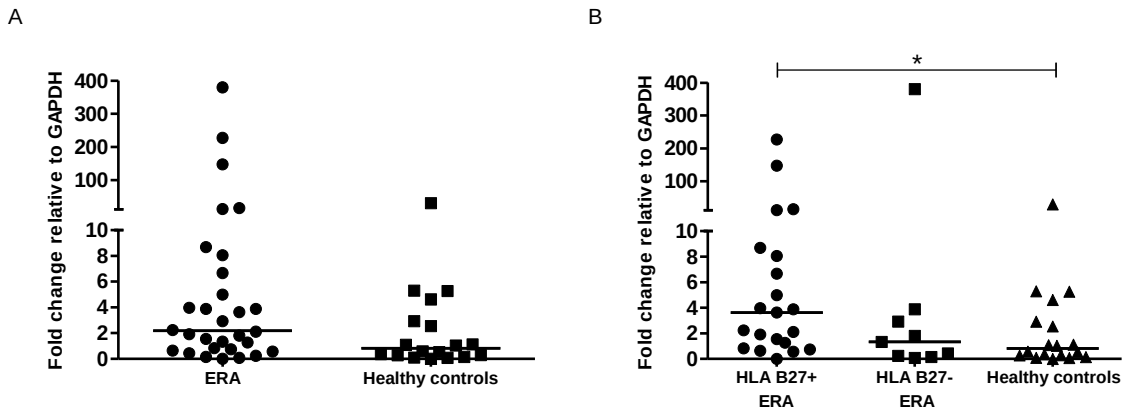


Figure 6.1 Gene expression of BiP from MDMs without LPS or TM stimulation is higher in HLA B27 positive patients with ERA compared to healthy controls. RNA was isolated from MDMs cultured for 24 hours without LPS or TM stimulation and transcribed in to cDNA. BiP was amplified using qPCR and normalised against GAPDH. Fold change was calculated by comparing the expression of BiP from patient and healthy control MDMs to the control sample. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann Whitney U test (HLA B27+ ERA n=21, HLA B27- ERA n=9, adolescent healthy controls n=18).

Interestingly, and similar to the results for TNFα gene expression from LPS-stimulated MDMs, there was a strong trend towards higher expression of BiP in MDMs without LPS or TM stimulation from patients without active enthesitis at the time of sample compared to healthy controls (median fold change 4.442, IQR 1.008-110.30, p=0.058) but this was not significantly higher than those patients with active enthesitis (median fold change 1.867, IQR 0.6681-3.952, figure 6.2 A). In addition, there was a trend towards higher BiP expression in MDMs without LPS or TM stimulation from patients with axial ERA and peripheral ERA compared to healthy controls (median fold change axial ERA: 2.250, IQR 0.7442-7.682, p=0.092; peripheral ERA: 4.993, IQR 1.043-194.0, p=0.057) but there was no significant difference between the ERA subtype categories (peripheral, indeterminate and axial, figure 6.2 B, table 6.1).

In contrast to XBP1 and CHOP, a high level of disease activity in patients with ERA was associated with high levels of BiP gene expression from MDMs without LPS or TM stimulation (median fold change 116.3, IQR 4.166-341.9) compared to patients with moderate and low levels of disease activity by JADAS criteria (median fold change moderate: 2.016, IQR 0.6058-7.035, p=0.045; low: 1.412, IQR 0.4914-5.918, p=0.034) and compared to healthy controls (p=0.015, figure 6.2 C).

Other features such as male sex, the presence of active peripheral arthritis and treatment did not did not associate with differential BiP expression and no difference was seen between these groups and healthy controls (table 6.1).

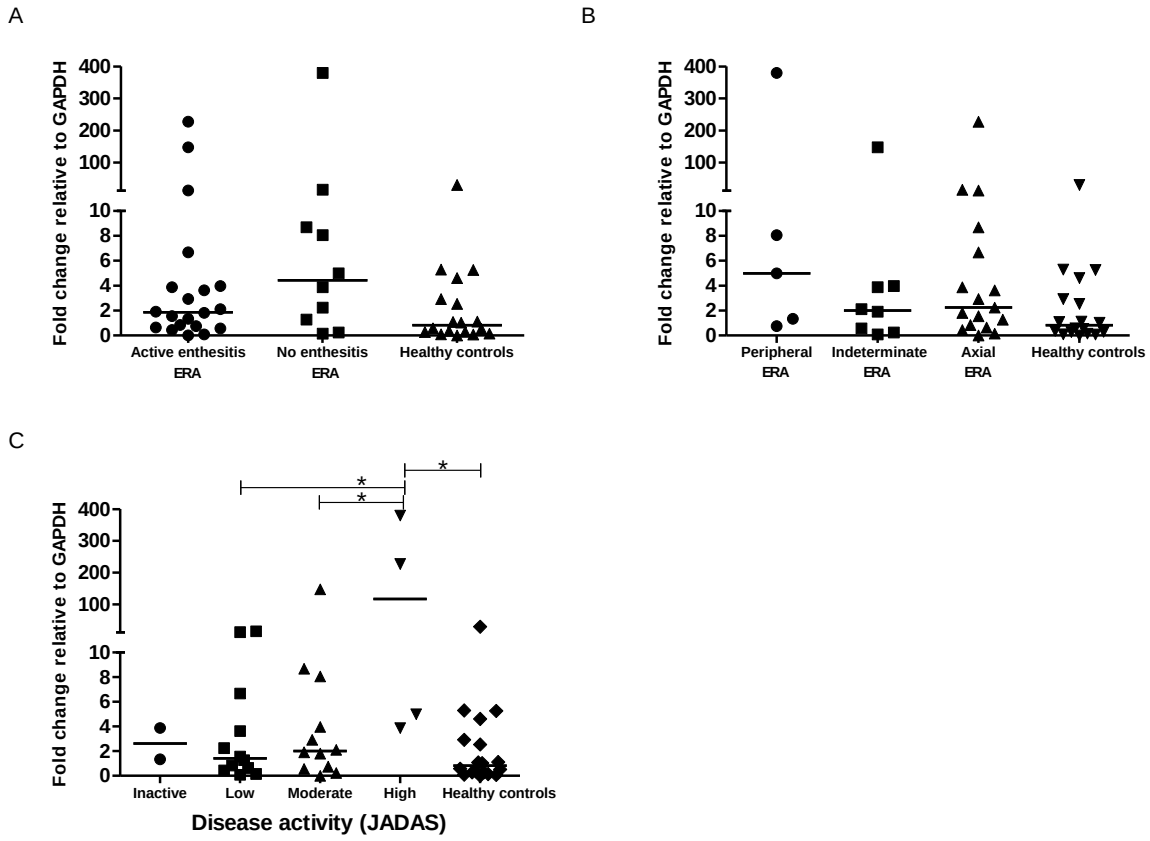


Figure 6.2 High disease activity is associated with increased BiP expression in MDMs without LPS and TM stimulation in patients with ERA. BiP expression was analysed from RNA from MDMs using qPCR and normalised against GAPDH. Fold change was calculated relative to the control sample. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann-Whitney U test or Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA with active enthesitis n=20, ERA with no enthesitis n=10, peripheral ERA n=5, indeterminate ERA n=8, axial ERA n=7, adolescent healthy controls n=18, inactive ERA n=2, ERA with low disease activity n=12, moderate disease activity n=12, high disease activity n=4, *p<0.05).

		Spliced XBP1	CHOP	BiP
HLA B27	Positive	1.035 (0.6807-1.668)	0.9829 (0.5548-2.040)	3.630 (1.056-8.375) *
	Negative	0.8591 (0.6572-1.244)	1.729 (0.6731-7.319)	1.338 (0.1977-3.409)
Sex	Male	1.000 (0.6818-1.556)	1.189 (0.5587-2.799)	2.114 (0.6946-7.368)
	Female	0.8645 (0.4879-1.121)	1.558 (0.5354-3.374)	3.891 (0.9611-8.476)
Active enthesitis	Yes	0.8767 (0.6795-1.198)	0.9931 (0.5510-2.144)	1.867 (0.6681-3.952)
	No	1.125 (0.6926-1.892)	2.355 (0.2690-6.956)	4.442 (1.008-110.30)
ERA subtype	Peripheral	0.7738 (0.5490-3.319)	1.189 (0.3466-1.666)	4.993 (1.043-194.0)
	Indeterminate	0.9878 (0.7249-1.932)	0.9931 (0.5510-3.784)	2.016 (0.3158-3.952)
	Axial	1.00 (0.6902-1.378)	1.516 (0.6041-4.344)	2.250 (0.7442-7.682)
Active peripheral arthritis	Yes	0.9621 (0.7249-1.902)	0.9931 (0.5510-2.144)	3.554 (0.8986-112.3)
	No	0.9727 (0.6830-1.357)	1.189 (0.5722-3.220)	2.033 (0.7210-4.649)
Disease activity (JADAS)	Low	1.028 (0.6878-1.624)	2.380 (0.5886-11.62)	1.412 (0.4914-5.918)
	Moderate	0.9186 (0.7170-1.991)	0.9931 (0.3896-1.558)	2.016 (0.6058-7.035)
	High	0.7912 (0.4565-1.169)	1.079 (0.4096-1.987)	116.3 (4.166-341.9) *
TNF inhibitor	Yes	0.7738 (0.6713-1.206)	1.374 (0.6533-5.189)	2.182 (1.161-8.000)
	No	1.004 (0.7170-2.489)	0.9931 (0.3753-2.355)	2.423 (0.2865-7.920)
DMARD	Yes	0.8888 (0.6830-1.137)	1.102 (0.5626-2.554)	2.589 (0.8219-5.415)
	No	1.253 (0.6265-2.937)	1.516 (0.3322-3.031)	1.738 (0.4914-172.8)

Table 6.1 Summary of spliced XBP1, CHOP and BiP gene expression from MDMs without LPS and TM stimulation in patients and healthy controls. Gene expression was analysed from RNA from MDMs using qPCR and normalised against unspliced XBP1 or GAPDH. Fold change was calculated relative to an unstimulated control for each patient and healthy control. Table shows median fold change and IQR in brackets, statistical analysis by Mann-Whitney U test. Key:

Significant difference between groups (p<0.05)	* Difference between ERA group and healthy controls (p<0.05)

6.1.4 Expression of spliced XBP1, CHOP and BiP is not significantly different between MDMs stimulated with LPS and TM and those treated with TM alone

Next, gene expression of spliced XBP1, CHOP and BiP was analysed in MDMs following induction of the UPR with TM. MDMs were cultured for 24 hours with either a combination of LPS and TM (LPS+TM) or TM alone, and then RNA was extracted and transcribed in to cDNA as described in section 2.5. qPCR was then performed and the results for CHOP and BiP expression were normalised against GAPDH, after which a fold change was calculated relative to the control sample. The results for spliced XBP1 were normalised against unspliced XBP1. As described above, subgroup analysis was performed but the group sizes were small and therefore the study was perhaps underpowered for differences to be observed between these subgroups.

As demonstrated in section 3.2.5, TM induced the UPR effectively in this assay independent of LPS stimulation. However, previous studies have shown that induction of the UPR with TM alone does not significantly enhance cytokine production in monocyte-derived dendritic cells (Goodall, Wu et al. 2010). Preliminary experiments for this assay demonstrated this was also the case for the production of cytokines TNFα and IL23 from MDMs (figure 3.8). For the markers of the UPR analysed, no significant difference in expression was noted between MDMs treated with both LPS and TM compared to those treated with TM alone (figure 6.3). The results shown in subsequent sections are therefore for MDMs following a combination of LPS and TM stimulation but the pattern of results seen was similar for MDMs stimulated with TM alone.

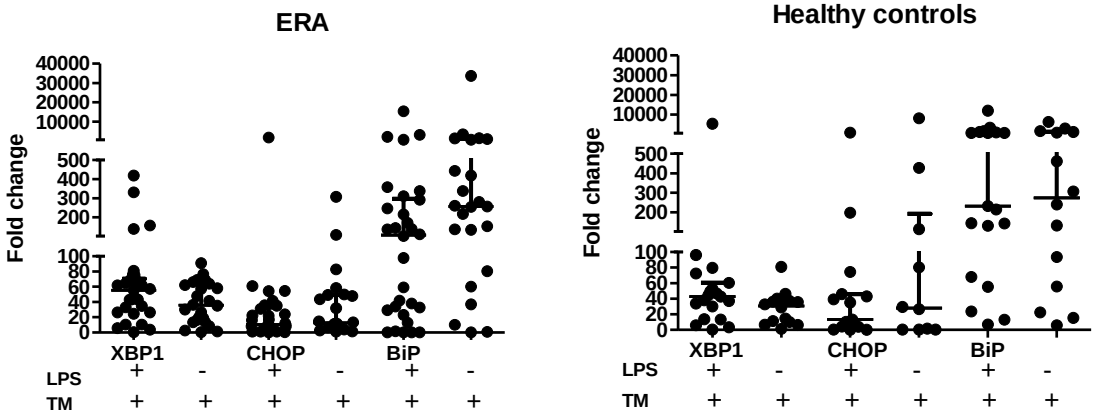


Figure 6.3 Gene expression of spliced XBP1, CHOP and BiP does not differ significantly between MDMs stimulated with both LPS and TM or TM alone in patients with ERA and healthy controls. Spliced XBP1, CHOP and BiP expression were analysed from RNA from using qPCR and normalised against unspliced XBP1 or GAPDH. Fold change was calculated relative to the control sample. Bar graph shows median with interquartile range, statistical analysis by Mann-Whitney U test between MDMs treated with LPS + TM and TM alone for all genes analysed.

6.1.5 Spliced XBP1 and CHOP gene expression are not significantly different from LPS + TM-stimulated MDMs in patients with ERA compared to healthy controls

Spliced XBP1 and CHOP gene expression were significantly enhanced in LPS + TM-stimulated MDMs compared to MDMs without LPS and TM stimulation in both patients and healthy controls. However, no difference in expression was noted between LPS + TM-stimulated MDMs from patients with ERA compared to age-matched healthy controls (XBP1: median fold change 55.33, IQR 26.35-70.75 vs 42.81, IQR 13.55-60.55, figure 6.4 A; CHOP: median fold change 10.41, IQR 5.464-33.19 vs 13.27, IQR 4.084-45.89, figure 6.4 B).

As with spliced XBP1 and CHOP expression from MDMs without LPS and TM stimulation, there was no significant difference in gene expression from LPS + TM-stimulated MDMs associated with clinical features such as HLA B27, male sex, active peripheral arthritis, enthesitis and disease activity by JADAS category or between these groups and healthy controls (table 6.2)

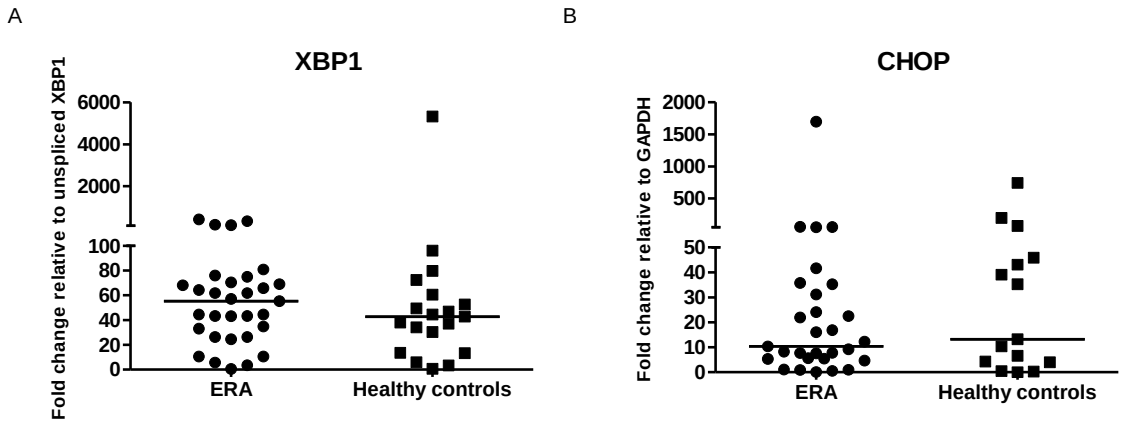


Figure 6.4 Gene expression of spliced XBP1 and CHOP in LPS + TM-stimulated MDMs is not significantly different between patients with ERA and healthy controls. RNA was isolated from MDMs stimulated with LPS + TM for 24 hours and transcribed in to cDNA. Spliced XBP1 and CHOP were amplified using qPCR and normalised against unspliced XBP1 or GAPDH. Fold change was calculated by comparing the expression of the gene of interest to the control sample. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann-Whitney U test (ERA n=30, adolescent healthy controls n=18).

A trend was seen towards higher spliced XBP1 expression in patients with peripheral ERA compared to healthy controls (median fold change 65.80, IQR 53.23-205.9, p=0.051) but again this group was small and no significant difference was observed compared to those with indeterminate and axial ERA (axial ERA: median fold change 44.02, IQR 31.44-71.92; indeterminate ERA 40.94, IQR 6.941-67.26, figure 6.5 A). There was no difference between these groups for CHOP (peripheral ERA: median fold change 7.621, IQR 0.9678-41.49; indeterminate ERA: 12.30, IQR 8.225-22.47; axial ERA: 9.254, IQR 5.464-38.45, figure 6.5 B).

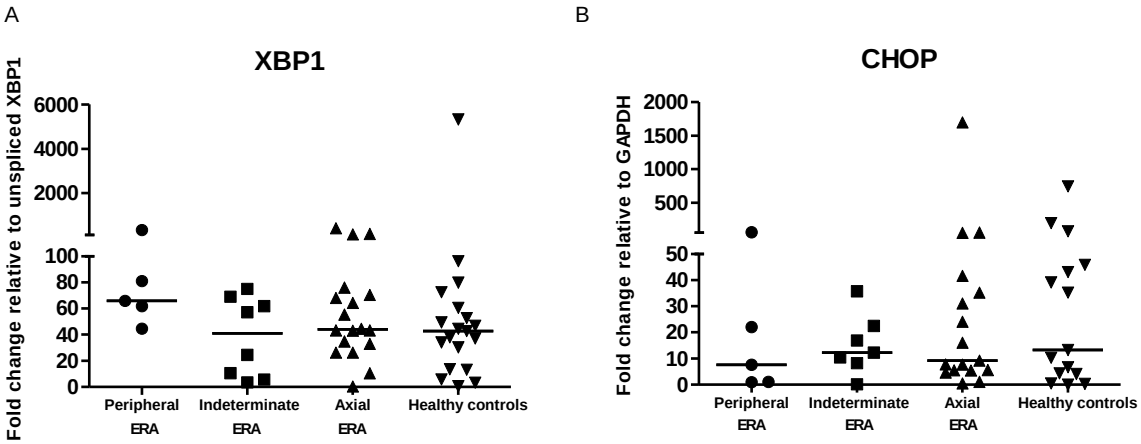


Figure 6.5 Trend towards higher spliced XBP1 gene expression in LPS + TM-stimulated MDMs from patients with peripheral ERA compared to healthy controls. Spliced XBP1and CHOP expression were analysed from RNA from LPS + TM-stimulated MDMs using qPCR and normalised against unspliced XBP1 or GAPDH. Fold change was calculated relative to the control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (peripheral ERA n=5, indeterminate ERA n=8, axial ERA n=7, THC = teenage healthy control n=18).

Interestingly, XBP1 expression from LPS + TM-stimulated MDMs for patients on DMARDS was higher compared to those not on DMARDs (median fold change 61.82, IQR 43.11-75.06 vs 18.46, IQR 4.100-64.92, p=0.042, table 6.2) but this may have been a chance finding and there was no difference observed for patients on and off TNF inhibitors (median fold change 55.33, IQR 43.26-72.07 vs 52.62, IQR 21.08-71.92). In addition, no difference was seen in CHOP expression between those patients taking DMARDs compared to those not taking them (median fold change 11.35, IQR 7.130-32.16 vs 5.502, IQR 1.028-54.19) and for those taking TNF inhibitors compared to those not on them (median fold change 11.35, IQR 5.567-28.85 vs 9.254, IQR 1.057-38.70).

6.1.6 Fold change in BiP gene expression from LPS + TM-stimulated MDMs is higher in adolescent healthy controls compared to patients with ERA

As seen in MDMs without LPS and TM stimulation, a different pattern of expression was observed for BiP from LPS + TM-stimulated MDMs compared to spliced XBP1 and CHOP. Interestingly, the fold change in gene expression of BiP was higher in adolescent healthy controls compared to patients with ERA (median fold change 232.3, IQR 68.12-1167 vs 106.2, IQR 27.67-268.8, p=0.044, figure 6.6 A). No difference was seen in BiP expression from LPS + TM-stimulated MDMs between patients with ERA who were HLA B27 positive compared to those who were HLA B27 negative (median fold change 101.8, IQR 27.92-324.3 vs 110.7, IQR 21.03-193.3) although a trend towards lower expression was seen in HLA B27 positive patients compared to healthy controls (p=0.081, figure 6.6 B).

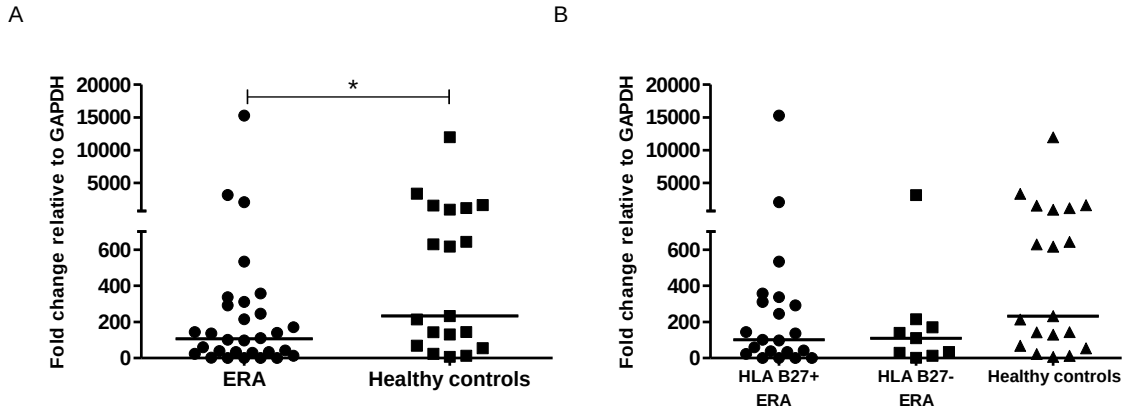


Figure 6.6 Gene expression of BiP in LPS + TM-stimulated MDMs is higher in adolescent healthy controls compared to patients with ERA. RNA was isolated from MDMs stimulated with LPS + TM for 24 hours and transcribed in to cDNA. BiP was amplified using qPCR and normalised against GAPDH. Fold change was calculated by comparing the expression of BiP to the control sample. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann-Whitney U test (ERA n=30, adolescent healthy controls n=18, HLA B27+ ERA n=21, HLA B27- ERA n=9, *p<0.05).

The gene expression of BiP from LPS + TM-stimulated MDMs from those with axial ERA was significantly lower than healthy controls (p=0.028, figure 6.7 A). However, no significant difference was noted between the peripheral, indeterminate and axial ERA subtypes (table 6.2). There was a trend to lower expression of BiP in those with moderate and low disease activity by JADAS (moderate disease activity 67.87, IQR 8.440-267.9, low disease activity 101.7, IQR 15.35-280.4, figure 6.7 B) compared to healthy controls (p=0.092 and p=0.057 respectively) but overall no significant difference was observed between the disease activity groups (table 6.2).

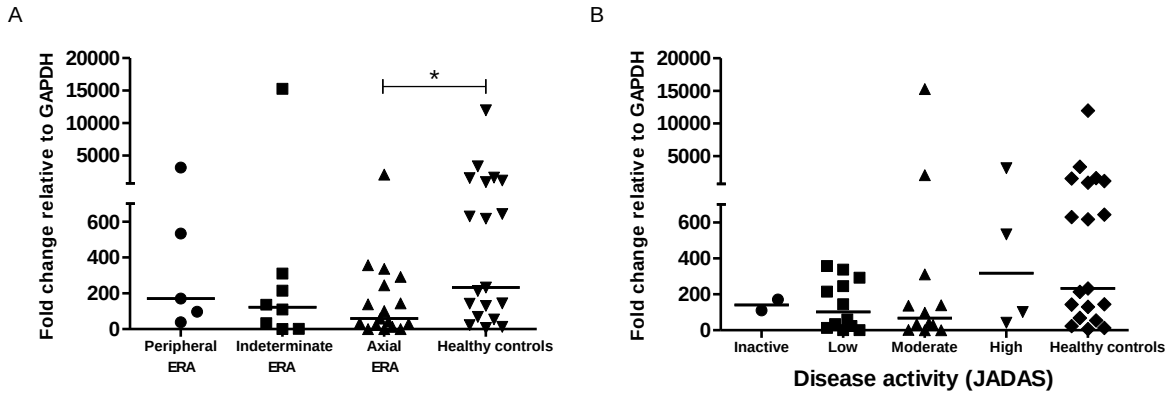


Figure 6.7 Gene expression of BiP is lower in LPS + TM-stimulated MDMs from patients with axial ERA compared to healthy controls. BiP expression was analysed from RNA from MDMs using qPCR and normalised against GAPDH. Fold change was calculated relative to an unstimulated control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (peripheral ERA n=5, indeterminate ERA n=8, axial ERA n=7, adolescent healthy controls n=18, inactive ERA n=2, ERA with low disease activity n=12, moderate disease activity n=12, high disease activity n=4, *p<0.05).

No difference in BiP expression from LPS + TM stimulated-MDMs was observed between those with ERA with and without active peripheral arthritis (median fold change 128.6, IQR 34.19-478.0 vs 106.2, IQR 20.41-257.2) or enthesitis at the time of sample (median fold change 119.0, IQR 33.13-238.0 vs 74.36, IQR 9.989-915.9) although expression in patients without peripheral arthritis was significantly lower than healthy controls (p=0.039) and there was a trend to lower expression in patients with active enthesitis compared to healthy controls (p=0.056). These differences may simply represent the overall difference observed between patients with ERA and healthy controls.

LPS + TM-stimulated MDMs from patients on treatment with DMARDs and TNF inhibitors did not express higher levels of BiP compared to those not taking these medications (DMARDs: median fold change 99.75, IQR 20.41-222.8 vs 227.4, IQR 35.85-1636; TNF inhibitors: median fold change 123.5, IQR 32.03-314.7 vs 71.88, IQR 25.14-296.7) although expression was lower in those on DMARDs compared to healthy controls (p=0.019) and a trend towards lower levels was noted in those not on TNF inhibitors compared to healthy controls (p=0.056).

		Spliced XBP1	CHOP	BiP
HLA B27	Positive	44.63 (25.47-67.43)	13.26 (6.148-40.05)	101.8 (27.92-324.3)
	Negative	64.97 (43.34-77.33)	9.254 (0.7839-23.05)	110.7 (21.03-193.3)
Sex	Male	56.31 (31.44-71.92)	8.225 (5.043-27.60)	101.8 (27.92-324.3)
	Female	34.78 (16.05-70.43)	35.34 (13.25-59.37)	110.7 (0.1675-422.3)
Active enthesitis	Yes	49.98 (25.03-67.20)	10.41 (5.657-24.08)	119.0 (33.13-238.0)
	No	65.80 (33.13-139.1)	10.04 (0.8155-41.69)	74.36 (9.989-915.9)
ERA subtype	Peripheral	65.80 (53.23-205.9)	7.621 (0.9678-41.49)	171.3 (67.87-1852)
	Indeterminate	40.94 (6.941-67.26)	12.30 (8.225-22.47)	123.5 (9.354-286.9)
	Axial	44.02 (31.44-71.92)	9.254 (5.464-38.45)	59.30 (17.88-268.8) *
Active peripheral arthritis	Yes	59.55 (10.45-68.25)	10.41 (1.087-35.75)	128.6 (34.19-478.0)
	No	44.63 (33.13-75.06)	10.71 (5.483-32.16)	106.2 (20.41-257.2)
Disease activity (JADAS)	Low	55.33 (38.94-103.6)	20.10 (6.175-35.63)	101.7 (15.35-280.4)
	Moderate	34.88 (14.06-67.26)	9.254 (5.426-22.47)	67.87 (8.440-267.9)
	High	68.16 (16.83-78.39)	2.873 (1.043-46.89)	317.8 (56.91-2510)
TNF inhibitor	Yes	55.33 (43.26-72.07)	11.35 (5.567-28.85)	123.5 (32.03-314.7)
	No	52.62 (21.08-71.92)	9.254 (1.057-38.70)	71.88 (25.14-296.7)
DMARD	Yes	61.82 (43.11-75.06)	11.35 (7.130-32.16)	99.75 (20.41-222.8) *
	No	18.46 (4.100-64.92)	5.502 (1.028-54.19)	227.4 (35.85-1636)

Table 6.2 Summary of spliced XBP1, CHOP and BiP gene expression from LPS + TM-stimulated MDMs in patients and healthy controls. Gene expression was analysed from RNA from MDMs stimulated with LPS + TM for 24 hours using qPCR and normalised against unspliced XBP1 or GAPDH. Fold change was calculated relative to the control sample for each patient and healthy control. Table shows median fold change and IQR in brackets, statistical analysis by Mann-Whitney U test. Key:

Significant difference between groups (p<0.05)	* Difference between ERA group and healthy controls (p<0.05)

6.1.7 Gene expression of spiced XBP1, CHOP and BiP is no different in MDMs stimulated with LPS + TM for 4 hours in patients with ERA and healthy controls

Optimisation experiments described in chapter 3 (3.2.5) had determined that the expression of spliced XBP1 was higher in MDMs after 24 hours incubation with TM compared to 4 hours. However, in view of the importance of investigating the UPR in patients with ERA and the hypothesis that induction of the UPR may precede the release of pro-inflammatory cytokines, it was decided to also analyse spliced XBP1, CHOP and BiP in MDMs after 4 hours incubation with LPS + TM. As discussed above, it was not possible to collect 4 hour samples for RNA for many patients and healthy controls because of the small amount of blood collected and thus the number of monocytes available for culture. However, RNA was available on the samples used to analyse IL17 gene expression (section 5.3.2, ERA n=15, adolescent healthy control n=5).

MDMs without LPS and TM stimulation had low levels of spliced XBP1, CHOP and BiP gene expression and no difference was seen between patients and healthy controls. When MDMs from patients with ERA were stimulated with LPS + TM for 4 hours, gene expression of spliced XBP1, CHOP and BiP was generally lower than expression in MDMs stimulated with LPS + TM for 24 hours although the difference was only significant for XBP1 expression (fold change 18.00, IQR 10.35-21.22 vs 49.98, IQR 26.35-71.66, p= 0.0002, figure 6.8 A). No significant difference was observed between patients and healthy controls (spliced XBP1: median fold change 18.00, IQR 10.35-21.22 vs 11.88, IQR 6.151-12.82, p=0.076; CHOP: median fold change 16.63, IQR 9.579-36.83 vs 8.459, IQR 5.667-16.61; BiP: median fold change 82.71, IQR 7.674-481.0 vs 203.7, IQR 27.83-1157, figure 6.8 B).

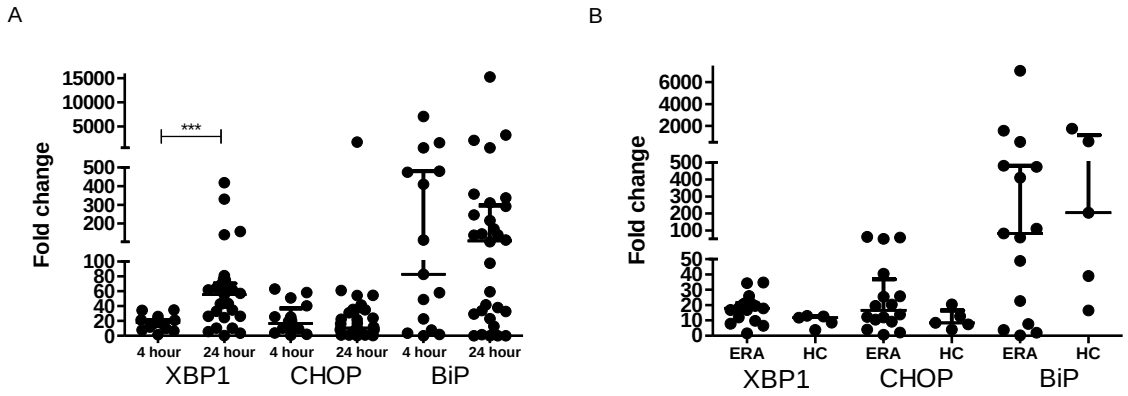


Figure 6.8 XBP1, CHOP and BiP gene expression is no different between patients with ERA and healthy controls. Expression of spliced XBP1, CHOP and BiP was analysed from RNA from LPS + TM-stimulated MDMs using qPCR and normalised against GAPDH or unspliced XBP1. Fold change was calculated relative to the control sample. Bar graphs show median with interquartile range, statistical analysis by Mann-Whitney U test (ERA 4 hour samples n=15, ERA 24 hour samples n=30, HC= adolescent healthy control n=5, *** p<0.001).

6.1.8 XBP1, CHOP and BiP gene expression in SFMCs from HLA B27 positive patients with ERA and HLA B27 negative patients with oligoarticular JIA

To determine whether the trends observed in LPS + TM-stimulated MDMs from patients with ERA were mirrored in an ex vivo setting, spliced XBP1, CHOP and BiP expression was measured from SFMCs using qPCR. SFMCs from HLA B27 positive patients with ERA were isolated from synovial fluid collected at the time of knee aspiration (as described in section 2.2.6). Ten samples from patients with ERA (HLA B27 positive) were compared to samples from a group of 10 patients with oligoarticular JIA who were HLA B27 negative in the absence of a suitable non-inflammatory control group.

No significant differences were noted between gene expression of spliced XBP1, CHOP and BiP in SFMCs between patients with ERA and patients with oligoarticular JIA (XBP1: median fold change 2.219, IQR 1.505-2.694 vs 1.790, IQR 1.464-2.732; CHOP: median fold change 2.196, IQR 1.297-2.2558 vs 2.107, IQR 1.532-3.127; BiP: 704.3, IQR 99.04-1226 vs 714.1, IQR 160.9-2937, figure 6.9).

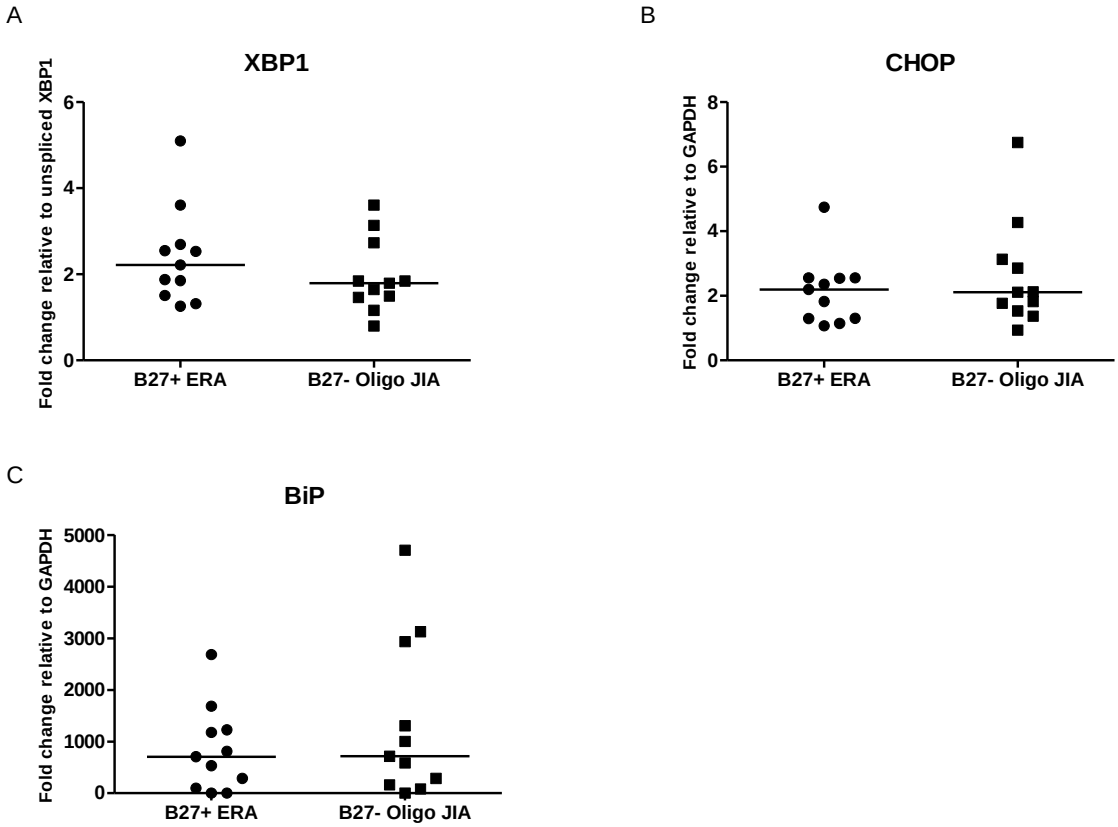


Figure 6.9 Spliced XBP1, CHOP and BiP gene expression is no different between patients with ERA and healthy controls. RNA was extracted from SFMCs and transcribed in to cDNA. Spliced XBP1, CHOP and BiP expression were analysed using qPCR and normalised against GAPDH or unspliced XBP1. Fold change was calculated relative to a control value (the average ΔCt of the lowest ERA and oligoarticular JIA sample) in the absence of a non-inflammatory control sample. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann-Whitney test (HLA B27 positive ERA n=10, HLA B27 negative oligoarticular JIA n=10).

6.1.9 Gene expression of spliced XBP1, CHOP and BiP from MDMs do not correlate

In view of the correlations observed in the production and gene expression of some pro-inflammatory cytokines (chapters 4 and 5), it was of interest to determine whether there was any correlation in the gene expression of the 3 markers of the UPR tested. For MDMs without LPS and TM stimulation, no correlations were observed between the gene expression of spliced XBP1, CHOP and BiP in patients with ERA or healthy controls. Similarly, for MDMs stimulated for 4 and 24 hours with LPS + TM, no correlations between the 3 markers were observed.

6.2 Effect of UPR induction on IL23 family gene expression and cytokine production in LPS-stimulated MDMs

6.2.1 Introduction

The next experiments were to determine the effect of inducing the UPR on the gene expression and production of cytokines in the IL23 family in this assay. It was hypothesised that inducing the UPR with TM in MDMs would augment the expression of pro-inflammatory cytokines compared to stimulation of MDMs with LPS alone. There is significant evidence to suggest that the UPR modulates cytokine production (Smith 2018) but also that the UPR may be induced by pro-inflammatory cytokines (Chae, Park et al. 2012). For IL23 in particular, there is evidence of upregulation with UPR induction (Colbert, DeLay et al. 2010, Goodall, Wu et al. 2010). Summary tables of the results can be found at the end of section 6.5.

6.2.2 Gene expression of IL23p19, IL12/23p40 and IL12p35 is not significantly enhanced by UPR induction

First, the effect of UPR induction on gene expression of IL23p19, IL12/23p40 and IL12p35 was analysed using qPCR. Interestingly, no significant increase in gene expression was noted between MDMs stimulated for 24 hours with LPS alone compared to MDMs stimulated with both LPS and TM (LPS + TM) for patients with ERA and healthy controls (table 6.3, figure 6.10). In addition, there was no difference in IL23p19, IL12/23p40 or IL12p35 expression from LPS + TM-stimulated-MDMs between patients with ERA and healthy controls. The median fold change in gene expression between LPS-stimulated MDMs and LPS + TM-stimulated MDMs was also calculated (by subtracting gene expression for LPS-stimulated MDMs from gene expression for LPS + TM-stimulated MDMs and dividing by gene expression for LPS-stimulated MDMs). The median fold increase in IL23p19 was 0.5625 for patients with ERA and 2.387 for adolescent healthy controls and for IL12/23p40, a median fold increase of 4.776 for patients with ERA and 5.435 for healthy controls was seen. For gene expression of IL12p35, a small decrease in median fold change was observed for patients with ERA (-0.00863) and a median fold increase of 0.9119 was seen for adolescent healthy controls. There was no significant difference in the fold change in gene expression

for IL23p19, IL12/23p40 or IL12p35 between LPS-stimulated MDMs and LPS + TM-stimulated MDMs for patients with ERA or healthy controls.

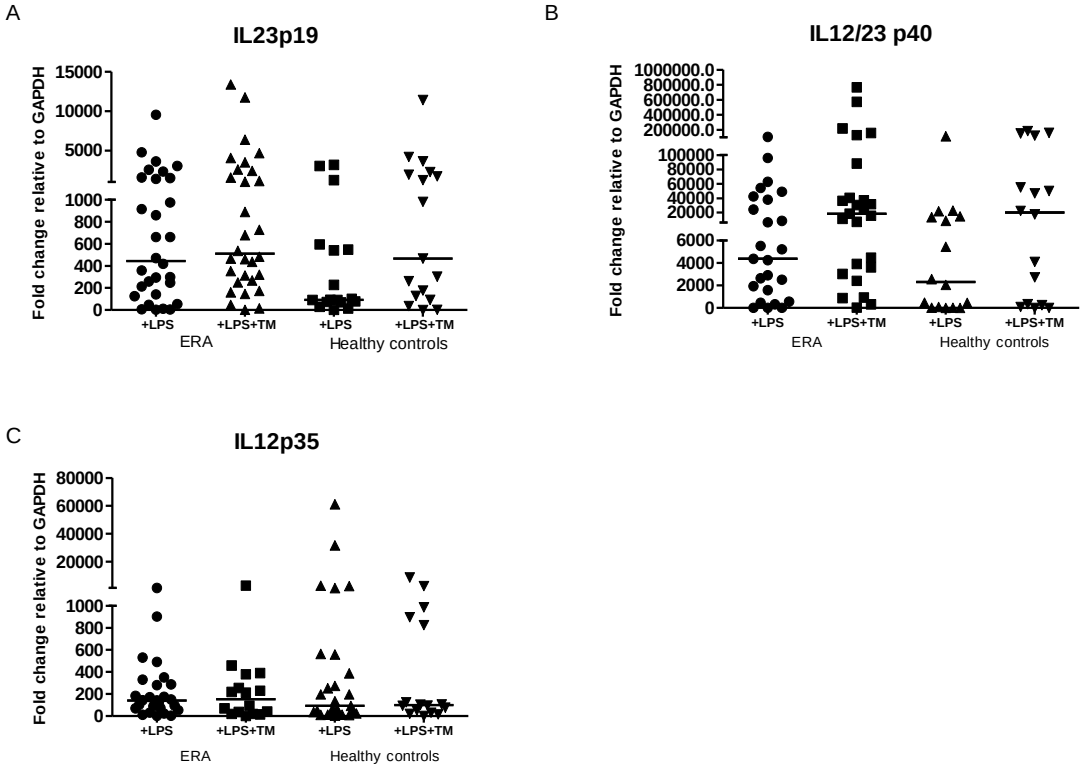


Figure 6.10 Gene expression of IL23p19, IL12/23p40 and IL12p35 is not significantly enhanced by UPR induction. Gene expression of IL23p19, IL12/23p40 and IL12p35 was analysed from RNA from MDMs following 24 hours stimulation with either LPS alone or LPS + TM using qPCR and normalised against GAPDH. Fold change was calculated relative to the control sample. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA n=30, adolescent healthy control n=18).

In view of the associations observed between clinical features of ERA such as HLA B27, enthesitis, male sex and high levels of certain pro-inflammatory cytokines such as IL23, TNFα, IL27, GMCSF, subgroup analysis of IL23p19, IL12/23p40 and IL12p35 gene expression was undertaken. Interestingly, the only clinical feature associated with enhanced gene expression was HLA B27. For IL23p19, gene expression was upregulated in HLA B27 positive patients with ERA compared to HLA B27 negative patients with ERA (median fold change 680.3, IQR 380.7-3792 vs 315.2, IQR 30.9-964.9, p=0.037, figure 6.11 A). A significant difference was also observed in fold increase in gene expression between MDMs stimulated with LPS alone compared to LPS + TM-stimulated MDMs (median fold increase 1.809 vs -0.2154, p=0.037). There was a suggestion that this enhanced IL23p19 expression in LPS + TM-stimulated MDMs in HLA B27 positive patients was modulated by treatment with TNF inhibitors. The fold increase in IL23p19 from LPS-stimulated MDMs to LPS + TM-stimulated MDMs was lower in those on treatment with TNF inhibitors compared to those not on treatment (median fold increase 0.7019 vs 2.182, p=0.091), although the difference was not significant perhaps because the numbers in each group were relatively small (n=10 and n=11 respectively). It was also noted that the gene expression of IL23p19 in HLA B27 positive patients on TNF inhibitors was lower than those not on them although again the difference was not significant (median fold change 502.7, IQR 410.1-2844 vs 729.1, IQR 173.6-4663, p=0.091).

For IL12/23p40, gene expression for LPS + TM-stimulated MDMs was not significantly different in HLA B27 positive patients with ERA compared to HLA B27 negative patients (median fold change 16840, IQR 3830-97871 vs 25180, IQR 922.9-36358, figure 6.11 B), although there was a trend towards a larger fold increase from LPS stimulated-MDMs to LPS + TM-stimulated MDMs in HLA B27 positive patients compared to those who were HLA B27 negative (median fold increase 6.298 vs 0.6742, p=0.074). Interestingly, as with IL23p19, a pattern of lower expression was observed for IL12/23p40 in LPS + TM-stimulated MDMs for HLA B27 positive patients on TNF inhibition compared to HLA B27 positive patients not on TNF inhibition, although the difference was not significant (median fold change 11194, IQR 4047-102546 vs 27651, IQR 3466-209650; median fold increase in IL12/23p40 from LPS-stimulated MDMs to LPS + TM-stimulated MDMs 2.557 vs 10.86).

The pattern of IL12p35 gene expression was similar to that of IL23p19 from LPS + TM-stimulated MDMs, with enhanced gene expression in HLA B27 positive patients compared to HLA B27 negative patients (median fold change 166.5, IQR 49.35-677.4 vs 13.14, IQR 10.13-200.5, p=0.011, figure 6.11 C). Interestingly, a fold increase in IL12p35 from LPS-stimulated to LPS + TM-stimulated MDMs was seen in patients who were HLA B27 positive but a fold decrease was seen for HLA B27 negative patients (median 0.5476 vs -0.5793, p=0.018). There was also a suggestion that treatment with TNF inhibitors modulated IL12p35 expression from LPS + TM-stimulated MDMs in HLA B27 positive patients with ERA, with a lower fold increase from LPS-stimulated to LPS + TM-stimulated MDMs observed in patients on TNF inhibition compared to those not on them (median fold increase 0.006437

vs 2.074) although the difference was not statistically significant and there was no difference between gene expression of IL12p35 between these groups (median fold change 136.4, IQR 64.89-274.4 vs 196.7, IQR 48.84-1017).

No significant associations were noted with other clinical features for IL23p19, IL12/23p40 or IL12p35 (data shown table 6.3).

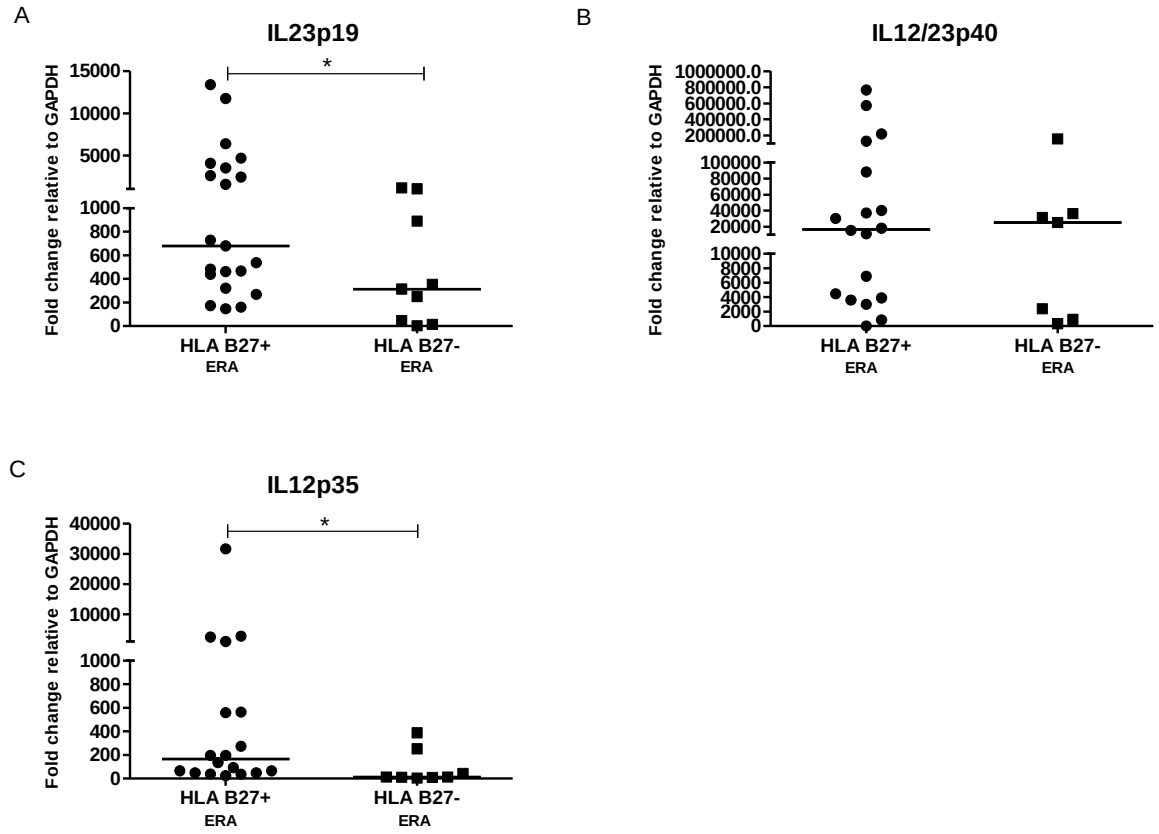


Figure 6.11 Gene expression of IL23p19 and IL12p35 is higher in HLA B27 positive patients with ERA compared to HLA B27 negative patients. RNA was isolated from MDMs stimulated with LPS + TM for 24 hours and transcribed in to cDNA. Genes were amplified using qPCR and normalised against GAPDH. Fold change was calculated by comparing the gene expression from LPS + TM-stimulated MDMs to the control sample for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis Mann Whitney test (B27+ ERA n=21, B27- ERA n=9, *p<0.05).

6.2.3 Gene expression of IL23p19, IL12/23p40 and IL12p35 from LPS + TM-stimulated MDMs correlate

In view of the similar pattern of gene expression between IL23p19, IL12/23p40 and IL12p35 in LPS + TM-stimulated MDMs, analysis was undertaken to determine whether there was correlation between them. Using Spearman's rank correlation coefficient, a strong correlation was observed between expression of IL23p19 and IL12/23p40 from LPS + TM-stimulated MDMs (r=0.64, p=0.0005). This was also the case for IL23p19 and IL12p35 (r=0.51, p=0.0071) and IL12/23p40 and IL12p35 (r=0.58, p=0.0028). It was also of interest to determine whether there was a correlation with markers of the UPR. Contrary to expectation, a negative correlation was noted between IL23p19 expression from LPS + TM-stimulated MDMs and spliced XBP1 (r=-0.50, p=0.0047, figure 6.18). A trend was noted towards a positive correlation between IL23p19 and CHOP gene expression (r=0.36, p=0.059, figure 6.18). In MDMs without LPS and TM stimulation, a significant correlation was found between CHOP and IL23p19 (r=0.42, p=0.023), IL12/23p40 (r=0.57, p=0.0027) and IL12p35 (r=0.45, p=0.045) in MDMs.

6.2.4 IL23 production from LPS + TM-stimulated MDMs is higher in patients with ERA compared to healthy controls and is associated with HLA B27 and enthesitis

Next, it was important to determine the effect of UPR induction on cytokine production and whether the patterns seen in gene expression were also observed at the protein level. IL23 was measured in cell culture supernatants from MDMs stimulated with LPS + TM for 24 hours in the larger final group of patients with ERA and also in patients with polyarticular JIA, AS, in addition to the teenage and adult healthy controls (table 3.1). Interestingly, IL23 production was significantly lower in MDMs stimulated with LPS + TM compared to those stimulated with LPS alone in patients with ERA (median 12620 pg/mL, IQR 6197-20735 pg/mL (decreased from 53580 pg/mL, IQR 35735-83945 pg/mL), p=<0.0001) and healthy controls (6122 pg/mL, IQR 252.3-16170 pg/mL (decreased from 32110 pg/mL, IQR 13745-48235 pg/mL), p=0.0006, figure 6.12 A). In fact levels were lower in all the patient and healthy control groups tested (table 6.5). However, as seen with LPS-stimulated MDMs, levels remained higher in patients with ERA compared to adolescent healthy controls (p=0.017, figure 6.12 B). Conversely, and in contrast to LPS-stimulated MDMs, no significant difference was observed in IL23 production from LPS + TM-stimulated MDMs between patients with polyarticular JIA and adolescent healthy controls and between adult patients with AS and adult healthy controls.

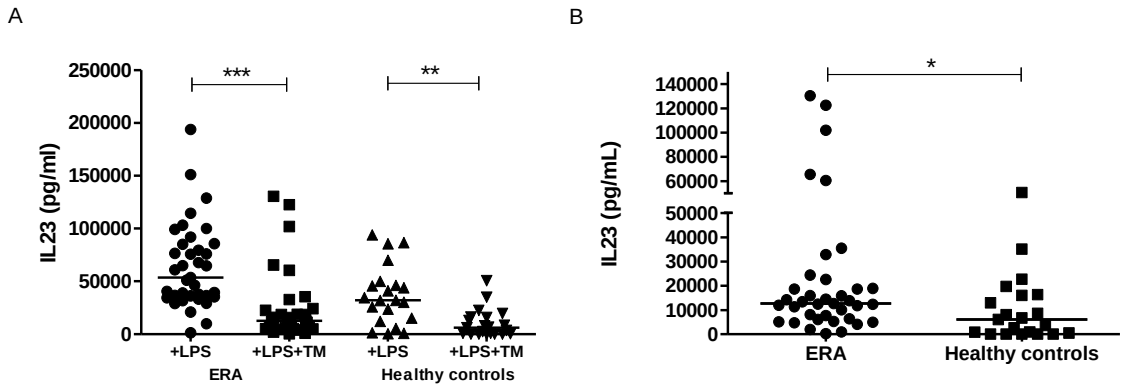


Figure 6.12 IL23 production is lower from LPS + TM-stimulated MDMs compared to MDMs stimulated with LPS alone but is higher in patients with ERA compared to healthy controls. MDMs were differentiated as described in section 2.2 and stimulated with LPS + TM for 24 hours. IL23 was measured in cell culture supernatants by ELISA. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann Whitney test (ERA n=37, adolescent healthy controls n=21, *p<0.05, **p<0.01, ***p<0.001).

Consistent with gene expression of IL23p19 and IL23 protein levels from LPS-stimulated MDMs, IL23 production from LPS + TM-stimulated MDMs was significantly higher in HLA B27 positive patients with ERA compared to those who were HLA B27 negative (median 15820 pg/mL, IQR 12025-28605 pg/mL vs 5725 pg/mL, IQR 2625-11470 pg/mL, p=0.0012) and healthy controls (p=0.0027, figure 6.13 A). LPS + TM-stimulated MDMs from patients with enthesitis also produced higher levels of IL23 in comparison to patients without active enthesitis (median 15820 pg/mL, IQR 10060-35480 pg/mL vs 10197 pg/mL, IQR 4714-13990 pg/mL, p=0.023) and healthy controls (p=0.0036, figure 6.13 B). However, no difference was observed in IL23 levels in male patients as compared to female patients with ERA (12620 pg/mL, IQR 6058-24350 pg/mL vs 12975 pg/mL, IQR 7387-16585 pg/mL).

There was no difference in IL23 production from LPS + TM-stimulated MDMs between those with peripheral ERA compared to those with axial ERA (peripheral ERA: 14440 pg/mL, IQR 9538-68690 pg/mL; indeterminate ERA: 18560 pg/mL, IQR 6407-63085 pg/mL; axial: 12290 pg/mL, IQR 6058-15940 pg/mL), although IL23 in all the ERA categories tended to be higher than healthy controls and, as with LPS-stimulated MDMs, IL23 production in the indeterminate ERA category was the highest and significantly higher than healthy controls (p=0.047, axial p=0.082, peripheral p=0.069, figure 6.13 C). There was also a trend towards higher IL23 production in the moderate and high disease activity groups by JADAS (low disease activity: median 11310 pg/mL, IQR 6058-14190 pg/mL; moderate disease activity: median 17190 pg/mL, IQR 5013-61818 pg/mL; high disease activity: median 13230 pg/mL, IQR 10609-47658 pg/mL) and production in the moderate disease activity group was significantly higher than healthy controls (p=0.034, high p=0.096, figure 6.13 D).

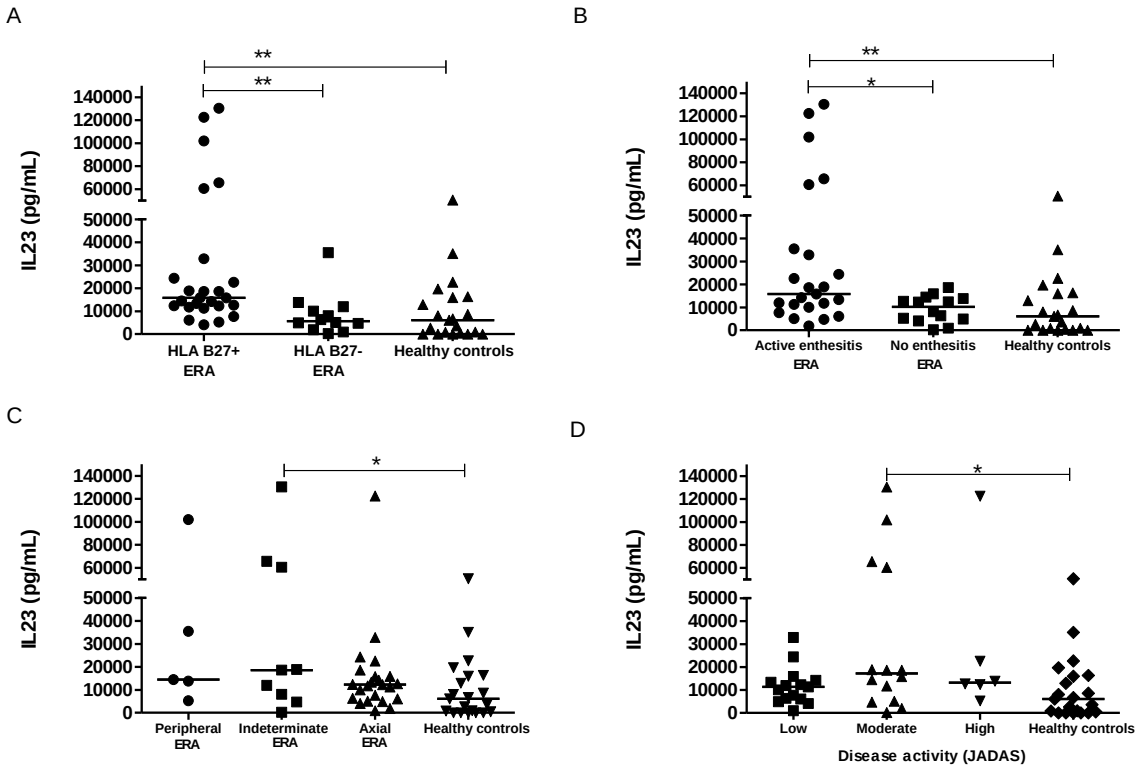


Figure 6.13 High levels of IL23 from LPS + TM-stimulated MDMs are associated with HLA B27 and enthesitis. MDMs were differentiated as described in section 2.2 and stimulated with LPS + TM for 24 hours. IL23 was measured from cell culture supernatants by ELISA. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (HLA B27 positive ERA n=25, HLA B27 negative ERA n=12, ERA with active enthesitis n=23, ERA with no enthesitis n=14, peripheral ERA n=5, indeterminate ERA n=9, axial ERA n=23, inactive ERA n=2, low disease activity n=15, moderate disease activity n=14, high disease activity n=6, adolescent healthy controls n=21, *p<0.05, **p<0.01).

Interestingly, and in contrast to IL23 production from LPS-stimulated MDMs, there was no difference between LPS + TM-stimulated MDMs in those patients with ERA on TNF inhibitors compared to those not on TNF inhibitors (15940 pg/mL, IQR 8104-35480 pg/mL vs 12170 pg/mL, IQR 5205-15473 pg/mL). In addition, treatment with TNF inhibitors did not have a significant modulating effect on IL23 levels from LPS + TM-stimulated MDMs from patients who were HLA B27 positive (as seen with gene expression of IL23p19), with no significant difference in IL23 production from HLA B27 positive patients with ERA on TNF inhibitors compared to those not on TNF inhibitors (median 18560 pg/mL, IQR 12290-60550 pg/mL vs 13805 pg/mL, IQR 10754-19808 pg/mL).

6.2.5 IL12 production is inhibited by UPR induction in LPS-stimulated MDMs

Next, analysis of IL12, another member of the IL23 family was undertaken. IL12 was measured in cell culture supernatants by Luminex assay from MDMs stimulated with LPS + TM for 24 hours. IL12 levels were significantly lower compared to levels from MDMs stimulated with LPS alone in patients with ERA (median 100.2 pg/mL, IQR 45.75-270.7 pg/mL (decreased from 3753 pg/mL, IQR 2055-7688 pg/mL), p=<0.0001) and adolescent healthy controls (median 303.5 pg/mL, IQR 127.4-440.1 pg/mL (decreased from 2733 pg/mL, IQR 1327-4309 pg/mL), p=<0.0001). Levels were also very low in the other patient and healthy control groups analysed (table 6.5) and in fact fell below the detection range in 54.1% of patients with ERA, 28.6% of adolescent healthy controls, 53.3% of polyarticular JIA, 47.6% of adult healthy controls and 28.6% of patients with AS. It was concluded that the addition of TM to the assay inhibited the production of IL12 and therefore further analysis of IL12 production was not undertaken in LPS + TM-stimulated MDMs.

6.2.6 IL27 production from LPS + TM-stimulated MDMs is higher in patients with ERA compared to healthy controls and is associated with enthesitis

The final cytokine in the IL23 family to be analysed was IL27. IL27 was measured in cell culture supernatants from LPS + TM-stimulated MDMs and analysed by Luminex assay. A reduction in IL27 levels was also seen compared to MDMs stimulated with LPS alone in patients with ERA (median 1523 pg/mL, IQR 984.7-1807 pg/mL, (decreased from 1986 pg/mL, IQR 1505-2268 pg/mL), p=0.0092) and healthy controls (median 952.5 pg/mL, IQR 560.8-1341 pg/mL, (decreased from 1603 pg/mL, IQR 1121-1918 pg/mL), p=0.015, figure 6.14 A). However, as with LPS-stimulated MDMs, IL27 production from LPS + TM-stimulated MDMs was significantly higher in patients with ERA compared to healthy controls (p=0.018, figure 6.14 B). The production of IL27 production from LPS + TM-stimulated MDMs was also lower in the other patient and healthy controls groups compared to levels from LPS-stimulated MDMs (table 6.5) but no significant difference was noted in levels between patients with polyarticular JIA and adolescent healthy controls and between patients with AS and adult healthy controls (p=0.067).

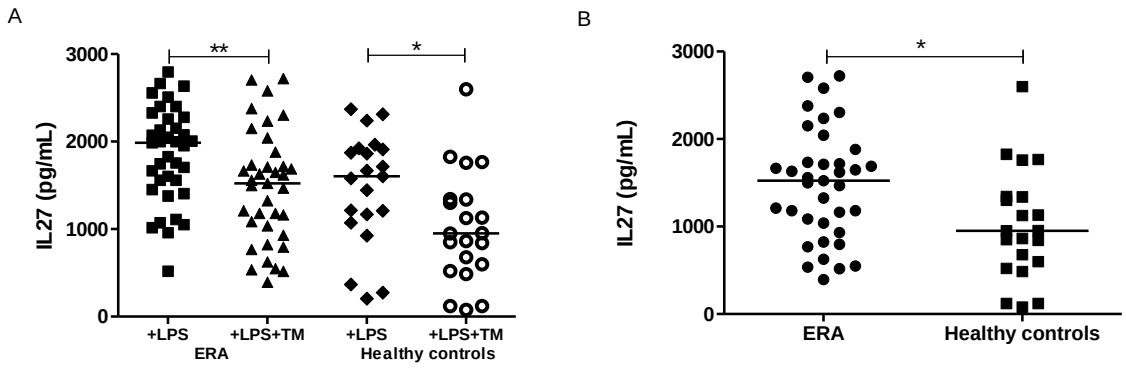


Figure 6.14 IL27 production from LPS + TM-stimulated MDMs is higher in patients with ERA compared to healthy controls. MDMs were differentiated as described in section 2.2 and stimulated with LPS + TM for 24 hours. IL27 was measured in cell culture supernatants by Luminex assay. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann Whitney test (ERA n=37, adolescent healthy controls n=21, *p<0.05, **p<0.01).

There was a trend towards higher IL27 production in HLA B27 positive patients with ERA compared to HLA B27 negative patients with ERA (median 1630 pg/mL, IQR 1173-2193 pg/mL vs 1206 pg/mL, IQR 776.3-1695 pg/mL, p=0.095) and levels in HLA B27 positive patients were significantly higher than healthy controls (p=0.0058, figure 6.15 A). Active enthesitis in patients with ERA was also associated with higher levels of IL27 from LPS + TM-stimulated MDMs (median 1666 pg/mL, IQR 1325-2150 pg/mL vs 1063 pg/mL, IQR 711.3-1547 pg/mL, p=0.0051) and patients with active enthesitis had higher levels of IL27 compared to healthy controls (p=0.0016, figure 6.15 B).

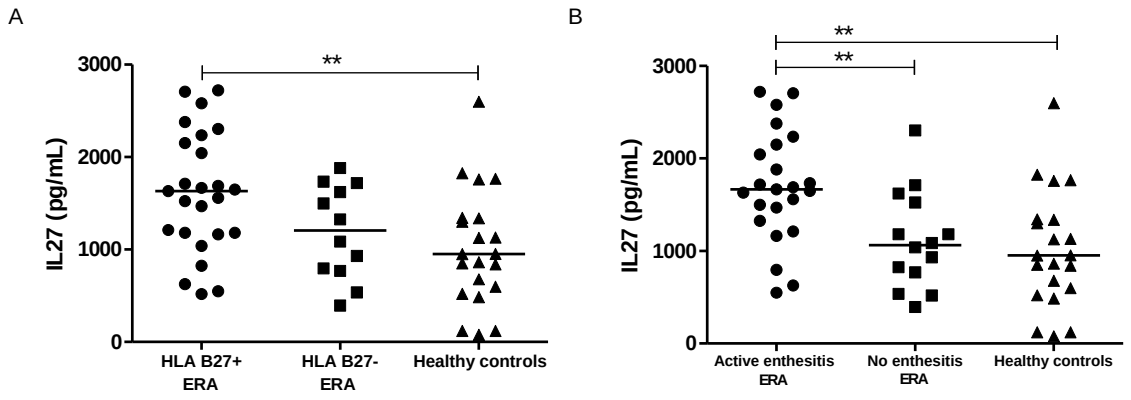


Figure 6.15 Higher IL27 levels from LPS + TM-stimulated MDMs are associated with HLA B27 and active enthesitis in patients with ERA. MDMs were differentiated as described in section 2.2 and stimulated with LPS + TM for 24 hours. IL27 was measured from cell culture supernatants by Luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (HLA B27 positive ERA n=25, HLA B27 negative ERA n=12, ERA with active enthesitis n=23, ERA with no enthesitis n=14, adolescent healthy controls n=21, *p<0.05, **p<0.01).

No significant difference was noted between patients with peripheral and axial ERA (median 1711 pg/mL, IQR 975.8-2157 pg/mL vs 1469 pg/mL, IQR 823.4-1688 pg/mL) and only levels from LPS + TM-stimulated MDMs in those with indeterminate ERA were significantly higher than healthy controls (median 1666 pg/mL, IQR 1150-1962 pg/mL, p=0.037). Similarly, no significant difference was observed between the low, moderate and high disease activity groups in patients with ERA (low: median 1325 pg/mL, 823.4-1688 pg/mL; moderate: 1689 pg/mL, IQR 1108-2253 pg/mL; high: 1352 pg/mL, IQR 971.3-1903 pg/mL), although levels from those in the moderate disease activity group were higher than healthy controls (p=0.021). As with IL23 from LPS + TM-treated MDMs, no significant difference was noted between male and female patients and between those on treatment with TNF inhibitors and those not on treatment (table 6.6).

6.3 Effect of UPR induction on the gene expression and cytokine production of other pro-inflammatory cytokines

6.3.1 Introduction

The next experiments focussed on the effect of UPR induction in LPS-stimulated MDMs on levels of other pro-inflammatory cytokines including TNFα, IL1β, IL6, IL17, GMCSF and IFNγ. As with LPS-stimulated MDMs, gene expression of TNFα, IL1β, IL6 and IL17 was analysed first using qPCR. Cytokine production was then measured in cell culture supernatants. As with the IL23 family of cytokines, it was hypothesised that induction of the UPR using TM would augment pro-inflammatory cytokine gene expression and protein production compared to stimulation with LPS alone.

6.3.2 Gene expression of TNFα, IL1β and IL6 is not significantly enhanced by UPR induction in patients with ERA

The gene expression of TNFα, IL1β and IL6 in MDMs stimulated for 24 hours with LPS + TM was not significantly different compared to MDMs stimulated with LPS alone in patients with ERA (table 6.3, figure 6.16). In LPS + TM-stimulated MDMs from adolescent healthy controls, there was a trend towards increased TNFα gene expression compared to MDMs stimulated with LPS alone (median fold change 9.714, IQR 4.141-14.52, (increased from 2.142, IQR 0.933-13.93), p=0.077) but no significant increase in IL1β or IL6 gene expression (table 6.3). No significant difference was observed between patients with ERA and healthy controls in TNFα, IL1β and IL6 gene expression from LPS + TM-stimulated MDMs (table 6.3, figure 6.16).

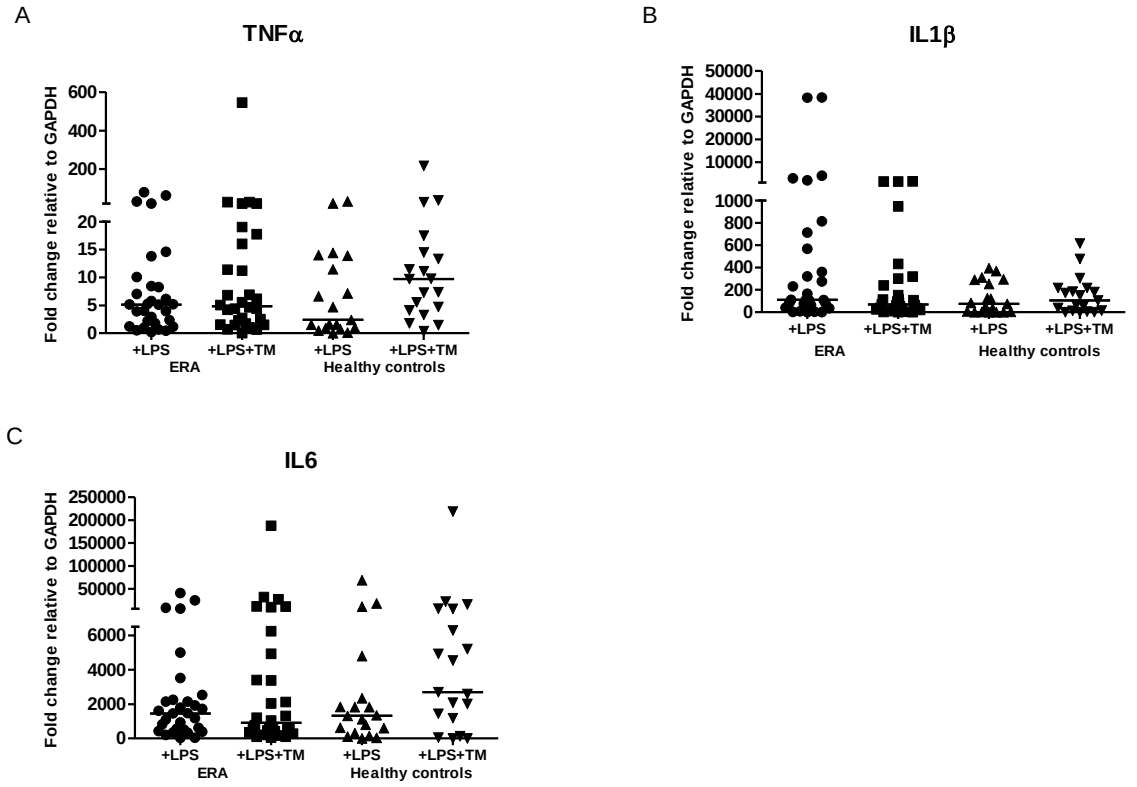


Figure 6.16 Gene expression of TNFα, IL1β and IL6 is no different in LPS and TM-stimulated MDMs compared to LPS-stimulated MDMs in patients with ERA and healthy controls. RNA was isolated from MDMs stimulated with LPS alone or LPS + TM for 24 hours and transcribed in to cDNA. TNFα, IL1β and IL6 were amplified using qPCR and normalised against a housekeeping gene (GAPDH). Fold change was calculated by comparing the expression of TNFα, IL1β or IL6 from stimulated MDMs to the control sample for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann-Whitney U test (ERA n=30, teenage healthy control n=18).

As with cytokines in the IL23 family, subgroup analysis was undertaken to determine whether similar associations in gene expression were observed with clinical features such as HLA B27. In fact, only gene expression of IL6 in LPS + TM-stimulated MDMs was significantly enhanced in HLA B27 positive patients with ERA compared to HLA B27 negative patients (median fold change 1209, IQR 543-110866 vs 415.9, IQR 131.5-1717, p=0.037, figure 6.17 C). For TNFα gene expression, a trend towards higher fold increase from LPS-stimulated MDMs to LPS + TM-stimulated MDMs was observed in patients with ERA who were HLA B27 positive compared to those who were HLA B27 negative where a fold decrease was observed (median 0.8921 vs -0.3229, p=0.094). However, no significant difference was noted in TNFα gene expression between HLA B27 positive and negative patients with ERA (median fold change 6.190, IQR 2.154-19.00 vs 2.549, IQR 1.223-8.407, figure 6.17 A). For IL1β, there was no significant difference in gene expression or fold increase between HLA B27 positive and negative patients with ERA (median fold change 63.12, IQR 33.56-367.9 vs 68.59, IQR 15.30-178.30, figure 6.18 B; fold decrease -0.5949 vs -0.7585). However, as seen with IL23p19 and IL12p35, there was a suggestion that treatment with TNF inhibitors modulated IL1β gene expression from LPS + TM-stimulated MDMs in HLA B27 positive patients with ERA. A fold decrease was noted between LPS-stimulated MDMs and LPS + TM-stimulated MDMs in patients on TNF inhibition compared to a small fold increase in IL1β expression which was seen in those not on TNF inhibitors (-0.7852 vs 0.08079, p=0.034).

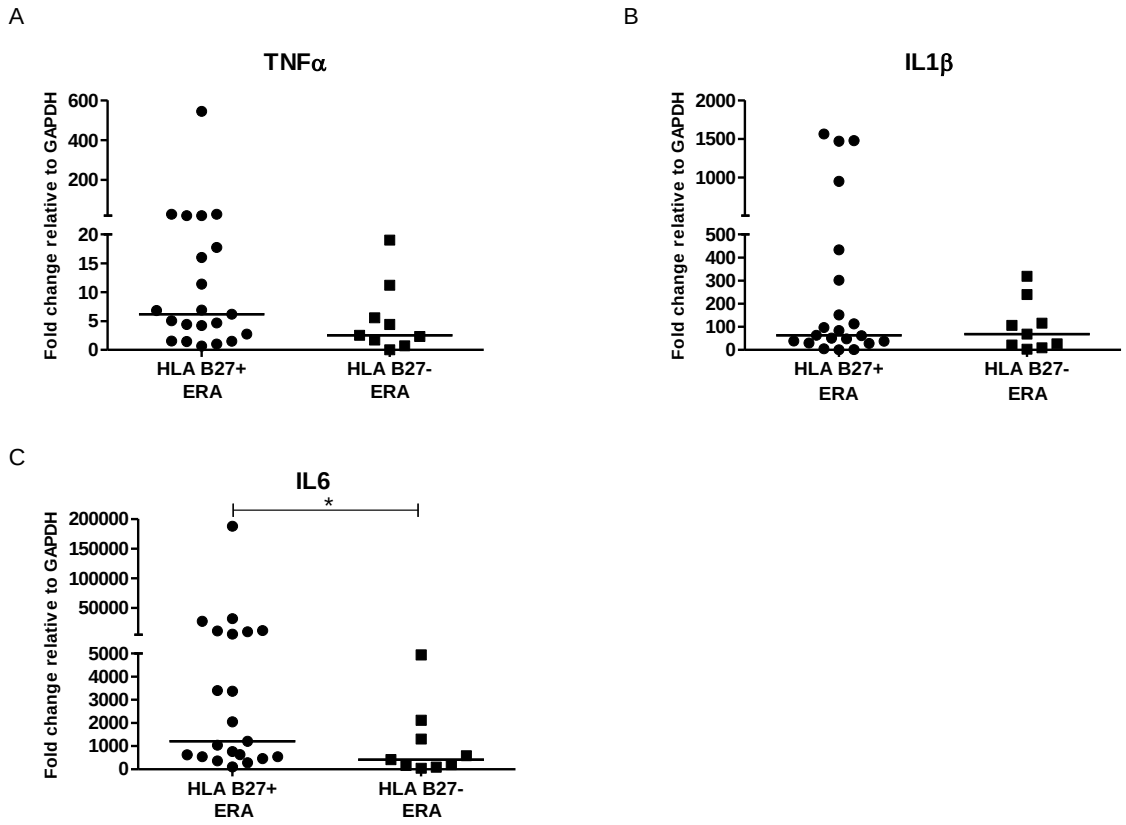


Figure 6.17 Gene expression of IL6 in LPS + TM-stimulated MDMs is higher in patients with ERA who are HLA B27 positive compared to those who are HLA B27 negative. RNA was isolated from MDMs stimulated with LPS + TM for 24 hours and transcribed in to cDNA. TNFα, IL1β and IL6 were amplified using qPCR and normalised against GAPDH. Fold change was calculated by comparing the expression of the gene of interest from LPS + TM-stimulated MDMs to the control sample for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann Whitney U test (HLA B27+ ERA n=21, HLA B27- ERA n=9).

No significant associations were noted with other clinical features analysed for TNFα, IL1β and IL6 gene expression (data shown in summary data table 6.4).

6.3.3 Correlations in gene expression between pro-inflammatory cytokines and markers of the UPR

To assess correlations in gene expression of pro-inflammatory cytokines and also markers of the UPR, Spearman's rank correlation was used. A heat map summarising the results can be found below (figure 6.18). Significant correlations were noted between the expression of many of the pro-inflammatory cytokines, in particular TNFα with IL6 and IL17 and IL6 with IL23p19 and IL12/23p40. As observed above with IL23p19, it was also interesting to note the significant negative correlations between the expression of spliced XBP1 and IL1β and IL6. There was a positive correlation in gene expression between CHOP and TNFα and also BiP with IL1β.

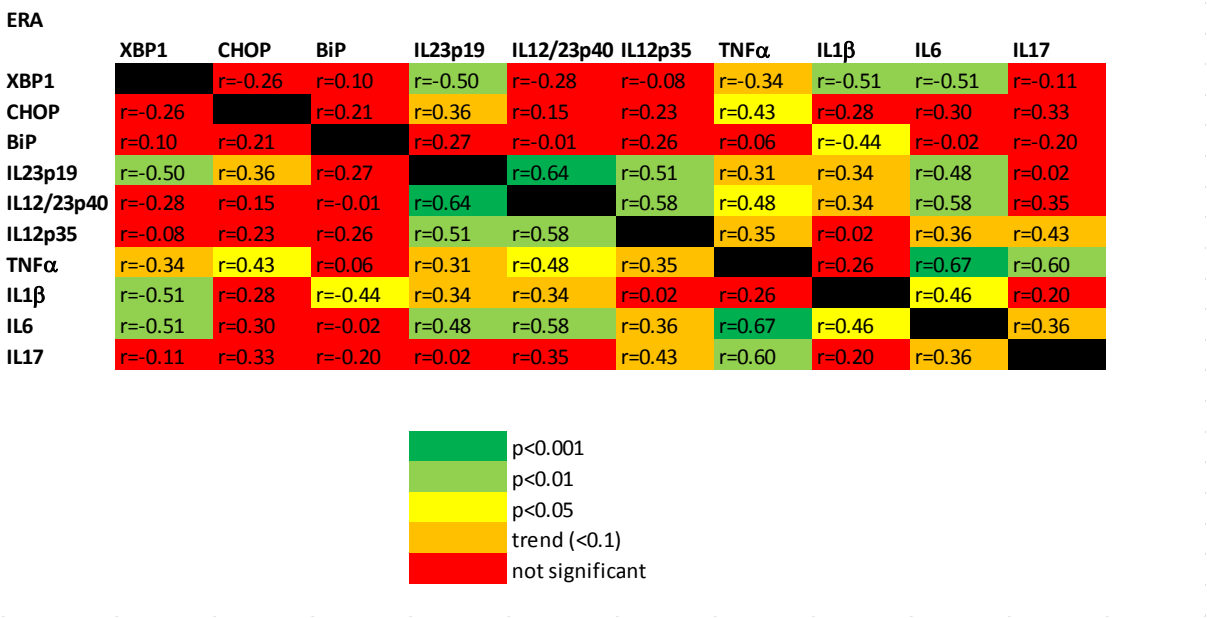


Figure 6.18 Heat map summarising correlations in gene expression between pro-inflammatory cytokines and markers of the UPR in patients with ERA. Gene expression was analysed using qPCR extracted from MDMs stimulated with LPS + TM for 24 hours. Spearman's rank correlation coefficient was used to analyse correlations in fold change of gene expression (ERA n=30).

6.3.4 TNFα and IL1β levels from LPS + TM-stimulated MDMs are higher in patients with ERA compared to healthy controls

Next, the production of TNFα, IL1β and IL6 was analysed in cell culture supernatants from MDMs stimulated with LPS + TM. As with MDMs stimulated with LPS alone, TNFα was measured after 4 hours stimulation with LPS + TM by ELISA (as discussed in section 5.2.1) whereas IL1β and IL6 were measured by Luminex assay after 24 hours stimulation with LPS + TM.

As observed with IL23, TNFα production was significantly lower in LPS + TM-stimulated MDMs compared to TNFα levels from MDMs stimulated with LPS alone in both patients with ERA (median 22025 pg/mL, IQR 13498-32860 pg/mL (decreased from 52445 pg/mL, IQR 28483-67190 pg/mL), p=<0.0001) and adolescent healthy controls (median 14230 pg/mL, IQR 4301-26480 pg/mL (decreased from 29090 pg/mL, IQR 14845-47785 pg/mL), p=0.012). However, TNFα levels from LPS + TM-stimulated MDMs remained higher in patients with ERA compared to healthy controls (p=0.041, figure 6.19 A) as observed in MDMs stimulated with LPS alone. Levels of TNFα production were also lower in LPS + TM-stimulated MDMs in the other patient and healthy control groups analysed compared to levels from MDMs stimulated with LPS alone and no significant differences were noted between these groups (table 6.5).

IL1β production from LPS + TM-stimulated MDMs was not significantly different compared to MDMs stimulated with LPS alone in patients with ERA and healthy controls (table 6.5) although as with TNFα, levels were higher from MDMs in patients with ERA compared to healthy controls (p=0.043). Interestingly, levels were also significantly higher from LPS + TM-stimulated MDMs from patients with ERA compared to patients with polyarticular JIA (p=0.023, figure 6.19 B). No significant difference was observed between LPS-stimulated MDMs and LPS + TM-stimulated MDMs in the other patient and healthy control groups analysed (table 6.5).

Similarly, IL6 production from LPS + TM-stimulated MDMs was not significantly higher than from MDMs stimulated with LPS alone (table 6.5) and as observed in IL6 production from LPS-stimulated MDMs, no difference was seen between patients with ERA and healthy controls (figure 6.19 C). No significant increase in IL6 production was observed between LPS-stimulated and LPS + TM-stimulated MDMs in the other patient and healthy control groups analysed and no significant difference was noted between these groups (table 6.5), although there was a trend to higher levels in patients with AS compared to adult healthy controls (p=0.057).

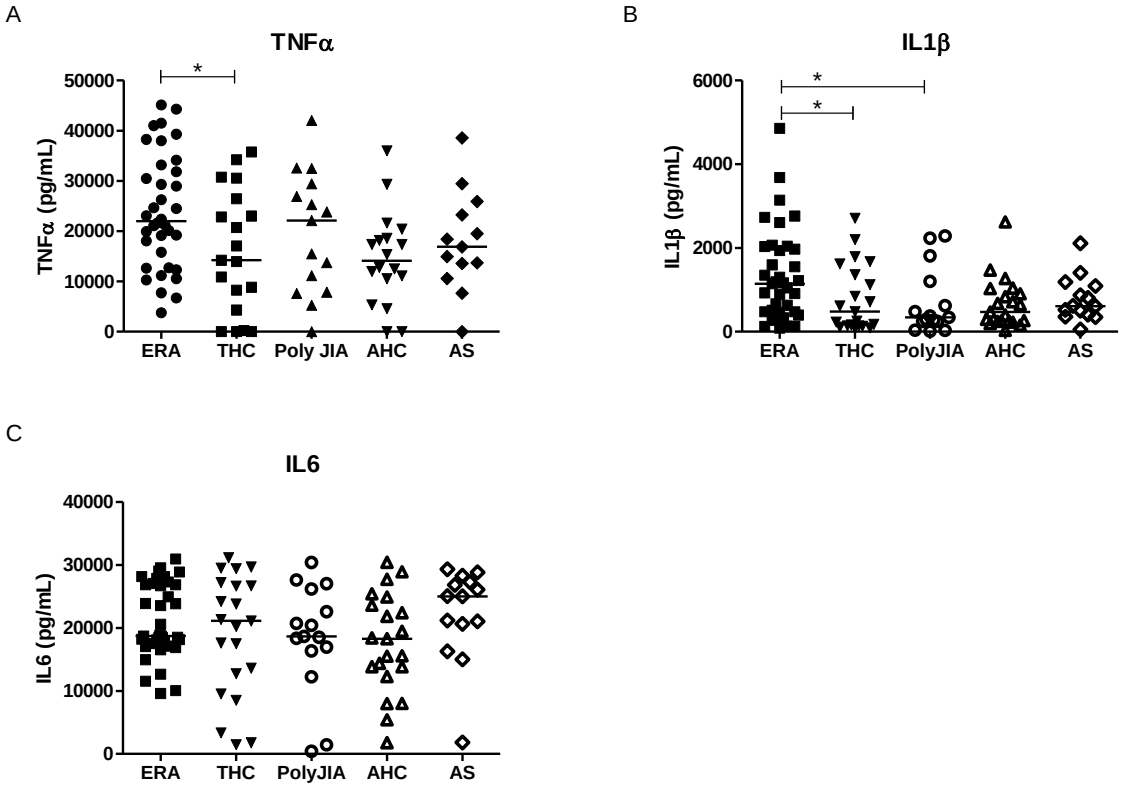


Figure 6.19 TNFα and IL1β production is higher from LPS + TM-stimulated MDMs from patients with ERA compared to healthy controls. MDMs were stimulated with LPS + TM for 4 or 24 hours. TNFα, IL1β and IL6 were measured in cell culture supernatants by ELISA or luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA n=37, THC=teenage healthy controls n=21, Poly JIA=polyarticular JIA, n=15, AS n=14, AHC=adult healthy controls n=21,*$p<0.05$).

6.3.5 Higher levels of TNFα, IL1β and IL6 are associated with active enthesitis from LPS + TM-stimulated MDMs in patients with ERA

Associations between clinical features and levels of TNFα, IL1β and IL6 production from LPS + TM-stimulated MDMs were investigated next. The results are summarised in table 6.6. In keeping with the results for gene expression, only IL6 levels from LPS + TM-stimulated MDMs were higher in HLA B27 positive patients with ERA compared to those who were HLA B27 negative (20574 pg/mL, IQR 18131-28001 pg/mL vs 17986 pg/mL, IQR 12395-23871 pg/mL, p=0.031, figure 6.20 C). Although TNFα and IL1β levels were not significantly different between HLA B27 positive and negative patients with ERA from LPS + TM-stimulated MDMs, TNFα and IL1β were both higher from MDMs from HLA B27 positive patients compared to healthy controls (p=0.037 and p=0.023 respectively, figure 6.20 A and B).

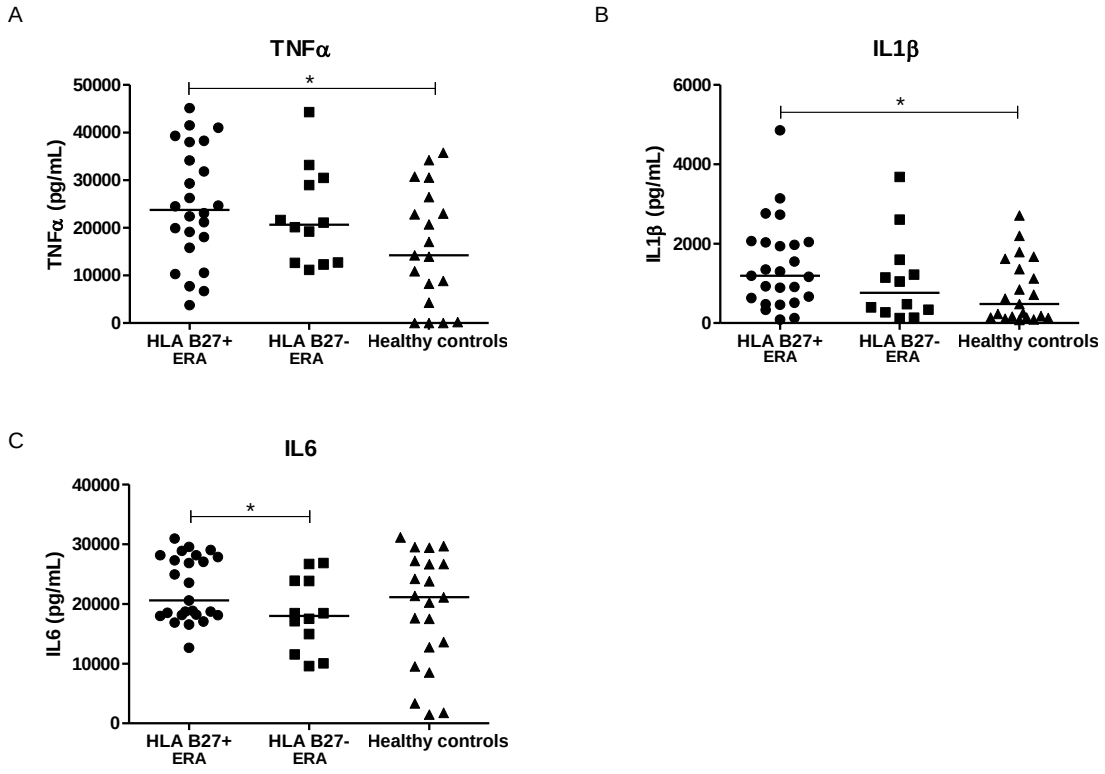


Figure 6.20 IL6 production from LPS + TM-stimulated MDMs is higher in HLA B27 positive patients with ERA. MDMs were differentiated as described in section 2.2 and stimulated with LPS + TM for 4 or 24 hours. TNFα, IL1β and IL6 were measured from cell culture supernatants by ELISA or luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (HLA B27 positive ERA=25, HLA B27 negative ERA =12, *p<0.05).

The presence of active enthesitis in patients with ERA was associated with significantly higher levels of TNFα, IL1β and IL6 compared to those without enthesitis at the time of sample from LPS + TM-stimulated MDMs (TNFα: median 29150 pg/mL, IQR 18863-38333 pg/mL vs 19690 pg/mL, IQR 12033-22570 pg/mL, p=0.036 figure 6.21 A; IL1β: median 1598 pg/mL, IQR 894.7-2610 pg/mL vs 471.6 pg/mL, IQR 284.3-1175 pg/mL, p=0.0023, figure 6.21 B; IL6: median 23546 pg/mL, IQR 18446-28156 pg/mL vs 17758 pg/mL, IQR 14379-23855 pg/mL, p=0.0098, figure 6.21 C). Levels of TNFα and IL1β from those with active enthesitis were also significantly higher than healthy controls (p=0.011 and p=0.0028 respectively).

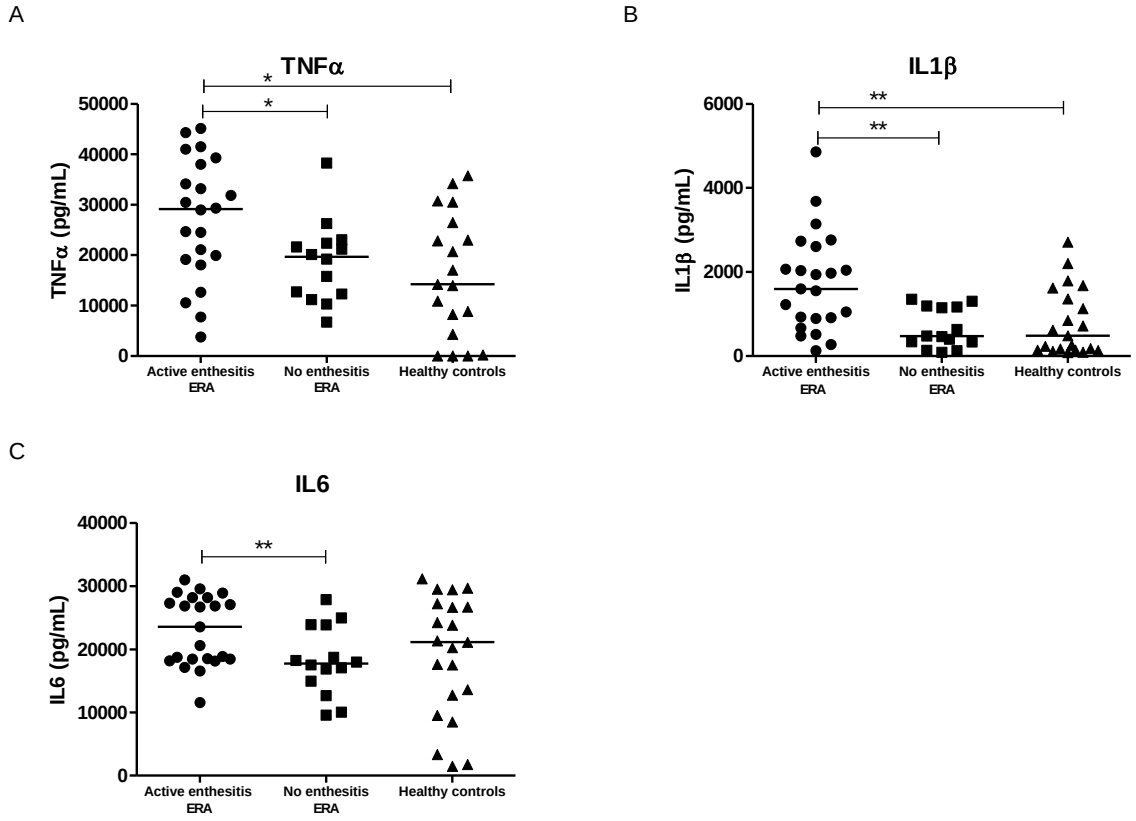


Figure 6.21 TNFα, IL1β and IL6 levels are significantly higher from LPS + TM-stimulated MDMs in patients with ERA with active enthesitis compared to those with no enthesitis. MDMs from patients with ERA and age-matched healthy controls were stimulated with LPS + TM for 4 or 24 hours and cytokines were detected in the cell culture supernatants by ELISA or luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA with active enthesitis n=23, ERA with no enthesitis n=14, healthy controls n=21, *p<0.05, **p<0.01).

There were no other significant differences in the production of TNFα, IL1β and IL6 from LPS + TM-stimulated MDMs between subgroups for clinical features such as male sex, ERA subtype and disease activity although some differences were seen between subgroups and healthy controls, reflecting overall differences observed between patients with ERA and healthy controls (table 6.6).

6.3.6 IL17 gene expression is enhanced by induction of the UPR in LPS-stimulated MDMs from adolescent healthy controls

As described in section 3.5.2, IL17 assays consistently produced negative results in cell culture supernatants from MDMs after 4 and 24 hours LPS stimulation. However, fold increase in gene expression was detected following LPS stimulation, especially after 24 hours. It was therefore decided to investigate the effect of UPR induction on the gene expression of IL17 in LPS-stimulated MDMs. In patients with ERA, there was no significant enhancement with UPR induction (median fold change 9.182, IQR 1.782-115.0, increased from 3.909, IQR 0.343-18.35) but in MDMs from adolescent healthy controls, gene expression was significantly enhanced by UPR induction (median fold change 16.66, IQR 4.986-104.4, increased from 1.642, IQR 0.144-10.00, p=0.014, figure 6.22). In addition, there were fewer patients and healthy controls where no expression was seen at all compared to LPS-stimulated MDMs (13.6% patients with ERA, 8.3% healthy controls compared to 36.3% of patients with ERA and 33.3% healthy controls). However, despite this, IL17 was not detected in cell culture supernatants from MDMs following LPS + TM stimulation after either 4 or 24 hours. In addition, there was no significant difference between IL17 gene expression from LPS + TM-stimulated MDMs from patients with ERA and healthy controls and no associations noted with the clinical features analysed (table 6.4).

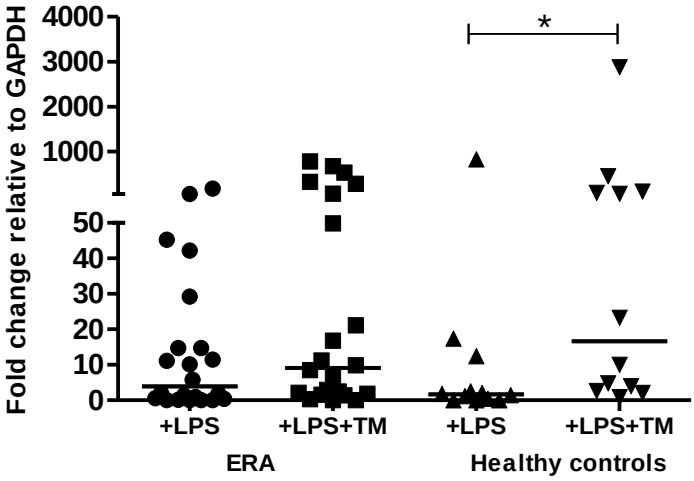


Figure 6.22 Gene expression of IL17 is enhanced by induction of the UPR in LPS-stimulated MDMs from adolescent healthy controls. IL17 expression was analysed from RNA from MDMs stimulated with LPS alone or LPS + TM using qPCR and normalised against GAPDH. Fold change was calculated relative to the control sample for each patient and healthy control. Box and whisker plots show median with range (minimum to maximum), statistical analysis by Mann-Whitney U test (ERA n=22, adolescent healthy controls n=12).

6.3.7 GMCSF production from LPS-stimulated MDMs is significantly enhanced by UPR induction and is higher in patients with ERA compared to healthy controls and patients with polyarticular JIA

Next, the production of GMCSF from LPS + TM-stimulated MDMs was analysed in cell culture supernatants by Luminex assay. Interestingly, GMCSF was the only cytokine analysed to be significantly increased by the induction of the UPR in patients with ERA (median 9473 pg/mL, IQR 4746-13961 pg/mL, increased from 1853 pg/mL, IQR 1125-3061 pg/mL, p<0.0001) and adolescent healthy controls (median 3834 pg/mL, IQR 1603-9158 pg/mL, increased from 1175 pg/mL, IQR 607.0-1779 pg/mL, p=0.0015, figure 6.23 A). As with GMCSF production from LPS-stimulated MDMs, levels were significantly higher from LPS + TM-stimulated MDMs in patients with ERA compared to healthy controls (p=0.0084, figure 6.23 B) and interestingly, patients with polyarticular JIA (p=0.039). However, there was no significant difference between any of the other patient and healthy control groups (figure 6.23 B), although the production of GMCSF was significantly enhanced by UPR induction compared to stimulation with LPS alone across all the groups analysed (table 6.5).

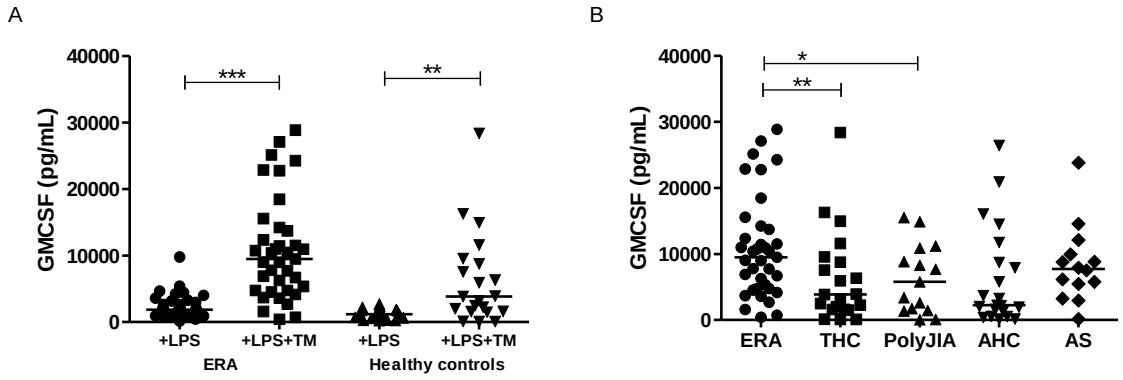


Figure 6.23 GMCSF production from LPS-stimulated MDMs is enhanced by induction of the UPR and is higher in patients with ERA compared to healthy controls and patients with polyarticular JIA. MDMs were cultured as per the protocol in section 2.2 and stimulated with LPS alone or LPS + TM for 24 hours. Cell culture supernatants were analysed by Luminex assay. Box and whisker plots show median (+minimum to maximum range), statistical analysis by Mann-Whitney test or Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA n=37, THC=teenage healthy controls n=21, polyarticular JIA n=15, AHC=adult healthy controls n=21, AS n=14, *p<0.05, **p<0.01, ***p<0.001).

6.3.8 Higher GMCSF production from LPS + TM-stimulated MDMs is associated with active enthesitis in patients with ERA

Analyses were then undertaken to determine whether any clinical features in patients with ERA were associated with higher levels of GMCSF production from LPS + TM-stimulated MDMs. Active enthesitis was found to be associated with significantly higher levels of GMCSF in patients with ERA compared to levels from MDMs from patients with no enthesitis at the time of sample (median 10948 pg/mL, IQR 6670-15561 pg/mL vs 5818 pg/mL, IQR 2423-10321 pg/mL, p=0.020, figure 6.24 A) and compared to healthy controls (p=0.0012). There was a trend towards higher levels of GMCSF from LPS + TM-stimulated MDMs from HLA B27 positive patients with ERA compared to patients who were HLA B27 negative (median 10451 pg/mL, IQR 5501-20599 pg/mL vs 7335 pg/mL, IQR 2941-10895 pg/mL, p=0.072, figure 6.24 B) and levels from HLA B27 positive patients were significantly higher than healthy controls (p=0.0025). There was no significant difference in GMCSF production from LPS + TM-stimulated MDMs between other ERA subgroups, such as ERA subtype, disease activity by JADAS and treatment with and without TNF inhibitors although in some subgroups levels were higher compared to healthy controls reflecting higher level of GMCSF overall from patients with ERA (see table 6.6 for a summary of results).

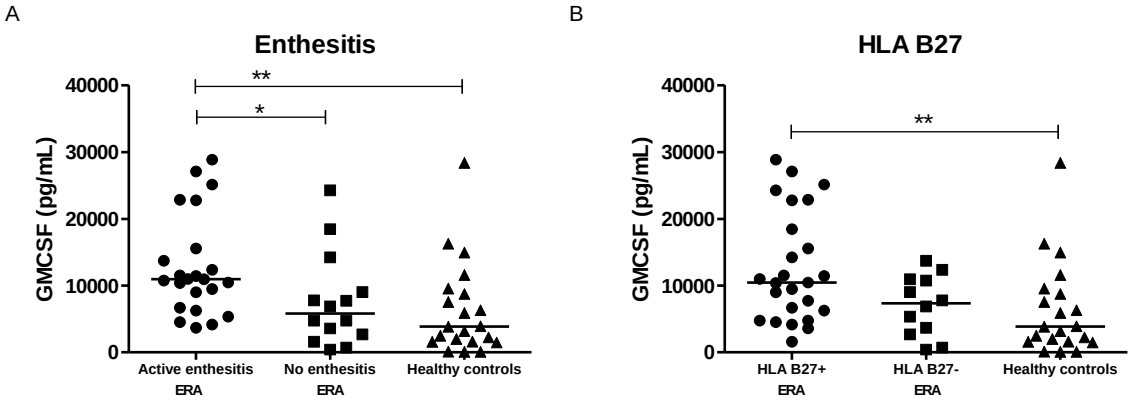


Figure 6.24 GMCSF production from LPS + TM-stimulated MDMs is higher in patients with ERA with active enthesitis. MDMs were differentiated as previously described (section 2.2) and stimulated with LPS + TM for 24 hours. GMCSF was measured in cell culture supernatants by Luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (HLA B27+ ERA n=25, HLA B27- ERA n=12, ERA with active enthesitis n=23, ERA without enthesitis n=14, adolescent healthy controls n=21, *p<0.05, **p<0.01).

6.3.9 IFNγ production from LPS + TM-stimulated MDMs is lower in patients with ERA compared to healthy controls and patients with polyarticular JIA

Finally, the production of IFNγ from LPS + TM-stimulated MDMs was analysed in cell culture supernatants by Luminex assay. In keeping with most other cytokines measured, levels were significantly lower compared to levels from MDMs stimulated with LPS alone in patients with ERA (6881 pg/mL, IQR 5533-9087 pg/mL, from 8836 pg/mL, IQR 7240-11255 pg/mL, p=0.0024, figure 6.25 A). However, no significant difference was noted between levels from LPS-stimulated and LPS + TM-stimulated MDMs in adolescent healthy controls (9337 pg/mL, IQR 7400-12770 pg/mL, from 11693 pg/mL, IQR 9481-13435 pg/mL). Levels were also lower from LPS + TM-stimulated MDMs compared to MDMs stimulated with LPS alone in patients with polyarticular JIA (p=0.034) but not significantly different in patients with AS and adult healthy controls (table 6.5). Interestingly, as with LPS-stimulated MDMs, IFNγ production from LPS + TM-stimulated MDMs was significantly lower from patients with ERA compared to healthy controls (p=0.0048) and patients with polyarticular JIA (p=0.0022, figure 6.25 B).

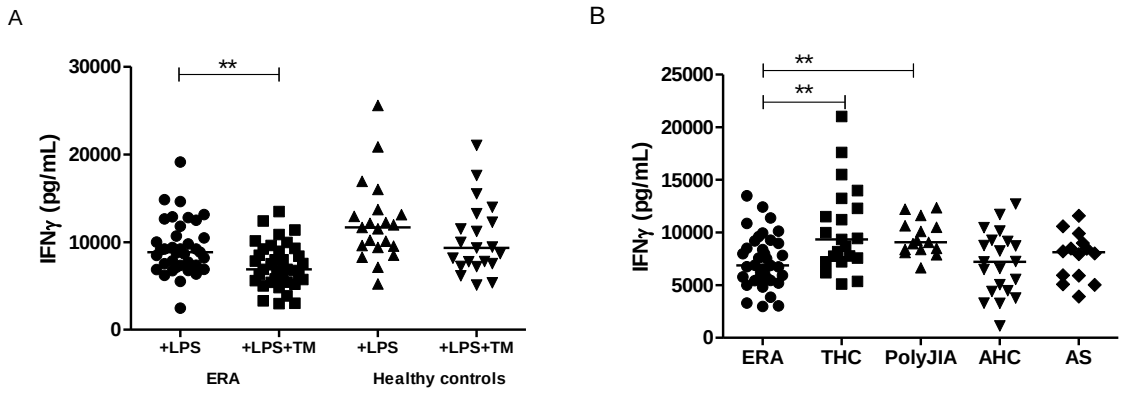


Figure 6.25 IFNγ production from LPS + TM-stimulated MDMs is lower in patients with ERA compared to healthy controls and patients with polyarticular JIA. MDMs were cultured as per the protocol in section 2.2 and stimulated for 24 hours with LPS alone or LPS + TM after which cell culture supernatants were collected and analysed by Luminex assay. Box and whisker plots show median (+minimum to maximum range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (ERA n=37, THC=teenage healthy controls n=21, polyarticular JIA n=15, AHC=adult healthy controls n=21, AS n=14, **p<0.01).

Further analysis was undertaken to determine whether clinical features such as HLA B27 or enthesitis were associated with lower levels of IFNγ from LPS + TM-stimulated MDMs. No significant associations were noted between any clinical features and levels of IFNγ (table 6.6). However, significant differences were observed between both HLA B27 positive patients and HLA B27 negative patients with ERA and healthy controls (both p=0.016, figure 6.26 A) but there was no difference between the patient groups. This was also the case for patients with ERA with and without active enthesitis and healthy controls (p=0.012 and 0.023, figure 6.26 B) and for other clinical features such as ERA subtype where no difference was noted between patients groups but levels of IFNγ were significantly lower than healthy controls (figure 6.26 C). It was concluded that these differences represented lower levels of IFNγ in patients with ERA overall.

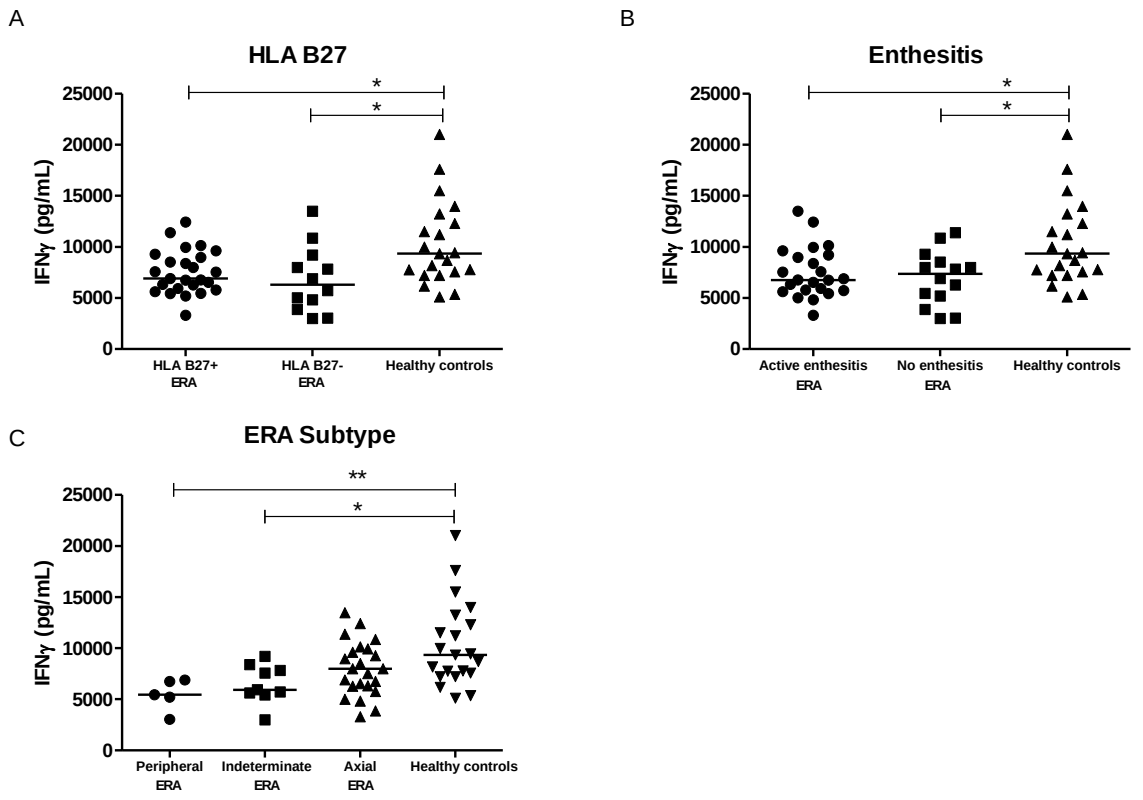


Figure 6.26 Lower IFNγ production is not associated with HLA B27, active enthesits or disease subtype in patients with ERA. MDMs were differentiated as described in the protocol above (section 2.2) and stimulated with LPS + TM for 24 hours. IFNγ was measured in cell culture supernatants by Luminex assay. Box and whisker plots show median and minimum to maximum range, statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups (HLA B27+ ERA n=25, HLA B27- ERA n=12, ERA with active enthesitis n=23, ERA without enthesitis n=14, peripheral ERA n=5, indeterminate ERA n= 9, axial ERA n=23, adolescent healthy controls n=21,*p<0.05, **p<0.01).

6.4 Correlations between cytokines from LPS + TM-stimulated MDMs

As with LPS-stimulated MDMs, significant correlations were noted between levels of pro-inflammatory cytokines from LPS + TM-stimulated MDMs. In particular, strong correlations were observed between levels of IL23, IL27 and GMCSF across all the patient and healthy control groups (figure 6.27). Fewer correlations were observed for IFNγ compared to other cytokines across all groups and, in keeping with the results for LPS-stimulated MDMs, fewer correlations were noted from MDMs from patients with AS compared to the other groups. This was perhaps due to the smaller sample size in this group.

ERA

	IL23	IL27	TNFα	IL1β	IL6	GMCSF	IFNγ
IL23		r=0.58	r=0.38	r=0.49	r=0.46	r=0.60	r=0.21
IL27	r=0.58		r=0.62	r=0.81	r=0.64	r=0.83	r=0.36
TNFα	r=0.38	r=0.62		r=0.64	r=0.51	r=0.52	r=0.43
IL1β	r=0.49	r=0.81	r=0.64		r=0.63	r=0.71	r=0.44
IL6	r=0.46	r=0.64	r=0.51	r=0.63		r=0.69	r=0.33
GMCSF	r=0.60	r=0.83	r=0.52	r=0.71	r=0.69		r=0.32
IFNγ	r=0.21	r=0.36	r=0.43	r=0.44	r=0.33	r=0.32	

Adolescent healthy controls

	IL23	IL27	TNFα	IL1β	IL6	GMCSF	IFNγ
IL23		r=0.65	r=0.43	r=0.39	r=0.66	r=0.76	r=0.12
IL27	r=0.65		r=0.23	r=0.40	r=0.86	r=0.93	r=0.16
TNFα	r=0.43	r=0.23		r=0.30	r=0.47	r=0.38	r=0.30
IL1β	r=0.39	r=0.80	r=0.30		r=0.82	r=0.70	r=0.22
IL6	r=0.66	r=0.86	r=0.47	r=0.82		r=0.85	r=0.21
GMCSF	r=0.76	r=0.93	r=0.38	r=0.70	r=0.85		r=0.19
IFNγ	r=0.12	r=0.16	r=0.30	r=0.22	r=0.21	r=0.19	

Polyarticular JIA

	IL23	IL27	TNFα	IL1β	IL6	GMCSF	IFNγ
IL23		r=0.76	r=0.39	r=0.73	r=0.52	r=0.80	r=-0.12
IL27	r=0.76		r=0.55	r=0.82	r=0.67	r=0.94	r=0.10
TNFα	r=0.39	r=0.55		r=0.55	r=0.49	r=0.52	r=-0.22
IL1β	r=0.73	r=0.82	r=0.55		r=0.79	r=0.78	r=-0.01
IL6	r=0.52	r=0.67	r=0.49	r=0.79		r=0.65	r=0.23
GMCSF	r=0.80	r=0.94	r=0.52	r=0.78	r=0.65		r=0.14
IFNγ	r=-0.12	r=0.10	r=-0.22	r=-0.01	r=0.23	r=0.14	

Adult healthy controls

	IL23	IL27	TNFα	IL1β	IL6	GMCSF	IFNγ
IL23		r=0.81	r=0.51	r=0.69	r=0.68	r=0.84	r=0.22
IL27	r=0.81		r=0.46	r=0.79	r=0.85	r=0.98	r=0.32
TNFα	r=0.51	r=0.46		r=0.64	r=0.36	r=0.44	r=-0.10
IL1β	r=0.69	r=0.79	r=0.64		r=0.67	r=0.73	r=0.30
IL6	r=0.68	r=0.85	r=0.36	r=0.67		r=0.84	r=0.54
GMCSF	r=0.84	r=0.98	r=0.44	r=0.73	r=0.84		r=0.32
IFNγ	r=0.22	r=0.32	r=-0.10	r=0.30	r=0.54	r=0.32	

AS

	IL23	IL27	TNFα	IL1β	IL6	GMCSF	IFNγ
IL23		r=0.52	r=0.35	r=0.34	r=0.53	r=0.75	r=0.08
IL27	r=0.52		r=0.15	r=0.49	r=0.67	r=0.71	r=0.42
TNFα	r=0.35	r=0.15		r=0.46	r=0.31	r=0.15	r=-0.12
IL1β	r=0.34	r=0.49	r=0.46		r=0.59	r=0.38	r=0.21
IL6	r=0.53	r=0.67	r=0.31	r=0.59		r=0.53	r=0.50
GMCSF	r=0.75	r=0.71	r=0.15	r=0.38	r=0.53		r=0.43
IFNγ	r=0.08	r=0.42	r=-0.12	r=0.21	r=0.50	r=0.43	

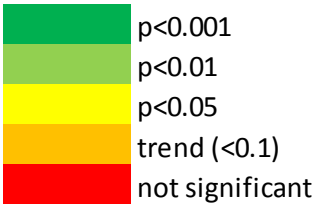


Figure 6.27 Correlations between levels of pro-inflammatory cytokines from LPS + TM-stimulated MDMs. Cytokines were measured in cell culture supernatants by ELISA or Luminex assay and correlations assessed using Spearman's rank correlation. The results are shown as heat maps.

6.5 Results summary for chapter 6

In view of the large number of results discussed in this chapter, tables were constructed to summarise the results.

Cytokines gene expression	Groups	LPS-stimulated MDMs	LPS + TM-stimulated MDMs	Difference between LPS-stimulated and LPS + TM-stimulated MDMs
IL23p19	ERA	445.0 (137.1-1515)	510.9 (264.2-2475)	Not significant
	Healthy controls	91.0 (58.91-560.6)	407.9 (111.0-2112)	Not significant
IL12/23p40	ERA	4360 (1072-40125)	18179 (3317-64317)	Not significant
	Healthy controls	2311 (46.77-14471)	19910 (281.8-106136)	Not significant
IL12p35	ERA	141.0 (69.07-288.0)	92.41 (36.25-560.3)	Not significant
	Healthy controls	152.3 (23.43-348.6)	100.2 (36.32-789.2)	Not significant
TNFα	ERA	5.152 (1.633-8.856)	4.861 (1.681-16.44)	Not significant
	Healthy controls	2.142 (0.933-13.93)	9.714 (4.141-14.52)	Trend to increase (p=0.077)
IL1β	ERA	111.4 (52.2-641.1)	68.59 (27.58-271.4)	Not significant
	Healthy controls	75.06 (2.362-292.0)	106.2 (13.09-218.3)	Not significant
IL6	ERA	1463 (490.2-2327)	904.5 (402.4-5276)	Not significant
	Healthy controls	1314 (308.7-2353)	2402 (1193-6608)	Not significant
IL17	ERA	3.909 (0.343-18.35)	9.182 (1.782-115.0)	Not significant
	Healthy controls	1.642 (0.144-10.00)	16.66 (4.986-104.4)	Increase (p=0.014)

Table 6.3 Summary of IL23p19, IL12/23p40, IL12p35, TNFα, IL1β, IL6 and IL17 gene expression comparing LPS-stimulated and LPS + TM-stimulated MDMs in patients and healthy controls. Gene expression was analysed from RNA from MDMs stimulated with LPS alone or LPS + TM for 24 hours using qPCR and normalised against GAPDH. Fold change was calculated relative to the control sample for each patient and healthy control. Table shows median fold change and IQR in brackets, statistical analysis by Mann-Whitney U test. Key:

Trend towards difference between groups (p<0.1)
Significant difference between groups (p<0.05)

		IL23p19	IL12/23p40	IL12p35	TNFα	IL1β	IL6	IL17
HLA B27	Positive (n=21)	680.3 (380.7-3792)	16840 (3830-97871)	166.5 (49.35-677.4)	6.190 (2.154-19.00)	63.12 (33.56-367.9)	1209 (543.1-10866)	8.335 (1.640-226.4)
	Negative (n=9)	315.2 (30.9-964.9)	25180 (922.9-36358)	13.14 (10.13-2005)	2.549 (1.223-8.407)	68.59 (15.30-178.3)	415.9 (131.5-1717)	9.875 (1.868-146.0)
Sex	Male (n=25)	484.4 (292.0-2005)	25180 (3759-83475)	114.3 (33.34-432.1)	4.659 (1.516-13.70)	68.59 (27.58-271.4)	770.7 (389.0-7583)	3.909 (0.4587-14.72)
	Female (n=5)	729.1 (110.4-5523)	9551 (8810-70764)	49.52 (25.93-788.6)	6.821 (3.271-23.86)	61.82 (23.53-790.7)	1038 (342.8-4812)	8.624 (0.0304-17.24)
Active Enthesitis	Yes (n=20)	510.9 (325.0-2220)	15500 (3019-88292)	92.41 (36.25-560.3)	4.408 (1.585-19.19)	73.78 (27.24-218.4)	904.5 (486.2-5532)	9.849 (1.569-282.1)
	No (n=10)	525.5 (39.04-5983)	21680 (3592-194400)	131.5 (22.54-23802)	5.885 (1.898-9.626)	65.21 (30.99-562.4)	922.0 (102.6-6581)	8.515 (1.853-49.87)
ERA subtype	Peripheral (n=5)	729.1 (300.8-1018)	25180 (12534-36740)	196.7 (57.21-320.2)	6.821 (5.321-15.74)	61.82 (20.71-168.8)	634.7 (317.2-2759)	12.42 (2.532-37.62)
	Indeterminate (n=8)	502.7 (124.0-3031)	9701 (775.4-166203)	80.41 (10.42-811.9)	4.318 (2.604-18.27)	33.56 (12.54-99.08)	830.0 (289.0-10306)	2.042 (1.602-20.20)
	Axial (n=17)	484.4 (259.7-3330)	21383 (2865-134467)	79.33 (37.80-1118)	4.408 (1.229-14.57)	106.9 (39.40-691.2)	1209 (389.0-8239)	0.9013 (0.0756-28.47)
Disease activity (JADAS)	Low (n=12)	408.0 (280.3-3660)	4482 (1676-142327)	274.4 (40.44-1671)	4.535 (1.467-10.27)	76.52 (28.26-255.0)	990.0 (222.8-3042)	6.399 (2.071-293.1)
	Moderate (n=12)	502.7 (192.9-3031)	22931 (3527-171273)	100.6 (15.69-811.9)	10.53 (3.135-19.95)	72.42 (31.71-277.7)	2218 (555.8-10684)	18.95 (1.938-56.94)
	High (n=4)	936.6 (692.0-2230)	21680 (12880-36552)	70.97 (49.01-212.5)	3.537 (1.134-6.511)	45.03 (9.584-1124)	588.0 (260.2-7828)	1.569 (1.424-8.515)
TNF inhibitor	Yes (n=12)	502.7 (351.2-2220)	11194 (3157-58921)	166.5 (54.82-985.6)	6.553 (2.428-14.85)	80.06 (14.80-286.9)	990.0 (525.7-9159)	13.97 (2.127-481.9)
	No (n=18)	582.3 (170.7-2823)	25180 (3019-88292)	49.52 (24.49-562.2)	4.533 (1.482-18.07)	65.21 (27.91-167.0)	836.5 (341.6-5267)	9.182 (1.533-52.23)

Table 6.4 Summary of results for gene expression of IL23p19, IL12/23p40, IL12p35, TNFα, IL1β and IL6 from LPS + TM-stimulated MDMs and clinical features in patients with ERA. Gene expression was analysed from RNA from MDMs stimulated with LPS + TM for 24 hours using qPCR and normalised against GAPDH. Fold change was calculated relative to the control sample for each patient and healthy control. Table shows median fold change and IQR in brackets, statistical analysis by Mann-Whitney U test or Kruskal-Wallis test as appropriate. Key:

Trend towards difference between groups ($p<0.1$)

Significant difference between groups ($p<0.05$)

Cytokine protein		LPS-stimulated MDMs (pg/mL)	LPS + TM-stimulated MDMs (pg/mL)	Difference between LPS-stimulated and LPS and TM-stimulated MDMs
IL23	ERA	53580 (35735-83945) *	12620 (6197-20735) *	Decrease (p<0.0001)
	Adolescent healthy controls	32110 (13745-48235)	6122 (252.3-16170)	Decrease (p=0.0006)
	Polyarticular JIA	51340 (36390-77830) *	10450 (4065-18720)	Decrease (p<0.0001)
	Adult healthy controls	34180 (16628-49403)	6319 (1280-13805)	Decrease (p=0.0004)
	AS	53835 (35305-80648) *	10090 (5389-15005)	Decrease (p=0.0001)
IL12	ERA	3753 (2055-7688) *	100.2 (45.75-270.7)	Decrease (p<0.0001)
	Adolescent healthy controls	2733 (1327-4309)	303.5 (127.4-440.1)	Decrease (p<0.0001)
	Polyarticular JIA	4533 (1683-6128)	112.5 (36.06-415.3)	Decrease (p=0.0006)
	Adult healthy controls	2300 (608.3-3579)	132.7 (5.780-319.8)	Decrease (p<0.0001)
	AS	4134 (2716-5794) *	322.9 (65.22-439.4)	Decrease (p=0.0001)
IL27	ERA	1986 (1505-2268) *	1523 (984.7-1807) *	Decrease (p=0.0092)
	Adolescent healthy controls	1603 (1121-1918)	952.5 (560.8-1341)	Decrease (p=0.015)
	Polyarticular JIA	1747 (1356-2366)	1182 (770.0-1614)	Decrease (p=0.034)
	Adult healthy controls	1379 (1039-1889)	823.4 (427.4-1332)	Decrease (p=0.024)
	AS	1854 (1333-1992)	1318 (858.3-1721)	Decrease (p=0.029)
TNFα	ERA	52445 (28483-67190) *	22025 (13498-32860) *	Decrease (p<0.0001)
	Adolescent healthy controls	29090 (14845-47785)	14230 (4301-26480)	Decrease (p=0.012)
	Polyarticular JIA	41220 (18680-54590)	22160 (7887-29420)	Decrease (p=0.024)
	Adult healthy controls	25110 (17970-39425)	14095 (9273-20730)	Decrease (p=0.024)
	AS	36510 (24745-48570)	16900 (12110-24595)	Decrease (p=0.0066)
IL1β	ERA	802.2 (339.6-1318)	1147 (471.3-2003) *	Not significant
	Adolescent healthy controls	458.6 (190.9-768.6)	484.5 (141.8-1494)	Not significant
	Polyarticular JIA	496.7 (209.7-1150)	343.0 (247.5-1201)	Not significant
	Adult healthy controls	347.5 (147.4-475.3)	471.1 (251.8-970.8)	Not significant
	AS	689.4 (424.3-1057) *	614.3 (396.3-1117)	Not significant
IL6	ERA	19892 (18289-27100)	18732 (17317-26955)	Not significant
	Adolescent healthy controls	24597 (17147-26290)	21132 (11140-26938)	Not significant
	Polyarticular JIA	23214 (18207-28004)	18657 (16364-26177)	Not significant
	Adult healthy controls	20913 (14045-23588)	18274 (13060-24258)	Not significant
	AS	26342 (21294-28006) *	25033 (19565-27533)	Not significant
GMCSF	ERA	1853 (1125-3061) *	9473 (4746-13961) **	Increase (p<0.0001)
	Adolescent healthy controls	1175 (607.0-1779)	3834 (1603-9158)	Increase (p=0.0015)
	Polyarticular JIA	1536 (727.1-2267)	5776 (1449-10898)	Increase (p=0.020)
	Adult healthy controls	1044 (433.9-1510)	2242 (925.2-10244)	Increase (p=0.0035)
	AS	1578 (992.7-2703) *	7718 (4896-10494)	Increase (p=0.0002)
IFNγ	ERA	8836 (7240-11255) **	6881 (5533-9087) **	Decrease (p=0.0024)
	Adolescent healthy controls	11693 (9481-13435)	9337 (7400-12770)	Not significant
	Polyarticular JIA	11584 (9149-14984)	9073 (8404-10655)	Decrease (p=0.034)
	Adult healthy	9485 (6391-11770)	7222 (4445-9235)	Not significant (p=0.07)

	controls			
	AS	9253 (7897-10684)	8126 (5709-9233)	Not significant

Table 6.5 Summary table of the effect of UPR induction on cytokine production from LPS-stimulated MDMs. Cytokine levels were measured in cell culture supernatants following incubation of MDMs with LPS alone or LPS + TM for 4 or 24 hours using ELISA or Luminex assay. Table shows median cytokine level for each group with IQR in brackets, statistical analysis by Mann-Whitney U test or Kruskal-Wallis test as appropriate. Key:

Significant increase between LPS and LPS and TM stimulated MDMs (p<0.001)	
Significant increase between LPS and LPS and TM stimulated MDMs (p<0.01)	* Difference between ERA group and healthy controls (p<0.05)
Significant increase between LPS and LPS and TM stimulated MDMs (p<0.05)	** Difference between ERA group and healthy controls (p<0.01)
Significant decrease between LPS and LPS and TM stimulated MDMs (p<0.05)	
Significant decrease between LPS and LPS and TM stimulated MDMs (p<0.01)	
Significant decrease between LPS and LPS and TM stimulated MDMs (p<0.001)	

		IL23 (pg/mL)	IL27 (pg/mL)	TNFα (pg/mL)	IL1β (pg/mL)	IL6 (pg/mL)	GMCSF (pg/mL)	IFNγ (pg/mL)
HLA B27	Positive	15820 (12025-28605) **	1630 (1173-2193) **	23775 (16365-37045) *	1194 (572.7-2038) *	20574 (18131-28001)	10451 (5501-20599) **	6897 (5853-9125) *
	Negative	5725 (2652-11470)	1206 (776.3-1695)	20635 (12655-30095)	763.4 (286.9-1503)	17986 (12395-23871)	7335 (2941-10895)	6305 (4104-8902) *
Sex	Male	12620 (6058-24350)	1559 (1039-1881)	23775 (16728-33430) *	1169 (479.8-2032)	18732 (17109-24043)	10738 (5341-15561) *	6881 (5728-9203)
	Female	12975 (7387-16585)	1134 (606.8-1809)	17865 (9872-25733)	495.3 (330.0-1494)	19364 (17627-24293)	6266 (4433-10412)	6625 (4726-9260)
Active enthesitis	Yes	15820 (10060-35480) **	1666 (1325-2150 **	29150 (18863-38333) *	1598 (894.7-2610) **	23546 (18446-28156)	10948 (6670-15561) **	6757 (5728-9203) *
	No	10197 (4715-13990)	1063 (711.3-1547)	19690 (12033-22570)	471.6 (284.3-1175)	17758 (14379-23855)	5818 (2423-10321)	7364 (4865-8707) *
ERA subtype	Peripheral	14440 (9538-68690)	1711 (975.8-2157)	18500 (13183-28153)	1303 (765.7-3734)	23847 (18423-27261)	12360 (5818-20645) *	5447 (4120-6812) **
	Indeterminate	18560 (6407-63085) *	1666 (1150-1962) *	28970 (20900-41805) *	1555 (454.3-2381)	18504 (18300-25373)	10738 (8628-17136) *	5923 (5519-8111) *
	Axial	12290 (6058-15940)	1469 (823.4-1688)	21110 (11200-33190)	911.7 (463.4-1935)	18720 (16898-27278)	7715 (4142-13717)	7996 (6288-9950)
Disease activity (JADAS)	Low	11310 (6058-14190)	1325 (823.4-1688)	19220 (10580-34150)	894.7 (479.3-1598)	18448 (16898-27278)	6670 (4530-10980)	7535 (6288-9950)
	Moderate	17190 (5013-61818) *	1689 (1108-2253) *	24500 (20045-35600) *	1388 (451.0-2834) *	18726 (17749-27111)	11235 (8140-22773) **	5826 (5270-8094) **
	High	13239 (10609-47568)	1352 (971.3-1903)	22700 (14928-31568)	1107 (431.9-1705)	20978 (17753-26444)	7934 (4462-12119)	8019 (4659-10051)
TNF inhibitor	Yes	15940 (8104-35480)	1559 (1087-2043)	24595 (16995-32440)	1220 (397.3-2043)	18856 (17526-26866)	9473 (6253-22844)	6881 (5923-7999) *
	No	12170 (5205-15473)	1511 (897.3-1754)	20635 (12705-34395)	987.3 (475.3-1682)	18726 (17092-27244)	9690 (4314-13839)	7146 (5150-9361) *

Table 6.6 Summary of results for cytokine production from LPS + TM-stimulated MDMs and clinical features in patients with ERA. Cytokine levels were measured in cell culture supernatants following incubation of MDMs with LPS + TM for 4 or 24 hours using ELISA or Luminex assay. Table shows median cytokine level for each group with IQR in brackets, statistical analysis by Mann-Whitney U test or Kruskal-Wallis test as appropriate. Key:

Trend towards difference between groups (p<0.1)	*	Difference between group and healthy controls (p<0.05)
Significant difference between groups (p<0.05)	**	Difference between group and healthy controls (p<0.01)
Significant difference between groups (p<0.01)		

6.6 Discussion

The results in this chapter indicate that, in this assay, markers of the UPR are not significantly enhanced in MDMs and SFMCs from patients with ERA compared to controls and in general, induction of the UPR results in a reduction or no significant change in cytokine levels, rather than an increase, with the significant exception of GMCSF. However, as with MDMs stimulated with LPS alone, higher levels of IL23, IL27, TNFα and GMCSF are produced by LPS + TM-stimulated MDMs from patients with ERA compared to adolescent healthy controls and the production of IFNγ remains significantly lower. The associations with HLA B27 and active enthesitis are also maintained.

The chapter begins by analysing three markers of the UPR, spliced XBP1, CHOP and BiP in MDMs without stimulation with LPS + TM. The evidence for the involvement of the UPR in the pathogenesis of SpA stemmed from the observation that HLA B27 misfolds or folds slowly in the ER compared to other HLA A and B alleles (Mear, Schreiber et al. 1999). These findings were confirmed by the observation that BiP, an ER chaperone protein, was found to be associated with HLA B27 in a steady state (Colbert, DeLay et al. 2010). BiP usually binds transiently with newly synthesised MHC class I molecules and only forms a stable association with proteins that misfold (Nossner and Parham 1995). Thus the prolonged interaction of HLA B27 with BiP implied heavy chain misfolding resulting in the accumulation of proteins within the ER and therefore induction of the UPR (Dufey, Sepulveda et al. 2014).

There is a clear link between the UPR and pro-inflammatory cytokine production, for example IL6 and TNFα, (Li, Schwabe et al. 2005) and components of the UPR are required for the full expression of these cytokines (Martinon and Glimcher 2011). In the context of SpA, cells from HLA B27 transgenic rats show evidence of UPR activation (Turner, Sowders et al. 2005) and this results in increased IL23 production and subsequent enrichment of Th17 cells (DeLay, Turner et al. 2009). However, the development of disease does not occur in germ-free conditions (Taurog, Richardson et al. 1994) and also correlates with the level of HLA B27 expression (Taurog, Maika et al. 1993, Turner, Delay et al. 2007). In addition, even when the UPR is switched off in these rats, inflammatory disease still occurs and maybe more severe (Tran, Dorris et al. 2006) indicating that other factors are likely to be important in disease pathogenesis.

It is not surprising therefore that the evidence for HLA B27 induction of the UPR is inconsistent in humans. Goodall et al demonstrated the importance of the UPR transcription factor CHOP in the production of IL23 in monocyte-derived dendritic cells from healthy volunteers (Goodall, Wu et al. 2010). However, Zeng et al (Zeng, Lindstrom et al. 2011) did not find evidence of enhanced UPR induction (including XBP1 and CHOP) in MDMs from patients with AS despite noting increased IL23 production in patients. Conversely, two studies demonstrated enhanced expression of UPR markers in SFMCs from patients with AS compared to controls (Dong, Zhang et al. 2008, Feng, Ding et al. 2012) but a third more

recent study which compared SFMCs from patients with AS, rheumatoid arthritis and healthy controls did not (Neerinckx, Carter et al. 2014).

The findings from this last study are consistent with the results in this chapter for SFMCs in HLA B27 positive patients with ERA which did not demonstrate enhanced expression of markers of the UPR compared to HLA B27 negative patients with oligoarticular JIA. This was also the case in MDMs with and without stimulation with LPS + TM, where no significant differences were noted in spliced XBP1 and CHOP expression between patients with ERA and adolescent healthy controls. However, it was interesting to observe the expression of BiP was different with a trend towards increased expression from MDMs without LPS + TM stimulation in patients with ERA compared to healthy controls and significantly enhanced expression in HLA B27 positive patients with ERA compared to healthy controls. BiP, as a key regulator of the UPR through its interactions with all three UPR pathways (Bertolotti, Zhang et al. 2000), associates with HLA B27 in a steady state (Colbert, DeLay et al. 2010) and a previous study noted that BiP was highly expressed in the adherent fraction of SFMCs from 5 patients with SpA (Gu, Rihl et al. 2002). Thus, the finding of enhanced expression of BiP in MDMs from HLA B27 positive patients is in keeping with these studies. However, the findings were reversed in LPS + TM-stimulated MDMs with higher fold change in BiP expression in adolescent healthy controls compared to patients with ERA and no association with HLA B27. In fact, lower fold change in expression or a trend towards lower fold change in BiP expression compared to healthy controls was noted across many of the ERA subgroups which was likely to represent lower fold change overall in patients with ERA rather than associations with specific clinical features. The explanation for this is perhaps that in patients with ERA, BiP is already upregulated without UPR induction and therefore further stimulation of MDMs with TM does not result in as significant an increase in BiP expression as it does in healthy controls. It would be of interest to investigate this further and also expand the numbers of patients and healthy controls tested at different time points.

The next experiments were carried out to analyse the effect of inducing the UPR on the IL23 cytokine family. It was expected that an increase in IL23 levels would be seen in view of data from previous studies demonstrating increased IL23p19 gene expression and enhanced IL23 production with induction of the UPR (Goodall, Wu et al. 2010). However, in this assay, levels of IL23p19 were not significantly increased and production of IL23 was significantly lower from LPS + TM-stimulated MDMs compared to MDMs stimulated with LPS alone. In the study by Goodall et al, RNA was analysed after 7 hours of stimulation with LPS + TM in myeloid dendritic cells and cytokine production after 48 hours stimulation which may explain some of the differences observed.

Although no difference was found overall between patients with ERA and healthy controls, it was interesting to note enhanced expression of IL23p19 and IL12p35 in HLA B27 positive patients and the fact that this seemed to be modified in MDMs derived from patients on

TNF inhibitors. There is some evidence for this from animal models. Colbert et al demonstrated upregulation of IL23p19 and IL12p35 expression in mouse bone marrow-derived macrophages after induction of the UPR with thapsigargin (Colbert, DeLay et al. 2010) but only IL23p19 in rat macrophages undergoing UPR secondary to HLA B27 misfolding (DeLay, Turner et al. 2009). The modifying effect of TNF inhibition on the UPR has also been observed in animal models although not in the context of SpA. Experiments on cardiomyocytes demonstrated that etanercept inhibits tribbles 3 expression (which is induced by the UPR) and thus CHOP expression leading to a reduction in apoptosis in cardiomyocytes undergoing mechanical stress (Cheng, Wang et al. 2015). These experiments are of limited relevance for this assay especially as CHOP expression did not appear to be modified in patients on TNF inhibitors but the effect of TNF inhibition on the UPR warrants further investigation. It would also be interesting to confirm these findings longitudinally by analysis samples from patients before and after treatment with TNF inhibition.

The production of IL23, IL12 and IL27 in cell culture supernatants from LPS + TM-stimulated MDMs was analysed next and interestingly, for IL23 and IL27, many of the associations seen in LPS-stimulated MDMs were also seen in LPS + TM-stimulated MDMs. For IL12 however, the findings were similar to those of Goodall et al, with much lower levels from LPS + TM-stimulated MDMs compared to MDMs stimulated by LPS alone. This is most likely due to the fact that N-glycosylation is required in the formation of IL12 and therefore the addition of TM to the assay impairs the production of the active form of IL12 (Murphy, Hayes et al. 2000). It was noted that a significant number of MDMs from patients and healthy controls produced little or no IL12 and therefore IL12 was not analysed further in this chapter.

IL23 and IL27 production was also significantly reduced compared to levels from MDMs stimulated with LPS alone. This was the case for most cytokines analysed. The reason for this is not clear but is perhaps because TM also interfered with the production of these cytokines or that the time point of measurement differed from other assays reported in the literature. TM was dissolved in DMSO but the concentration was low (1:10000) and DMSO alone did not affect cytokine production. Other agents that induced the UPR in an alternative manner such as thapsigargin were considered but because of the limited number of MDMs available for each patient and healthy control, these were not investigated in this assay.

Despite lower levels of production, IL23 remained significantly higher than healthy controls in LPS + TM-stimulated MDMs and again significant associations were noted with HLA B27 and enthesitis. Interestingly, in contrast to LPS-stimulated MDMs, the association with male sex was not seen. Similarly, levels of IL23 production from LPS + TM-stimulated MDMs from patients with polyarticular JIA and AS were not significantly different to healthy controls, which was perhaps due to increased variation in results and smaller size of these groups.

For IL27, as with LPS-stimulated MDMs, levels were significantly higher in patients with ERA compared to healthy controls but no difference was noted between HLA B27 positive and

negative patients with ERA, although levels in those who were HLA B27 positive were higher than healthy controls. The findings for enthesitis also mirrored those of LPS-stimulated MDMs, with significantly higher levels observed from LPS + TM-stimulated MDMs from those patients with active enthesitis at the time of sample compared to those without enthesitis and healthy controls.

In contrast to the gene expression of IL23p19 and IL12p35, treatment with TNF inhibitors did not have a significant modulating effect on IL23 or IL27 levels from LPS + TM-stimulated MDMs, with no difference in IL23 or IL27 between HLA B27 positive patients with ERA on TNF inhibitors compared to those not on TNF inhibitors.

The next section focussed on the effect of UPR induction on the gene expression of TNFα, IL1β and IL6. Given the evidence that the UPR induces pro-inflammatory cytokine production, in particular TNFα and IL6 (Li, Schwabe et al. 2005), it was perhaps surprising that no significant increase in expression was seen with UPR induction compared to expression with LPS stimulation alone. As discussed in previous chapters, it would be of interest to investigate gene expression at different time points in this assay to see whether any differences are observed after a shorter incubation period with TM. One explanation maybe that the pathways induced by the UPR resulting in cytokine gene expression and production were already activated by LPS stimulation in this assay and therefore no increase in production was seen with the addition of TM, despite clear evidence that the UPR was being induced.

No difference was noted between the gene expression of TNFα, IL1β and IL6 from LPS + TM-stimulated MDMs in patients with ERA and healthy controls and only IL6 gene expression was enhanced in HLA B27 positive patients with ERA compared to those who were HLA B27 negative. Interestingly, as with IL23p19 and IL12p35, there was a suggestion that IL1β expression was modified by treatment with TNF inhibitors in HLA B27 positive patients but not TNFα or IL6.

In the cell culture supernatants from LPS + TM-stimulated MDMs, the findings for TNFα were similar to IL23 and IL27, with a significant reduction in levels noted compared to MDMs stimulated with LPS alone and higher levels of production in patients with ERA compared to healthy controls. The production of IL1β and IL6 was not significantly different between LPS-stimulated and LPS + TM-stimulated MDMs and IL6 levels were no different between patients and healthy controls, as observed in MDMs treated with LPS alone. Interestingly, in contrast to the findings for LPS-stimulated MDMs, IL1β production was higher in patients with ERA compared to healthy controls and patients with polyarticular JIA.

The difference in pattern in IL1β production compared to TNFα and IL6 may be as a result of the different mechanisms by which the UPR regulates the secretion of pro-inflammatory cytokines. All pathways of the UPR activate NFκB which, in conjunction with other factors such as MAP kinases, results in the production of many pro-inflammatory cytokines (Darling

and Cook 2014, Smith 2018). In addition, UPR transcription factors such as XBP1 and CHOP bind directly to the promoter regions for certain cytokines such as TNFα and IL6 (XBP1) (Martinon, Chen et al. 2010) and IL23p19 (CHOP) (Goodall, Wu et al. 2010) thus increasing production. The UPR has also been shown to activate the inflammasome and this provides an alternative mechanism for IL1β production (Kim, Joe et al. 2014) and perhaps IL23p19 (Cowardin, Kuehne et al. 2015, Wang, Zhong et al. 2019).

The strong association with active enthesitis and pro-inflammatory cytokine production in patients with ERA was maintained in TNFα and IL1β from LPS + TM-stimulated MDMs and was also seen with IL6 production, in contrast to the findings with MDMs stimulated with LPS alone. Similarly and mirroring the results for IL6 gene expression, IL6 protein levels were also higher in HLA B27 positive patients with ERA compared to those who were HLA B27 negative. TNFα and IL1β levels from LPS + TM-stimulated MDMs from HLA B27 positive patients with ERA were higher than healthy controls but not significantly different from HLA B27 negative patients with ERA in keeping with the results from MDMs stimulated with LPS alone.

In view of the results above, the finding that the fold change in IL17 gene expression was significantly increased in LPS + TM-stimulated MDMs compared to LPS-stimulated MDMs from healthy controls was interesting. The increase in fold change for patients with ERA was not significant, no associations were noted with any of the clinical features analysed and IL17 was not detected in the cell culture supernatants from LPS + TM-stimulated MDMs after either 4 or 24 hours stimulation in either patients or healthy controls. The significance of the increase in gene expression in healthy controls is unclear but given the importance of IL17 in the pathogenesis of SpA, it would be of interest to investigate further in larger numbers of patients with ERA and healthy controls and in the other patient groups. It has been shown that IL17 may induce the UPR in a murine model of lung injury (Kim, Kim et al. 2015) and in in vitro studies of mouse bone marrow derived macrophages (Yang, Liu et al. 2018). In one study of murine macrophages in the context of retinal neovascularisation, there was a suggestion of a positive feedback loop between IL17 and the UPR (Wang, Gao et al. 2021). However, given that the enhanced gene expression of IL17 did not translate in to increased IL17 protein production with UPR induction, the significance in the assay described in this thesis is uncertain. Other cell types may be the predominant producers of IL17 in ERA and SpA and the interaction between the UPR has been investigated in T lymphocytes in patients with ulcerative colitis showing enhanced gene expression of IL17 with induction of the UPR in patients compared to healthy controls (Li, Wang et al. 2016). It would be of interest to investigate this in other cell types from patients with ERA and SpA.

The next cytokine to be analysed was GMCSF. GMCSF was the only cytokine to be significantly increased by induction of the UPR with higher levels seen across all patient and healthy control groups in LPS + TM-stimulated MDMs compared to LPS-stimulated MDMs. Interestingly, the increase was most significant in the ERA and AS patient groups. In patients

with ERA, active enthesitis was associated with higher levels of GMCSF compared to those without active enthesitis and healthy controls, whereas in LPS-stimulated MDMs only a trend to higher levels was seen with active enthesitis. Similarly a trend was observed towards higher levels from LPS + TM-stimulated MDMs in HLA B27 positive patients with ERA compared to HLA B27 negative patients, with significantly higher levels than healthy controls; whereas no difference was seen between HLA B27 positive and negative patients with ERA in LPS-stimulated MDMs.

The increase in levels GMCSF production with UPR induction was striking when compared to the production of other cytokines and is supported by evidence from recent studies in murine models of cardiovascular disease which demonstrate upregulation of GMCSF as a result of the UPR (Ying, Li et al. 2018). This study reported that a possible mechanism for this was induction of NFκB signalling by the UPR and showed NFκB could bind to a promoter region of GMCSF thus enhancing production. However, in this assay, if UPR induction resulted in increased NFκB signalling sufficient to enhance GMCSF production, it would be expected that other pro-inflammatory cytokines would also be significantly increased by this mechanism but this was not the case. It would therefore be of interest to investigate this further by analysing the expression of NFκB and other possible mechanisms of GMCSF production. Al-Mossawi et al showed that in GMCSF-producing CD4 T cells, high levels of the G-protein coupled receptor, GPR65, were expressed and that silencing this receptor led to a reduction in GMCSF (Al-Mossawi, Chen et al. 2017). They also demonstrated culturing CD4 T cells in an acidic environment significantly increased GMCSF and therefore it would be important to exclude this as a potential mechanism for enhanced GMCSF expression in this assay when TM is added.

IFNγ was the final cytokine to be analysed in LPS + TM-stimulated MDMs and the findings, as with the majority of cytokines analysed, were similar to those in MDMs stimulated with LPS alone with no association with clinical features such as HLA B27 and active enthesitis. There was a reduction in levels produced by LPS + TM-stimulated MDMs compared to LPS-stimulated MDMs in patients with ERA and polyarticular JIA and no difference in production noted for both healthy control groups and patients with AS. Interestingly, lower levels of IFNγ production were still seen in patients with ERA compared to healthy controls and patients with polyarticular JIA which in view of the inhibitory effect of IFNγ on the IL23-driven development of Th17 cells (Harrington, Hatton et al. 2005), may be an important factor in implicating the IL23/IL17 axis in patients with ERA compared to other JIA subtypes.

As with LPS-stimulated MDMs, analysis of correlations between cytokines and also with markers of the UPR from LPS + TM-stimulated MDMs was undertaken. A particularly close correlation was noted between levels of IL23 production and both IL27 and GMCSF. Interestingly, GMCSF is a key driver of IL23 production in myeloid cells by inducing a more pro-inflammatory cell phenotype (McGeachy 2011). This is perhaps of significance in view of the results from this assay indicating that induction of the UPR enhances the production of

GMCSF. As suggested by others (Al-Mossawi, Chen et al. 2017), GMCSF may represent an important treatment target in SpA and therefore ERA.

Fewer correlations were noted between markers of the UPR and cytokine gene expression but contrary to expectations, negative correlations were seen between the expression of spliced XBP1 and IL23p19, IL1β and IL6 suggesting a negative feedback loop between some pro-inflammatory cytokines and spliced XBP1. This is perhaps explained by the fact that although all three pathways of the UPR are activated early, the IRE1 pathway (which includes XBP1) is rapidly repressed and spliced XBP1 degraded (Yoshida, Oku et al. 2006, Hetz, Martinon et al. 2011). The trend observed between CHOP and IL23p19 in LPS + TM-stimulated MDMs is supported by the study by Goodall et al suggesting that CHOP was required for the induction of IL23 (Goodall, Wu et al. 2010).

The limitations of the experiments in this chapter include the mechanism of action of induction of the UPR using TM-induced inhibition of N-glycosylation which undoubtedly affected the gene expression and production of IL12 and may also have had a negative impact on the expression of other cytokines. Other methods of inducing the UPR were considered (such as thapsigargin) but insufficient numbers of MDMs were available for each patient and healthy control to use both methods of UPR induction. As discussed in other chapters it would be of interest to analyse gene expression and cytokine production at different time points but this was also restricted due to limited numbers of MDMs. It would also be interesting to confirm these in vitro findings in an ex vivo setting and to expand the experiments in SF.

Overall this chapter indicates that, in this assay, induction of the UPR results in enhanced expression of the UPR markers spliced XBP1, CHOP and BiP but that this is not significantly different between patients and healthy controls, although the expression of BiP may be higher in MDMs without UPR induction in HLA B27 positive patients with ERA. In addition, the gene expression and production of the majority of cytokines is not enhanced by the UPR in this assay and in fact, cytokine production is no different or lower with UPR induction when compared to MDMs stimulated with LPS alone and this may be due to the mechanism of action of TM. The exception to this is the production of GMCSF, which is significantly increased by UPR induction in this assay. Thus the UPR may not be the primary cause of increased IL23 and other pro-inflammatory cytokines in patients with ERA but may be a contributing factor by inducing the production of GMCSF. Despite this, the pattern of cytokine production is maintained, with associations seen with HLA B27 and active enthesitis in patients with ERA, although the association of higher levels of cytokine production in male patients with ERA was lost. Interestingly, differences were observed between levels of GMCSF, IFNγ and IL1β from LPS + TM-stimulated MDMs in patients with ERA compared to patients with polyarticular JIA, potentially marking these three cytokines as important in the pathogenesis of ERA compared to other subtypes of JIA.

The finding that markers of the UPR are not significantly different between patients with ERA and healthy controls is in keeping with other similar studies in patients with AS (Zeng, Lindstrom et al. 2011, Neerinckx, Carter et al. 2014). Alternative mechanisms such as autophagy, which is also linked to protein misfolding and the UPR, may play a role but although there is evidence for this in related conditions such as inflammatory bowel disease, the evidence in SpA is inconsistent. Ciccia et al demonstrated that IL23 regulation in gut biopsies from patients with AS was related to activation of autophagy (Ciccia, Accardo-Palumbo et al. 2014). However, Neerinckx et al did not find any evidence of activation of autophagy-associated genes in synovial tissues and PBMCs in patients with AS (Neerinckx, Carter et al. 2014). The ability of HLA B27 to form free heavy chains at the cell surface which can stimulate Th17 cells provides another mechanism for pro-inflammatory cytokine production (Wong-Baeza, Ridley et al. 2013). Gut dysbiosis may also play a role (Wendling 2016). Thus, despite the clear evidence that HLA B27 misfolds, which can induce the UPR, this may not be the main mechanism for pro-inflammatory cytokine production in ERA and indeed SpA, although it may contribute to it.

CHAPTER 7: SERUM BIOMARKERS AND A PILOT STUDY INVESTIGATING THE EFFECT OF OESTROGEN ON LPS-STIMULATED MONOCYTE DERIVED MACROPHAGES FROM PATIENTS WITH ERA

Overview of chapter 7

The aim of this chapter was to study levels of serum biomarkers in ERA and to determine whether differences in clinical phenotype resulted in differences in biomarker levels.

The first part of the chapter focusses on the analysis of several biomarkers including CRP, calprotectin, MMP3 and Dkk1 and their associations with clinical features in ERA. Serum levels of IL23 and IL17 are also discussed. Analyses of correlations between biomarkers are also included.

The second part of the chapter describes a preliminary investigation in to the effect of oestrogen on cytokine production from LPS-stimulated MDMs.

Hypothesis for Chapter 7

Serum biomarkers measured from patients with ERA will be higher than healthy controls

The addition of oestrogen to the MDM bioassay will reduce cytokine production from MDMs from patients with ERA and AS.

7.1 Measurement of serum biomarkers in patients with ERA

7.1.1 Introduction

The search for biomarkers indicating disease activity, prognosis and radiographic progression in SpA has been extensive but as yet no single reliable marker has been identified. In view of the similarities between adult SpA and ERA, clinically and in the results from the MDM bioassay, it was of interest to determine whether certain biomarkers studied in adult SpA were helpful in ERA. Biomarkers were analysed in serum from patients who also had samples analysed as part of the MDM bioassay where serum was available. Certain biomarkers relevant to SpA were also analysed in a larger group of patients with ERA having similar clinical characteristics to the group analysed for the MDM assay with serum samples available. The demographics and clinical features of the larger group analysed are summarised in table 7.1. Data was available for some biomarkers in 18 of these patients at two time points: pre- and post-treatment with TNF inhibitors. Serum biomarkers were also tested in a group of age and gender-matched healthy controls, some of whom also had samples analysed as part of the MDM assay. Although the numbers tested were larger, fewer clinical details were available for the larger group of patients and therefore it was not possible to analyse subgroups of patients with and without enthesitis, active peripheral arthritis or hip arthritis. However, it was possible to calculate JADAS in 50% of these patients. It was not possible to measure all biomarkers in all the patients recruited for the MDM assay because of lack of availability of serum samples and due to the amount of serum available, not all biomarkers were tested in all patients and healthy controls in the larger group. The exact number of patients and healthy controls tested are detailed in each section below.

	ERA	Healthy Controls
Number in final analysis	94*	50*
Number also analysed in MDM assay	Varied depending on available sample (detailed below)	Varied depending on available sample (detailed below)
Age (median)	17 yrs	17 yrs 4 mths
Male (%)	78.7	75
HLA B27 + (%)	63.8	0**
CRP (mg/dL, median)	1.7	0.18
Disease duration (median)	5 yrs 4 mths	-
Axial arthritis (%)	68.1	-
Pain VAS (median)	3**	-
Physician VAS (median)	2**	-
(C)HAQ (median)	0**	-
Swollen joint count (median)	0**	-
Restricted joint count (median)	1**	-
Active joint count (median)	1**	-
Recent steroid treatment (%) * **	13.8	-
Current NSAID (%)	43.6	-
Current DMARD (%)	61.7	-
Current TNF inhibitor (%)	39.4	-

Table 7.1 Patients and healthy volunteers included in biomarker analysis. Each patient and healthy volunteer donated a serum sample from which biomarkers were analysed by ELISA. Basic demographics were collected at the time of sample collection and clinical data for patients was collected retrospectively from clinic letters (*= number varied depending on biomarker tested due to availability of serum (detailed below)**=some data missing (HLA B27 status healthy controls 58%, pain VAS patient 29%, physician VAS 16%, CHAQ 12%, swollen joint count 16%, restricted joint count 16%, active joint count 30%), ***=recent steroid treatment included oral, intra-articular or intravenous).

7.1.2 HLA B27: trend towards higher CRP in HLA B27 positive patients with ERA

HLA B27 is the strongest genetic marker in SpA, accounting for around 20% of the genetic susceptibility to disease (Smith 2015, Ellinghaus, Jostins et al. 2016). However, not all patients with SpA or ERA are HLA B27 positive. In the MDM bioassay in this thesis, for patients with ERA, HLA B27 positivity was associated with higher levels of IL23, IL12, IL27, TNFα, IL1β, GMCSF and lower levels of IFNγ from LPS-stimulated MDMs compared to healthy controls. Additional stimulation with TM resulted in similar associations for MDMs from HLA B27 positive patients with IL27, TNFα, IL1, GMCSF and IFNγ and significantly higher levels of IL23 and IL6, as well as IL23p19, IL12p35 and IL6 gene expression compared to MDMs from HLA B27 negative patients. In both AS and early SpA, HLA B27 has been associated with younger age of onset (Feldtkeller, Khan et al. 2003, Chung, Machado et al. 2011), and in early inflammatory back pain, with increased severity and number of SIJ lesions at baseline MRI and persistent inflammation at 1 year (Marzo-Ortega, McGonagle et al. 2009). We have previously noted that patients with the axial ERA subtype are more likely to be HLA B27 positive compared to those with peripheral ERA (Fisher, Ioannou et al. 2012) in a large single centre cohort. In JIA, HLA B27 is associated with a reduced chance of treatment-free remission after 8 years of disease (Berntson, Nordal et al. 2013).

In the group of patients with ERA who had samples analysed for the MDM assay (table 3.1), despite the associations of HLA B27 and high levels of certain cytokines, no significant associations between HLA B27 and clinical features were found (table 7.1). However, this group was small in comparison to the cohort previously analysed (Fisher, Ioannou et al. 2012) which may explain the lack of clinical associations. A trend was noted towards higher CRP in those patients with ERA who were HLA B27 positive but the proportion of patients overall with a clinically significant rise in their CRP (>5mg/dL) was low (28% of HLA B27 positive ERA vs 16.7% of HLA B27 negative patients) and as previously noted in adult patients with AS, CRP may not be a good predictor of disease activity (Claushuis, de Vries et al. 2015).

		HLA B27 positive (n=25)	HLA B27 negative (n=12)	Difference
Age of onset (median)		12 years 11 months (IQR 10 years 3 months-14 years 3 months)	12 years 3 months (IQR 10 years 10 months-14 years)	Not significant
CRP (mg/dL)		1.100 (IQR 0.6-5.55)	0.600 (0.600-2.925)	Trend (p=0.078)
Sex	Male (%)	80	91.7	Not significant
	Female (%)	20	8.3	
Active Enthesitis	Yes (%)	68	50	Not significant
	No (%)	32	50	
Active peripheral arthritis	Yes (%)	28	8.3	Not significant
	No (%)	72	91.7	
Active hip arthritis	Yes (%)	52	33.3	Not significant
	No (%)	48	66.7	
ERA subtype	Peripheral (%)	12	16.7	Not significant
	Indeterminate (%)	20	33.3	
	Axial (%)	68	50	
Disease activity (JADAS)	Inactive and low (%)	40	58.3	Not significant
	Moderate (%)	40	33.3	
	High (%)	20	8.3	
DMARD	Yes (%)	72	75	Not significant
	No (%)	28	25	
TNF inhibitor	Yes (%)	44	33.3	Not significant
	No (%)	56	66.7	

Table 7.2 Summary of associations between clinical features and HLA B27 in patients with ERA in those with samples analysed in the MDM bioassay. HLA B27 status was recorded as part of routine clinical care and compared to clinical features, disease phenotype and treatment. Table shows percentage, statistical analysis by Fisher's exact test or Chi squared test as appropriate. Key:

Trend towards difference between groups ($p<0.1$)

In the larger group of patients with ERA with serum analysed for other biomarkers (table 7.1), no additional associations with clinical features were found with HLA B27.

7.1.3 CRP is significantly higher in patients with inflammatory arthritis compared to healthy controls

Despite the fact that CRP may not be a good marker of disease activity in all patients with AS (Claushuis, de Vries et al. 2015), there is strong evidence in adult patients that elevated CRP in early (non-radiographic) SpA and axial SpA predicts radiographic progression (Poddubnyy, Rudwaleit et al. 2011, Ramiro, van der Heijde et al. 2014, Poddubnyy, Protopopov et al. 2016) and also good response to TNF inhibitors (Sieper, van der Heijde et al. 2015). However, CRP may only be increased in around 40% of patients with SpA (Maksymowych 2017) and was not found to be significantly different in adult patients with inflammatory back pain compared to those with mechanical back pain in one recent study (Turina, Yeremenko et al. 2017).

For those patients studied as part of the bioassay, despite the low proportion of patients with ERA with an elevated CRP (21.6% >5 mg/dL), levels were significantly higher compared to healthy controls (median 0.900 mg/dL, IQR 0.600-5.150 vs 0.15 mg/dL, IQR 0.020-0.480 mg/dL, p=0.0008). Levels were also significantly higher in patients with polyarticular JIA compared to healthy controls (median 1.00 mg/dL, IQR 0.600-3.200 mg/dL, p=0.0078) and patients with AS compared to healthy controls (median 3.550 mg/dL, IQR 1.425-6.325 mg/dL, p<0.0001). Although CRP did not correlate with levels of cytokine production from the MDM bioassay, it was interesting to note higher CRP in male patients with ERA compared to female patients (median 1.2 mg/dL, IQR 0.6-5.7 vs 0.6 mg/dL, IQR 0.0-0.75 mg/dL, p=0.046). A trend towards higher CRP was also observed in male patients with AS compared to female patients (median 4.850 mg/dL, IQR 2.075-11.58 vs 1.900 mg/dL, IQR 0.8250-4.000, p=0.093). However, no associations were observed between disease duration, age and CRP in any of the inflammatory arthritis groups. In addition, no significant associations between CRP and other clinical features were found (table 7.3) although there was a trend towards higher levels in those categorised as having high disease activity by JADAS (p=0.057) which was expected given that CRP is a component of this disease activity score (Consolaro, Ruperto et al. 2009).

		CRP (mg/dL)	Difference
Age of onset		No correlation	
Disease duration		No correlation	
Sex	Male	1.200 (IQR 0.600-5.700)	P=0.046
	Female	0.600 (IQR 0.000-0.750)	
Active Enthesitis	Yes	0.700 (IQR 0.600-5.400)	Not significant
	No	1.000 (IQR 0.600-5.150)	
Active peripheral arthritis	Yes	0.750 (IQR 0.150-11.10)	Not significant
	No	1.000 (IQR 0.600-4.550)	
Active hip arthritis	Yes	1.600 (IQR 0.650-7.450)	Not significant
	No	0.600 (IQR 0.150-3.125)	
ERA subtype	Peripheral	0.900 (IQR 0.300-8.950)	Not significant
	Indeterminate	0.600 (IQR 0.000-4.450)	
	Axial	1.100 (IQR 0.600-5.700)	
Disease activity (JADAS)	Inactive and low	0.900 (IQR 0.600-1.800)	Trend (p=0.057)
	Moderate	0.650 (IQR 0.450-6.350)	
	High	8.500 (IQR 0.825-61.53)	
DMARD	Yes	0.900 (IQR 0.600-5.400)	Not significant
	No	0.800 (IQR 0.450-14.55)	
TNF inhibitor	Yes	0.800 (IQR 0.600-2.000)	Not significant
	No	1.100 (IQR 0.600-9.425)	

Table 7.3 Summary of associations of CRP and clinical features in patients with ERA with samples analysed in the MDM bioassay. CRP was tested as part of routine clinical care and compared between categories of sex, active arthritis/ enthesitis, disease phenotype, disease activity and treatment. Table shows median CRP (mg/dL) and IQR in brackets, statistical analysis by Mann-Whitney U test or Kruskal Wallis test as appropriate. Key:

Trend towards difference between groups (p<0.1)
Significant difference between groups (p<0.05)

CRP was further analysed in conjunction with the other biomarkers measured below in a larger cohort of patients (table 7.1) (n=94) and a summary of the results are shown in table 7.5. The results did not identify any additional associations between CRP and the clinical features available but levels of CRP were significantly higher in patients compared to healthy controls (median 1.7 mg/dL, IQR 0.0-8.4 mg/dL vs 0.18 mg/dL, IQR 0.03-0.61 mg/dL, p=0.0001).

7.1.4 Male sex is not associated with different clinical features compared to female patients with ERA

In view of the above findings of higher CRP in male patients with ERA and also the findings in chapters 4 and 5 demonstrating higher levels of certain cytokines such as IL23, TNFα, IL1 and GMCSF from LPS-stimulated MDMs from male patients with ERA compared to healthy controls and lower levels of IFNγ, it was of interest to investigate whether male patients with ERA had different clinical features compared to female patients. However, no such differences were observed (table 7.4), although as discussed previously, the number of female patients recruited was significantly lower than the number of males and this may explain why no differences were seen.

		Male (n=31)	Female (n=6)	Difference
Age of onset (median)		12 years 6 months (IQR 10 years 7 months- 14 years 1 month	11 years 10 months (IQR 10 years- 13 years 3 months)	Not significant
CRP (mg/dL)		1.200 (IQR 0.600-5.700)	0.600 (IQR 0.000-0.750)	p=0.046
Active Enthesitis	Yes (%)	61.3	66.7	Not significant
	No (%)	38.7	33.3	
Active peripheral arthritis	Yes (%)	19.4	33.3	Not significant
	No (%)	80.6	66.7	
Active hip arthritis	Yes (%)	45.2	50	Not significant
	No (%)	54.8	50	
ERA subtype	Peripheral (%)	12.9	16.7	Not significant
	Indeterminate (%)	22.6	33.3	
	Axial (%)	64.5	50	
Disease activity (JADAS)	Inactive and low (%)	48.4	33.3	Not significant
	Moderate (%)	35.5	50	
	High (%)	16.1	16.7	
DMARD	Yes (%)	74.2	66.7	Not significant
	No (%)	25.8	33.3	
TNF inhibitor	Yes (%)	41.9	33.3	Not significant
	No (%)	58.1	66.7	

Table 7.4 Summary of associations of sex and clinical features in patients with ERA with samples analysed in the MDM bioassay. Data was collected as part of routine clinical care and compared between categories of active arthritis/ enthesitis, disease phenotype, disease activity and treatment. Table shows percentage, statistical analysis by Fisher's exact test or Chi squared test as appropriate.

Further study in a larger patient cohort was of interest in view of the findings in this thesis and adult SpA demonstrating higher levels of pro-inflammatory cytokines such as TNFα, IL6 and IL17 in males with SpA and different clinical characteristics between males and females (Rusman, van Vollenhoven et al. 2018). Clinical associations with male sex were therefore analysed in the larger group of patients in which biomarker analysis was undertaken (table 7.1). However, no other associations were identified.

7.1.5 Levels of serum calprotectin are higher in patients with ERA compared to healthy controls

Calprotectin, also known as MRP8/14, is another biomarker of inflammation studied in adult SpA with mixed results (Maksymowych 2017). Calprotectin is released from macrophages during infiltration of inflamed tissues (Frosch, Strey et al. 2000) and is recognised as an acute-phase protein with normal serum levels in healthy individuals being reported as below 1000ng/mL (Ometto, Friso et al. 2017). Although not sufficiently sensitive to be a diagnostic marker (Turina, Yeremenko et al. 2017), levels were shown to be higher in adult patients with axial SpA compared to healthy controls (Gupta, Bhattacharya et al. 2016) and predicted radiographic progression in some studies (Turina, Sieper et al. 2014). However, levels do not necessarily correlate with disease activity in AS (Oktayoglu, Bozkurt et al. 2014). Serum calprotectin levels were available on 14 patients with ERA and 10 healthy controls that were recruited for the MDM assay. Higher levels were observed in these patients compared to healthy controls (median 2093 ng/mL, IQR 1223.0-3806 ng/mL vs 715.5 ng/mL, IQR 365.0-1528 ng/mL, p=0.015). Levels were therefore analysed in the larger group of patients with ERA (table 7.1) (n=74). In this group, serum calprotectin levels were significantly higher in patients with ERA (median 1998 ng/mL, IQR 1489-3213 ng/mL vs 457.0 ng/mL, IQR 360.0-1188 ng/mL, p<0.0001, figure 7.1). However, no associations were noted between calprotectin and the clinical features analysed (table 7.5).

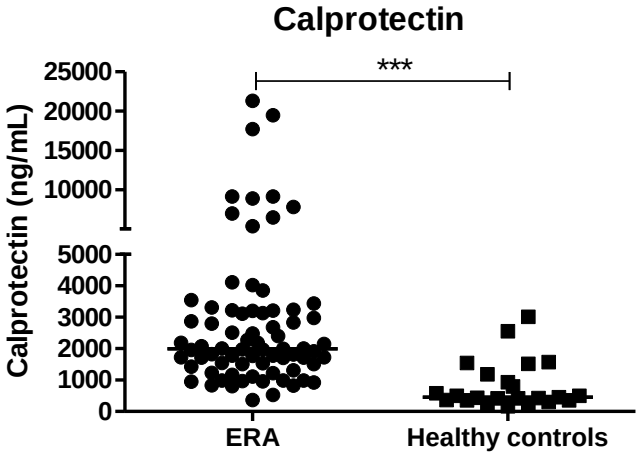


Figure 7.1 Calprotectin (also known as MRP 8/14) is higher in patients with ERA compared to healthy controls. Serum calprotectin was measured by ELISA in patients with ERA and healthy controls. Box and whisker plot shows median (+ interquartile range), statistical analysis by Mann Whitney U Test (ERA n=74, healthy controls n=23, ***p<0.001).

7.1.6 Serum MMP3 is higher in HLA B27 positive and male patients with ERA

MMP3 is a metalloproteinase produced in response to inflammatory stimuli in the joints of patients with inflammatory arthritis (Moz, Aita et al. 2017). MMP3 has been studied in adult SpA and higher serum levels have been reported compared to healthy controls (Gao, Zhang et al. 2015) and also in a cohort of patients with juvenile onset AS (Mou, Zhang et al. 2015). Some studies have demonstrated a correlation between serum MMP3 levels and disease activity (Maksymowych, Rahman et al. 2008, Soliman, Labib et al. 2012) but this has not been demonstrated conclusively (Wendling, Cedoz et al. 2008, Arends, van der Veer et al. 2011, Mattey, Packham et al. 2012). In AS, MMP3 levels may predict radiographic progression better than CRP (Maksymowych, Landewe et al. 2007, Vosse, Landewe et al. 2008).

Only 8 patients with ERA with samples analysed in the MDM bioassay had sufficient serum for testing MMP3. This was therefore analysed in serum from the larger cohort of patients with ERA described in table 7.1 (n=78). Overall, no difference between the patients with ERA and healthy controls was noted (median 15.84 ng/mL, IQR 7.783-39.01 ng/mL vs 15.10 ng/mL, IQR 8.000-19.93 ng/mL). However, patients with ERA who were HLA B27 positive had higher serum levels than those who were HLA B27 negative (median 24.16 ng/mL, IQR 10.28-55.33 ng/mL vs 11.60 ng/mL, IQR 3.984-22.98 ng/mL, p=0.012, figure 7.2A). Higher levels were also found in male patients with ERA compared to female patients (median 19.47 ng/mL, IQR 9.432-43.72 ng/mL vs 11.50 ng/mL, IQR 4.050-27.52 ng/mL, p=0.046, figure 7.2B) and a trend towards higher levels was also noted in patients with axial ERA compared to those with peripheral ERA (median 22.61 ng/mL, IQR 10.90-40.12 ng/mL vs 10.84 ng/mL, IQR 3.453-37.39 ng/mL, p=0.08). No other associations were noted with disease activity or treatment (table 7.5). Serum MMP3 was also available on 20 patients with polyarticular JIA and no significant difference was noted between these patients and healthy controls (median 10.22 ng/mL, IQR 5.268-30.40 ng/mL).

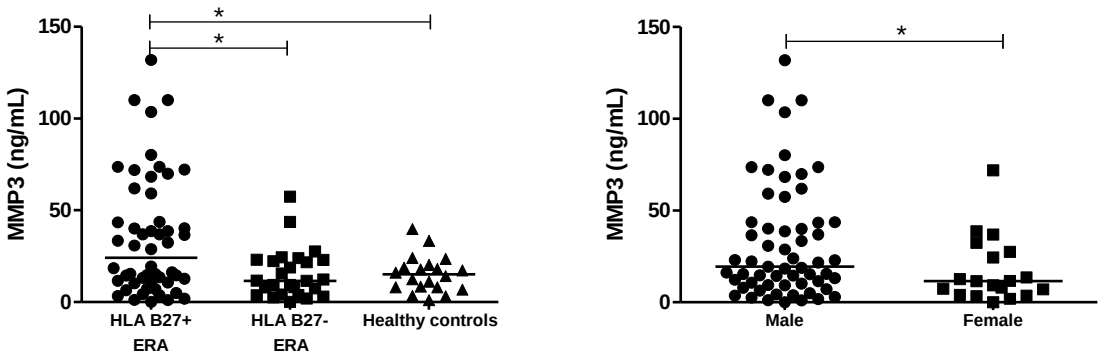


Figure 7.2 Serum MMP3 is higher in HLA B27 positive patients and male patients with ERA. Serum MMP3 was measured by ELISA in patients with ERA and healthy controls. Box and whisker plots show median (+ interquartile range), statistical analysis by Mann Whitney U Test or Kruskal-Wallis test as appropriate (HLA B27+ ERA n=52, HLA B27- ERA n=26, healthy controls n=20, male ERA n=59, female ERA n=19 *$p<0.05$).

7.1.7 Serum IL23 and IL17 are no different in patients with ERA compared to healthy controls

In view of the importance of IL23 and IL17 in the pathogenesis of SpA, several studies analysing serum levels have been published with mixed results (Reveille 2015). This is unsurprising given that analysis of cytokines in serum and plasma produces highly variable results influenced by diurnal rhythm as well as patient factors, sample collection and storage techniques (Aziz 2015). However, some studies in SpA have demonstrated significantly higher levels of serum IL23 in patients compared to healthy controls (Mei, Pan et al. 2011, Romero-Sanchez, Jaimes et al. 2011, Andersen, Rasmussen et al. 2012, Chen, Chang et al. 2012) but this has not been consistently shown and association with disease activity has not been demonstrated (Sveaas, Berg et al. 2015, Maksymowych 2017). Similarly, for serum IL17, some studies have shown elevated serum levels in patients with SpA (Mei, Pan et al. 2011, Romero-Sanchez, Jaimes et al. 2011, Chen, Chang et al. 2012) but others have not (Andersen, Rasmussen et al. 2012, Sveaas, Berg et al. 2015).

In view of the findings in the MDM assay analysed in previous chapters, it was of interest to determine whether levels of serum IL23 correlated with IL23 production in cell culture supernatants in those patients and healthy controls tested as part of the MDM assay. Serum IL23 tested by ELISA was available on 27 patients and 20 healthy controls who also had IL23 supernatant levels available from LPS-stimulated MDMs. However, no correlation between serum and supernatant levels was found in either group and no difference noted between patients and healthy controls (median 775 pg/mL, IQR 388.9-4832 pg/mL vs 3185 pg/mL, IQR 37.04-11472 pg/mL). Serum IL23 was subsequently measured in the larger cohort of patients described in table 7.1 (n=92) but no difference was noted between patients and healthy controls and no significant associations with clinical features were identified (table 7.5). Similarly no significant difference was noted between serum IL23 in a group of patients with polyarticular JIA compared to healthy controls (median 1461 pg/mL, IQR 138.0-7472 pg/mL).

Serum IL17 was also tested in the large cohort of patients with ERA (table 7.1) (n=92) but in general levels were below the detection range for the assay and in those with detectable levels, these were highly variable. No difference was found between patients and healthy controls.

7.1.8 Serum Dkk1 is higher in patients with ERA compared to healthy controls but lower in those with SIJ fusion

The final biomarker analysed was Dkk1. Dkk1 is an inhibitor of the Wnt signalling pathway which is key for new bone formation. The process of new bone formation in addition to bone loss distinguishes SpA from other forms of inflammatory arthritis. Consequently the investigation of markers involved in bone remodelling, including the Wnt pathway, has been of great interest in SpA. Dkk1 has been studied in patients with AS and found to be lower in some cohorts (Kwon, Lim et al. 2012) but normal or high in others (Daoussis, Liossis et al.

2010, Nocturne, Pavy et al. 2015). Interestingly, patients with higher levels do not have evidence of syndesmophyte formation (Yucong, Lu et al. 2014), suggesting that this may protect against new bone formation in patients with AS.

Serum Dkk1 was analysed by ELISA in the large cohort of patients described in table 7.1 (n=78) as serum was only available for 8 patients with samples analysed in the MDM bioassay. Dkk1 was found to be significantly higher in patients with ERA compared to healthy controls (median 2912 pg/mL, IQR 2201-3569 pg/mL vs 1806 pg/mL, IQR 1307-2950 pg/mL, p=0.0018, figure 7.3A). Levels were also significantly higher in a group of patients with polyarticular JIA (n=20) compared to healthy controls (median 3007 pg/mL, IQR 2208-3623 pg/mL, p=0.0114). For patients with ERA, levels were higher in those who were HLA B27 positive compared to negative (median 3081 pg/mL, IQR 2326-3694 pg/mL vs 2445 pg/mL, IQR 1895-3347 pg/mL, p=0.026, figure 7.3B) and a trend was noted towards higher levels in male patients compared to females (3059 pg/mL, IQR 2298-3608 pg/mL vs 2342 pg/mL, IQR 1961-3143 pg/mL, p=0.055). No other associations were noted between disease activity or treatment and Dkk1 levels.

In view of the role of Dkk1 within the Wnt signalling pathway and bone remodelling, Dkk1 was analysed in a subset of patients from those described in table 7.1 who already had evidence of new bone formation (SIJ fusion) on MRI scan (n=11). This is a relatively unusual finding in patients with early ERA and therefore unsurprisingly this group of patients were older compared to those without SIJ fusion (median 20 years 5 months vs 16 years 9 months) and had a longer disease duration (median 9 years 8 months vs 5 years 1 month). Interestingly, in those with evidence of SIJ fusion, serum Dkk1 levels were lower compared to those without SIJ fusion on MRI (median 2214 pg/mL, IQR 2103-2618 pg/mL vs 3059 pg/mL, IQR 2321-3608 pg/mL, p=0.037, figure 7.3C).

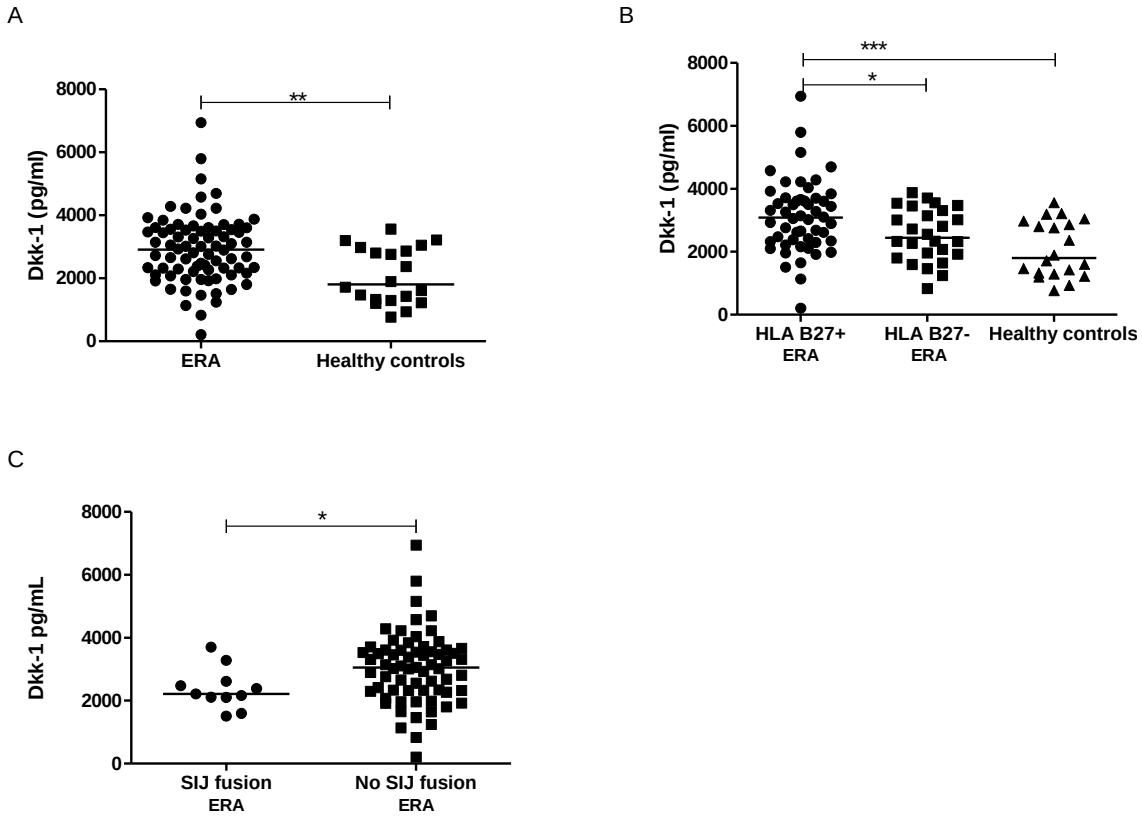


Figure 7.3 Serum Dkk1 is higher in patients with ERA compared to healthy controls but lower in those with SIJ fusion. Serum Dkk1 was measured by ELISA in patients with ERA and healthy controls. Box and whisker plots show median (+ interquartile range), statistical analysis by Mann Whitney U Test or Kruskal-Wallis test as appropriate (ERA n=78, healthy controls n=20, HLA B27+ ERA n=52, HLA B27- ERA n=26, healthy controls n=20, ERA with SIJ fusion n=11, ERA without SIJ fusion n=67 *p<0.05, **p<0.01, ***p<0.001).

		CRP (mg/dL)	Calprotectin (ng/mL)	MMP3 (ng/mL)	Dkk1 (pg/mL)	IL23 (pg/mL)
ERA vs healthy controls	ERA (all)	1.700 (IQR 0.0-8.400)	1998 (IQR 1489-3213)	15.84 (IQR 7.783-39.01)	2912 (IQR 2201-3569)	1028 (IQR 257.9-4832)
	Healthy controls	0.1813 (IQR 0.0337-0.6065	457.0 (IQR 360.0-1188)	15.10 (IQR 8.000-19.93)	1806 (IQR 1307-2950)	2226 (IQR 15.26-10865)
Age of onset		No correlation	No correlation	No correlation	No correlation	No correlation
Disease duration		No correlation	No correlation	No correlation	No correlation	No correlation
Sex	Male	1.600 (IQR 0.0-7.250)	2000 (IQR 1412-3274)	19.47 (IQR 9.432-43.72)	3059 (IQR 2298-3608)	1046 (IQR 209.9-5389)
	Female	4.400 (IQR 0.5250-23.80)	1819 (IQR 1485-2899)	11.50 (IQR 4.050-27.52)	2342 (IQR 1961-3143)	848.7 (IQR 498.3-3178)
HLA B27	Positive	2.300 (IQR 0.1250-9.275)	2184 (IQR 1524-3263)	24.16 (IQR 10.28-55.33)	3081 (IQR 2326-3694)	1054 (IQR 282.1-5513)
	Negative	1.200 (IQR 0.00-5.350)	1957 (IQR 1369-2840)	11.60 (IQR 3.984-22.98)	2445 (IQR 1895-3347)	840.1 (IQR 153.7-3212)
ERA subtype	Axial	1.700 (IQR 0.600-7.600)	2134 (IQR 1341-3291)	22.61 (IQR 10.90-40.12)	2710 (IQR 2121-3607)	1505 (IQR 186.7-5646)
	Peripheral	1.650 (IQR 0.00-18.28)	1937 (IQR 1531-2576)	10.84 (IQR 3.453-37.39)	3020 (IQR 2498-3483)	743.6 (IQR 310.7-2288)
Disease activity (JADAS)	Inactive	1.000 (IQR 0.300-2.350)	1586 (IQR 748-3379)	19.24 (IQR 8.849-44.35)	3429 (IQR 2554-4972)	1796 (IQR 194.5-12174)
	Low	4.300 (IQR 0.350-11.30)	2439 (IQR 1143-3391)	14.52 (IQR 9.279-32.24)	3311 (IQR 2352-3622)	3150 (IQR 499.6-10055)
	Moderate	1.650 (IQR 0.0-6.675)	1996 (IQR 1721-2801)	15.55 (IQR 7.177-66.98)	3059 (IQR 2187-3770)	1028 (IQR 513.1-3178)
	High	31.20 (IQR 1.000-97.65)	9127 (IQR 1716-19460)	23.99 (IQR 7.940-62.07)	2637 (IQR 2396-2851)	833.3 (IQR 229.6-2047)
Treatment	Nil/NSAID only	2.800 (IQR 0.570-18.28)	1964 (IQR 1510-4826)	21.41 (IQR 7.899-59.89)	3383 (IQR 2496-3963)	840.1 (IQR 472.0-3270)
	DMARD	1.850 (IQR 0.00-7.650)	1996 (IQR 1112-3441)	15.55 (IQR 6.470-38.65)	3012 (IQR 1961-3498)	1046 (IQR 213.4-4459)
	TNF Inhibitor	1.300 (IQR 0.0-5.700)	2145 (IQR 1367-3047)	16.13 (IQR 9.735-35.01)	2654 (IQR 2105-3484)	848.7 (IQR 142.6-5873)
Pre- and post-TNF inhibitor	Pre-TNF inhibition	3.200 (IQR 0.0-11.03)	2805 (IQR 1707-7058)	Samples not available	Samples not available	1213 (IQR 658.7-4853)
	Post-TNF inhibition	0.800 (IQR 0.600-1.825)	2256 (IQR 1122-4162)			796.4 (399.8-2183)

Table 7.5 Summary of results for serum biomarkers in patients with ERA. Serum was collected from patients with ERA and healthy controls and analysed for biomarkers by ELISA. Table shows median plus interquartile range. Statistical analysis by Mann Whitney U test or Kruskal-Wallis test as appropriate. Key:

Trend towards difference between groups (p<0.1)
Significant difference between groups (p<0.05)
Significant difference between groups (p<0.01)
Significant difference between groups (p<0.001)

7.1.9 Correlations between serum biomarkers in patients with ERA

Analysis of correlations between the biomarkers measured was undertaken in view of the differences seen in the levels of biomarkers such as CRP, calprotectin and Dkk1 between patients with ERA and healthy controls and also the similarities in associations with male sex and HLA B27. A significant correlation was seen between serum MMP3 and Dkk1 in patients with ERA ($r=0.40$, $p=0.0003$). MMP3 was also weakly correlated with number of swollen joints in patients with ERA ($r=0.32$, $p=0.023$). No other correlations between biomarkers were noted.

As discussed above, no correlation was observed between serum IL23 levels and IL23 production from LPS-stimulated MDMs in the MDM assay. In fact no correlation was observed between any of the biomarkers tested and cytokines analysed from the MDM assay.

7.2 Effect of oestrogen on cytokine production from LPS-stimulated MDMs

7.2.1 Introduction

Despite the evidence in adult SpA of differences in clinical phenotype and serum cytokine levels between males and females (Rusman, van Vollenhoven et al. 2018), evidence for the involvement of sex hormones in the pathogenesis of SpA is conflicting. One study suggested that low oestrogen was associated with active disease and demonstrated an improvement in arthritis after oestrogen therapy (Jimenez-Balderas, Tapia-Serrano et al. 1990). A more recent study also found that oestrogen attenuated arthritis in an animal model of SpA (Jeong, Bae et al. 2017). However, other studies have not demonstrated any differences in sex hormones in patients with SpA (Straub, Struharova et al. 2002). No such studies have been undertaken in patients with ERA.

In this thesis, differences were seen in serum CRP, MMP3 and production of some cytokines from LPS-stimulated MDMs between male and females and therefore a preliminary investigation was carried out to determine the effect of oestrogen on cytokine production from LPS-stimulated MDMs.

7.2.2 Fold change in IL23 production between LPS-stimulated MDMs with and without oestrogen is lower in patients with SpA compared to healthy controls

Oestrogen (β-oestradiol) was added at a dose of 10 nM (taken from previous studies in the literature as a physiological level of oestrogen in pre-menopausal females (Celojevic, Petersen et al. 2011)) to LPS-stimulated MDMs for 24 hours in a subgroup of male, HLA B27 positive patients with ERA and AS (referred to as the SpA group in these experiments). A comparison group of male, age-matched healthy controls was also tested. Cytokine levels in the cell culture supernatants were subsequently analysed by Luminex assay or ELISA and, for each patient, compared to levels from LPS-stimulated MDMs without the addition of

oestrogen. Fold change in cytokine production between LPS-stimulated MDMs with and without oestrogen was calculated.

The numbers in each group were small and therefore it was difficult to draw definite conclusions from the results which are summarised in table 7.6. There was no significant difference in overall cytokine production between MDMs stimulated with LPS alone and LPS-stimulated MDMs with oestrogen added but IL23 and IL12 production tended to be lower with the addition of oestrogen in patients with SpA (figure 7.4 A and C). It was therefore interesting to note the fold change in IL23 production was significantly lower in patients with SpA compared to healthy controls (median fold change -0.087, IQR -0.021- -0.033 vs 0.138, IQR 0.043-0.498, p=0.03, figure 7.4 B).

		SpA (n=7)		**Healthy controls** (n=3)	
MDM stimulation		LPS alone	LPS + oestrogen	LPS alone	LPS + oestrogen
IL23	Concentration (pg/mL)	52650 (34510-78740)	48060 (27370-58840)	29335 (12260-46410)	33395 (18370-48420)
	Fold change	-0.087 (-0.021- -0.033)		0.138 (0.043-0.498)	
IL12	Concentration (pg/mL)	5277 (2808-6690)	4211 (3170-5771)	4321 (1997-6644)	4495 (2144-6845)
	Fold change	-0.074 (-0.014-0.094)		0.040 (0.030-0.073)	
IL27	Concentration (pg/mL)	1953 (1756-2132)	1924 (1686-1977)	2118 (1863-2372)	1783 (1730-1836)
	Fold change	-0.015 (-0.156-0.048)		-0.158 (-0.271- -0.015)	
IL1β	Concentration (pg/mL)	687.6 (480.7-1537)	1005 (562.2-1589)	709.5 (538.3-880.3)	1011 (686.0-1337)
	Fold change	0.304 (0.044-0.615		0.425 (0.274-0.518)	
IL6	Concentration (pg/mL)	27279 (26080-28384)	28268 (28041-28738)	26231 (25049-27412)	27575 (27384-27767)
	Fold change	0.058 (0.006-0.094)		0.051 (-0.001-0.109)	
GMCSF	Concentration (pg/mL)	1613 (1202-2947)	1553 (907.0-2903)	1609 (1496-1721)	1882 (1597-2167)
	Fold change	0.158 (-0.078-0.247)		0.170 (0.068-0.259)	
IFNγ	Concentration (pg/mL)	8764 (7338-10047)	13139 (9379-13718)	9364 (8512-10217)	11206 (9054-13359)
	Fold change	0.333 (0.265-0.372)		0.197 (0.064-0.308)	

Table 7.6 Summary table of the effect of oestrogen on cytokine production from LPS-stimulated MDMs. Cytokine levels were measured in cell culture supernatants following incubation of MDMs with LPS with and without oestrogen (β-oestradiol 10 nM) for 24 hours using ELISA or Luminex assay. Table shows median cytokine level for each group with IQR in brackets, statistical analysis by Mann-Whitney U test or Kruskal-Wallis test as appropriate. Key:

Trend towards difference between groups (p<0.1)

Significant difference between groups (p<0.05)

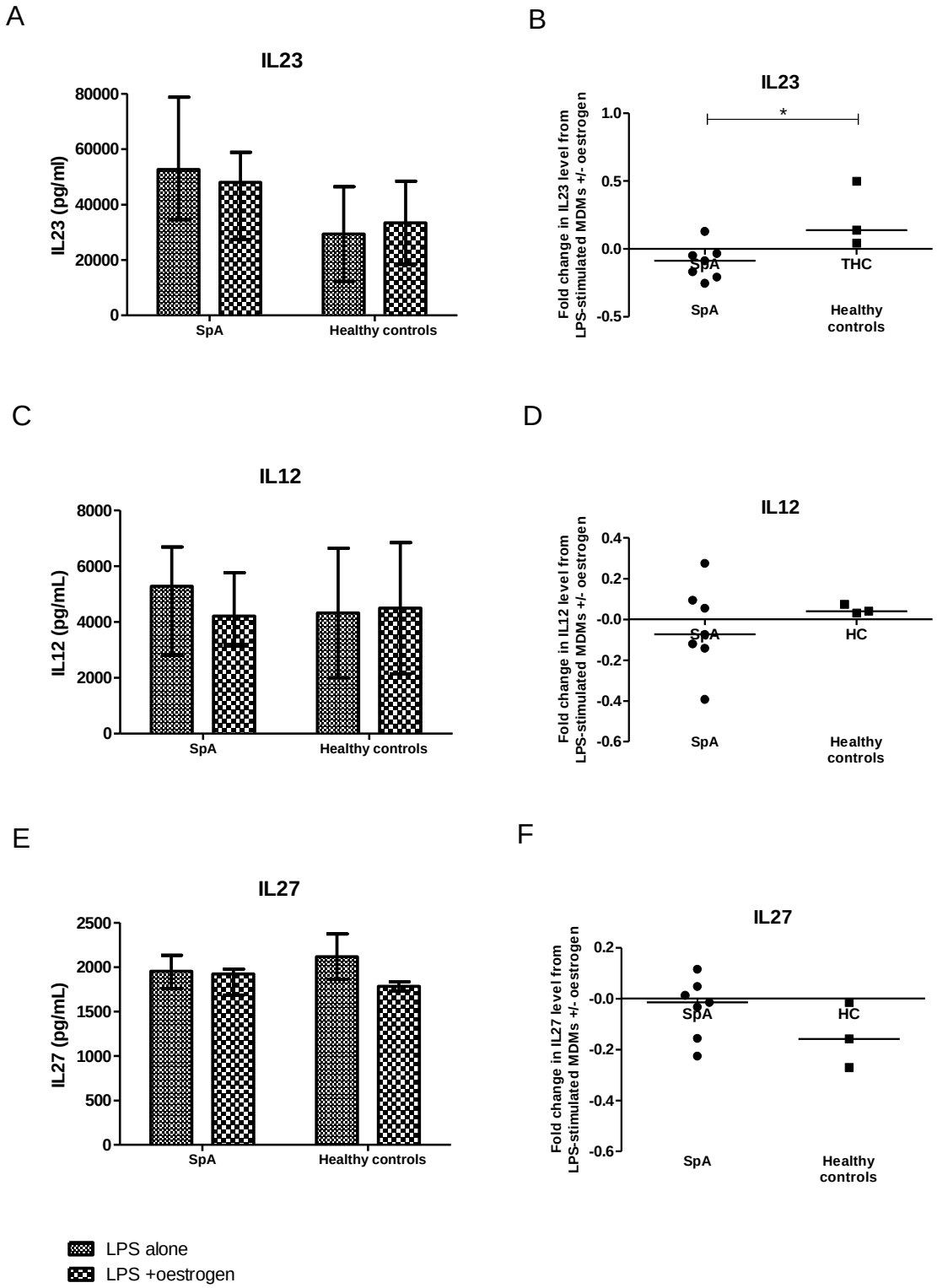


Figure 7.4 Fold change in IL23 production from LPS-stimulated MDMs with and without oestrogen is lower in patients with SpA (ERA and AS) compared to healthy controls. MDMs were cultured as per the protocol in section 2.2 and stimulated for 24 hours with LPS with and without oestrogen after which cell culture supernatants were collected and analysed by ELISA or Luminex assay. Bar charts and box and whisker plots show median (+ interquartile

range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups or Mann Whitney U Test. Fold change was calculated by dividing the difference in cytokine production between LPS-stimulated MDMs with and without oestrogen by cytokine levels from LPS-stimulated MDMs without oestrogen (SpA=patients with ERA n=4 and AS n=3, healthy controls n=3, *p<0.05).

Interestingly, the addition of oestrogen to LPS-stimulated MDMs resulted in increased IFNγ production, particularly in patients with SpA (median 8764 pg/mL, IQR 7338-10047 pg/mL increased to 13139 pg/mL, IQR 9379-13718 pg/mL, p=0.05). In healthy controls the increase was not statistically significant (median 9364 pg/mL, IQR 8512-10217 pg/mL increased to 11206 pg/mL, IQR 9054-13359 pg/mL, figure 7.5 G).

In view of this preliminary data, it would be of interest to expand the numbers in future work to investigate the potential benefits of oestrogen on cytokine production in SpA and, in addition, investigate the effects of other sex hormones such as testosterone and progesterone.

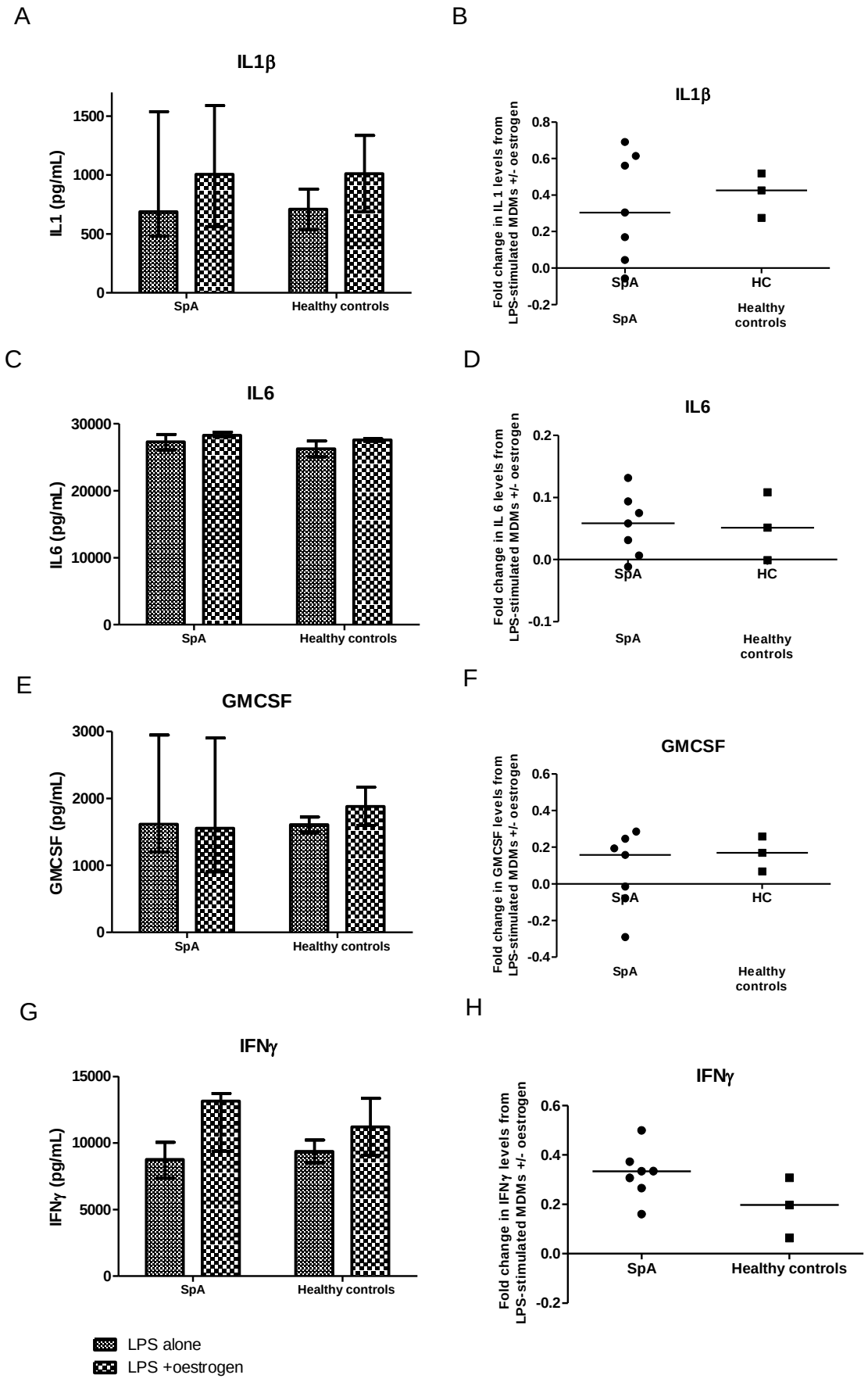

A
IL1β
IL1 (pg/mL)
1500
1000
500
0
SpA
Healthy controls
B
IL1β
Fold change in IL 1 levels from LPS-stimulated MDMs +/- oestrogen
0.8
0.6
0.4
0.2
0.0
-0.2
SpA
HC
SpA
Healthy controls
C
IL6
IL6 (pg/mL)
30000
20000
10000
0
SpA
Healthy controls
D
IL6
Fold change in IL 6 levels from LPS-stimulated MDMs +/- oestrogen
0.2
0.1
0.0
-0.1
SpA
HC
SpA
Healthy controls
E
GMCSF
GMCSF (pg/mL)
3000
2000
1000
0
SpA
Healthy controls
F
GMCSF
Fold change in GMCSF levels from LPS-stimulated MDMs +/- oestrogen
0.6
0.4
0.2
0.0
-0.2
-0.4
SpA
HC
SpA
Healthy controls
G
IFNγ
IFNγ (pg/mL)
15000
10000
5000
0
SpA
Healthy controls
LPS alone
LPS +oestrogen
H
IFNγ
Fold change in IFNγ levels from LPS-stimulated MDMs +/- oestrogen
0.6
0.4
0.2
0.0
-0.2
SpA
Healthy controls

Figure 7.5 IFNγ production from LPS-stimulated MDMs is increased by the addition of oestrogen to LPS-stimulated MDMs in patients with SpA (ERA and AS). MDMs were cultured as per the protocol in section 2.2 and stimulated for 24 hours with LPS with and without oestrogen after which cell culture supernatants were collected and analysed by Luminex assay. Bar charts and box and whisker plots show median (+ interquartile range), statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test to compare all groups or Mann Whitney U Test. Fold change was calculated by dividing the difference in cytokine production between LPS-stimulated MDMs with and without oestrogen by cytokine levels from LPS-stimulated MDMs without oestrogen (SpA=patients with ERA n=4 and AS n=3, healthy controls n=3, *p<0.05).

7.3 Discussion

The findings in this chapter demonstrate higher levels of certain biomarkers such as CRP, calprotectin and Dkk1 in patients with ERA compared to healthy controls. In addition, the differences noted in previous chapters between HLA B27 positive and negative patients and male and female patients with ERA were also observed with some of the biomarkers tested, in particular CRP and MMP3. A pilot study into the effect of oestrogen on LPS-stimulated MDMs from patients with SpA (both ERA and AS) also demonstrated potential differences in the production of certain cytokines (IL23 and IFNγ) with the addition of oestrogen.

The study of biomarkers in adults with SpA has yielded some promising results but further research is required to identify reliable biomarkers for diagnosis, disease activity, response to treatment or prediction of radiographic progression (Maksymowych 2017). In ERA, the identification of one or more biomarkers to aid with diagnosis and predict prognosis would be invaluable in view of the fluctuating nature of the condition, difficulties in interpreting SIJ MRI in the maturing skeleton (Vendhan, Sen et al. 2014) and the dearth of information on long term outcomes (Fisher, Ioannou et al. 2012).

HLA B27 forms part of the classification criteria for ERA (Petty, Southwood et al. 2004) and part of the ASAS criteria for the diagnosis of adult SpA (Rudwaleit, van der Heijde et al. 2011). However, not all patients with ERA or adult SpA are HLA B27 positive and prevalence varies with ethnicity (Reveille, Hirsch et al. 2012). Therefore, although a strong risk factor, HLA B27 alone is not considered an adequate biomarker for SpA. Distinctions between HLA B27 positive and negative patients have been observed in adult SpA such as earlier age of onset and perhaps more severe disease (Marzo-Ortega, McGonagle et al. 2009) with worse radiographic progression (Ramiro, van der Heijde et al. 2014) although this is not universally found (Cortes, Maksymowych et al. 2015). In this study of biomarkers in ERA, it was interesting to note a trend towards higher CRP in those who were HLA B27 positive although this may have been a chance finding as the numbers analysed were small and this was not replicated in the larger cohort of patients with ERA or in studies of adult SpA (Arevalo, Gratacos Masmitja et al. 2018).

When CRP was analysed in this study, levels were found to be higher in patients with ERA and, as expected, in the other forms of inflammatory arthritis compared to healthy controls. However, the proportion of patients with a clinically significant rise in CRP was low (ERA 21.6%, polyarticular JIA 13.3%, AS 21.4%) which has been previously observed in adult SpA making the use of CRP as a biomarker for diagnosis problematic (Turina, Yeremenko et al. 2017). In terms of prognosis, elevated CRP has been associated with radiographic progression in adults with both non-radiographic SpA (Poddubnyy, Rudwaleit et al. 2011) and radiographic axial SpA (Ramiro, van der Heijde et al. 2014, Poddubnyy, Protopopov et al. 2016). Radiographic evidence of sacroiliitis is unusual in ERA and the effect of CRP on prognosis has not been studied. Interestingly, no significant difference was found in CRP pre- and post- treatment with TNF inhibitors in this study. However, CRP was significantly higher in males with ERA compared to females with ERA which is consistent with data from studies of adult SpA (Tournadre, Pereira et al. 2013, van der Horst-Bruinsma, Zack et al. 2013, Webers, Essers et al. 2016). Analysis of longitudinal data in patients with ERA would be of interest to determine whether baseline CRP is predictive of development or progression of axial disease.

Another marker of inflammation, calprotectin, was found to be elevated in patients with ERA compared to healthy controls but no differences were noted between male and female patients or HLA B27 positive and negative patients. In the context of ERA and SpA, calprotectin may be important because it promotes T cell differentiation towards a Th17 phenotype and enhances the production of IL17 in an IL6-dependent manner (Lee, Woo et al. 2013). Faecal calprotectin is already an established biomarker in inflammatory bowel disease (Lopez, Leach et al. 2017). In JIA, serum calprotectin has shown some promise in predicting likelihood of response to treatment and of flare on stopping treatment (Anink, Van Suijlekom-Smit et al. 2015). However, in this study, no differences in calprotectin were observed before and after treatment with TNF inhibitors. This may be explained by the small sample size tested. In adult SpA, levels of calprotectin have been shown to decrease with TNF inhibition (Turina, Yeremenko et al. 2014). In addition, elevated serum calprotectin is associated with more severe radiographic progression in axial SpA and therefore longitudinal study of calprotectin in patients with ERA would be of interest to determine whether there is a correlation with progression to axial arthritis.

MMPs are a group of 28 zinc-dependent proteases involved in the degradation of the extra-cellular matrix (Moz, Aita et al. 2017). Several MMPs have been studied in SpA as potential biomarkers, including MMP8, MMP9 and MMP3. Interestingly, one study has linked the production of MMP8 and MMP9 to calprotectin (Moz, Basso et al. 2016). However, MMP3 is perhaps the most promising MMP studied as a biomarker in SpA with serum levels in general higher than healthy controls (Gao, Zhang et al. 2015). MMP3 has also been studied previously in ERA with levels shown to correlate with measures of disease activity including swollen joint count, BASDAI, BASFI, physician global assessment, patient global and pain scores (Viswanath, Myles et al. 2011). In this study however, no difference in serum level

was found between patients with ERA and healthy controls or indeed between patients with polyarticular JIA and healthy controls and no correlation was observed with the JADAS. However, a weak correlation was found between MMP3 and the swollen joint count which is consistent with the previous study in ERA (Viswanath, Myles et al. 2011) and literature in adult SpA and AS demonstrating higher levels in those with peripheral arthritis (Maksymowych, Rahman et al. 2008, Almodovar, Rios et al. 2014). The missing data for clinical features such as swollen and active joint count reduced the likelihood of seeing a positive correlation with disease activity measures in this study.

Higher levels of MMP3 were found in patients with ERA who were HLA B27 positive compared to those who were HLA B27 negative which has been observed in a previous study (Mou, Zhang et al. 2015). Higher serum levels were also observed in male patients with ERA compared to female patients with ERA which has also been demonstrated in studies of adult SpA (Arends, van der Veer et al. 2011, Almodovar, Rios et al. 2014). The observation of higher levels of MMP3 and CRP in male patients with ERA was not explained by higher JADAS score in male patients although as discussed previously in chapter 4 (section 4.6), JADAS may not accurately reflect disease activity in patients with ERA. It would be of interest to correlate MMP3, CRP levels and male sex with the JSpADA to see whether higher levels of disease activity in males explain the higher levels of these potential biomarkers. In addition, some studies in adult SpA have shown an association between higher MMP3 levels and radiographic progression (Maksymowych, Landewe et al. 2007, Vosse, Landewe et al. 2008) and it would be of interest to investigate this in a longitudinal study of patients with ERA.

The analysis of serum cytokines, in particular IL23 and IL17, as biomarkers in adult SpA has not yielded consistent results (Maksymowych 2017) and therefore it was not surprising that no differences were found in serum levels of IL23 between patients and healthy controls in this study. IL17, as noted before in the MDM bioassay, was difficult to detect in the serum of patients and healthy controls. Further analysis using high sensitivity assays was considered but was not pursued in this study in view of the inconsistency of results from adult studies.

In view of the importance of new bone formation in the pathogenesis of SpA, there has been considerable interest in bone turnover markers as biomarkers. The process of new bone formation in SpA is thought to involve interaction between chronic inflammation and the Wnt pathway (Lories and Haroon 2014) and appears to be sequential with new bone formation occurring on resolution of inflammation (Tseng, Pitt et al. 2016). The Wnt signalling pathway is involved in many aspects of cell function and promotes new bone formation by osteoblastogenesis (Pinzone, Hall et al. 2009). Inhibitors of this pathway include Dkk1 which has been studied in patients with AS with mixed results (Daoussis, Liossis et al. 2010, Kwon, Lim et al. 2012, Klingberg, Nurkkala et al. 2014). This may be explained by differences in the disease duration and treatment in these patient cohorts. In this study of patients with ERA with a median disease duration of 5 years 4 months, Dkk1

was significantly higher compared to healthy controls and similar to patients with polyarticular JIA. This is consistent with studies in adults with rheumatoid arthritis (Wang, Liu et al. 2011, Seror, Boudaoud et al. 2016) and a study from a cohort of patients with early SpA which found levels of Dkk1 to be higher than in healthy controls (Nocturne, Pavy et al. 2015). This study also found that Dkk1 was associated with high levels of inflammation and the presence of sacroiliitis.

In the study for this thesis, Dkk1 was higher in those patients with ERA who were HLA B27 positive compared to those who were HLA B27 negative. There was also a trend towards higher levels in male patients compared to female patients with ERA. A correlation between Dkk1 and MMP3 was noted and it would be of interest to investigate associations between JSpADA or MRI inflammation and Dkk1 in this cohort of patients. However, no significant difference was found between those with axial ERA and those with peripheral ERA.

Dkk1 production is strongly induced by TNFα (Yeremenko, Zwerina et al. 2015) providing a potential mechanism for the persistence of new bone formation in patients treated with TNF inhibitors (Bray, Lopes et al. 2019). High levels of Dkk1 appear to protect against new bone formation (Heiland, Appel et al. 2012, Yucong, Lu et al. 2014) and blockade of Dkk1 was found to induce SIJ fusion in an animal model of SpA (Uderhardt, Diarra et al. 2010). Thus, the finding of lower Dkk1 levels in patients with ERA with bony fusion of their SIJs was interesting especially as the majority of these patients were treated with TNF inhibitors (81.8% vs 39.4% of the cohort overall). This group was also older with longer disease duration compared to the rest of the cohort analysed. Lower levels of Dkk1 have been found in some cohorts of adult patients with AS (Kwon, Lim et al. 2012, Lories and Haroon 2014, Yucong, Lu et al. 2014) and it is possible that these studies included patients with longer disease duration and higher rates of new bone formation compared to the studies in early SpA which found higher levels of Dkk1 compared to healthy controls (Nocturne, Pavy et al. 2015). Longitudinal studies of Dkk1 would be of interest in patients with ERA and SpA to investigate whether there is a time point at which patients 'switch' from expressing high levels of Dkk1 to lower levels and whether this is influenced by treatment or associated with new bone formation becoming the predominant disease feature (as opposed to inflammation in early disease).

In view of the findings from the MDM assay and in levels of serum biomarkers suggesting differences between male and female patients with ERA, the final part of the chapter focussed on the effect of oestrogen on the production of cytokines from LPS-stimulated MDMs. Historically, a male predominance is seen in both ERA and adult SpA although it has been acknowledged that the increased prevalence seen in males may not be as great as previously thought (Rusman, van Vollenhoven et al. 2018). In ERA this is further complicated by the fact that male sex forms part of the classification criteria thus creating a bias towards diagnosis in males (Petty, Southwood et al. 2004). Females with SpA are under-represented in studies in SpA (Rusman, van Vollenhoven et al. 2018) and this was also the case in this

thesis in both the MDM bioassay study and the larger cohort of patients with ERA analysed for serum biomarkers. In adult studies of SpA, clinical characteristics may differ between males and females for example the time to diagnosis is significantly longer in females (Jovani, Blasco-Blasco et al. 2017), higher disease activity and pain scores are found in females (de Carvalho, Bortoluzzo et al. 2012, Tournadre, Pereira et al. 2013, van der Horst-Bruinsma, Zack et al. 2013, Landi, Maldonado-Ficco et al. 2016, Kilic, Kilic et al. 2017) but less severe radiographic progression occurs in females compared to males (Lee, Reveille et al. 2007, Vosse, Landewe et al. 2008, Baraliakos, van den Berg et al. 2012, van Tubergen, Ramiro et al. 2012, Maas, Spoorenberg et al. 2015, Landi, Maldonado-Ficco et al. 2016). Differences have also been reported in response to treatment with significantly lower efficacy of TNF inhibitors in females compared to males (Arends, Brouwer et al. 2011, van der Horst-Bruinsma, Zack et al. 2013, Gremese, Bernardi et al. 2014, Lubrano, Perrotta et al. 2017).

As discussed above, it is evident that females with SpA tend to have a lower CRP and also lower levels of certain pro-inflammatory cytokines such as TNFα, IL17, IL6, IL18 (Huang, Tso et al. 2012, Gracey, Yao et al. 2016). In addition, Gracey et al reported lower levels of Th17 cells in female patients with AS compared to male patients (Gracey, Yao et al. 2016). Furthermore, there is a suggestion that oestrogen may have a beneficial effect on disease activity in both females with AS (Jimenez-Balderas, Tapia-Serrano et al. 1990) and in an animal model (Jeong, Bae et al. 2017) although this is not universally found (Mahendira, Thavaneswaran et al. 2014). Sex is known to influence immune responses with a bias towards Th1 in females and Th17 in males (Oertelt-Prigione 2012, Zhang, Rego et al. 2012).

The above information prompted the design of a pilot study to investigate the effect of oestrogen on cytokine production from LPS-stimulated MDMs in patients with ERA and SpA with the hypothesis that oestrogen may reduce the production of pro-inflammatory cytokines. The numbers recruited were small because few patients and healthy controls had the required number of monocytes for all the MDM assay conditions both with and without oestrogen. In addition, as discussed in section 3.6, the in vitro assay has the disadvantage of being an artificial system; therefore it is difficult to draw definite conclusions from this pilot data. However, the finding of an overall median decrease in IL23 production in patients with SpA compared to an increase in production for healthy controls, resulting in a significant difference in fold change in IL23 production with the addition of oestrogen to the assay was interesting. Taken together with the trend towards increased IFNγ production (potentially suppressing IL17) with the addition of oestrogen to LPS-stimulated MDMs in patients with SpA, may go some way to explaining the differences seen between the sexes in SpA and ERA. The findings for IFNγ are supported by a study investigating CD4 T cell responses in males and females which found higher levels of IFNγ production in females. This warrants further study in a larger number of patients and healthy controls, if possible in an in vivo setting as it may indicate the need for different treatment strategies between males and females. Other sex hormones such as testosterone and progesterone have not been shown

to be significantly different between patients with SpA and healthy controls (Jimenez-Balderas, Tapia-Serrano et al. 1990, Giltay, Popp-Snijders et al. 1998, Straub, Struharova et al. 2002) and therefore were not studied as part of this thesis but would be of interest to include in future studies.

In summary, this chapter identifies potential biomarkers in ERA that need further evaluation and correlation with markers of disease activity (such as the JSpADA) and MRI inflammation. The data analysed was mostly cross-sectional, apart from the 18 patients with serum samples collected before and after treatment with TNF inhibitors. Further longitudinal study is key to determining the usefulness of these biomarkers in predicting clinical phenotype such as the development of axial disease, response to treatment and likelihood of remission in patients with ERA. The lack of correlation between the serum biomarkers measured and outputs from the MDM bioassay may relate to the difficulty in measuring cytokines and other analytes in serum and plasma due to the effect of diurnal variation and storage conditions. It may also reflect the artificial nature of the in vitro bioassay used to study MDMs. For certain biomarkers tested such as MMP3 and Dkk1, it may be due to low serum sample numbers available for testing from those who also had samples analysed as part of the in vitro bioassay. However, the study of these biomarkers reinforced some of the findings from the MDM assay identifying potential differences between HLA B27 positive and negative patients and male and female patients which prompted the pilot study investigating the effect of oestrogen on cytokine production. It is not possible to draw definite conclusions from the results of this small study which indicate potential differences in key IL23/17 related cytokine production with the addition of oestrogen to the assay. However, with important implications for treatment strategies, this work will be developed further following the completion of this thesis.

CHAPTER 8: OVERALL FINDINGS AND FUTURE WORK

Overview of Chapter 8

This chapter provides a summary of the findings from this thesis. Firstly, the results of the bioassay for LPS-stimulated MDMs are discussed, highlighting the important clinical factors which were associated with higher levels of pro-inflammatory cytokine production. Secondly, the findings from the bioassay on induction of the UPR are described. This is followed by a summary of the results from the analysis of certain biomarkers in patients with ERA and of the pilot study investigating the effect of the addition of oestrogen to the bioassay.

The final part of this chapter will describe the potential future directions for the work described in this thesis.

8.1 Overall findings

ERA is a subtype of JIA with distinct clinical features compared to other subtypes, sharing many similarities with adult SpA. The peak age of onset is early adolescence and differences in clinical presentation between the adult and paediatric population may relate to differences in the immune system as well as body composition and skeletal maturation. ERA is an under researched subtype of JIA and studies comparing the immune response between adult SpA and ERA are scarce.

The aim of this thesis was to investigate the biology of IL23 and other pro-inflammatory cytokines from MDMs in patients with ERA. IL23 was chosen because of the importance of the IL23/IL17 axis in the pathogenesis of SpA and in particular because IL23 has a key role in enthesitis (Sherlock, Joyce-Shaikh et al. 2012, Bridgewood, Sharif et al. 2020). Initial findings showed that MDMs from patients with active enthesitis exhibited an enhanced pro-inflammatory response with LPS stimulation, demonstrated by measuring phosphorylation of p38 MAPK. Subsequently, IL23 production was also shown to be elevated in LPS-stimulated MDMs from patients with active enthesitis compared to those without enthesitis and healthy controls. In fact, IL23 production was significantly higher in LPS-stimulated MDMs from all patient groups with inflammatory arthritis compared to age-matched healthy controls. This confirmed the hypothesis that IL23 gene expression and production would be similar in patients with ERA and AS and higher than healthy controls. In ERA, two other subgroups of patients exhibited significantly higher levels of IL23 production: those who were positive for HLA B27 and males. This was also the case for male patients with AS. The gene expression of the IL23p19 subunit was also higher in male patients with ERA compared to male healthy controls and trends were seen towards higher expression in those who were HLA B27 positive in both LPS-stimulated MDMs and SFMCs. The association of higher IL23 with these two subgroups was interesting given that HLA B27 and male sex are both associated a worse prognosis in ERA and AS. Male patients with ERA are more likely to develop sacroiliitis and males with AS exhibit worse radiographic progression of axial disease (Flato, Hoffmann-Vold et al. 2006, de Carvalho, Bortoluzzo et al. 2012, Landi, Maldonado-Ficco et al. 2016, Webers, Essers et al. 2016). HLA B27 is associated with a higher chance of developing inflammatory back pain and a lower chance of achieving drug free remission in patients with ERA and with more severe radiographic changes in patients with AS (Bennett, McGonagle et al. 2008, Berntson, Damgard et al. 2008, Berntson, Nordal et al. 2013).

Another interesting finding was that treatment with TNF inhibitors appeared to significantly influence IL23 cytokine production and gene expression from LPS-stimulated MDMs for both patients with ERA and AS with increased levels suggesting a paradoxical upregulation of the IL23/IL17 pathway as shown in other studies (Zakharova and Ziegler 2005, Notley, Inglis et al. 2008). This may form part of the mechanism for radiographic progression despite treatment with TNF inhibitors which is seen in patients with ERA and AS (van der Heijde, Landewe et al. 2008, Hugle, Burgos-Vargas et al. 2014). Higher levels of IL12 were also noted

from LPS-stimulated MDMs in patients with ERA on TNF inhibitors and correlated strongly with IL23, as did levels of IL27. Both were higher in those patients with enthesitis and who were HLA B27 positive.

Further analysis of other pro-inflammatory cytokines from LPS-stimulated MDMs in patients with ERA revealed similar associations of higher levels with HLA B27, enthesitis and male sex, in particular for TNFα, IL1β and GMCSF. This confirmed the hypothesis that pro-inflammatory cytokine gene expression and production at the protein level would be similar in patients with ERA and AS and higher than healthy controls. The findings were consistent with previous studies in AS which have shown higher levels of some pro-inflammatory cytokines in male patients compared to female patients (Gracey, Yao et al. 2016) (Huang, Tso et al. 2012). In general, strong correlations were found between these cytokines and IL23. GMCSF, in particular, correlated strongly with IL23, IL12 and IL1β across all patient and healthy control groups. This was interesting because of the role of GMCSF in promoting a pro-inflammatory macrophage phenotype thus creating a positive feedback loop and further driving pro-inflammatory cytokine production, especially IL23. Another cytokine likely to contribute to the pathogenic role of IL23 was IFNγ which was produced at lower levels in LPS-stimulated MDMs from patients with ERA and to a lesser extent AS, compared to healthy controls and patients with polyarticular JIA. A reverse IFNγ signature has previously been reported in macrophages from patients with AS (Smith, Barnes et al. 2008). IFNγ has an important role in the inhibition of IL23-driven Th17 cell development (Harrington, Hatton et al. 2005, Lee, Lee et al. 2013) and therefore lower levels would promote the pathogenicity of this pathway. The distinction between ERA, AS and polyarticular JIA in this study may provide one explanation as to why the IL23/IL17 axis is so important in the pathogenesis of SpA compared to other forms if inflammatory arthritis but this needs further study.

The mechanism of association between HLA B27 and both adult SpA and ERA has not been fully explained. In this thesis, one potential mechanism - the induction of the UPR - was investigated but there was no evidence of upregulation of UPR markers in patients with ERA or AS compared to healthy controls, suggesting that the UPR may not have a significant role in pathogenesis. This was at odds with the hypothesis for this thesis that UPR markers would be higher in patients with ERA and AS but is consistent with some other studies in adult SpA (Zeng, Lindstrom et al. 2011, Neerinckx, Carter et al. 2014). In addition, and contrary to the hypothesis for chapter 6, UPR induction did not enhance cytokine production from MDMs as expected but reduced levels in most cases, with the exception of GMCSF. Despite the lower levels of cytokine production and gene expression from MDMs stimulated with both LPS and TM, similar patterns were seen compared to MDMs stimulated with LPS alone, including higher levels of IL23, IL27, TNFα and IL1β in patients with ERA compared to healthy controls and associations with enthesitis and HLA B27 in particular. In contrast to other cytokines, GMCSF production from LPS+TM-stimulated MDMs compared to MDMs stimulated by LPS alone was significantly higher across all study groups but especially in MDMs from patients

with ERA and AS and again a positive association was noted with enthesitis and to a lesser extent HLA B27. GMCSF has been shown to be upregulated by the UPR in animal models (Ying, Li et al. 2018) and, as a potential therapeutic target in SpA, this result warrants further study.

The identification of biomarkers to aid diagnosis, predict the development of axial disease and prognosis is invaluable in patients with ERA. The final results chapter in this thesis analyses a number of biomarkers in patients with ERA. CRP, calprotectin and Dkk1 were all higher in patients with ERA compared to healthy controls, thus proving the first hypothesis for this chapter. Higher serum levels of some biomarkers were noted in male patients compared to female patients, in particular with CRP and MMP3 which was consistent with studies in adult SpA (Huang, Tso et al. 2012, Gracey, Yao et al. 2016). The presence of HLA B27 was associated with higher levels of MMP3 and Dkk1. Dkk1 production is driven by TNFα (Yeremenko, Zwerina et al. 2015) and the finding of lower levels of Dkk1 levels in patients with evidence of SIJ fusion (who had longer disease duration and were mostly treated with TNF inhibitors) aligned with previous studies in animal models suggesting that blockade of Dkk1 was associated with SIJ fusion (Uderhardt, Diarra et al. 2010). This, and the paradoxical upregulation of the IL23/IL17 pathway seen with TNF inhibitors are therefore likely to contribute to radiographic progression in patients with SpA despite treatment. The differences between male and female patients were explored further in a pilot study adding oestrogen to the in vitro bioassay. The findings included a median decease in IL23 production following the addition of oestrogen in the SpA group and a trend towards increased IFNγ production. These results may explain some of the differences seen between males and females with both ERA and adult SpA but need further investigation with larger numbers of patients.

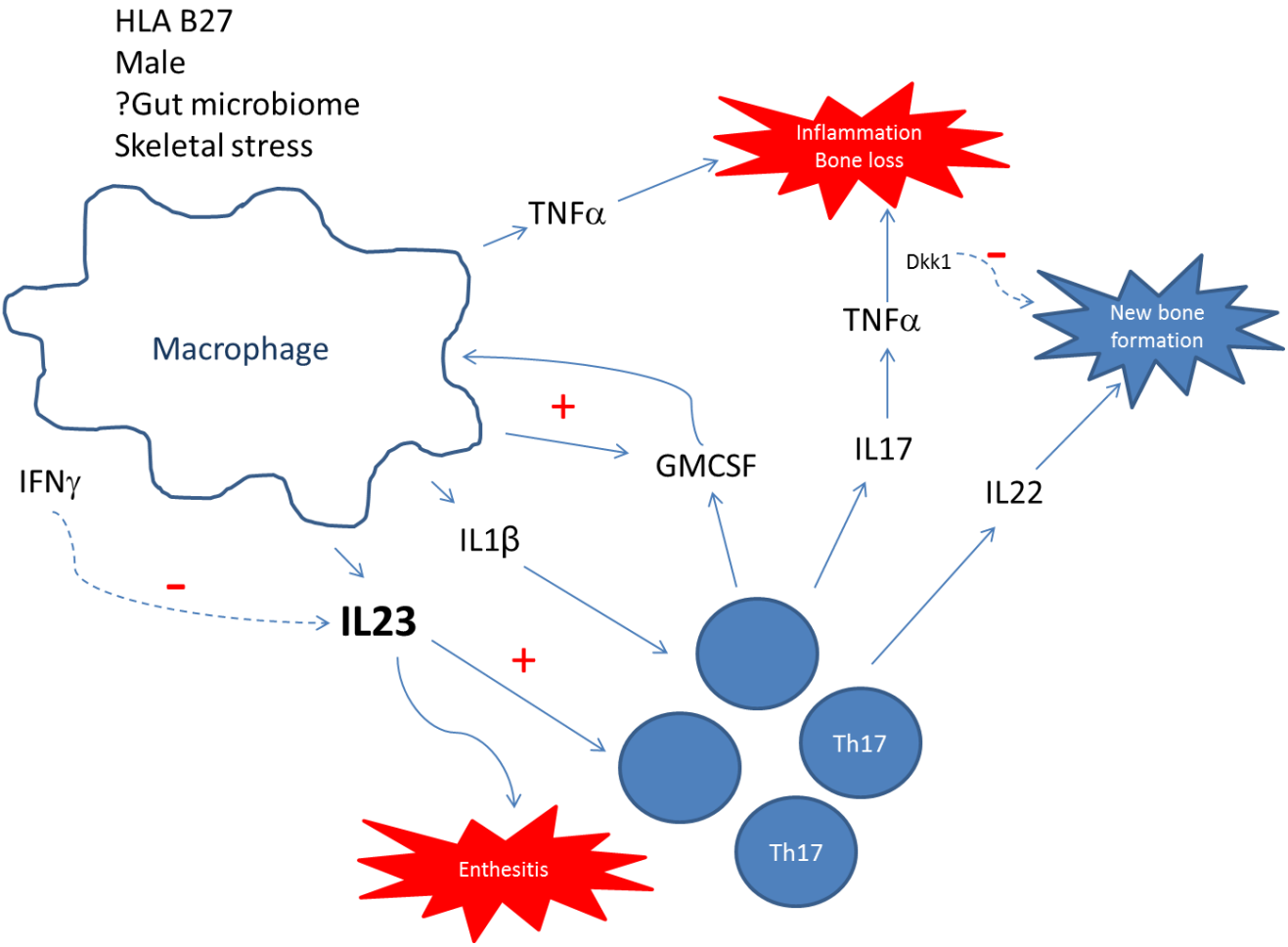


Figure 8.1 Summary of the theory of the pathogenesis of ERA from the findings in this thesis. MDMs from patients with ERA produced higher levels of IL23 and other pro-inflammatory cytokines including TNFα, IL1β and GMCSF compared to healthy controls, but lower levels of IFNγ. This may be influenced by HLA B27 and sex. Upregulation of the IL23/17 axis without the modulating effect of IFNγ and with further enhancement from the positive feedback of GMCSF, results in enthesitis, arthritis and subsequently axial arthritis, perhaps influenced by changes in the gut microbiome and body composition with growth and development.

8.2 Conclusions and future work

The results of this thesis contribute significantly to the knowledge of the pathogenesis of ERA and show many similarities between ERA and AS in the in vitro bioassay. In future work, increased sample numbers and better characterisation of patients in the AS group would help to confirm this and therefore to determine whether novel treatments for AS should also be effective for patients with ERA. In addition, although similarities were noted between the results for patients with polyarticular JIA and ERA, important differences such as the production of IFNγ were also noted. Increased sample numbers and reduced heterogeneity in the polyarticular JIA group would help to confirm these findings which may in part explain the differences in pathogenesis and clinical features between these JIA subtypes.

Using the in vitro bioassay, future experiments could designed to investigate novel therapeutic strategies in patients with ERA, such as the addition of an IL23p19 inhibitor to the assay to measure the effect on IL23 production from MDMs, especially in those groups with higher IL23 (including those with enthesitis, HLA B27 positive and male patients). Similarly the addition of a GMCSF inhibitor or IFNγ to the MDM bioassay would provide an insight in to the potential mechanisms and effects in vivo. Further investigation into the mechanism by which treatment with TNF inhibitors causes an increase in IL23 production and into the positive feedback loop between GMCSF and IL23 could also be explored using the bioassay. Co-culture experiments of MDMs from patients with ERA with T cells were considered during this thesis to investigate the effect on IL17 production and would be an important part of future work. In addition, validation of the findings from this thesis in other cell types such as dendritic cells and synovial fibroblasts would be extremely valuable in informing novel therapeutic options.

In the clinical setting, there were key features, such as enthesitis, HLA B27 and male sex, which were associated with higher levels of pro-inflammatory cytokine production and certain biomarkers. If these findings were confirmed in an ex vivo setting, then stratification of patients based on these factors may be possible which would facilitate earlier or different treatment strategies. In particular, the association of enthesitis and higher levels of IL23 production may warrant the study of a potential early window of opportunity to treat with IL23 blockade in some patients with ERA.

Future work to investigate some of the potential pathogenic mechanisms identified by the results in this thesis is important. This would include further study of the interaction between treatment with TNF inhibition, the possible paradoxical upregulation of the IL23/IL17 axis and the link with radiographic progression. Further study of Dkk1 in relation to this would also be important to explore the mechanism of new bone formation in SpA and therefore potiential therapeutic targets for this. The strong correlation between GMCSF and IL23 in the in vitro bioassay and its role in promoting a pro-inflammatory macrophage phenotype identifies it as a potential therapeutic target in patients with ERA which warrants

further investigation and potentially therapeutic clinical trials. The significance of the enhanced production of GMCSF with induction of the UPR is unclear in the context of ERA given that the UPR does not appear to differ between patients and healthy controls in this study but may have implications for other diseases. The mechanism for this warrants further study.

IFNγ production from MDMs in this assay did not correlate with other pro-inflammatory cytokines studied suggesting a different mechanism of production. In view of its inhibitory role on the development of pathogenic Th17 cells, and the lower levels in patients with ERA and AS compared to healthy controls and those patients with polyarticular JIA, it would be particularly important to investigate this further to determine whether these differences explain the importance of the IL23/IL17 axis in SpA compared to other forms of inflammatory arthritis.

As well as decreasing IL23 production, the addition of oestrogen to the in vitro bioassay appeared to increase IFNγ production which may explain some of the differences between the findings in males and females for ERA and SpA but the numbers were small and would certainly need expanding to draw any conclusions from these results.

The identification of reliable biomarkers in adult SpA has proved challenging but those identified in this study for patients with ERA warrant further investigation. Longitudinal study would be particularly important to identify those that could help with diagnosis but especially prognosis and the development of axial disease which would help to stratify treatment.

The study of other pathogenic mechanisms investigating the role of HLA B27 and SpA is important, not just for ERA, but also AS and other HLA B27-associated conditions. The gut microbiome, which appears to be significantly affected by the presence of HLA B27 (Lin, Bach et al. 2014, Asquith, Davin et al. 2017, Asquith, Sternes et al. 2019), would be of particular interest given the differences across the ages and sexes which might influence clinical presentation of disease (Bartlett, Schleifer et al. 1998, Yurkovetskiy, Burrows et al. 2013).

In conclusion, this thesis significantly adds to the knowledge of the pathogenesis of ERA. Further work is important to validate the findings and investigate the pathogenic mechanisms discussed above. To that end, future studies encompassing the whole age spectrum of SpA are crucial to further the understanding of the pathogenesis of both ERA and adult SpA.

Bibliography

Abdel-Haq, R., J. C. M. Schlachetzki, C. K. Glass and S. K. Mazmanian (2019). "Microbiome-microglia connections via the gut-brain axis." J Exp Med **216**(1): 41-59.

Abdollahi-Roodsaz, S., L. A. Joosten, M. F. Roelofs, T. R. Radstake, G. Matera, C. Popa, J. W. van der Meer, M. G. Netea and W. B. van den Berg (2007). "Inhibition of Toll-like receptor 4 breaks the inflammatory loop in autoimmune destructive arthritis." Arthritis Rheum **56**(9): 2957-2967.

Abel, A. M., C. Yang, M. S. Thakar and S. Malarkannan (2018). "Natural Killer Cells: Development, Maturation, and Clinical Utilization." Front Immunol **9**: 1869.

Abildtrup, M., G. H. Kingsley and D. L. Scott (2015). "Calprotectin as a biomarker for rheumatoid arthritis: a systematic review." J Rheumatol **42**(5): 760-770.

Adorini, L. (1999). "Interleukin-12, a key cytokine in Th1-mediated autoimmune diseases." Cell Mol Life Sci **55**(12): 1610-1625.

Agarwal, P., A. Raghavan, S. L. Nandiwada, J. M. Curtsinger, P. R. Bohjanen, D. L. Mueller and M. F. Mescher (2009). "Gene regulation and chromatin remodeling by IL-12 and type I IFN in programming for CD8 T cell effector function and memory." J Immunol **183**(3): 1695-1704.

Agere, S. A., N. Akhtar, J. M. Watson and S. Ahmed (2017). "RANTES/CCL5 Induces Collagen Degradation by Activating MMP-1 and MMP-13 Expression in Human Rheumatoid Arthritis Synovial Fibroblasts." Front Immunol **8**: 1341.

Aggarwal, A., A. N. Sarangi, P. Gaur, A. Shukla and R. Aggarwal (2017). "Gut microbiome in children with enthesitis-related arthritis in a developing country and the effect of probiotic administration." Clin Exp Immunol **187**(3): 480-489.

Aggarwal, A., R. Srivastava, S. Singh and P. Kumar Dubey (2012). "IL1 gene polymorphisms in enthesitis related arthritis category of juvenile idiopathic arthritis (ERA-JIA)." Clin Rheumatol **31**(4): 607-611.

Aissaoui, N., S. Rostom, J. Hakkou, K. Berrada Ghziouel, R. Bahiri, R. Abouqal and N. Hajjaj-Hassouni (2012). "Fatigue in patients with ankylosing spondylitis: prevalence and relationships with disease-specific variables, psychological status, and sleep disturbance." Rheumatol Int **32**(7): 2117-2124.

Ajrawat, P., Z. Touma, I. Sari, C. Taheri, J. P. Diaz Martinez and N. Haroon (2020). "Effect of TNF-inhibitor therapy on spinal structural progression in ankylosing spondylitis patients: A systematic review and meta-analysis." Int J Rheum Dis **23**(6): 728-743.

Al-Mossawi, M. H., L. Chen, H. Fang, A. Ridley, J. de Wit, N. Yager, A. Hammitzsch, I. Pulyakhina, B. P. Fairfax, D. Simone, Y. Yi, S. Bandyopadhyay, K. Doig, R. Gundle, B. Kendrick, F. Powrie, J. C. Knight and P. Bowness (2017). "Unique transcriptome signatures and GM-CSF expression in lymphocytes from patients with spondyloarthritis." Nat Commun **8**(1): 1510.

Allen, R. L., C. A. O'Callaghan, A. J. McMichael and P. Bowness (1999). "Cutting edge: HLA-B27 can form a novel beta 2-microglobulin-free heavy chain homodimer structure." J Immunol **162**(9): 5045-5048.

Almodovar, R., V. Rios, S. Ocana, M. Gobbo, M. L. Casas, P. Zarco-Montejo and X. Juanola (2014). "Association of biomarkers of inflammation, cartilage and bone turnover with gender, disease activity, radiological damage and sacroiliitis by magnetic resonance imaging in patients with early spondyloarthritis." Clin Rheumatol **33**(2): 237-241.

Alvarez-Navarro, C. and J. A. Lopez de Castro (2014). "ERAP1 structure, function and pathogenetic role in ankylosing spondylitis and other MHC-associated diseases." Mol Immunol **57**(1): 12-21.

Alvaro-Gracia, J. M., C. Yu, N. J. Zvaifler and G. S. Firestein (1993). "Mutual antagonism between interferon-gamma and tumor necrosis factor-alpha on fibroblast-like synoviocytes: paradoxical induction of IFN-gamma and TNF-alpha receptor expression." J Clin Immunol **13**(3): 212-218.

Alzabin, S., S. M. Abraham, T. E. Taher, A. Palfreeman, D. Hull, K. McNamee, A. Jawad, E. Pathan, A. Kinderlerer, P. C. Taylor, R. Williams and R. Mageed (2012). "Incomplete response of inflammatory

arthritis to TNFalpha blockade is associated with the Th17 pathway." Ann Rheum Dis **71**(10): 1741-1748.
Ambarus, C. A., S. Krausz, M. van Eijk, J. Hamann, T. R. Radstake, K. A. Reedquist, P. P. Tak and D. L. Baeten (2012). "Systematic validation of specific phenotypic markers for in vitro polarized human macrophages." J Immunol Methods **375**(1-2): 196-206.
Ambarus, C. A., N. Yeremenko and D. L. Baeten (2018). "Altered cytokine expression by macrophages from HLA-B27-positive spondyloarthritis patients without evidence of endoplasmic reticulum stress." Rheumatol Adv Pract **2**(1): rky014.
Amor, B., M. Dougados and M. Mijiyawa (1990). "[Criteria of the classification of spondylarthropathies]." Rev Rhum Mal Osteoartic **57**(2): 85-89.
Amor, B., R. S. Santos, R. Nahal, V. Listrat and M. Dougados (1994). "Predictive factors for the longterm outcome of spondyloarthropathies." J Rheumatol **21**(10): 1883-1887.
Andersen, T., R. D. Ostgard, M. P. Aspari, T. W. Kragstrup, H. Glerup, A. G. Jurik, C. Johansen, M. Hvid and B. W. Deleuran (2019). "Anti-tumor necrosis factor treatment increases both the Th17 and Th22 T helper subsets in spondyloarthritis." APMIS **127**(12): 789-796.
Andersen, T., T. K. Rasmussen, M. Hvid, C. K. Holm, K. J. Madsen, A. G. Jurik, M. Hokland, K. Stengaard-Pedersen, B. Schiottz-Christensen and B. Deleuran (2012). "Increased plasma levels of IL-21 and IL-23 in spondyloarthritis are not associated with clinical and MRI findings." Rheumatol Int **32**(2): 387-393.
Anink, J., L. W. Van Suijlekom-Smit, M. H. Otten, F. H. Prince, M. A. van Rossum, K. M. Dolman, E. P. Hoppenreijs, R. ten Cate, S. Ursu, L. R. Wedderburn, G. Horneff, M. Frosch, T. Vogl, F. Gohar, D. Foell, J. Roth and D. Holzinger (2015). "MRP8/14 serum levels as a predictor of response to starting and stopping anti-TNF treatment in juvenile idiopathic arthritis." Arthritis Res Ther **17**: 200.
Annunziato, F., L. Cosmi, F. Liotta, E. Maggi and S. Romagnani (2008). "The phenotype of human Th17 cells and their precursors, the cytokines that mediate their differentiation and the role of Th17 cells in inflammation." Int Immunol **20**(11): 1361-1368.
Antoniou, A. N., S. J. Powis and T. Elliott (2003). "Assembly and export of MHC class I peptide ligands." Curr Opin Immunol **15**(1): 75-81.
Appel, H., W. Kuon, M. Kuhne, P. Wu, S. Kuhlmann, S. Kollnberger, A. Thiel, P. Bowness and J. Sieper (2004). "Use of HLA-B27 tetramers to identify low-frequency antigen-specific T cells in Chlamydia-triggered reactive arthritis." Arthritis Res Ther **6**(6): R521-534.
Appel, H., R. Maier, J. Bleil, A. Hempfing, C. Loddenkemper, U. Schlichting, U. Syrbe and J. Sieper (2013). "In situ analysis of interleukin-23- and interleukin-12-positive cells in the spine of patients with ankylosing spondylitis." Arthritis Rheum **65**(6): 1522-1529.
Appel, H., R. Maier, P. Wu, R. Scheer, A. Hempfing, R. Kayser, A. Thiel, A. Radbruch, C. Loddenkemper and J. Sieper (2011). "Analysis of IL-17(+) cells in facet joints of patients with spondyloarthritis suggests that the innate immune pathway might be of greater relevance than the Th17-mediated adaptive immune response." Arthritis Res Ther **13**(3): R95.
Arango Duque, G. and A. Descoteaux (2014). "Macrophage cytokines: involvement in immunity and infectious diseases." Front Immunol **5**: 491.
Araujo, E. G., M. Englbrecht, S. Hoepken, S. Finzel, E. Kampylafka, A. Kleyer, S. Bayat, V. Schoenau, A. Hueber, J. Rech and G. Schett (2019). "Effects of ustekinumab versus tumor necrosis factor inhibition on enthesitis: Results from the enthesial clearance in psoriatic arthritis (ECLIPSA) study." Semin Arthritis Rheum **48**(4): 632-637.
Arends, S., E. Brouwer, E. van der Veer, H. Groen, M. K. Leijsma, P. M. Houtman, A. J. T. L. Th, C. G. Kallenberg and A. Spoorenberg (2011). "Baseline predictors of response and discontinuation of tumor necrosis factor-alpha blocking therapy in ankylosing spondylitis: a prospective longitudinal observational cohort study." Arthritis Res Ther **13**(3): R94.
Arends, S., E. van der Veer, H. Groen, P. M. Houtman, T. L. Jansen, M. K. Leijsma, J. Bijzet, P. C. Limburg, C. G. Kallenberg, A. Spoorenberg and E. Brouwer (2011). "Serum MMP-3 level as a

biomarker for monitoring and predicting response to etanercept treatment in ankylosing spondylitis." J Rheumatol **38**(8): 1644-1650.
Arevalo, M., J. Gratacos Masmitja, M. Moreno, J. Calvet, C. Orellana, D. Ruiz, C. Castro, P. Carreto, M. Larrosa, E. Collantes, P. Font and R. group (2018). "Influence of HLA-B27 on the Ankylosing Spondylitis phenotype: results from the REGISPONSER database." Arthritis Res Ther **20**(1): 221.
Arthur, J. S. and S. C. Ley (2013). "Mitogen-activated protein kinases in innate immunity." Nat Rev Immunol **13**(9): 679-692.
Asquith, M., S. Davin, P. Stauffer, C. Michell, C. Janowitz, P. Lin, J. Ensign-Lewis, J. M. Kinchen, D. R. Koop and J. T. Rosenbaum (2017). "Intestinal Metabolites Are Profoundly Altered in the Context of HLA-B27 Expression and Functionally Modulate Disease in a Rat Model of Spondyloarthritis." Arthritis Rheumatol **69**(10): 1984-1995.
Asquith, M., P. R. Sternes, M. E. Costello, L. Karstens, S. Diamond, T. M. Martin, Z. Li, M. S. Marshall, T. D. Spector, K. A. le Cao, J. T. Rosenbaum and M. A. Brown (2019). "HLA Alleles Associated With Risk of Ankylosing Spondylitis and Rheumatoid Arthritis Influence the Gut Microbiome." Arthritis Rheumatol **71**(10): 1642-1650.
Assassi, S., J. D. Reveille, F. C. Arnett, M. H. Weisman, M. M. Ward, S. K. Agarwal, P. Gourh, J. Bhula, R. Sharif, K. Sampat, M. D. Mayes and F. K. Tan (2011). "Whole-blood gene expression profiling in ankylosing spondylitis shows upregulation of toll-like receptor 4 and 5." J Rheumatol **38**(1): 87-98.
Atsumi, T., K. Ishihara, D. Kamimura, H. Ikushima, T. Ohtani, S. Hirota, H. Kobayashi, S. J. Park, Y. Saeki, Y. Kitamura and T. Hirano (2002). "A point mutation of Tyr-759 in interleukin 6 family cytokine receptor subunit gp130 causes autoimmune arthritis." J Exp Med **196**(7): 979-990.
Aziz, N. (2015). "Measurement of Circulating Cytokines and Immune-Activation Markers by Multiplex Technology in the Clinical Setting: What Are We Really Measuring?" For Immunopathol Dis Therap **6**(1-2): 19-22.
Baeten, D., X. Baraliakos, J. Braun, J. Sieper, P. Emery, D. van der Heijde, I. McInnes, J. M. van Laar, R. Landewe, P. Wordsworth, J. Wollenhaupt, H. Kellner, J. Paramarta, J. Wei, A. Brachat, S. Bek, D. Laurent, Y. Li, Y. A. Wang, A. P. Bertolino, S. Gsteiger, A. M. Wright and W. Hueber (2013). "Anti-interleukin-17A monoclonal antibody secukinumab in treatment of ankylosing spondylitis: a randomised, double-blind, placebo-controlled trial." Lancet **382**(9906): 1705-1713.
Baeten, D., P. Demetter, C. A. Cuvelier, E. Kruithof, N. Van Damme, M. De Vos, E. M. Veys and F. De Keyser (2002). "Macrophages expressing the scavenger receptor CD163: a link between immune alterations of the gut and synovial inflammation in spondyloarthropathy." J Pathol **196**(3): 343-350.
Baeten, D., E. Kruithof, F. Van den Bosch, P. Demetter, N. Van Damme, C. Cuvelier, M. De Vos, H. Mielants, E. M. Veys and F. De Keyser (2001). "Immunomodulatory effects of anti-tumor necrosis factor alpha therapy on synovium in spondylarthropathy: histologic findings in eight patients from an open-label pilot study." Arthritis Rheum **44**(1): 186-195.
Baeten, D., M. Ostergaard, J. C. Wei, J. Sieper, P. Jarvinen, L. S. Tam, C. Salvarani, T. H. Kim, A. Solinger, Y. Datsenko, C. Pamulapati, S. Visvanathan, D. B. Hall, S. Aslanyan, P. Scholl and S. J. Padula (2018). "Risankizumab, an IL-23 inhibitor, for ankylosing spondylitis: results of a randomised, double-blind, placebo-controlled, proof-of-concept, dose-finding phase 2 study." Ann Rheum Dis.
Baeten, D., J. Sieper, J. Braun, X. Baraliakos, M. Dougados, P. Emery, A. Deodhar, B. Porter, R. Martin, M. Andersson, S. Mpofu, H. B. Richards, M. S. Group and M. S. Group (2015). "Secukinumab, an Interleukin-17A Inhibitor, in Ankylosing Spondylitis." N Engl J Med **373**(26): 2534-2548.
Bal, A., E. Unlu, G. Bahar, E. Aydog, E. Eksioglu and R. Yorgancioglu (2007). "Comparison of serum IL-1 beta, sIL-2R, IL-6, and TNF-alpha levels with disease activity parameters in ankylosing spondylitis." Clin Rheumatol **26**(2): 211-215.
Baraliakos, X., J. Braun, A. Deodhar, D. Poddubnyy, A. Kivitz, H. Tahir, F. Van den Bosch, E. M. Delicha, Z. Talloczy and A. Fierlinger (2019). "Long-term efficacy and safety of secukinumab 150 mg in ankylosing spondylitis: 5-year results from the phase III MEASURE 1 extension study." RMD Open **5**(2): e001005.

Baraliakos, X., U. Kiltz, S. Peters, H. Appel, F. Dybowski, M. Igelmann, L. Kalthoff, D. Krause, H. J. Menne, E. Saracbasi-Zender, E. Schmitz-Bortz, M. Vigneswaran and J. Braun (2017). "Efficiency of treatment with non-steroidal anti-inflammatory drugs according to current recommendations in patients with radiographic and non-radiographic axial spondyloarthritis." Rheumatology (Oxford) **56**(1): 95-102.
Baraliakos, X., J. Listing, J. Brandt, A. Zink, R. Alten, G. Burmester, E. Gromnica-Ihle, H. Kellner, M. Schneider, H. Sorensen, H. Zeidler, M. Rudwaleit, J. Sieper and J. Braun (2005). "Clinical response to discontinuation of anti-TNF therapy in patients with ankylosing spondylitis after 3 years of continuous treatment with infliximab." Arthritis Res Ther **7**(3): R439-444.
Baraliakos, X., R. van den Berg, J. Braun and D. van der Heijde (2012). "Update of the literature review on treatment with biologics as a basis for the first update of the ASAS/EULAR management recommendations of ankylosing spondylitis." Rheumatology (Oxford) **51**(8): 1378-1387.
Barkham, N., H. I. Keen, L. C. Coates, P. O'Connor, E. Hensor, A. D. Fraser, L. S. Cawkwell, A. Bennett, D. McGonagle and P. Emery (2009). "Clinical and imaging efficacy of infliximab in HLA-B27-Positive patients with magnetic resonance imaging-determined early sacroiliitis." Arthritis Rheum **60**(4): 946-954.
Barkhuizen, A., S. Steinfeld, J. Robbins, C. West, J. Coombs and S. Zwillich (2006). "Celecoxib is efficacious and well tolerated in treating signs and symptoms of ankylosing spondylitis." J Rheumatol **33**(9): 1805-1812.
Barros, M. H., F. Hauck, J. H. Dreyer, B. Kempkes and G. Niedobitek (2013). "Macrophage polarisation: an immunohistochemical approach for identifying M1 and M2 macrophages." PLoS One **8**(11): e80908.
Barry, M. and R. C. Bleackley (2002). "Cytotoxic T lymphocytes: all roads lead to death." Nat Rev Immunol **2**(6): 401-409.
Bartlett, J. A., S. J. Schleifer, M. K. Demetrikopoulos, B. R. Delaney, S. C. Shiflett and S. E. Keller (1998). "Immune function in healthy adolescents." Clin Diagn Lab Immunol **5**(1): 105-113.
Bay-Jensen, A. C., S. Wichuk, I. Byrjalsen, D. J. Leeming, N. Morency, C. Christiansen, M. A. Karsdal and W. P. Maksymowych (2013). "Circulating protein fragments of cartilage and connective tissue degradation are diagnostic and prognostic markers of rheumatoid arthritis and ankylosing spondylitis." PLoS One **8**(1): e54504.
Benham, H., L. M. Rehaume, S. Z. Hasnain, J. Velasco, A. C. Baillet, M. Ruutu, K. Kikly, R. Wang, H. W. Tseng, G. P. Thomas, M. A. Brown, G. Strutton, M. A. McGuckin and R. Thomas (2014). "Interleukin-23 mediates the intestinal response to microbial beta-1,3-glucan and the development of spondyloarthritis pathology in SKG mice." Arthritis Rheumatol **66**(7): 1755-1767.
Benjamin, M. and D. McGonagle (2001). "The anatomical basis for disease localisation in seronegative spondyloarthropathy at entheses and related sites." J Anat **199**(Pt 5): 503-526.
Benjamin, R. and P. Parham (1990). "Guilt by association: HLA-B27 and ankylosing spondylitis." Immunol Today **11**(4): 137-142.
Bennett, A. N., D. McGonagle, P. O'Connor, E. M. Hensor, F. Sivera, L. C. Coates, P. Emery and H. Marzo-Ortega (2008). "Severity of baseline magnetic resonance imaging-evident sacroiliitis and HLA-B27 status in early inflammatory back pain predict radiographically evident ankylosing spondylitis at eight years." Arthritis Rheum **58**(11): 3413-3418.
Berntson, L., M. Damgard, B. Andersson-Gare, T. Herlin, S. Nielsen, E. Nordal, M. Rygg, M. Zak, A. Fasth and G. Nordic Paediatric Rheumatology Study (2008). "HLA-B27 predicts a more extended disease with increasing age at onset in boys with juvenile idiopathic arthritis." J Rheumatol **35**(10): 2055-2061.
Berntson, L., E. Nordal, K. Aalto, S. Peltoniemi, T. Herlin, M. Zak, S. Nielsen and M. Rygg (2013). "HLA-B27 predicts a more chronic disease course in an 8-year followup cohort of patients with juvenile idiopathic arthritis." J Rheumatol **40**(5): 725-731.

Berntson, L., E. Nordal, K. Aalto, S. Peltoniemi, T. Herlin, M. Zak, S. Nielsen, M. Rygg and R. Nordic Study Group of Paediatric (2013). "HLA-B27 predicts a more chronic disease course in an 8-year followup cohort of patients with juvenile idiopathic arthritis." J Rheumatol **40**(5): 725-731.
Bertolotti, A., Y. Zhang, L. M. Hendershot, H. P. Harding and D. Ron (2000). "Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response." Nat Cell Biol **2**(6): 326-332.
Bettigole, S. E., R. Lis, S. Adoro, A. H. Lee, L. A. Spencer, P. F. Weller and L. H. Glimcher (2015). "The transcription factor XBP1 is selectively required for eosinophil differentiation." Nat Immunol **16**(8): 829-837.
Beziat, V., H. G. Hilton, P. J. Norman and J. A. Traherne (2017). "Deciphering the killer-cell immunoglobulin-like receptor system at super-resolution for natural killer and T-cell biology." Immunology **150**(3): 248-264.
Bianchi, E. and L. Rogge (2019). "The IL-23/IL-17 pathway in human chronic inflammatory diseases-new insight from genetics and targeted therapies." Genes Immun **20**(5): 415-425.
Bianchi, M. E. (2007). "DAMPs, PAMPs and alarmins: all we need to know about danger." J Leukoc Biol **81**(1): 1-5.
Bird, L. A., C. A. Peh, S. Kollnberger, T. Elliott, A. J. McMichael and P. Bowness (2003). "Lymphoblastoid cells express HLA-B27 homodimers both intracellularly and at the cell surface following endosomal recycling." Eur J Immunol **33**(3): 748-759.
Boel, A., C. Lopez-Medina, D. van der Heijde and F. A. van Gaalen (2022). "Age at onset in axial spondyloarthritis around the world: data from the Assessment in SpondyloArthritis international Society Peripheral Involvement in Spondyloarthritis study." Rheumatology (Oxford) **61**(4): 1468-1475.
Boersma, J. W. (1976). "Retardation of ossification of the lumbar vertebral column in ankylosing spondylitis by means of phenylbutazone." Scand J Rheumatol **5**(1): 60-64.
Bollow, M., J. Braun, T. Biedermann, S. Mutze, S. Paris, C. Schauer-Petrowskaja, K. Minden, S. A. Schmitz, M. Schontube and B. Hamm (1998). "Use of contrast-enhanced MR imaging to detect sacroiliitis in children." Skeletal Radiol **27**(11): 606-616.
Bollow, M., T. Fischer, H. Reisshauer, M. Backhaus, J. Sieper, B. Hamm and J. Braun (2000). "Quantitative analyses of sacroiliac biopsies in spondyloarthropathies: T cells and macrophages predominate in early and active sacroiliitis- cellularity correlates with the degree of enhancement detected by magnetic resonance imaging." Ann Rheum Dis **59**(2): 135-140.
Boniface, K., W. M. Blumenschein, K. Brovont-Porth, M. J. McGeachy, B. Basham, B. Desai, R. Pierce, T. K. McClanahan, S. Sadekova and R. de Waal Malefyt (2010). "Human Th17 cells comprise heterogeneous subsets including IFN-gamma-producing cells with distinct properties from the Th1 lineage." J Immunol **185**(1): 679-687.
Boonen, A., V. Patel, S. Traina, C. F. Chiou, A. Maetzel and W. Tsuji (2008). "Rapid and sustained improvement in health-related quality of life and utility for 72 weeks in patients with ankylosing spondylitis receiving etanercept." J Rheumatol **35**(4): 662-667.
Bowness, P. (2015). "Hla-B27." Annu Rev Immunol **33**: 29-48.
Bowness, P., A. Ridley, J. Shaw, A. T. Chan, I. Wong-Baeza, M. Fleming, F. Cummings, A. McMichael and S. Kollnberger (2011). "Th17 cells expressing KIR3DL2+ and responsive to HLA-B27 homodimers are increased in ankylosing spondylitis." J Immunol **186**(4): 2672-2680.
Boyette, L. B., C. Macedo, K. Hadi, B. D. Elinoff, J. T. Walters, B. Ramaswami, G. Chalasani, J. M. Taboas, F. G. Lakkis and D. M. Metes (2017). "Phenotype, function, and differentiation potential of human monocyte subsets." PLoS One **12**(4): e0176460.
Braem, K., F. P. Luyten and R. J. Lories (2012). "Blocking p38 signalling inhibits chondrogenesis in vitro but not ankylosis in a model of ankylosing spondylitis in vivo." Ann Rheum Dis **71**(5): 722-728.
Braun, J., X. Baraliakos, B. Buehring, U. Kiltz and M. Fruth (2018). "Imaging of axial spondyloarthritis. New aspects and differential diagnoses." Clin Exp Rheumatol **36 Suppl 114**(5): 35-42.
Braun, J., X. Baraliakos, A. Deodhar, D. Baeten, J. Sieper, P. Emery, A. Readie, R. Martin, S. Mpofu, H. B. Richards and M. s. group (2017). "Effect of secukinumab on clinical and radiographic outcomes in

ankylosing spondylitis: 2-year results from the randomised phase III MEASURE 1 study." Ann Rheum Dis **76**(6): 1070-1077.
Braun, J., X. Baraliakos, A. Deodhar, D. Poddubnyy, P. Emery, E. M. Delicha, Z. Talloczy and B. Porter (2019). "Secukinumab shows sustained efficacy and low structural progression in ankylosing spondylitis: 4-year results from the MEASURE 1 study." Rheumatology (Oxford) **58**(5): 859-868.
Braun, J., M. Bollow, L. Neure, E. Seipelt, F. Seyrekbasan, H. Herbst, U. Eggens, A. Distler and J. Sieper (1995). "Use of immunohistologic and in situ hybridization techniques in the examination of sacroiliac joint biopsy specimens from patients with ankylosing spondylitis." Arthritis Rheum **38**(4): 499-505.
Braun, J., J. Zochling, X. Baraliakos, R. Alten, G. Burmester, K. Grasedyck, J. Brandt, H. Haibel, M. Hammer, A. Krause, F. Mielke, H. P. Tony, W. Ebner, B. Gomor, J. Hermann, H. Zeidler, E. Beck, M. Baumgaertner and J. Sieper (2006). "Efficacy of sulfasalazine in patients with inflammatory back pain due to undifferentiated spondyloarthritis and early ankylosing spondylitis: a multicentre randomised controlled trial." Ann Rheum Dis **65**(9): 1147-1153.
Bray, T. J., K. Vendhan, J. Roberts, D. Atkinson, S. Punwani, D. Sen, Y. Ioannou and M. A. Hall-Craggs (2016). "Association of the apparent diffusion coefficient with maturity in adolescent sacroiliac joints." J Magn Reson Imaging **44**(3): 556-564.
Bray, T. J. P., A. Lopes, C. Fisher, C. Ciurtin, D. Sen and M. A. Hall-Craggs (2019). "Sacroiliac Joint Ankylosis in Young Spondyloarthritis Patients Receiving Biologic Therapy: Observation of Serial Magnetic Resonance Imaging Scans." Arthritis Rheumatol **71**(4): 594-598.
Breban, M., J. Tap, A. Leboime, R. Said-Nahal, P. Langella, G. Chiocchia, J. P. Furet and H. Sokol (2017). "Faecal microbiota study reveals specific dysbiosis in spondyloarthritis." Ann Rheum Dis **76**(9): 1614-1622.
Bredella, M. A., L. S. Steinbach, S. Morgan, M. Ward and J. C. Davis (2006). "MRI of the sacroiliac joints in patients with moderate to severe ankylosing spondylitis." AJR Am J Roentgenol **187**(6): 1420-1426.
Brentano, F., O. Schorr, R. E. Gay, S. Gay and D. Kyburz (2005). "RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3." Arthritis Rheum **52**(9): 2656-2665.
Brewerton, D. A., F. D. Hart, A. Nicholls, M. Caffrey, D. C. James and R. D. Sturrock (1973). "Ankylosing spondylitis and HL-A 27." Lancet **1**(7809): 904-907.
Bridgewood, C., A. Alase, A. Watad, M. Wittmann, R. Cuthbert and D. McGonagle (2019). "The IL-23p19/EBI3 heterodimeric cytokine termed IL-39 remains a theoretical cytokine in man." Inflamm Res **68**(6): 423-426.
Bridgewood, C., K. Sharif, J. Sherlock, A. Watad and D. McGonagle (2020). "Interleukin-23 pathway at the enthesis: The emerging story of enthesitis in spondyloarthropathy." Immunol Rev.
Bridgewood, C., A. Watad, T. Russell, T. M. Palmer, H. Marzo-Ortega, A. Khan, P. A. Millner, R. Dunsmuir, A. Rao, P. Loughenbury, M. Wittmann, R. J. Cuthbert and D. G. McGonagle (2019). "Identification of myeloid cells in the human enthesis as the main source of local IL-23 production." Ann Rheum Dis **78**(7): 929-933.
Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch and A. Zychlinsky (2004). "Neutrophil extracellular traps kill bacteria." Science **303**(5663): 1532-1535.
Brionez, T. F., S. Assassi, J. D. Reveille, C. Green, T. Learch, L. Diekman, M. M. Ward, J. C. Davis, Jr., M. H. Weisman and P. Nicassio (2010). "Psychological correlates of self-reported disease activity in ankylosing spondylitis." J Rheumatol **37**(4): 829-834.
Brown, M. A., T. Kenna and B. P. Wordsworth (2016). "Genetics of ankylosing spondylitis--insights into pathogenesis." Nat Rev Rheumatol **12**(2): 81-91.
Brown, M. A., L. G. Kennedy, A. J. MacGregor, C. Darke, E. Duncan, J. L. Shatford, A. Taylor, A. Calin and P. Wordsworth (1997). "Susceptibility to ankylosing spondylitis in twins: the role of genes, HLA, and the environment." Arthritis Rheum **40**(10): 1823-1828.

Brown, M. A., S. H. Laval, S. Brophy and A. Calin (2000). "Recurrence risk modelling of the genetic susceptibility to ankylosing spondylitis." Ann Rheum Dis **59**(11): 883-886.
Bryan, A. R. and C. E. Rabinovich (2014). "Enthesitis-related arthritis: time to re-define?" Curr Rheumatol Rep **16**(12): 466.
Burgos-Vargas, R., S. M. Tse, G. Horneff, A. L. Pangan, J. Kalabic, S. Goss, K. Unnebrink and J. K. Anderson (2015). "A Randomized, Double-Blind, Placebo-Controlled Multicenter Study of Adalimumab in Pediatric Patients With Enthesitis-Related Arthritis." Arthritis Care Res (Hoboken) **67**(11): 1503-1512.
Burgos-Vargas, R., J. Vazquez-Mellado, C. Pacheco-Tena, A. Hernandez-Garduno and M. V. Goycochea-Robles (2002). "A 26 week randomised, double blind, placebo controlled exploratory study of sulfasalazine in juvenile onset spondyloarthropathies." Ann Rheum Dis **61**(10): 941-942.
Burmester, G. R., I. B. McInnes, J. M. Kremer, P. Miranda, J. Vencovsky, A. Godwood, M. Albulescu, M. A. Michaels, X. Guo, D. Close and M. Weinblatt (2018). "Mavrilimumab, a Fully Human Granulocyte-Macrophage Colony-Stimulating Factor Receptor alpha Monoclonal Antibody: Long-Term Safety and Efficacy in Patients With Rheumatoid Arthritis." Arthritis Rheumatol **70**(5): 679-689.
Butbul Aviel, Y., P. Tyrrell, R. Schneider, S. Dhillon, B. M. Feldman, R. Laxer, R. K. Saurenmann, L. Spiegel, B. Cameron, S. M. Tse and E. Silverman (2013). "Juvenile Psoriatic Arthritis (JPsA): juvenile arthritis with psoriasis?" Pediatr Rheumatol Online J **11**(1): 11.
Caffrey, M. F. and D. C. James (1973). "Human lymphocyte antigen association in ankylosing spondylitis." Nature **242**(5393): 121.
Calin, A., S. Garrett, H. Whitelock, L. G. Kennedy, J. O'Hea, P. Mallorie and T. Jenkinson (1994). "A new approach to defining functional ability in ankylosing spondylitis: the development of the Bath Ankylosing Spondylitis Functional Index." J Rheumatol **21**(12): 2281-2285.
Cambre, I., D. Gaublomme, A. Burssens, P. Jacques, N. Schryvers, A. De Muynck, L. Meuris, S. Lambrecht, S. Carter, P. de Bleser, Y. Saeys, L. Van Hoorebeke, G. Kollias, M. Mack, P. Simoens, R. Lories, N. Callewaert, G. Schett and D. Elewaut (2018). "Mechanical strain determines the site-specific localization of inflammation and tissue damage in arthritis." Nat Commun **9**(1): 4613.
Campbell, I. K., A. Bendele, D. A. Smith and J. A. Hamilton (1997). "Granulocyte-macrophage colony stimulating factor exacerbates collagen induced arthritis in mice." Ann Rheum Dis **56**(6): 364-368.
Campbell, I. K., M. J. Rich, R. J. Bischof, A. R. Dunn, D. Grail and J. A. Hamilton (1998). "Protection from collagen-induced arthritis in granulocyte-macrophage colony-stimulating factor-deficient mice." J Immunol **161**(7): 3639-3644.
Canete, J. D., S. E. Martinez, J. Farres, R. Sanmarti, M. Blay, A. Gomez, G. Salvador and J. Munoz-Gomez (2000). "Differential Th1/Th2 cytokine patterns in chronic arthritis: interferon gamma is highly expressed in synovium of rheumatoid arthritis compared with seronegative spondyloarthropathies." Ann Rheum Dis **59**(4): 263-268.
Cannon, G. W., S. H. Pincus, R. D. Emkey, A. Denes, S. A. Cohen, F. Wolfe, P. A. Saway, A. M. Jaffer, A. L. Weaver, L. Cogen and et al. (1989). "Double-blind trial of recombinant gamma-interferon versus placebo in the treatment of rheumatoid arthritis." Arthritis Rheum **32**(8): 964-973.
Carr, E. J., J. Dooley, J. E. Garcia-Perez, V. Lagou, J. C. Lee, C. Wouters, I. Meyts, A. Goris, G. Boeckxstaens, M. A. Linterman and A. Liston (2016). "The cellular composition of the human immune system is shaped by age and cohabitation." Nat Immunol **17**(4): 461-468.
Celojevic, D., A. Petersen, J. O. Karlsson, A. Behndig and M. Zetterberg (2011). "Effects of 17beta-estradiol on proliferation, cell viability and intracellular redox status in native human lens epithelial cells." Mol Vis **17**: 1987-1996.
Chae, M. K., S. G. Park, S. O. Song, E. S. Kang, B. S. Cha, H. C. Lee and B. W. Lee (2012). "Pentoxifylline attenuates methionine- and choline-deficient-diet-induced steatohepatitis by suppressing TNF-alpha expression and endoplasmic reticulum stress." Exp Diabetes Res **2012**: 762565.
Chamberlain, L. M., D. Holt-Casper, M. Gonzalez-Juarrero and D. W. Grainger (2015). "Extended culture of macrophages from different sources and maturation results in a common M2 phenotype." J Biomed Mater Res A **103**(9): 2864-2874.

Chan, A., A. Filer, G. Parsonage, S. Kollnberger, R. Gundle, C. D. Buckley and P. Bowness (2008). "Mediation of the proinflammatory cytokine response in rheumatoid arthritis and spondylarthritis by interactions between fibroblast-like synoviocytes and natural killer cells." Arthritis Rheum **58**(3): 707-717.
Chan, A. T., S. D. Kollnberger, L. R. Wedderburn and P. Bowness (2005). "Expansion and enhanced survival of natural killer cells expressing the killer immunoglobulin-like receptor KIR3DL2 in spondylarthritis." Arthritis Rheum **52**(11): 3586-3595.
Chauvin, N. A., V. Ho-Fung, D. Jaramillo, J. C. Edgar and P. F. Weiss (2015). "Ultrasound of the joints and entheses in healthy children." Pediatr Radiol **45**(9): 1344-1354.
Chauvin, N. A., R. Xiao, T. G. Brandon, D. M. Biko, M. Francavilla, D. Khrichenko and P. F. Weiss (2019). "MRI of the Sacroiliac Joint in Healthy Children." AJR Am J Roentgenol: 1-7.
Chavez-Galan, L., M. L. Olleros, D. Vesin and I. Garcia (2015). "Much More than M1 and M2 Macrophages, There are also CD169(+) and TCR(+) Macrophages." Front Immunol **6**: 263.
Chen, H. A., C. H. Chen, H. T. Liao, Y. J. Lin, P. C. Chen, W. S. Chen, C. C. Chien and C. T. Chou (2012). "Clinical, functional, and radiographic differences among juvenile-onset, adult-onset, and late-onset ankylosing spondylitis." J Rheumatol **39**(5): 1013-1018.
Chen, J., S. Lin and C. Liu (2014). "Sulfasalazine for ankylosing spondylitis." Cochrane Database Syst Rev(11): CD004800.
Chen, L. and D. B. Flies (2013). "Molecular mechanisms of T cell co-stimulation and co-inhibition." Nat Rev Immunol **13**(4): 227-242.
Chen, W. S., Y. S. Chang, K. C. Lin, C. C. Lai, S. H. Wang, K. H. Hsiao, H. T. Lee, M. H. Chen, C. Y. Tsai and C. T. Chou (2012). "Association of serum interleukin-17 and interleukin-23 levels with disease activity in Chinese patients with ankylosing spondylitis." J Chin Med Assoc **75**(7): 303-308.
Cheng, W. P., B. W. Wang, H. M. Lo and K. G. Shyu (2015). "Mechanical Stretch Induces Apoptosis Regulator TRB3 in Cultured Cardiomyocytes and Volume-Overloaded Heart." PLoS One **10**(4): e0123235.
Cho, I. and M. J. Blaser (2012). "The human microbiome: at the interface of health and disease." Nat Rev Genet **13**(4): 260-270.
Chowers, Y., A. Sturm, M. Sans, K. Papadakis, M. Gazouli, M. Harbord, J. Jahnel, G. J. Mantzaris, J. Meier, C. Mottet, L. Peyrin-Biroulet and M. Allez (2010). "Report of the ECCO workshop on anti-TNF therapy failures in inflammatory bowel diseases: biological roles and effects of TNF and TNF antagonists." J Crohns Colitis **4**(4): 367-376.
Chu, C. Q., D. Swart, D. Alcorn, J. Tocker and K. B. Elkon (2007). "Interferon-gamma regulates susceptibility to collagen-induced arthritis through suppression of interleukin-17." Arthritis Rheum **56**(4): 1145-1151.
Chung, H. Y., P. Machado, D. van der Heijde, M. A. D'Agostino and M. Dougados (2011). "HLA-B27 positive patients differ from HLA-B27 negative patients in clinical presentation and imaging: results from the DESIR cohort of patients with recent onset axial spondyloarthritis." Ann Rheum Dis **70**(11): 1930-1936.
Chung, Y., S. H. Chang, G. J. Martinez, X. O. Yang, R. Nurieva, H. S. Kang, L. Ma, S. S. Watowich, A. M. Jetten, Q. Tian and C. Dong (2009). "Critical regulation of early Th17 cell differentiation by interleukin-1 signaling." Immunity **30**(4): 576-587.
Ciccia, F., A. Accardo-Palumbo, R. Alessandro, A. Rizzo, S. Principe, S. Peralta, F. Raiata, A. Giardina, G. De Leo and G. Triolo (2012). "Interleukin-22 and interleukin-22-producing NKp44+ natural killer cells in subclinical gut inflammation in ankylosing spondylitis." Arthritis Rheum **64**(6): 1869-1878.
Ciccia, F., A. Accardo-Palumbo, A. Rizzo, G. Guggino, S. Raimondo, A. Giardina, A. Cannizzaro, R. A. Colbert, R. Alessandro and G. Triolo (2014). "Evidence that autophagy, but not the unfolded protein response, regulates the expression of IL-23 in the gut of patients with ankylosing spondylitis and subclinical gut inflammation." Ann Rheum Dis **73**(8): 1566-1574.
Ciccia, F., M. Bombardieri, A. Principato, A. Giardina, C. Tripodo, R. Porcasi, S. Peralta, V. Franco, E. Giardina, A. Craxi, C. Pitzalis and G. Triolo (2009). "Overexpression of interleukin-23, but not

interleukin-17, as an immunologic signature of subclinical intestinal inflammation in ankylosing spondylitis." Arthritis Rheum **60**(4): 955-965.
Ciccia, F., G. Guggino, A. Rizzo, L. Saieva, S. Peralta, A. Giardina, A. Cannizzaro, G. Sireci, G. De Leo, R. Alessandro and G. Triolo (2015). "Type 3 innate lymphoid cells producing IL-17 and IL-22 are expanded in the gut, in the peripheral blood, synovial fluid and bone marrow of patients with ankylosing spondylitis." Ann Rheum Dis **74**(9): 1739-1747.
Claushuis, T. A., M. K. de Vries, M. A. van der Weijden, I. M. Visman, M. T. Nurmohamed, J. W. Twisk, I. E. Van der Horst-Bruinsma and J. B. Crusius (2015). "C-reactive protein polymorphisms influence serum CRP-levels independent of disease activity in ankylosing spondylitis." Clin Exp Rheumatol **33**(2): 159-165.
Codarri, L., G. Gyulveszi, V. Tosevski, L. Hesske, A. Fontana, L. Magnenat, T. Suter and B. Becher (2011). "RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation." Nat Immunol **12**(6): 560-567.
Colbert, R. A. (2010). "Classification of juvenile spondyloarthritis: Enthesitis-related arthritis and beyond." Nat Rev Rheumatol **6**(8): 477-485.
Colbert, R. A., M. L. DeLay, E. I. Klenk and G. Layh-Schmitt (2010). "From HLA-B27 to spondyloarthritis: a journey through the ER." Immunol Rev **233**(1): 181-202.
Collison, L. W., C. J. Workman, T. T. Kuo, K. Boyd, Y. Wang, K. M. Vignali, R. Cross, D. Sehy, R. S. Blumberg and D. A. Vignali (2007). "The inhibitory cytokine IL-35 contributes to regulatory T-cell function." Nature **450**(7169): 566-569.
Colonna, M., H. Nakajima, F. Navarro and M. Lopez-Botet (1999). "A novel family of Ig-like receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells." J Leukoc Biol **66**(3): 375-381.
Consolaro, A., N. Ruperto, A. Bazso, A. Pistorio, S. Magni-Manzoni, G. Filocamo, C. Malattia, S. Viola, A. Martini, A. Ravelli and O. Paediatric Rheumatology International Trials (2009). "Development and validation of a composite disease activity score for juvenile idiopathic arthritis." Arthritis Rheum **61**(5): 658-666.
Constantin, T., I. Foeldvari, J. Vojinovic, G. Horneff, R. Burgos-Vargas, I. Nikishina, J. D. Akikusa, T. Avcin, J. Chaitow, E. Koskova, B. R. Lauwerys, I. Calvo Penades, B. Flato, M. L. Gamir, H. I. Huppertz, J. J. Raad, K. Jarosova, J. Anton, M. Macku, W. J. Otero Escalante, L. Rutkowska-Sak, R. Trauzeddel, P. J. Velez-Sanchez, C. Wouters, J. Wajdula, C. Zang, J. Bukowski, D. Woodworth, B. Vlahos, A. Martini and N. Ruperto (2016). "Two-year Efficacy and Safety of Etanercept in Pediatric Patients with Extended Oligoarthritis, Enthesitis-related Arthritis, or Psoriatic Arthritis." J Rheumatol **43**(4): 816-824.
Conway, R. and F. D. O'Shea (2012). "Juvenile versus adult-onset ankylosing spondylitis: are we comparing apples and oranges?" J Rheumatol **39**(5): 887-889.
Cook, A. D., E. L. Braine, I. K. Campbell, M. J. Rich and J. A. Hamilton (2001). "Blockade of collagen-induced arthritis post-onset by antibody to granulocyte-macrophage colony-stimulating factor (GM-CSF): requirement for GM-CSF in the effector phase of disease." Arthritis Res **3**(5): 293-298.
Cornelissen, F., P. S. Asmawidjaja, A. M. Mus, O. Corneth, K. Kikly and E. Lubberts (2013). "IL-23 dependent and independent stages of experimental arthritis: no clinical effect of therapeutic IL-23p19 inhibition in collagen-induced arthritis." PLoS One **8**(2): e57553.
Cornelissen, F., A. M. Mus, P. S. Asmawidjaja, J. P. van Hamburg, J. Tocker and E. Lubberts (2009). "Interleukin-23 is critical for full-blown expression of a non-autoimmune destructive arthritis and regulates interleukin-17A and RORgammat in gammadelta T cells." Arthritis Res Ther **11**(6): R194.
Cornish, A. L., I. K. Campbell, B. S. McKenzie, S. Chatfield and I. P. Wicks (2009). "G-CSF and GM-CSF as therapeutic targets in rheumatoid arthritis." Nat Rev Rheumatol **5**(10): 554-559.
Cortes, A., J. Hadler, J. P. Pointon, P. C. Robinson, T. Karaderi, P. Leo, K. Cremin, K. Pryce, J. Harris, S. Lee, K. B. Joo, S. C. Shim, M. Weisman, M. Ward, X. Zhou, H. J. Garchon, G. Chiocchia, J. Nossent, B. A. Lie, O. Forre, J. Tuomilehto, K. Laiho, L. Jiang, Y. Liu, X. Wu, L. A. Bradbury, D. Elewaut, R. Burgos-Vargas, S. Stebbings, L. Appleton, C. Farrah, J. Lau, T. J. Kenna, N. Haroon, M. A. Ferreira, J. Yang, J. Mulero, J. L. Fernandez-Sueiro, M. A. Gonzalez-Gay, C. Lopez-Larrea, P. Deloukas, P. Donnelly, P.

Bowness, K. Gafney, H. Gaston, D. D. Gladman, P. Rahman, W. P. Maksymowych, H. Xu, J. B. Crusius, I. E. van der Horst-Bruinsma, C. T. Chou, R. Valle-Onate, C. Romero-Sanchez, I. M. Hansen, F. M. Pimentel-Santos, R. D. Inman, V. Videm, J. Martin, M. Breban, J. D. Reveille, D. M. Evans, T. H. Kim, B. P. Wordsworth and M. A. Brown (2013). "Identification of multiple risk variants for ankylosing spondylitis through high-density genotyping of immune-related loci." Nat Genet **45**(7): 730-738.
Cortes, A., W. P. Maksymowych, B. P. Wordsworth, R. D. Inman, P. Danoy, P. Rahman, M. A. Stone, M. Corr, L. S. Gensler, D. Gladman, A. Morgan, H. Marzo-Ortega, M. M. Ward, Sparcc, Tasc, T. J. Learch, J. D. Reveille, M. A. Brown and M. H. Weisman (2015). "Association study of genes related to bone formation and resorption and the extent of radiographic change in ankylosing spondylitis." Ann Rheum Dis **74**(7): 1387-1393.
Costello, M. E., F. Ciccia, D. Willner, N. Warrington, P. C. Robinson, B. Gardiner, M. Marshall, T. J. Kenna, G. Triolo and M. A. Brown (2015). "Brief Report: Intestinal Dysbiosis in Ankylosing Spondylitis." Arthritis Rheumatol **67**(3): 686-691.
Coudert, P., R. Dube-Cyr, K. Chaumoitre, O. Gille, J. M. Vital, J. L. Jouve, P. J. Arnoux, M. Panuel and M. Evin (2019). "Sacroiliac joint morphologic changes from infancy to adulthood." Spine J **19**(10): 1730-1738.
Cowardin, C. A., S. A. Kuehne, E. L. Buonomo, C. S. Marie, N. P. Minton and W. A. Petri, Jr. (2015). "Inflammasome activation contributes to interleukin-23 production in response to Clostridium difficile." mBio **6**(1).
Croft, M. (2003). "Co-stimulatory members of the TNFR family: keys to effective T-cell immunity?" Nat Rev Immunol **3**(8): 609-620.
Croxford, A. L., P. Kulig and B. Becher (2014). "IL-12-and IL-23 in health and disease." Cytokine Growth Factor Rev **25**(4): 415-421.
Croxford, A. L., F. Mair and B. Becher (2012). "IL-23: one cytokine in control of autoimmunity." Eur J Immunol **42**(9): 2263-2273.
Cua, D. J., J. Sherlock, Y. Chen, C. A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S. A. Lira, D. Gorman, R. A. Kastelein and J. D. Sedgwick (2003). "Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain." Nature **421**(6924): 744-748.
Cuenda, A. and S. Rousseau (2007). "p38 MAP-kinases pathway regulation, function and role in human diseases." Biochim Biophys Acta **1773**(8): 1358-1375.
Cui, J., Y. Li, L. Zhu, D. Liu, Z. Songyang, H. Y. Wang and R. F. Wang (2012). "NLRP4 negatively regulates type I interferon signaling by targeting the kinase TBK1 for degradation via the ubiquitin ligase DTX4." Nat Immunol **13**(4): 387-395.
Cuthbert, R. J., E. M. Fragkakis, R. Dunsmuir, Z. Li, M. Coles, H. Marzo-Ortega, P. V. Giannoudis, E. Jones, Y. M. El-Sherbiny and D. McGonagle (2017). "Brief Report: Group 3 Innate Lymphoid Cells in Human Enthesis." Arthritis Rheumatol **69**(9): 1816-1822.
Cuthbert, R. J., A. Watad, E. M. Fragkakis, R. Dunsmuir, P. Loughenbury, A. Khan, P. A. Millner, A. Davison, H. Marzo-Ortega, D. Newton, C. Bridgewood and D. G. McGonagle (2019). "Evidence that tissue resident human enthesis gammadeltaT-cells can produce IL-17A independently of IL-23R transcript expression." Ann Rheum Dis **78**(11): 1559-1565.
Dagfinrud, H., T. K. Kvien and K. B. Hagen (2008). "Physiotherapy interventions for ankylosing spondylitis." Cochrane Database Syst Rev(1): CD002822.
Dangoria, N. S., M. L. DeLay, D. J. Kingsbury, J. P. Mear, B. Uchanska-Ziegler, A. Ziegler and R. A. Colbert (2002). "HLA-B27 misfolding is associated with aberrant intermolecular disulfide bond formation (dimerization) in the endoplasmic reticulum." J Biol Chem **277**(26): 23459-23468.
Daoussis, D., S. N. Liossis, E. E. Solomou, A. Tsanaktsi, K. Bounia, M. Karampetsou, G. Yiannopoulos and A. P. Andonopoulos (2010). "Evidence that Dkk-1 is dysfunctional in ankylosing spondylitis." Arthritis Rheum **62**(1): 150-158.
Darling, N. J. and S. J. Cook (2014). "The role of MAPK signalling pathways in the response to endoplasmic reticulum stress." Biochim Biophys Acta **1843**(10): 2150-2163.

Dashti, N., M. Mahmoudi, S. Aslani and A. Jamshidi (2018). "HLA-B*27 subtypes and their implications in the pathogenesis of ankylosing spondylitis." Gene **670**: 15-21.
Davidson, S. I., Y. Liu, P. A. Danoy, X. Wu, G. P. Thomas, L. Jiang, L. Sun, N. Wang, J. Han, H. Han, C. Australo-Anglo-American Spondyloarthritis, P. M. Visscher, M. A. Brown and H. Xu (2011). "Association of STAT3 and TNFRSF1A with ankylosing spondylitis in Han Chinese." Ann Rheum Dis **70**(2): 289-292.
Davis, J. C., Jr., D. Van Der Heijde, J. Braun, M. Dougados, J. Cush, D. O. Clegg, A. Kivitz, R. Fleischmann, R. Inman, W. Tsuji and G. Enbrel Ankylosing Spondylitis Study (2003). "Recombinant human tumor necrosis factor receptor (etanercept) for treating ankylosing spondylitis: a randomized, controlled trial." Arthritis Rheum **48**(11): 3230-3236.
de Andrade, K. R., G. R. de Castro, G. Vicente, J. S. da Rosa, M. Nader, I. A. Pereira and T. S. Frode (2014). "Evaluation of circulating levels of inflammatory and bone formation markers in axial spondyloarthritis." Int Immunopharmacol **21**(2): 481-486.
de Carvalho, H. M., A. B. Bortoluzzo, C. R. Goncalves, J. A. da Silva, A. C. Ximenes, M. B. Bertolo, S. L. Ribeiro, M. Keiserman, R. Menin, T. L. Skare, S. Carneiro, V. F. Azevedo, W. P. Vieira, E. N. Albuquerque, W. A. Bianchi, R. Bonfiglioli, C. Campanholo, I. P. Costa, A. P. Duarte, M. B. Gavi, C. L. Kohem, N. H. Leite, S. A. Lima, E. S. Meirelles, I. A. Pereira, M. M. Pinheiro, E. Polito, G. G. Resende, F. A. Rocha, M. B. Santiago, F. Sauma Mde, P. D. Sampaio-Barros and S. Brazilian Registry on (2012). "Gender characterization in a large series of Brazilian patients with spondyloarthritis." Clin Rheumatol **31**(4): 687-695.
De Rycke, L., B. Vandooren, E. Kruithof, F. De Keyser, E. M. Veys and D. Baeten (2005). "Tumor necrosis factor alpha blockade treatment down-modulates the increased systemic and local expression of Toll-like receptor 2 and Toll-like receptor 4 in spondylarthropathy." Arthritis Rheum **52**(7): 2146-2158.
Deepak, P. and W. J. Sandborn (2017). "Ustekinumab and Anti-Interleukin-23 Agents in Crohn's Disease." Gastroenterol Clin North Am **46**(3): 603-626.
DeLay, M. L., M. J. Turner, E. I. Klenk, J. A. Smith, D. P. Sowders and R. A. Colbert (2009). "HLA-B27 misfolding and the unfolded protein response augment interleukin-23 production and are associated with Th17 activation in transgenic rats." Arthritis Rheum **60**(9): 2633-2643.
Delves, P. J. and I. M. Roitt (2000). "The immune system. First of two parts." N Engl J Med **343**(1): 37-49.
Delves, P. J. and I. M. Roitt (2000). "The immune system. Second of two parts." N Engl J Med **343**(2): 108-117.
Deodhar, A., S. D. Chakravarty, C. Cameron, S. Peterson, R. Hensman, S. Fogarty, P. Spin, S. Kafka, S. Nair and L. S. Gensler (2020). "A systematic review and network meta-analysis of current and investigational treatments for active ankylosing spondylitis." Clin Rheumatol **39**(8): 2307-2315.
Di Paola, M., D. Cavalieri, D. Albanese, M. Sordo, M. Pindo, C. Donati, I. Pagnini, T. Giani, G. Simonini, A. Paladini, P. Lionetti, C. De Filippo and R. Cimaz (2016). "Alteration of Fecal Microbiota Profiles in Juvenile Idiopathic Arthritis. Associations with HLA-B27 Allele and Disease Status." Front Microbiol **7**: 1703.
Dibra, D., X. Xia, M. Gagea, G. Lozano and S. Li (2018). "A spontaneous model of spondyloarthropathies that develops bone loss and pathological bone formation: A process regulated by IL27RA-/- and mutant-p53." PLoS One **13**(3): e0193485.
Ding, M., T. J. Guan, C. Y. Wei and B. H. Chen (2018). "Identification of pathways significantly associated with spondyloarthropathy/ankylosing spondylitis using the subpathway method." Mol Med Rep **18**(4): 3825-3833.
Dong, C., R. J. Davis and R. A. Flavell (2002). "MAP kinases in the immune response." Annu Rev Immunol **20**: 55-72.
Dong, W., Y. Zhang, M. Yan, H. Liu, Z. Chen and P. Zhu (2008). "Upregulation of 78-kDa glucose-regulated protein in macrophages in peripheral joints of active ankylosing spondylitis." Scand J Rheumatol **37**(6): 427-434.

Donnithorne, K. J., R. Q. Cron and T. Beukelman (2011). "Attainment of inactive disease status following initiation of TNF-alpha inhibitor therapy for juvenile idiopathic arthritis: enthesitis-related arthritis predicts persistent active disease." J Rheumatol **38**(12): 2675-2681.
Dougados, M. and D. Baeten (2011). "Spondyloarthritis." Lancet **377**(9783): 2127-2137.
Dougados, M., S. van der Linden, R. Juhlin, B. Huitfeldt, B. Amor, A. Calin, A. Cats, B. Dijkmans, I. Olivieri, G. Pasero and et al. (1991). "The European Spondylarthropathy Study Group preliminary criteria for the classification of spondylarthropathy." Arthritis Rheum **34**(10): 1218-1227.
Dougados, M., J. C. Wei, R. Landewe, J. Sieper, X. Baraliakos, F. Van den Bosch, W. P. Maksymowych, J. Ermann, J. A. Walsh, T. Tomita, A. Deodhar, D. van der Heijde, X. Li, F. Zhao, C. C. Bertram, G. Gallo, H. Carlier, L. S. Gensler, V. Coast and C.-W. S. Groups (2020). "Efficacy and safety of ixekizumab through 52 weeks in two phase 3, randomised, controlled clinical trials in patients with active radiographic axial spondyloarthritis (COAST-V and COAST-W)." Ann Rheum Dis **79**(2): 176-185.
Drouart, M., P. Saas, M. Billot, J. P. Cedoz, P. Tiberghien, D. Wendling and E. Toussirot (2003). "High serum vascular endothelial growth factor correlates with disease activity of spondylarthropathies." Clin Exp Immunol **132**(1): 158-162.
Dufey, E., D. Sepulveda, D. Rojas-Rivera and C. Hetz (2014). "Cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. 1. An overview." Am J Physiol Cell Physiol **307**(7): C582-594.
El-Behi, M., B. Ciric, H. Dai, Y. Yan, M. Cullimore, F. Safavi, G. X. Zhang, B. N. Dittel and A. Rostami (2011). "The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF." Nat Immunol **12**(6): 568-575.
El-Zayadi, A. A., E. A. Jones, S. M. Churchman, T. G. Baboolal, R. J. Cuthbert, J. J. El-Jawhari, A. M. Badawy, A. A. Alase, Y. M. El-Sherbiny and D. McGonagle (2017). "Interleukin-22 drives the proliferation, migration and osteogenic differentiation of mesenchymal stem cells: a novel cytokine that could contribute to new bone formation in spondyloarthropathies." Rheumatology (Oxford) **56**(3): 488-493.
Eligini, S., M. Crisci, E. Bono, P. Songia, E. Tremoli, G. I. Colombo and S. Colli (2013). "Human monocyte-derived macrophages spontaneously differentiated in vitro show distinct phenotypes." J Cell Physiol **228**(7): 1464-1472.
Ellinghaus, D., L. Jostins, S. L. Spain, A. Cortes, J. Bethune, B. Han, Y. R. Park, S. Raychaudhuri, J. G. Pouget, M. Hubenthal, T. Folseraas, Y. Wang, T. Esko, A. Metspalu, H. J. Westra, L. Franke, T. H. Pers, R. K. Weersma, V. Collij, M. D'Amato, J. Halfvarson, A. B. Jensen, W. Lieb, F. Degenhardt, A. J. Forstner, A. Hofmann, I. B. D. G. C. International, C. International Genetics of Ankylosing Spondylitis, P. S. C. S. G. International, C. Genetic Analysis of Psoriasis, E. Psoriasis Association Genetics, S. Schreiber, U. Mrowietz, B. D. Juran, K. N. Lazaridis, S. Brunak, A. M. Dale, R. C. Trembath, S. Weidinger, M. Weichenthal, E. Ellinghaus, J. T. Elder, J. N. Barker, O. A. Andreassen, D. P. McGovern, T. H. Karlsen, J. C. Barrett, M. Parkes, M. A. Brown and A. Franke (2016). "Analysis of five chronic inflammatory diseases identifies 27 new associations and highlights disease-specific patterns at shared loci." Nat Genet **48**(5): 510-518.
Elliott, J. M. and W. M. Yokoyama (2011). "Unifying concepts of MHC-dependent natural killer cell education." Trends Immunol **32**(8): 364-372.
Ernst, M., M. Inglese, P. Waring, I. K. Campbell, S. Bao, F. J. Clay, W. S. Alexander, I. P. Wicks, D. M. Tarlinton, U. Novak, J. K. Heath and A. R. Dunn (2001). "Defective gp130-mediated signal transducer and activator of transcription (STAT) signaling results in degenerative joint disease, gastrointestinal ulceration, and failure of uterine implantation." J Exp Med **194**(2): 189-203.
Evans, D. M., C. C. Spencer, J. J. Pointon, Z. Su, D. Harvey, G. Kochan, U. Oppermann, A. Dilthey, M. Pirinen, M. A. Stone, L. Appleton, L. Moutsianas, S. Leslie, T. Wordsworth, T. J. Kenna, T. Karaderi, G. P. Thomas, M. M. Ward, M. H. Weisman, C. Farrar, L. A. Bradbury, P. Danoy, R. D. Inman, W. Maksymowych, D. Gladman, P. Rahman, A. Morgan, H. Marzo-Ortega, P. Bowness, K. Gaffney, J. S. Gaston, M. Smith, J. Bruges-Armas, A. R. Couto, R. Sorrentino, F. Paladini, M. A. Ferreira, H. Xu, Y. Liu, L. Jiang, C. Lopez-Larrea, R. Diaz-Pena, A. Lopez-Vazquez, T. Zayats, G. Band, C. Bellenguez, H.

Blackburn, J. M. Blackwell, E. Bramon, S. J. Bumpstead, J. P. Casas, A. Corvin, N. Craddock, P. Deloukas, S. Dronov, A. Duncanson, S. Edkins, C. Freeman, M. Gillman, E. Gray, R. Gwilliam, N. Hammond, S. E. Hunt, J. Jankowski, A. Jayakumar, C. Langford, J. Liddle, H. S. Markus, C. G. Mathew, O. T. McCann, M. I. McCarthy, C. N. Palmer, L. Peltonen, R. Plomin, S. C. Potter, A. Rautanen, R. Ravindrarajah, M. Ricketts, N. Samani, S. J. Sawcer, A. Strange, R. C. Trembath, A. C. Viswanathan, M. Waller, P. Weston, P. Whittaker, S. Widaa, N. W. Wood, G. McVean, J. D. Reveille, B. P. Wordsworth, M. A. Brown and P. Donnelly (2011). "Interaction between ERAP1 and HLA-B27 in ankylosing spondylitis implicates peptide handling in the mechanism for HLA-B27 in disease susceptibility." Nat Genet **43**(8): 761-767.
Fagerli, K. M., D. van der Heijde, M. S. Heiberg, A. Wierod, S. Kalstad, E. Rodevand, K. Mikkelsen, T. K. Kvien and E. Lie (2014). "Is there a role for sulphasalazine in axial spondyloarthritis in the era of TNF inhibition? Data from the NOR-DMARD longitudinal observational study." Rheumatology (Oxford) **53**(6): 1087-1094.
Faubion, W. A., M. Dubinsky, F. M. Ruemmele, J. Escher, J. Rosh, J. S. Hyams, S. Eichner, Y. Li, N. Reilly, R. B. Thakkar, A. M. Robinson and A. Lazar (2017). "Long-term Efficacy and Safety of Adalimumab in Pediatric Patients with Crohn's Disease." Inflamm Bowel Dis **23**(3): 453-460.
Feagan, B. G., J. Panes, M. Ferrante, A. Kaser, G. R. D'Haens, W. J. Sandborn, E. Louis, M. F. Neurath, D. Franchimont, O. Dewit, U. Seidler, K. J. Kim, C. Selinger, S. J. Padula, I. Herichova, A. M. Robinson, K. Wallace, J. Zhao, M. Minocha, A. A. Othman, A. Soaita, S. Visvanathan, D. B. Hall and W. O. Bocher (2018). "Risankizumab in patients with moderate to severe Crohn's disease: an open-label extension study." Lancet Gastroenterol Hepatol **3**(10): 671-680.
Feagan, B. G., W. J. Sandborn, C. Gasink, D. Jacobstein, Y. Lang, J. R. Friedman, M. A. Blank, J. Johanns, L. L. Gao, Y. Miao, O. J. Adedokun, B. E. Sands, S. B. Hanauer, S. Vermeire, S. Targan, S. Ghosh, W. J. de Villiers, J. F. Colombel, Z. Tulassay, U. Seidler, B. A. Salzberg, P. Desreumaux, S. D. Lee, E. V. Loftus, Jr., L. A. Dieleman, S. Katz, P. Rutgeerts and U.-I.-U. S. Group (2016). "Ustekinumab as Induction and Maintenance Therapy for Crohn's Disease." N Engl J Med **375**(20): 1946-1960.
Feldtkeller, E., M. A. Khan, D. van der Heijde, S. van der Linden and J. Braun (2003). "Age at disease onset and diagnosis delay in HLA-B27 negative vs. positive patients with ankylosing spondylitis." Rheumatol Int **23**(2): 61-66.
Feng, Y., J. Ding, C. M. Fan and P. Zhu (2012). "Interferon-gamma contributes to HLA-B27-associated unfolded protein response in spondyloarthropathies." J Rheumatol **39**(3): 574-582.
Fernandez-Carballido, C., C. Tornero, M. C. Castro-Villegas, E. Galindez, J. F. Garcia-Llorente, M. L. Garcia-Vivar, B. Joven-Ibanez, X. Juanola, C. Urrego-Laurin, C. Lopez-Medina, R. Almodovar, N. Martinez-Alberola, T. Ruiz-Jimeno, E. de Miguel and E. s. group (2020). "No radiographic sacroiliitis progression was observed in patients with early spondyloarthritis at 6 years: results of the Esperanza multicentric prospective cohort." RMD Open **6**(2).
Fert, I., N. Cagnard, S. Glatigny, F. Letourneur, S. Jacques, J. A. Smith, R. A. Colbert, J. D. Taurog, G. Chiocchia, L. M. Araujo and M. Breban (2014). "Reverse interferon signature is characteristic of antigen-presenting cells in human and rat spondyloarthritis." Arthritis Rheumatol **66**(4): 841-851.
Fisher, C., Y. Ioannou, M. Hall-Craggs and D. Sen (2012). "Enthesitis related arthritis; a new era of understanding." Ann Paediatr Rheum **1**(1): 8-16.
Fisher, C., A. Radziszewska, L. Suffield, M. Hall-Craggs, D. Sen and Y. Ioannou (2015). "O45. Tumour Necrosis Factor Inhibition in Enthesitis Related Arthritis and Disease Modifying Effects in Axial Disease." Rheumatology **54**(suppl 1): i45-i45.
Fisher, C. and D. Sen (2012). "Juvenile idiopathic arthritis: in adolescence and beyond." Br J Hosp Med (Lond) **73**(10): 564-570.
Fisher, C., L. Suffield, A. Radziszewska, D. Sen and Y. Ioannou (2014). "284. Enthesitis-Related Arthritis and Two Distinct Patterns of Disease: Clinical Characteristics and Outcomes in a Cohort of 109 Patients." Rheumatology **53**(suppl 1): i165-i165.

Fitzgerald, K. A., S. M. McWhirter, K. L. Faia, D. C. Rowe, E. Latz, D. T. Golenbock, A. J. Coyle, S. M. Liao and T. Maniatis (2003). "IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway." Nat Immunol **4**(5): 491-496.
Flato, B., A. M. Hoffmann-Vold, A. Reiff, O. Forre, G. Lien and O. Vinje (2006). "Long-term outcome and prognostic factors in enthesitis-related arthritis: a case-control study." Arthritis Rheum **54**(11): 3573-3582.
Flores, R. R., L. Carbo, E. Kim, M. Van Meter, C. M. L. De Padilla, J. Zhao, D. Colangelo, M. J. Yousefzadeh, L. A. Angelini, L. Zhang, E. Pola, N. Vo, C. H. Evans, A. Gambotto, L. J. Niedernhofer and P. D. Robbins (2019). "Adenoviral gene transfer of a single-chain IL-23 induces psoriatic arthritis-like symptoms in NOD mice." FASEB J **33**(8): 9505-9515.
Foeldvari, I., T. Constantin, J. Vojinovic, G. Horneff, V. Chasnyk, J. Dehoorne, V. Panaviene, G. Susic, V. Stanevicha, K. Kobusinska, Z. Zuber, B. Dobrzyniecka, I. Nikishina, B. Bader-Meunier, L. Breda, P. Dolezalova, C. Job-Deslandre, I. Rumba-Rozenfelde, N. Wulffraat, R. D. Pedersen, J. F. Bukowski, B. Vlahos, A. Martini, N. Ruperto and O. Paediatric Rheumatology International Trials (2019). "Etanercept treatment for extended oligoarticular juvenile idiopathic arthritis, enthesitis-related arthritis, or psoriatic arthritis: 6-year efficacy and safety data from an open-label trial." Arthritis Res Ther **21**(1): 125.
Foell, D., N. Wulffraat, L. R. Wedderburn, H. Wittkowski, M. Frosch, J. Gerss, V. Stanevicha, D. Mihaylova, V. Ferriani, F. K. Tsakalidou, I. Foeldvari, R. Cuttica, B. Gonzalez, A. Ravelli, R. Khubchandani, S. Oliveira, W. Armbrust, S. Garay, J. Vojinovic, X. Norambuena, M. L. Gamir, J. Garcia-Consuegra, L. Lepore, G. Susic, F. Corona, P. Dolezalova, A. Pistorio, A. Martini, N. Ruperto, J. Roth and O. Paediatric Rheumatology International Trials (2010). "Methotrexate withdrawal at 6 vs 12 months in juvenile idiopathic arthritis in remission: a randomized clinical trial." JAMA **303**(13): 1266-1273.
Francois, R. J., L. Neure, J. Sieper and J. Braun (2006). "Immunohistological examination of open sacroiliac biopsies of patients with ankylosing spondylitis: detection of tumour necrosis factor alpha in two patients with early disease and transforming growth factor beta in three more advanced cases." Ann Rheum Dis **65**(6): 713-720.
Frosch, M., A. Strey, T. Vogl, N. M. Wulffraat, W. Kuis, C. Sunderkotter, E. Harms, C. Sorg and J. Roth (2000). "Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis." Arthritis Rheum **43**(3): 628-637.
Furukawa, M., H. Takaishi, J. Takito, M. Yoda, S. Sakai, T. Hikata, A. Hakozaki, S. Uchikawa, M. Matsumoto, K. Chiba, T. Kimura, Y. Okada, K. Matsuo, H. Yoshida and Y. Toyama (2009). "IL-27 abrogates receptor activator of NF-kappa B ligand-mediated osteoclastogenesis of human granulocyte-macrophage colony-forming unit cells through STAT1-dependent inhibition of c-Fos." J Immunol **183**(4): 2397-2406.
Gaffen, S. L. (2009). "Structure and signalling in the IL-17 receptor family." Nat Rev Immunol **9**(8): 556-567.
Gao, J. W., K. F. Zhang, J. S. Lu and T. Su (2015). "Serum matrix metalloproteinases-3 levels in patients with ankylosing spondylitis." Genet Mol Res **14**(4): 17068-17078.
Gao, W., Y. Xiong, Q. Li and H. Yang (2017). "Inhibition of Toll-Like Receptor Signaling as a Promising Therapy for Inflammatory Diseases: A Journey from Molecular to Nano Therapeutics." Front Physiol **8**: 508.
Garrett, S., T. Jenkinson, L. G. Kennedy, H. Whitelock, P. Gaisford and A. Calin (1994). "A new approach to defining disease status in ankylosing spondylitis: the Bath Ankylosing Spondylitis Disease Activity Index." J Rheumatol **21**(12): 2286-2291.
Gaston, J. S., J. C. Goodall and D. Baeten (2011). "Interleukin-23: a central cytokine in the pathogenesis of spondylarthritis." Arthritis Rheum **63**(12): 3668-3671.
Gaur, P., R. Misra and A. Aggarwal (2016). "IL-27 levels are low in enthesitis-related arthritis category of juvenile idiopathic arthritis." Clin Exp Rheumatol **34**(2): 337-342.

Gaur, P., A. Myles, R. Misra and A. Aggarwal (2017). "Intermediate monocytes are increased in enthesitis-related arthritis, a category of juvenile idiopathic arthritis." Clin Exp Immunol **187**(2): 234-241.
Gensler, L. S., M. M. Ward, J. D. Reveille, T. J. Learch, M. H. Weisman and J. C. Davis, Jr. (2008). "Clinical, radiographic and functional differences between juvenile-onset and adult-onset ankylosing spondylitis: results from the PSOAS cohort." Ann Rheum Dis **67**(2): 233-237.
Ghoreschi, K., A. Laurence, X. P. Yang, C. M. Tato, M. J. McGeachy, J. E. Konkel, H. L. Ramos, L. Wei, T. S. Davidson, N. Bouladoux, J. R. Grainger, Q. Chen, Y. Kanno, W. T. Watford, H. W. Sun, G. Eberl, E. M. Shevach, Y. Belkaid, D. J. Cua, W. Chen and J. J. O'Shea (2010). "Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling." Nature **467**(7318): 967-971.
Giannini, E. H., N. Ruperto, A. Ravelli, D. J. Lovell, D. T. Felson and A. Martini (1997). "Preliminary definition of improvement in juvenile arthritis." Arthritis Rheum **40**(7): 1202-1209.
Gilis, E., C. Mortier, K. Venken, K. Debusschere, L. Vereecke and D. Elewaut (2018). "The Role of the Microbiome in Gut and Joint Inflammation in Psoriatic Arthritis and Spondyloarthritis." J Rheumatol Suppl **94**: 36-39.
Giltay, E. J., C. Popp-Snijders, D. van Schaardenburg, B. J. Dekker-Saeys, L. J. Gooren and B. A. Dijkmans (1998). "Serum testosterone levels are not elevated in patients with ankylosing spondylitis." J Rheumatol **25**(12): 2389-2394.
Ginhoux, F. and M. Guilliams (2016). "Tissue-Resident Macrophage Ontogeny and Homeostasis." Immunity **44**(3): 439-449.
Girardin, S. E., R. Tournebize, M. Mavris, A. L. Page, X. Li, G. R. Stark, J. Bertin, P. S. DiStefano, M. Yaniv, P. J. Sansonetti and D. J. Philpott (2001). "CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri." EMBO Rep **2**(8): 736-742.
Glintborg, B., M. Ostergaard, N. S. Krogh, U. Tarp, N. Manilo, A. G. Loft, A. Hansen, A. Schlemmer, V. Fana, H. M. Lindegaard, H. Nordin, C. Rasmussen, L. Ejstrup, D. V. Jensen, P. M. Petersen and M. L. Hetland (2013). "Clinical response, drug survival and predictors thereof in 432 ankylosing spondylitis patients after switching tumour necrosis factor alpha inhibitor therapy: results from the Danish nationwide DANBIO registry." Ann Rheum Dis **72**(7): 1149-1155.
Gmuca, S., R. Xiao, T. G. Brandon, I. Pagnini, T. B. Wright, T. Beukelman, E. M. Morgan and P. F. Weiss (2017). "Multicenter inception cohort of enthesitis-related arthritis: variation in disease characteristics and treatment approaches." Arthritis Res Ther **19**(1): 84.
Goirand, M., S. Breton, F. Chevallier, N. P. Duong, F. Uettwiller, I. Melki, R. Mouy, C. Wouters, B. Bader-Meunier, C. Job-Deslandre and P. Quartier (2018). "Clinical features of children with enthesitis-related juvenile idiopathic arthritis / juvenile spondyloarthritis followed in a French tertiary care pediatric rheumatology centre." Pediatr Rheumatol Online J **16**(1): 21.
Goldie, I. and A. Nachemson (1969). "Synovial pH in rheumatoid knee-joints. I. The effect of synovectomy." Acta Orthop Scand **40**(5): 634-641.
Gomez, K. S., K. Raza, S. D. Jones, L. G. Kennedy and A. Calin (1997). "Juvenile onset ankylosing spondylitis--more girls than we thought?" J Rheumatol **24**(4): 735-737.
Goodall, J. C., C. Wu, Y. Zhang, L. McNeill, L. Ellis, V. Saudek and J. S. Gaston (2010). "Endoplasmic reticulum stress-induced transcription factor, CHOP, is crucial for dendritic cell IL-23 expression." Proc Natl Acad Sci U S A **107**(41): 17698-17703.
Gordon, S. and A. Pluddemann (2017). "Tissue macrophages: heterogeneity and functions." BMC Biol **15**(1): 53.
Gordon, S., A. Pluddemann and F. Martinez Estrada (2014). "Macrophage heterogeneity in tissues: phenotypic diversity and functions." Immunol Rev **262**(1): 36-55.
Goriely, S., C. Molle, M. Nguyen, V. Albarani, N. O. Haddou, R. Lin, D. De Wit, V. Flamand, F. Willems and M. Goldman (2006). "Interferon regulatory factor 3 is involved in Toll-like receptor 4 (TLR4)- and TLR3-induced IL-12p35 gene activation." Blood **107**(3): 1078-1084.

Goto, Y., C. Panea, G. Nakato, A. Cebula, C. Lee, M. G. Diez, T. M. Laufer, L. Ignatowicz and Ivanov, II (2014). "Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation." Immunity **40**(4): 594-607.
Gracey, E., A. Burssens, I. Cambre, G. Schett, R. Lories, I. B. McInnes, H. Asahara and D. Elewaut (2020). "Tendon and ligament mechanical loading in the pathogenesis of inflammatory arthritis." Nat Rev Rheumatol **16**(4): 193-207.
Gracey, E., Y. Yao, B. Green, Z. Qaiyum, Y. Baglaenko, A. Lin, A. Anton, R. Ayearst, P. Yip and R. D. Inman (2016). "Sexual Dimorphism in the Th17 Signature of Ankylosing Spondylitis." Arthritis Rheumatol **68**(3): 679-689.
Granfors, K., S. Jalkanen, A. A. Lindberg, O. Maki-Ikola, R. von Essen, R. Lahesmaa-Rantala, H. Isomaki, R. Saario, W. J. Arnold and A. Toivanen (1990). "Salmonella lipopolysaccharide in synovial cells from patients with reactive arthritis." Lancet **335**(8691): 685-688.
Greenblatt, M. B., J. H. Shim, W. Zou, D. Sitara, M. Schweitzer, D. Hu, S. Lotinun, Y. Sano, R. Baron, J. M. Park, S. Arthur, M. Xie, M. D. Schneider, B. Zhai, S. Gygi, R. Davis and L. H. Glimcher (2010). "The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice." J Clin Invest **120**(7): 2457-2473.
Gremese, E., S. Bernardi, S. Bonazza, M. Nowik, G. Peluso, A. Massara, B. Tolusso, L. Messuti, M. C. Miceli, A. Zoli, F. Trotta, M. Govoni and G. Ferraccioli (2014). "Body weight, gender and response to TNF-alpha blockers in axial spondyloarthritis." Rheumatology (Oxford) **53**(5): 875-881.
Grootjans, J., A. Kaser, R. J. Kaufman and R. S. Blumberg (2016). "The unfolded protein response in immunity and inflammation." Nat Rev Immunol **16**(8): 469-484.
Gu, J., M. Rihl, E. Marker-Hermann, D. Baeten, J. G. Kuipers, Y. W. Song, W. P. Maksymowych, R. Burgos-Vargas, E. M. Veys, F. De Keyser, H. Deister, M. Xiong, F. Huang, W. C. Tsai and D. T. Yu (2002). "Clues to pathogenesis of spondyloarthropathy derived from synovial fluid mononuclear cell gene expression profiles." J Rheumatol **29**(10): 2159-2164.
Guedez, Y. B., K. B. Whittington, J. L. Clayton, L. A. Joosten, F. A. van de Loo, W. B. van den Berg and E. F. Rosloniec (2001). "Genetic ablation of interferon-gamma up-regulates interleukin-1beta expression and enables the elicitation of collagen-induced arthritis in a nonsusceptible mouse strain." Arthritis Rheum **44**(10): 2413-2424.
Guha, M. and N. Mackman (2001). "LPS induction of gene expression in human monocytes." Cell Signal **13**(2): 85-94.
Guilliams, M., A. Mildner and S. Yona (2018). "Developmental and Functional Heterogeneity of Monocytes." Immunity **49**(4): 595-613.
Guo, R., L. Cao, X. Kong, X. Liu, H. Xue, L. Shen and X. Li (2015). "Fever as an initial manifestation of enthesitis-related arthritis subtype of juvenile idiopathic arthritis: retrospective study." PLoS One **10**(6): e0128979.
Gupta, L., S. Bhattacharya, V. Agarwal and A. Aggarwal (2016). "Elevated levels of serum MRP8/14 in ankylosing spondylitis: associated with peripheral arthritis and active disease." Clin Rheumatol **35**(12): 3075-3079.
Guzman, J., K. Oen, L. B. Tucker, A. M. Huber, N. Shiff, G. Boire, R. Scuccimarri, R. Berard, S. M. Tse, K. Morishita, E. Stringer, N. Johnson, D. M. Levy, K. W. Duffy, D. A. Cabral, A. M. Rosenberg, M. Larche, P. Dancey, R. E. Petty, R. M. Laxer, E. Silverman, P. Miettunen, A. L. Chetaille, E. Haddad, K. Houghton, L. Spiegel, S. E. Turvey, H. Schmeling, B. Lang, J. Ellsworth, S. Ramsey, A. Bruns, S. Campillo, S. Benseler, G. Chedeville, R. Schneider, R. Yeung, C. M. Duffy and A.-O. i. Re (2015). "The outcomes of juvenile idiopathic arthritis in children managed with contemporary treatments: results from the ReACCh-Out cohort." Ann Rheum Dis **74**(10): 1854-1860.
Hacquard-Bouder, C., M. Ittah and M. Breban (2006). "Animal models of HLA-B27-associated diseases: new outcomes." Joint Bone Spine **73**(2): 132-138.
Haibel, H., H. C. Brandt, I. H. Song, A. Brandt, J. Listing, M. Rudwaleit and J. Sieper (2007). "No efficacy of subcutaneous methotrexate in active ankylosing spondylitis: a 16-week open-label trial." Ann Rheum Dis **66**(3): 419-421.

Haibel, H., M. Rudwaleit, J. Listing and J. Sieper (2005). "Open label trial of anakinra in active ankylosing spondylitis over 24 weeks." Ann Rheum Dis **64**(2): 296-298.
Hammer, R. E., S. D. Maika, J. A. Richardson, J. P. Tang and J. D. Taurog (1990). "Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human beta 2m: an animal model of HLA-B27-associated human disorders." Cell **63**(5): 1099-1112.
Haroon, N., R. D. Inman, T. J. Learch, M. H. Weisman, M. Lee, M. H. Rahbar, M. M. Ward, J. D. Reveille and L. S. Gensler (2013). "The impact of tumor necrosis factor alpha inhibitors on radiographic progression in ankylosing spondylitis." Arthritis Rheum **65**(10): 2645-2654.
Haroon, N., F. W. Tsui, B. Uchanska-Ziegler, A. Ziegler and R. D. Inman (2012). "Endoplasmic reticulum aminopeptidase 1 (ERAP1) exhibits functionally significant interaction with HLA-B27 and relates to subtype specificity in ankylosing spondylitis." Ann Rheum Dis **71**(4): 589-595.
Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy and C. T. Weaver (2005). "Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages." Nat Immunol **6**(11): 1123-1132.
Hashimoto, S., M. Yamada, K. Motoyoshi and K. S. Akagawa (1997). "Enhancement of macrophage colony-stimulating factor-induced growth and differentiation of human monocytes by interleukin-10." Blood **89**(1): 315-321.
Hayden, M. S. and S. Ghosh (2008). "Shared principles in NF-kappaB signaling." Cell **132**(3): 344-362.
Hayden, M. S. and S. Ghosh (2012). "NF-kappaB, the first quarter-century: remarkable progress and outstanding questions." Genes Dev **26**(3): 203-234.
Healey, E. L., K. L. Haywood, K. P. Jordan, A. Garratt and J. C. Packham (2011). "Impact of ankylosing spondylitis on work in patients across the UK." Scand J Rheumatol **40**(1): 34-40.
Heiland, G. R., H. Appel, D. Poddubnyy, J. Zwerina, A. Hueber, H. Haibel, X. Baraliakos, J. Listing, M. Rudwaleit, G. Schett and J. Sieper (2012). "High level of functional dickkopf-1 predicts protection from syndesmophyte formation in patients with ankylosing spondylitis." Ann Rheum Dis **71**(4): 572-574.
Hepworth, M. R. and G. F. Sonnenberg (2014). "Regulation of the adaptive immune system by innate lymphoid cells." Curr Opin Immunol **27**: 75-82.
Hermann, E., D. T. Yu, K. H. Meyer zum Buschenfelde and B. Fleischer (1993). "HLA-B27-restricted CD8 T cells derived from synovial fluids of patients with reactive arthritis and ankylosing spondylitis." Lancet **342**(8872): 646-650.
Hetz, C., F. Martinon, D. Rodriguez and L. H. Glimcher (2011). "The unfolded protein response: integrating stress signals through the stress sensor IRE1alpha." Physiol Rev **91**(4): 1219-1243.
Hinks, A., P. Martin, E. Flynn, S. Eyre, J. Packham, A. Barton, J. Worthington and W. Thomson (2011). "Subtype specific genetic associations for juvenile idiopathic arthritis: ERAP1 with the enthesitis related arthritis subtype and IL23R with juvenile psoriatic arthritis." Arthritis Res Ther **13**(1): R12.
Hinze, C. H., D. Foell, A. L. Johnson, S. J. Spalding, B. S. Gottlieb, P. W. Morris, Y. Kimura, K. Onel, S. C. Li, A. A. Grom, J. Taylor, H. I. Brunner, J. L. Huggins, J. J. Nocton, K. A. Haines, B. S. Edelheit, M. Shishov, L. K. Jung, C. B. Williams, M. S. Tesher, D. M. Costanzo, L. S. Zemel, J. A. Dare, M. H. Passo, K. C. Ede, J. C. Olson, E. A. Cassidy, T. A. Griffin, L. Wagner-Weiner, J. E. Weiss, L. B. Vogler, K. A. Rouster-Stevens, T. Beukelman, R. Q. Cron, D. Kietz, K. Schikler, J. Mehta, T. V. Ting, J. W. Verbsky, A. B. Eberhard, B. Huang, E. H. Giannini and D. J. Lovell (2019). "Serum S100A8/A9 and S100A12 Levels in Children With Polyarticular Forms of Juvenile Idiopathic Arthritis: Relationship to Maintenance of Clinically Inactive Disease During Anti-Tumor Necrosis Factor Therapy and Occurrence of Disease Flare After Discontinuation of Therapy." Arthritis Rheumatol **71**(3): 451-459.
Hirahara, K., K. Ghoreschi, A. Laurence, X. P. Yang, Y. Kanno and J. J. O'Shea (2010). "Signal transduction pathways and transcriptional regulation in Th17 cell differentiation." Cytokine Growth Factor Rev **21**(6): 425-434.
Ho, A. M., M. D. Johnson and D. M. Kingsley (2000). "Role of the mouse ank gene in control of tissue calcification and arthritis." Science **289**(5477): 265-270.

Hofer, M. F., R. Mouy and A. M. Prieur (2001). "Juvenile idiopathic arthritides evaluated prospectively in a single center according to the Durban criteria." J Rheumatol **28**(5): 1083-1090.
Honda, K. and T. Taniguchi (2006). "IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors." Nat Rev Immunol **6**(9): 644-658.
Horai, R., S. Saijo, H. Tanioka, S. Nakae, K. Sudo, A. Okahara, T. Ikuse, M. Asano and Y. Iwakura (2000). "Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice." J Exp Med **191**(2): 313-320.
Horneff, G., R. Burgos-Vargas, T. Constantin, I. Foeldvari, J. Vojinovic, V. G. Chasnyk, J. Dehoorne, V. Panaviene, G. Susic, V. Stanevica, K. Kobusinska, Z. Zuber, R. Mouy, I. Rumba-Rozenfelde, L. Breda, P. Dolezalova, C. Job-Deslandre, N. Wulffraat, D. Alvarez, C. Zang, J. Wajdula, D. Woodworth, B. Vlahos, A. Martini and N. Ruperto (2014). "Efficacy and safety of open-label etanercept on extended oligoarticular juvenile idiopathic arthritis, enthesitis-related arthritis and psoriatic arthritis: part 1 (week 12) of the CLIPPER study." Ann Rheum Dis **73**(6): 1114-1122.
Horneff, G., S. Fitter, I. Foeldvari, K. Minden, J. Kuemmerle-Deschner, N. Tzaribacev, A. Thon, M. Borte, G. Ganser, R. Trauzeddel and H. I. Huppertz (2012). "Double-blind, placebo-controlled randomized trial with adalimumab for treatment of juvenile onset ankylosing spondylitis (JoAS): significant short term improvement." Arthritis Res Ther **14**(5): R230.
Horneff, G., I. Foeldvari, K. Minden, R. Trauzeddel, J. B. Kummerle-Deschner, K. Tenbrock, G. Ganser and H. I. Huppertz (2015). "Efficacy and safety of etanercept in patients with the enthesitis-related arthritis category of juvenile idiopathic arthritis: results from a phase III randomized, double-blind study." Arthritis Rheumatol **67**(8): 2240-2249.
Hreggvidsdottir, H. S., T. Noordenbos and D. L. Baeten (2014). "Inflammatory pathways in spondyloarthritis." Mol Immunol **57**(1): 28-37.
Huang, W. N., T. K. Tso, Y. C. Kuo and G. J. Tsay (2012). "Distinct impacts of syndesmophyte formation on male and female patients with ankylosing spondylitis." Int J Rheum Dis **15**(2): 163-168.
Hueber, W., B. E. Sands, S. Lewitzky, M. Vandemeulebroecke, W. Reinisch, P. D. Higgins, J. Wehkamp, B. G. Feagan, M. D. Yao, M. Karczewski, J. Karczewski, N. Pezous, S. Bek, G. Bruin, B. Mellgard, C. Berger, M. Londei, A. P. Bertolino, G. Tougas, S. P. Travis and G. Secukinumab in Crohn's Disease Study (2012). "Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial." Gut **61**(12): 1693-1700.
Hugle, B., R. Burgos-Vargas, R. D. Inman, F. O'Shea, R. M. Laxer, J. Stimec, K. Whitney-Mahoney, M. Duvnjak, M. Anderson and S. M. Tse (2014). "Long-term outcome of anti-tumor necrosis factor alpha blockade in the treatment of juvenile spondyloarthritis." Clin Exp Rheumatol **32**(3): 424-431.
Inman, R. D., J. C. Davis, Jr., D. Heijde, L. Diekman, J. Sieper, S. I. Kim, M. Mack, J. Han, S. Visvanathan, Z. Xu, B. Hsu, A. Beutler and J. Braun (2008). "Efficacy and safety of golimumab in patients with ankylosing spondylitis: results of a randomized, double-blind, placebo-controlled, phase III trial." Arthritis Rheum **58**(11): 3402-3412.
Isailovic, N., K. Daigo, A. Mantovani and C. Selmi (2015). "Interleukin-17 and innate immunity in infections and chronic inflammation." J Autoimmun **60**: 1-11.
Itano, A. A., S. J. McSorley, R. L. Reinhardt, B. D. Ehst, E. Ingulli, A. Y. Rudensky and M. K. Jenkins (2003). "Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity." Immunity **19**(1): 47-57.
Ivanov, II, K. Atarashi, N. Manel, E. L. Brodie, T. Shima, U. Karaoz, D. Wei, K. C. Goldfarb, C. A. Santee, S. V. Lynch, T. Tanoue, A. Imaoka, K. Itoh, K. Takeda, Y. Umesaki, K. Honda and D. R. Littman (2009). "Induction of intestinal Th17 cells by segmented filamentous bacteria." Cell **139**(3): 485-498.
Iwakoshi, N. N., A. H. Lee, P. Vallabhajosyula, K. L. Otipoby, K. Rajewsky and L. H. Glimcher (2003). "Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1." Nat Immunol **4**(4): 321-329.
Iwakoshi, N. N., M. Pypaert and L. H. Glimcher (2007). "The transcription factor XBP-1 is essential for the development and survival of dendritic cells." J Exp Med **204**(10): 2267-2275.

Iwakura, Y., H. Ishigame, S. Saijo and S. Nakae (2011). "Functional specialization of interleukin-17 family members." Immunity **34**(2): 149-162.
Iwasaki, A. and R. Medzhitov (2010). "Regulation of adaptive immunity by the innate immune system." Science **327**(5963): 291-295.
Iwasaki, A. and R. Medzhitov (2015). "Control of adaptive immunity by the innate immune system." Nat Immunol **16**(4): 343-353.
Jacques, P., S. Lambrecht, E. Verheugen, E. Pauwels, G. Kollias, M. Armaka, M. Verhoye, A. Van der Linden, R. Achten, R. J. Lories and D. Elewaut (2014). "Proof of concept: enthesitis and new bone formation in spondyloarthritis are driven by mechanical strain and stromal cells." Ann Rheum Dis **73**(2): 437-445.
Jadon, D. R., A. V. Ramanan and R. Sengupta (2013). "Juvenile versus adult-onset ankylosing spondylitis -- clinical, radiographic, and social outcomes. a systematic review." J Rheumatol **40**(11): 1797-1805.
Jadon, D. R., G. Shaddick, A. Jobling, A. V. Ramanan and R. Sengupta (2015). "Clinical outcomes and progression to orthopedic surgery in juvenile- versus adult-onset ankylosing spondylitis." Arthritis Care Res (Hoboken) **67**(5): 651-657.
Jaguin, M., N. Houlbert, O. Fardel and V. Lecureur (2013). "Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin." Cell Immunol **281**(1): 51-61.
Jain, A. and C. Pasare (2017). "Innate Control of Adaptive Immunity: Beyond the Three-Signal Paradigm." J Immunol **198**(10): 3791-3800.
Jandus, C., G. Bioley, J. P. Rivals, J. Dudler, D. Speiser and P. Romero (2008). "Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondylarthritides." Arthritis Rheum **58**(8): 2307-2317.
Janeway, C. A., Jr. (1989). "Approaching the asymptote? Evolution and revolution in immunology." Cold Spring Harb Symp Quant Biol **54 Pt 1**: 1-13.
Janeway, C. A., P. Travers, M. Walport and J. D. Capra (2005). "Immunobiology: the immune system in health and disease."
Jarlborg, M., D. S. Courvoisier, C. Lamacchia, L. Martinez Prat, M. Mahler, C. Bentow, A. Finckh, C. Gabay, M. J. Nissen and r. physicians of the Swiss Clinical Quality Management (2020). "Serum calprotectin: a promising biomarker in rheumatoid arthritis and axial spondyloarthritis." Arthritis Res Ther **22**(1): 105.
Jeong, H., E. K. Bae, H. Kim, Y. H. Eun, I. Y. Kim, H. Kim, J. Lee, C. H. Jeon, E. M. Koh and H. S. Cha (2017). "Estrogen attenuates the spondyloarthritis manifestations of the SKG arthritis model." Arthritis Res Ther **19**(1): 198.
Jethwa, H. and P. Bowness (2016). "The interleukin (IL)-23/IL-17 axis in ankylosing spondylitis: new advances and potentials for treatment." Clin Exp Immunol **183**(1): 30-36.
Jimenez-Balderas, F. J., R. Tapia-Serrano, J. I. Madero-Cervera, S. Murrieta and G. Mintz (1990). "Ovarian function studies in active ankylosing spondylitis in women. Clinical response to estrogen therapy." J Rheumatol **17**(4): 497-502.
Jo, S., S. E. Wang, Y. L. Lee, S. Kang, B. Lee, J. Han, I. H. Sung, Y. S. Park, S. C. Bae and T. H. Kim (2018). "IL-17A induces osteoblast differentiation by activating JAK2/STAT3 in ankylosing spondylitis." Arthritis Res Ther **20**(1): 115.
Jones, A., T. J. P. Bray, P. Mandl, M. A. Hall-Craggs, H. Marzo-Ortega and P. M. Machado (2019). "Performance of magnetic resonance imaging in the diagnosis of axial spondyloarthritis: a systematic literature review." Rheumatology (Oxford) **58**(11): 1955-1965.
Jones, G. T., L. E. Dean, E. Pathan, R. J. Hollick and G. J. Macfarlane (2020). "Real-world evidence of TNF inhibition in axial spondyloarthritis: can we generalise the results from clinical trials?" Ann Rheum Dis **79**(7): 914-919.

Jones, J. A., D. T. Chang, H. Meyerson, E. Colton, I. K. Kwon, T. Matsuda and J. M. Anderson (2007). "Proteomic analysis and quantification of cytokines and chemokines from biomaterial surface-adherent macrophages and foreign body giant cells." J Biomed Mater Res A **83**(3): 585-596.
Jones, S. D., J. Porter, S. L. Garrett, L. G. Kennedy, H. Whitelock and A. Calin (1995). "A new scoring system for the Bath Ankylosing Spondylitis Metrology Index (BASMI)." J Rheumatol **22**(8): 1609.
Jostins, L., S. Ripke, R. K. Weersma, R. H. Duerr, D. P. McGovern, K. Y. Hui, J. C. Lee, L. P. Schumm, Y. Sharma, C. A. Anderson, J. Essers, M. Mitrovic, K. Ning, I. Cleynen, E. Theatre, S. L. Spain, S. Raychaudhuri, P. Goyette, Z. Wei, C. Abraham, J. P. Achkar, T. Ahmad, L. Amininejad, A. N. Ananthakrishnan, V. Andersen, J. M. Andrews, L. Baidoo, T. Balschun, P. A. Bampton, A. Bitton, G. Boucher, S. Brand, C. Buning, A. Cohain, S. Cichon, M. D'Amato, D. De Jong, K. L. Devaney, M. Dubinsky, C. Edwards, D. Ellinghaus, L. R. Ferguson, D. Franchimont, K. Fransen, R. Gearry, M. Georges, C. Gieger, J. Glas, T. Haritunians, A. Hart, C. Hawkey, M. Hedl, X. Hu, T. H. Karlsen, L. Kupcinskas, S. Kugathasan, A. Latiano, D. Laukens, I. C. Lawrance, C. W. Lees, E. Louis, G. Mahy, J. Mansfield, A. R. Morgan, C. Mowat, W. Newman, O. Palmieri, C. Y. Ponsioen, U. Potocnik, N. J. Prescott, M. Regueiro, J. I. Rotter, R. K. Russell, J. D. Sanderson, M. Sans, J. Satsangi, S. Schreiber, L. A. Simms, J. Sventoraityte, S. R. Targan, K. D. Taylor, M. Tremelling, H. W. Verspaget, M. De Vos, C. Wijmenga, D. C. Wilson, J. Winkelmann, R. J. Xavier, S. Zeissig, B. Zhang, C. K. Zhang, H. Zhao, I. B. D. G. C. International, M. S. Silverberg, V. Annese, H. Hakonarson, S. R. Brant, G. Radford-Smith, C. G. Mathew, J. D. Rioux, E. E. Schadt, M. J. Daly, A. Franke, M. Parkes, S. Vermeire, J. C. Barrett and J. H. Cho (2012). "Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease." Nature **491**(7422): 119-124.
Jounai, N., K. Kobiyama, M. Shiina, K. Ogata, K. J. Ishii and F. Takeshita (2011). "NLRP4 negatively regulates autophagic processes through an association with beclin1." J Immunol **186**(3): 1646-1655.
Jovani, V., M. Blasco-Blasco, M. T. Ruiz-Cantero and E. Pascual (2017). "Understanding How the Diagnostic Delay of Spondyloarthritis Differs Between Women and Men: A Systematic Review and Metaanalysis." J Rheumatol **44**(2): 174-183.
Jovanovic, D. V., J. A. Di Battista, J. Martel-Pelletier, F. C. Jolicoeur, Y. He, M. Zhang, F. Mineau and J. P. Pelletier (1998). "IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages." J Immunol **160**(7): 3513-3521.
June, C. H., J. A. Ledbetter, M. M. Gillespie, T. Lindsten and C. B. Thompson (1987). "T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression." Mol Cell Biol **7**(12): 4472-4481.
Kaeley, G. S. (2020). "Visualization of Enthesitis by Ultrasound: a Key Diagnostic Tool in Spondyloarthropathy Diagnosis and Management." Curr Rheumatol Rep **22**(9): 48.
Kavanaugh, A., L. Puig, A. B. Gottlieb, C. Ritchlin, Y. You, S. Li, M. Song, B. Randazzo, P. Rahman and I. B. McInnes (2016). "Efficacy and safety of ustekinumab in psoriatic arthritis patients with peripheral arthritis and physician-reported spondylitis: post-hoc analyses from two phase III, multicentre, double-blind, placebo-controlled studies (PSUMMIT-1/PSUMMIT-2)." Ann Rheum Dis.
Kawai, T. and S. Akira (2007). "TLR signaling." Semin Immunol **19**(1): 24-32.
Kayagaki, N., M. T. Wong, I. B. Stowe, S. R. Ramani, L. C. Gonzalez, S. Akashi-Takamura, K. Miyake, J. Zhang, W. P. Lee, A. Muszynski, L. S. Forsberg, R. W. Carlson and V. M. Dixit (2013). "Noncanonical inflammasome activation by intracellular LPS independent of TLR4." Science **341**(6151): 1246-1249.
Keat, A. C., R. N. Maini, G. C. Nkwazi, G. D. Pegrum, G. L. Ridgway and J. T. Scott (1978). "Role of Chlamydia trachomatis and HLA-B27 in sexually acquired reactive arthritis." Br Med J **1**(6113): 605-607.
Keidel, S., L. Chen, J. Pointon and P. Wordsworth (2013). "ERAP1 and ankylosing spondylitis." Curr Opin Immunol **25**(1): 97-102.
Kelchtermans, H., E. Schurgers, L. Geboes, T. Mitera, J. Van Damme, J. Van Snick, C. Uyttenhove and P. Matthys (2009). "Effector mechanisms of interleukin-17 in collagen-induced arthritis in the absence of interferon-gamma and counteraction by interferon-gamma." Arthritis Res Ther **11**(4): R122.

Kenna, T. J. and M. A. Brown (2013). "The role of IL-17-secreting mast cells in inflammatory joint disease." Nat Rev Rheumatol **9**(6): 375-379.
Khameneh, H. J., S. A. Isa, L. Min, F. W. Nih and C. Ruedl (2011). "GM-CSF signalling boosts dramatically IL-1 production." PLoS One **6**(7): e23025.
Khare, S. D., M. J. Bull, J. Hanson, H. S. Luthra and C. S. David (1998). "Spontaneous inflammatory disease in HLA-B27 transgenic mice is independent of MHC class II molecules: a direct role for B27 heavy chains and not B27-derived peptides." J Immunol **160**(1): 101-106.
Khare, S. D., J. Hansen, H. S. Luthra and C. S. David (1996). "HLA-B27 heavy chains contribute to spontaneous inflammatory disease in B27/human beta2-microglobulin (beta2m) double transgenic mice with disrupted mouse beta2m." J Clin Invest **98**(12): 2746-2755.
Kievits, F., P. Ivanyi, P. Krimpenfort, A. Berns and H. L. Ploegh (1987). "HLA-restricted recognition of viral antigens in HLA transgenic mice." Nature **329**(6138): 447-449.
Kilic, G., E. Kilic and S. Ozgocmen (2017). "Is there any gender-specific difference in the cut-off values of ankylosing spondylitis disease activity score in patients with axial spondyloarthritis?" Int J Rheum Dis **20**(9): 1201-1211.
Kim, E. Y., H. H. Chi, M. Bouziane, A. Gaur and K. D. Moudgil (2008). "Regulation of autoimmune arthritis by the pro-inflammatory cytokine interferon-gamma." Clin Immunol **127**(1): 98-106.
Kim, E. Y. and K. D. Moudgil (2017). "Immunomodulation of autoimmune arthritis by pro-inflammatory cytokines." Cytokine **98**: 87-96.
Kim, S., Y. Joe, S. O. Jeong, M. Zheng, S. H. Back, S. W. Park, S. W. Ryter and H. T. Chung (2014). "Endoplasmic reticulum stress is sufficient for the induction of IL-1beta production via activation of the NF-kappaB and inflammasome pathways." Innate Immun **20**(8): 799-815.
Kim, S. R., H. J. Kim, D. I. Kim, K. B. Lee, H. J. Park, J. S. Jeong, S. H. Cho and Y. C. Lee (2015). "Blockade of Interplay between IL-17A and Endoplasmic Reticulum Stress Attenuates LPS-Induced Lung Injury." Theranostics **5**(12): 1343-1362.
Kiuru, M. J., M. Niva, A. Reponen and H. K. Pihlajamaki (2005). "Bone stress injuries in asymptomatic elite recruits: a clinical and magnetic resonance imaging study." Am J Sports Med **33**(2): 272-276.
Klingberg, E., M. K. Magnusson, H. Strid, A. Deminger, A. Stahl, J. Sundin, M. Simren, H. Carlsten, L. Ohman and H. Forsblad-d'Elia (2019). "A distinct gut microbiota composition in patients with ankylosing spondylitis is associated with increased levels of fecal calprotectin." Arthritis Res Ther **21**(1): 248.
Klingberg, E., M. Nurkkala, H. Carlsten and H. Forsblad-d'Elia (2014). "Biomarkers of bone metabolism in ankylosing spondylitis in relation to osteoproliferation and osteoporosis." J Rheumatol **41**(7): 1349-1356.
Kobayashi, K. S. and P. J. van den Elsen (2012). "NLRC5: a key regulator of MHC class I-dependent immune responses." Nat Rev Immunol **12**(12): 813-820.
Koenders, M. I., I. Devesa, R. J. Marijnissen, S. Abdollahi-Roodsaz, A. M. Boots, B. Walgreen, F. E. di Padova, M. J. Nicklin, L. A. Joosten and W. B. van den Berg (2008). "Interleukin-1 drives pathogenic Th17 cells during spontaneous arthritis in interleukin-1 receptor antagonist-deficient mice." Arthritis Rheum **58**(11): 3461-3470.
Koenders, M. I., E. Lubberts, B. Oppers-Walgreen, L. van den Bersselaar, M. M. Helsen, J. K. Kolls, L. A. Joosten and W. B. van den Berg (2005). "Induction of cartilage damage by overexpression of T cell interleukin-17A in experimental arthritis in mice deficient in interleukin-1." Arthritis Rheum **52**(3): 975-983.
Koning, N., M. van Eijk, W. Pouwels, M. S. Brouwer, D. Voehringer, I. Huitinga, R. M. Hoek, G. Raes and J. Hamann (2010). "Expression of the inhibitory CD200 receptor is associated with alternative macrophage activation." J Innate Immun **2**(2): 195-200.
Kontoyiannis, D., M. Pasparakis, T. T. Pizarro, F. Cominelli and G. Kollias (1999). "Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies." Immunity **10**(3): 387-398.

Koo, B. S., J. S. Oh, S. Y. Park, J. H. Shin, G. Y. Ahn, S. Lee, K. B. Joo and T. H. Kim (2020). "Tumour necrosis factor inhibitors slow radiographic progression in patients with ankylosing spondylitis: 18-year real-world evidence." Ann Rheum Dis **79**(10): 1327-1332.
Kruger, P., M. Saffarzadeh, A. N. Weber, N. Rieber, M. Radsak, H. von Bernuth, C. Benarafa, D. Roos, J. Skokowa and D. Hartl (2015). "Neutrophils: Between host defence, immune modulation, and tissue injury." PLoS Pathog **11**(3): e1004651.
Kruithof, E., V. Van den Bossche, L. De Rycke, B. Vandooren, R. Joos, J. D. Canete, P. P. Tak, A. M. Boots, E. M. Veys and D. Baeten (2006). "Distinct synovial immunopathologic characteristics of juvenile-onset spondylarthritis and other forms of juvenile idiopathic arthritis." Arthritis Rheum **54**(8): 2594-2604.
Kuo, S. J., C. C. Huang, C. H. Tsai, H. C. Hsu, C. M. Su and C. H. Tang (2018). "Chemokine C-C Motif Ligand 4 Gene Polymorphisms Associated with Susceptibility to Rheumatoid Arthritis." Biomed Res Int **2018**: 9181647.
Kwon, S. R., M. J. Lim, C. H. Suh, S. G. Park, Y. S. Hong, B. Y. Yoon, H. A. Kim, H. J. Choi and W. Park (2012). "Dickkopf-1 level is lower in patients with ankylosing spondylitis than in healthy people and is not influenced by anti-tumor necrosis factor therapy." Rheumatol Int **32**(8): 2523-2527.
Lacey, D. C., A. Achuthan, A. J. Fleetwood, H. Dinh, J. Roiniotis, G. M. Scholz, M. W. Chang, S. K. Beckman, A. D. Cook and J. A. Hamilton (2012). "Defining GM-CSF- and macrophage-CSF-dependent macrophage responses by in vitro models." J Immunol **188**(11): 5752-5765.
Lamot, L., F. Borovecki, L. Tambic Bukovac, M. Vidovic, M. Perica, K. Gotovac and M. Harjacek (2014). "Aberrant expression of shared master-key genes contributes to the immunopathogenesis in patients with juvenile spondyloarthritis." PLoS One **9**(12): e115416.
Landewe, R., J. Braun, A. Deodhar, M. Dougados, W. P. Maksymowych, P. J. Mease, J. D. Reveille, M. Rudwaleit, D. van der Heijde, C. Stach, B. Hoepken, A. Fichtner, G. Coteur, M. de Longueville and J. Sieper (2014). "Efficacy of certolizumab pegol on signs and symptoms of axial spondyloarthritis including ankylosing spondylitis: 24-week results of a double-blind randomised placebo-controlled Phase 3 study." Ann Rheum Dis **73**(1): 39-47.
Landi, M., H. Maldonado-Ficco, R. Perez-Alamino, J. A. Maldonado-Cocco, G. Citera, P. Arturi, P. D. Sampaio-Barros, D. E. Flores Alvarado, R. Burgos-Vargas, E. Santos, D. Palleiro, M. A. Gutierrez, E. Vieira-Sousa, F. Pimentel-Santos, S. O. Paira, A. Berman, C. V. Barrezueta, J. Vazquez-Mellado, E. Collantes-Estevez and R. G. F. R. A. D. O. G. Morteo (2016). "Gender differences among patients with primary ankylosing spondylitis and spondylitis associated with psoriasis and inflammatory bowel disease in an iberoamerican spondyloarthritis cohort." Medicine (Baltimore) **95**(51): e5652.
Lavin, Y., A. Mortha, A. Rahman and M. Merad (2015). "Regulation of macrophage development and function in peripheral tissues." Nat Rev Immunol **15**(12): 731-744.
Lawrence, T. (2009). "The nuclear factor NF-kappaB pathway in inflammation." Cold Spring Harb Perspect Biol **1**(6): a001651.
Lee, D. G., J. W. Woo, S. K. Kwok, M. L. Cho and S. H. Park (2013). "MRP8 promotes Th17 differentiation via upregulation of IL-6 production by fibroblast-like synoviocytes in rheumatoid arthritis." Exp Mol Med **45**: e20.
Lee, J., J. Lee, M. K. Park, M. A. Lim, E. M. Park, E. K. Kim, E. J. Yang, S. Y. Lee, J. Y. Jhun, S. H. Park, H. Y. Kim and M. L. Cho (2013). "Interferon gamma suppresses collagen-induced arthritis by regulation of Th17 through the induction of indoleamine-2,3-deoxygenase." PLoS One **8**(4): e60900.
Lee, W., J. D. Reveille, J. C. Davis, Jr., T. J. Learch, M. M. Ward and M. H. Weisman (2007). "Are there gender differences in severity of ankylosing spondylitis? Results from the PSOAS cohort." Ann Rheum Dis **66**(5): 633-638.
Lemmel, E. M., D. Brackertz, M. Franke, W. Gaus, P. W. Hartl, K. Machalke, H. Mielke, H. J. Obert, H. H. Peter, J. Sieper and et al. (1988). "Results of a multicenter placebo-controlled double-blind randomized phase III clinical study of treatment of rheumatoid arthritis with recombinant interferon-gamma." Rheumatol Int **8**(2): 87-93.

Levin, A. D., M. E. Wildenberg and G. R. van den Brink (2016). "Mechanism of Action of Anti-TNF Therapy in Inflammatory Bowel Disease." J Crohns Colitis **10**(8): 989-997.
Levy-Clarke, G., D. A. Jabs, R. W. Read, J. T. Rosenbaum, A. Vitale and R. N. Van Gelder (2014). "Expert panel recommendations for the use of anti-tumor necrosis factor biologic agents in patients with ocular inflammatory disorders." Ophthalmology **121**(3): 785-796 e783.
Li, N., X. M. Wang, L. J. Jiang, M. Zhang, N. Li, Z. Z. Wei, N. Zheng and Y. J. Zhao (2016). "Effects of endoplasmic reticulum stress on the expression of inflammatory cytokines in patients with ulcerative colitis." World J Gastroenterol **22**(7): 2357-2365.
Li, X., K. W. Kim, M. L. Cho, J. H. Ju, C. M. Kang, H. J. Oh, J. K. Min, S. H. Lee, S. H. Park and H. Y. Kim (2010). "IL-23 induces receptor activator of NF-kappaB ligand expression in fibroblast-like synoviocytes via STAT3 and NF-kappaB signal pathways." Immunol Lett **127**(2): 100-107.
Li, Y., R. F. Schwabe, T. DeVries-Seimon, P. M. Yao, M. C. Gerbod-Giannone, A. R. Tall, R. J. Davis, R. Flavell, D. A. Brenner and I. Tabas (2005). "Free cholesterol-loaded macrophages are an abundant source of tumor necrosis factor-alpha and interleukin-6: model of NF-kappaB- and map kinase-dependent inflammation in advanced atherosclerosis." J Biol Chem **280**(23): 21763-21772.
Liang, H., H. Zhang, H. Ji and C. Wang (2015). "Effects of home-based exercise intervention on health-related quality of life for patients with ankylosing spondylitis: a meta-analysis." Clin Rheumatol **34**(10): 1737-1744.
Lin, C., M. Diab and D. Milojevic (2015). "Grey-scale ultrasound findings of lower extremity entheses in healthy children." Pediatr Rheumatol Online J **13**: 14.
Lin, P., M. Bach, M. Asquith, A. Y. Lee, L. Akileswaran, P. Stauffer, S. Davin, Y. Pan, E. D. Cambronne, M. Dorris, J. W. Debelius, C. L. Lauber, G. Ackermann, Y. V. Baeza, T. Gill, R. Knight, R. A. Colbert, J. D. Taurog, R. N. Van Gelder and J. T. Rosenbaum (2014). "HLA-B27 and human beta2-microglobulin affect the gut microbiota of transgenic rats." PLoS One **9**(8): e105684.
Lin, T. T., J. Lu, C. Y. Qi, L. Yuan, X. L. Li, L. P. Xia and H. Shen (2015). "Elevated serum level of IL-27 and VEGF in patients with ankylosing spondylitis and associate with disease activity." Clin Exp Med **15**(2): 227-231.
Lin, Y. C., T. H. Liang, W. S. Chen and H. Y. Lin (2009). "Differences between juvenile-onset ankylosing spondylitis and adult-onset ankylosing spondylitis." J Chin Med Assoc **72**(11): 573-580.
Lopez-Medina, C., S. Ramiro, D. van der Heijde, J. Sieper, M. Dougados and A. Molto (2019). "Characteristics and burden of disease in patients with radiographic and non-radiographic axial Spondyloarthritis: a comparison by systematic literature review and meta-analysis." RMD Open **5**(2): e001108.
Lopez, R. N., S. T. Leach, D. A. Lemberg, G. Duvoisin, R. B. Gearry and A. S. Day (2017). "Fecal biomarkers in inflammatory bowel disease." J Gastroenterol Hepatol **32**(3): 577-582.
Lories, R. J. and N. Haroon (2014). "Bone formation in axial spondyloarthritis." Best Pract Res Clin Rheumatol **28**(5): 765-777.
Lories, R. J. and I. B. McInnes (2012). "Primed for inflammation: enthesis-resident T cells." Nat Med **18**(7): 1018-1019.
Lubberts, E. (2015). "The IL-23-IL-17 axis in inflammatory arthritis." Nat Rev Rheumatol **11**(7): 415-429.
Lubberts, E., L. A. Joosten, F. A. van de Loo, P. Schwarzenberger, J. Kolls and W. B. van den Berg (2002). "Overexpression of IL-17 in the knee joint of collagen type II immunized mice promotes collagen arthritis and aggravates joint destruction." Inflamm Res **51**(2): 102-104.
Lubrano, E., F. M. Perrotta, M. Manara, S. D'Angelo, O. Addimanda, R. Ramonda, L. Punzi, I. Olivieri, C. Salvarani and A. Marchesoni (2017). "The Sex Influence on Response to Tumor Necrosis Factor-alpha Inhibitors and Remission in Axial Spondyloarthritis." J Rheumatol.
Lukas, C., C. Cyteval, M. Dougados and U. Weber (2018). "MRI for diagnosis of axial spondyloarthritis: major advance with critical limitations 'Not everything that glisters is gold (standard)'." RMD Open **4**(1): e000586.

Lukens, J. R., M. J. Barr, D. D. Chaplin, H. Chi and T. D. Kanneganti (2012). "Inflammasome-derived IL-1beta regulates the production of GM-CSF by CD4(+) T cells and gammadelta T cells." J Immunol **188**(7): 3107-3115.
Maas, F., A. Spoorenberg, E. Brouwer, R. Bos, M. Efde, R. N. Chaudhry, N. J. Veeger, P. M. van Ooijen, R. Wolf, H. Bootsma, E. van der Veer and S. Arends (2015). "Spinal radiographic progression in patients with ankylosing spondylitis treated with TNF-alpha blocking therapy: a prospective longitudinal observational cohort study." PLoS One **10**(4): e0122693.
Machado, P., R. Landewe, E. Lie, T. K. Kvien, J. Braun, D. Baker, D. van der Heijde and S. Assessment of SpondyloArthritis international (2011). "Ankylosing Spondylitis Disease Activity Score (ASDAS): defining cut-off values for disease activity states and improvement scores." Ann Rheum Dis **70**(1): 47-53.
Mahendira, D., A. Thavaneswaran, A. Carty, N. Haroon, A. Anton, L. Passalent, K. A. Alnaqbi, L. Savage, E. Aslanyan and R. D. Inman (2014). "Analysis of the effect of the oral contraceptive pill on clinical outcomes in women with ankylosing spondylitis." J Rheumatol **41**(7): 1344-1348.
Mahendra, A., R. Misra and A. Aggarwal (2009). "Th1 and Th17 Predominance in the Enthesitis-related Arthritis Form of Juvenile Idiopathic Arthritis." J Rheumatol **36**(8): 1730-1736.
Maksymowych, W. P. (2015). "Biomarkers in axial spondyloarthritis." Curr Opin Rheumatol **27**(4): 343-348.
Maksymowych, W. P. (2017). "An update on biomarker discovery and use in axial spondyloarthritis." Expert Rev Mol Diagn **17**(11): 965-974.
Maksymowych, W. P., R. Landewe, B. Conner-Spady, M. Dougados, H. Mielants, H. van der Tempel, A. R. Poole, N. Wang and D. van der Heijde (2007). "Serum matrix metalloproteinase 3 is an independent predictor of structural damage progression in patients with ankylosing spondylitis." Arthritis Rheum **56**(6): 1846-1853.
Maksymowych, W. P., P. Rahman, K. Shojania, W. P. Olszynski, G. T. Thomson, S. Ballal, R. L. Wong, R. D. Inman and M. S. Group (2008). "Beneficial effects of adalimumab on biomarkers reflecting structural damage in patients with ankylosing spondylitis." J Rheumatol **35**(10): 2030-2037.
Mannion, M. L., L. McAllister, R. Q. Cron and M. L. Stoll (2016). "Ustekinumab as a Therapeutic Option for Children With Refractory Enthesitis-Related Arthritis." J Clin Rheumatol **22**(5): 282-284.
Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi and M. Locati (2004). "The chemokine system in diverse forms of macrophage activation and polarization." Trends Immunol **25**(12): 677-686.
Marino, A., M. De Souza, T. Giani and R. Cimaz (2020). "Pharmacotherapy for juvenile spondyloarthritis: an overview of the available therapies." Expert Opin Pharmacother **21**(17): 2161-2168.
Marsh, S. G., E. D. Albert, W. F. Bodmer, R. E. Bontrop, B. Dupont, H. A. Erlich, M. Fernandez-Vina, D. E. Geraghty, R. Holdsworth, C. K. Hurley, M. Lau, K. W. Lee, B. Mach, M. Maiers, W. R. Mayr, C. R. Muller, P. Parham, E. W. Petersdorf, T. Sasazuki, J. L. Strominger, A. Svejgaard, P. I. Terasaki, J. M. Tiercy and J. Trowsdale (2010). "Nomenclature for factors of the HLA system, 2010." Tissue Antigens **75**(4): 291-455.
Martindale, J., J. Smith, C. J. Sutton, D. Grennan, L. Goodacre and J. A. Goodacre (2006). "Disease and psychological status in ankylosing spondylitis." Rheumatology (Oxford) **45**(10): 1288-1293.
Martinez-Gonzalez, O., J. Cantero-Hinojosa, P. Paule-Sastre, J. C. Gomez-Magan and D. Salvatierra-Rios (1994). "Intestinal permeability in patients with ankylosing spondylitis and their healthy relatives." Br J Rheumatol **33**(7): 644-647.
Martinez, F. O. and S. Gordon (2014). "The M1 and M2 paradigm of macrophage activation: time for reassessment." F1000Prime Rep **6**: 13.
Martinon, F., X. Chen, A. H. Lee and L. H. Glimcher (2010). "TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages." Nat Immunol **11**(5): 411-418.
Martinon, F. and L. H. Glimcher (2011). "Regulation of innate immunity by signaling pathways emerging from the endoplasmic reticulum." Curr Opin Immunol **23**(1): 35-40.

Marzo-Ortega, H., D. McGonagle, P. O'Connor, E. M. Hensor, A. N. Bennett, M. J. Green and P. Emery (2009). "Baseline and 1-year magnetic resonance imaging of the sacroiliac joint and lumbar spine in very early inflammatory back pain. Relationship between symptoms, HLA-B27 and disease extent and persistence." Ann Rheum Dis **68**(11): 1721-1727.
Mascanfroni, I. D., A. Yeste, S. M. Vieira, E. J. Burns, B. Patel, I. Sloma, Y. Wu, L. Mayo, R. Ben-Hamo, S. Efroni, V. K. Kuchroo, S. C. Robson and F. J. Quintana (2013). "IL-27 acts on DCs to suppress the T cell response and autoimmunity by inducing expression of the immunoregulatory molecule CD39." Nat Immunol **14**(10): 1054-1063.
Mathieu, A., F. Paladini, A. Vacca, A. Cauli, M. T. Fiorillo and R. Sorrentino (2009). "The interplay between the geographic distribution of HLA-B27 alleles and their role in infectious and autoimmune diseases: a unifying hypothesis." Autoimmun Rev **8**(5): 420-425.
Mattey, D. L., J. C. Packham, N. B. Nixon, L. Coates, P. Creamer, S. Hailwood, G. J. Taylor and A. K. Bhalla (2012). "Association of cytokine and matrix metalloproteinase profiles with disease activity and function in ankylosing spondylitis." Arthritis Res Ther **14**(3): R127.
May, E., M. L. Dorris, N. Satumtira, I. Iqbal, M. I. Rehman, E. Lightfoot and J. D. Taurog (2003). "CD8 alpha beta T cells are not essential to the pathogenesis of arthritis or colitis in HLA-B27 transgenic rats." J Immunol **170**(2): 1099-1105.
Mayordomo, A. C., J. E. Silva, C. V. Gorlino, J. L. Arias, W. Beron and M. S. Di Genaro (2018). "IL-12/23p40 overproduction by dendritic cells leads to an increased Th1 and Th17 polarization in a model of Yersinia enterocolitica-induced reactive arthritis in TNFRp55-/- mice." PLoS One **13**(3): e0193573.
McCormack, W. J., A. E. Parker and L. A. O'Neill (2009). "Toll-like receptors and NOD-like receptors in rheumatic diseases." Arthritis Res Ther **11**(5): 243.
McGeachy, M. J. (2011). "GM-CSF: the secret weapon in the T(H)17 arsenal." Nat Immunol **12**(6): 521-522.
McGeachy, M. J., Y. Chen, C. M. Tato, A. Laurence, B. Joyce-Shaikh, W. M. Blumenschein, T. K. McClanahan, J. J. O'Shea and D. J. Cua (2009). "The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo." Nat Immunol **10**(3): 314-324.
McGonagle, D., M. Benjamin, H. Marzo-Ortega and P. Emery (2002). "Advances in the understanding of entheseal inflammation." Curr Rheumatol Rep **4**(6): 500-506.
McGonagle, D., H. Marzo-Ortega, P. O'Connor, W. Gibbon, P. Hawkey, K. Henshaw and P. Emery (2002). "Histological assessment of the early enthesitis lesion in spondyloarthropathy." Ann Rheum Dis **61**(6): 534-537.
McHugh, K. and P. Bowness (2012). "The link between HLA-B27 and SpA--new ideas on an old problem." Rheumatology (Oxford) **51**(9): 1529-1539.
Mear, J. P., K. L. Schreiber, C. Munz, X. Zhu, S. Stevanovic, H. G. Rammensee, S. L. Rowland-Jones and R. A. Colbert (1999). "Misfolding of HLA-B27 as a result of its B pocket suggests a novel mechanism for its role in susceptibility to spondyloarthropathies." J Immunol **163**(12): 6665-6670.
Mease, P. (2019). "Ustekinumab Fails to Show Efficacy in a Phase III Axial Spondyloarthritis Program: The Importance of Negative Results." Arthritis Rheumatol **71**(2): 179-181.
Mease, P., L. C. Coates, P. S. Helliwell, M. Stanislavchuk, A. Rychlewska-Hanczewska, A. Dudek, W. Abi-Saab, C. Tasset, L. Meuleners, P. Harrison, R. Besuyen, A. Van der Aa, N. Mozaffarian, J. M. Greer, R. Kunder, F. Van den Bosch and D. D. Gladman (2018). "Efficacy and safety of filgotinib, a selective Janus kinase 1 inhibitor, in patients with active psoriatic arthritis (EQUATOR): results from a randomised, placebo-controlled, phase 2 trial." Lancet **392**(10162): 2367-2377.
Mease, P., S. Hall, O. FitzGerald, D. van der Heijde, J. F. Merola, F. Avila-Zapata, D. Cieslak, D. Graham, C. Wang, S. Menon, T. Hendrikx and K. S. Kanik (2017). "Tofacitinib or Adalimumab versus Placebo for Psoriatic Arthritis." N Engl J Med **377**(16): 1537-1550.
Mease, P. J., D. D. Gladman, A. Deodhar, D. G. McGonagle, P. Nash, W. H. Boehncke, A. Gottlieb, X. L. Xu, S. Xu, E. C. Hsia, C. S. Karyekar and P. S. Helliwell (2020). "Impact of guselkumab, an interleukin-

23 p19 subunit inhibitor, on enthesitis and dactylitis in patients with moderate to severe psoriatic arthritis: results from a randomised, placebo-controlled, phase II study." RMD Open **6**(2).
Mease, P. J., P. S. Helliwell, K. F. Hjuler, K. Raymond and I. McInnes (2020). "Brodalumab in psoriatic arthritis: results from the randomised phase III AMVISION-1 and AMVISION-2 trials." Ann Rheum Dis.
Mei, Y., F. Pan, J. Gao, R. Ge, Z. Duan, Z. Zeng, F. Liao, G. Xia, S. Wang, S. Xu, J. Xu, L. Zhang and D. Ye (2011). "Increased serum IL-17 and IL-23 in the patient with ankylosing spondylitis." Clin Rheumatol **30**(2): 269-273.
Melis, L., B. Vandooren, E. Kruithof, P. Jacques, M. De Vos, H. Mielants, G. Verbruggen, F. De Keyser and D. Elewaut (2010). "Systemic levels of IL-23 are strongly associated with disease activity in rheumatoid arthritis but not spondyloarthritis." Ann Rheum Dis **69**(3): 618-623.
Merilahti-Palo, R., K. O. Soderstrom, R. Lahesmaa-Rantala, K. Granfors and A. Toivanen (1991). "Bacterial antigens in synovial biopsy specimens in yersinia triggered reactive arthritis." Ann Rheum Dis **50**(2): 87-90.
Mescher, M. F., J. M. Curtsinger, P. Agarwal, K. A. Casey, M. Gerner, C. D. Hammerbeck, F. Popescu and Z. Xiao (2006). "Signals required for programming effector and memory development by CD8+ T cells." Immunol Rev **211**: 81-92.
Metcalf, D. (1995). "The granulocyte-macrophage regulators: reappraisal by gene inactivation." Exp Hematol **23**(7): 569-572.
Metcalf, D. (2008). "Hematopoietic cytokines." Blood **111**(2): 485-491.
Mia, S., A. Warnecke, X. M. Zhang, V. Malmstrom and R. A. Harris (2014). "An optimized protocol for human M2 macrophages using M-CSF and IL-4/IL-10/TGF-beta yields a dominant immunosuppressive phenotype." Scand J Immunol **79**(5): 305-314.
Midwood, K., S. Sacre, A. M. Piccinini, J. Inglis, A. Trebaul, E. Chan, S. Drexler, N. Sofat, M. Kashiwagi, G. Orend, F. Brennan and B. Foxwell (2009). "Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease." Nat Med **15**(7): 774-780.
Mielants, H., E. M. Veys, C. Cuvelier and M. de Vos (1988). "Ileocolonoscopic findings in seronegative spondylarthropathies." Br J Rheumatol **27 Suppl 2**: 95-105.
Milia, A. F., L. Ibba-Manneschi, M. Manetti, G. Benelli, S. Generini, L. Messerini and M. Matucci-Cerinic (2011). "Evidence for the prevention of enthesitis in HLA-B27/hbeta(2)m transgenic rats treated with a monoclonal antibody against TNF-alpha." J Cell Mol Med **15**(2): 270-279.
Mills, C. D., K. Kincaid, J. M. Alt, M. J. Heilman and A. M. Hill (2000). "M-1/M-2 macrophages and the Th1/Th2 paradigm." J Immunol **164**(12): 6166-6173.
Minden, K. (2009). "Adult outcomes of patients with juvenile idiopathic arthritis." Horm Res **72 Suppl 1**: 20-25.
Miossec, P. and J. K. Kolls (2012). "Targeting IL-17 and TH17 cells in chronic inflammation." Nat Rev Drug Discov **11**(10): 763-776.
Mistry, R. R., P. Patro, V. Agarwal and D. P. Misra (2019). "Enthesitis-related arthritis: current perspectives." Open Access Rheumatol **11**: 19-31.
Moll, J. M., I. Haslock, I. F. Macrae and V. Wright (1974). "Associations between ankylosing spondylitis, psoriatic arthritis, Reiter's disease, the intestinal arthropathies, and Behcet's syndrome." Medicine (Baltimore) **53**(5): 343-364.
Molnar, C., A. Scherer, X. Baraliakos, M. de Hooge, R. Micheroli, P. Exer, R. O. Kissling, G. Tamborrini, L. M. Wildi, M. J. Nissen, P. Zufferey, J. Bernhard, U. Weber, R. B. M. Landewe, D. van der Heijde, A. Ciurea and P. Rheumatologists of the Swiss Clinical Quality Management (2018). "TNF blockers inhibit spinal radiographic progression in ankylosing spondylitis by reducing disease activity: results from the Swiss Clinical Quality Management cohort." Ann Rheum Dis **77**(1): 63-69.
Monnet, D., A. Kadi, B. Izac, N. Lebrun, F. Letourneur, E. Zinovieva, R. Said-Nahal, G. Chiocchia and M. Breban (2012). "Association between the IL-1 family gene cluster and spondyloarthritis." Ann Rheum Dis **71**(6): 885-890.

Motta, V., F. Soares, T. Sun and D. J. Philpott (2015). "NOD-like receptors: versatile cytosolic sentinels." Physiol Rev **95**(1): 149-178.
Mou, Y. K., P. P. Zhang, Q. X. Li, Z. M. Lin, Z. T. Liao, Q. J. Wei and J. R. Gu (2015). "Changes of serum levels of MMP-3, sRANKL, and OPG in juvenile-onset ankylosing spondylitis patients carrying different HLA-B27 subtypes." Clin Rheumatol **34**(6): 1085-1089.
Moz, S., A. Aita, D. Basso, R. Ramonda, M. Plebani and L. Punzi (2017). "Spondyloarthritis: Matrix Metalloproteinasesas Biomarkers of Pathogenesis and Response to Tumor Necrosis Factor (TNF) Inhibitors." Int J Mol Sci **18**(4).
Moz, S., D. Basso, A. Padoan, D. Bozzato, P. Fogar, C. F. Zambon, M. Pelloso, C. Sperti, S. Vigili de Kreutzenberg, C. Pasquali, S. Pedrazzoli, A. Avogaro and M. Plebani (2016). "Blood expression of matrix metalloproteinases 8 and 9 and of their inducers S100A8 and S100A9 supports diagnosis and prognosis of PDAC-associated diabetes mellitus." Clin Chim Acta **456**: 24-30.
Murphy, C. A., C. L. Langrish, Y. Chen, W. Blumenschein, T. McClanahan, R. A. Kastelein, J. D. Sedgwick and D. J. Cua (2003). "Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation." J Exp Med **198**(12): 1951-1957.
Murphy, F. J., M. P. Hayes and P. R. Burd (2000). "Disparate intracellular processing of human IL-12 preprotein subunits: atypical processing of the P35 signal peptide." J Immunol **164**(2): 839-847.
Murray, P. J., J. E. Allen, S. K. Biswas, E. A. Fisher, D. W. Gilroy, S. Goerdt, S. Gordon, J. A. Hamilton, L. B. Ivashkiv, T. Lawrence, M. Locati, A. Mantovani, F. O. Martinez, J. L. Mege, D. M. Mosser, G. Natoli, J. P. Saeij, J. L. Schultze, K. A. Shirey, A. Sica, J. Suttles, I. Udalova, J. A. van Ginderachter, S. N. Vogel and T. A. Wynn (2014). "Macrophage activation and polarization: nomenclature and experimental guidelines." Immunity **41**(1): 14-20.
Murray, P. J. and T. A. Wynn (2011). "Protective and pathogenic functions of macrophage subsets." Nat Rev Immunol **11**(11): 723-737.
Myles, A. and A. Aggarwal (2011). "Expression of Toll-like receptors 2 and 4 is increased in peripheral blood and synovial fluid monocytes of patients with enthesitis-related arthritis subtype of juvenile idiopathic arthritis." Rheumatology (Oxford) **50**(3): 481-488.
Nakajima, H., H. Takamori, Y. Hiyama and W. Tsukada (1991). "The effect of treatment with recombinant gamma-interferon on adjuvant-induced arthritis in rats." Agents Actions **34**(1-2): 63-65.
Nash, P., B. Kirkham, M. Okada, P. Rahman, B. Combe, G. R. Burmester, D. H. Adams, L. Kerr, C. Lee, C. L. Shuler, M. Genovese and S.-P. S. Group (2017). "Ixekizumab for the treatment of patients with active psoriatic arthritis and an inadequate response to tumour necrosis factor inhibitors: results from the 24-week randomised, double-blind, placebo-controlled period of the SPIRIT-P2 phase 3 trial." Lancet **389**(10086): 2317-2327.
Nathan, C. F., H. W. Murray, M. E. Wiebe and B. Y. Rubin (1983). "Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity." J Exp Med **158**(3): 670-689.
Neerinckx, B., S. Carter and R. Lories (2014). "IL-23 expression and activation of autophagy in synovium and PBMCs of HLA-B27 positive patients with ankylosing spondylitis. Response to: 'Evidence that autophagy, but not the unfolded protein response, regulates the expression of IL-23 in the gut of patients with ankylosing spondylitis and subclinical gut inflammation' by Ciccia et al." Ann Rheum Dis **73**(11): e68.
Neerinckx, B., S. Carter and R. J. Lories (2014). "No evidence for a critical role of the unfolded protein response in synovium and blood of patients with ankylosing spondylitis." Ann Rheum Dis **73**(3): 629-630.
Neisig, A., R. Wubbolts, X. Zang, C. Melief and J. Neefjes (1996). "Allele-specific differences in the interaction of MHC class I molecules with transporters associated with antigen processing." J Immunol **156**(9): 3196-3206.
Nicholson, L. B. (2016). "The immune system." Essays Biochem **60**(3): 275-301.

Nistala, K., S. Adams, H. Cambrook, S. Ursu, B. Olivito, W. de Jager, J. G. Evans, R. Cimaz, M. Bajaj-Elliott and L. R. Wedderburn (2010). "Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment." Proc Natl Acad Sci U S A **107**(33): 14751-14756.
Nistala, K., H. Moncrieffe, K. R. Newton, H. Varsani, P. Hunter and L. R. Wedderburn (2008). "Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers." Arthritis Rheum **58**(3): 875-887.
Nocturne, G., S. Pavy, S. Boudaoud, R. Seror, P. Goupille, P. Chanson, D. van der Heijde, F. van Gaalen, F. Berenbaum, X. Mariette, K. Briot, A. Feydy, P. Claudepierre, P. Dieude, J. Nithitham, K. E. Taylor, L. A. Criswell, M. Dougados, C. Roux and C. Miceli-Richard (2015). "Increase in Dickkopf-1 Serum Level in Recent Spondyloarthritis. Data from the DESIR Cohort." PLoS One **10**(8): e0134974.
Noordenbos, T., N. Yeremenko, I. Gofita, M. van de Sande, P. P. Tak, J. D. Canete and D. Baeten (2012). "Interleukin-17-positive mast cells contribute to synovial inflammation in spondylarthritis." Arthritis Rheum **64**(1): 99-109.
Nossner, E. and P. Parham (1995). "Species-specific differences in chaperone interaction of human and mouse major histocompatibility complex class I molecules." J Exp Med **181**(1): 327-337.
Notley, C. A., J. J. Inglis, S. Alzabin, F. E. McCann, K. E. McNamee and R. O. Williams (2008). "Blockade of tumor necrosis factor in collagen-induced arthritis reveals a novel immunoregulatory pathway for Th1 and Th17 cells." J Exp Med **205**(11): 2491-2497.
Noureldin, B. and N. Barkham (2018). "The current standard of care and the unmet needs for axial spondyloarthritis." Rheumatology (Oxford) **57**(suppl_6): vi10-vi17.
O'Shea, F. D., E. Boyle, R. Riarh, S. M. Tse, R. M. Laxer and R. D. Inman (2009). "Comparison of clinical and radiographic severity of juvenile-onset versus adult-onset ankylosing spondylitis." Ann Rheum Dis **68**(9): 1407-1412.
Oeckinghaus, A., M. S. Hayden and S. Ghosh (2011). "Crosstalk in NF-kappaB signaling pathways." Nat Immunol **12**(8): 695-708.
Oertelt-Prigione, S. (2012). "The influence of sex and gender on the immune response." Autoimmun Rev **11**(6-7): A479-485.
Oktayoglu, P., M. Bozkurt, N. Mete, M. Caglayan, S. Em and K. Nas (2014). "Elevated serum levels of calprotectin (myeloid-related protein 8/14) in patients with ankylosing spondylitis and its association with disease activity and quality of life." J Investig Med **62**(6): 880-884.
Oliveira-Ramos, F., M. Eusebio, M. M. F, A. F. Mourao, C. Furtado, R. Campanilho-Marques, I. Cordeiro, J. Ferreira, M. Cerqueira, R. Figueira, I. Brito, H. Canhao, M. J. Santos, J. A. Melo-Gomes and J. E. Fonseca (2016). "Juvenile idiopathic arthritis in adulthood: fulfilment of classification criteria for adult rheumatic diseases, long-term outcomes and predictors of inactive disease, functional status and damage." RMD Open **2**(2): e000304.
Ometto, F., L. Friso, D. Astorri, C. Botsios, B. Raffeiner, L. Punzi and A. Doria (2017). "Calprotectin in rheumatic diseases." Exp Biol Med (Maywood) **242**(8): 859-873.
Oppmann, B., R. Lesley, B. Blom, J. C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, F. Zonin, E. Vaisberg, T. Churakova, M. Liu, D. Gorman, J. Wagner, S. Zurawski, Y. Liu, J. S. Abrams, K. W. Moore, D. Rennick, R. de Waal-Malefyt, C. Hannum, J. F. Bazan and R. A. Kastelein (2000). "Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12." Immunity **13**(5): 715-725.
Orecchioni, M., Y. Ghosheh, A. B. Pramod and K. Ley (2019). "Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS-) vs. Alternatively Activated Macrophages." Front Immunol **10**: 1084.
Orr, K. E., S. Andronikou, M. J. Bramham, I. Holjar-Erlic, F. Menegotto and A. V. Ramanan (2018). "Magnetic resonance imaging of sacroiliitis in children: frequency of findings and interobserver reliability." Pediatr Radiol **48**(11): 1621-1628.
Ospelt, C., F. Brentano, Y. Rengel, J. Stanczyk, C. Kolling, P. P. Tak, R. E. Gay, S. Gay and D. Kyburz (2008). "Overexpression of toll-like receptors 3 and 4 in synovial tissue from patients with early

rheumatoid arthritis: toll-like receptor expression in early and longstanding arthritis." Arthritis Rheum **58**(12): 3684-3692.
Ospelt, C. and S. Gay (2010). "TLRs and chronic inflammation." Int J Biochem Cell Biol **42**(4): 495-505.
Otten, M. H., F. H. Prince, M. Twilt, R. Ten Cate, W. Armbrust, E. P. Hoppenreijs, Y. Koopman-Keemink, N. M. Wulffraat, S. L. Gorter, K. M. Dolman, J. F. Swart, J. M. van den Berg, M. A. van Rossum and L. W. van Suijlekom-Smit (2011). "Tumor necrosis factor-blocking agents for children with enthesitis-related arthritis--data from the dutch arthritis and biologicals in children register, 1999-2010." J Rheumatol **38**(10): 2258-2263.
Packham, J. (2018). "Optimizing outcomes for ankylosing spondylitis and axial spondyloarthritis patients: a holistic approach to care." Rheumatology (Oxford) **57**(suppl_6): vi29-vi34.
Page, C. E., S. Smale, S. M. Carty, N. Amos, S. N. Lauder, R. M. Goodfellow, P. J. Richards, S. A. Jones, N. Topley and A. S. Williams (2010). "Interferon-gamma inhibits interleukin-1beta-induced matrix metalloproteinase production by synovial fibroblasts and protects articular cartilage in early arthritis." Arthritis Res Ther **12**(2): R49.
Pagnini, I., S. Savelli, M. Matucci-Cerinic, C. Fonda, R. Cimaz and G. Simonini (2010). "Early predictors of juvenile sacroiliitis in enthesitis-related arthritis." J Rheumatol **37**(11): 2395-2401.
Papp, K., D. Thaci, D. Marcoux, L. Weibel, S. Philipp, P. D. Ghislain, I. Landells, P. Hoeger, C. Kotkin, K. Unnebrink, M. Seyger and D. Williams (2017). "Efficacy and safety of adalimumab every other week versus methotrexate once weekly in children and adolescents with severe chronic plaque psoriasis: a randomised, double-blind, phase 3 trial." Lancet **390**(10089): 40-49.
Pedersen, S. J., I. J. Sorensen, P. Garnero, J. S. Johansen, O. R. Madsen, N. Tvede, M. S. Hansen, G. Thamsborg, L. S. Andersen, O. Majgaard, A. G. Loft, J. Erlendsson, K. Asmussen, A. G. Jurik, J. Moller, M. Hasselquist, D. Mikkelsen, T. Skjodt, R. Lambert, A. Hansen and M. Ostergaard (2011). "ASDAS, BASDAI and different treatment responses and their relation to biomarkers of inflammation, cartilage and bone turnover in patients with axial spondyloarthritis treated with TNFalpha inhibitors." Ann Rheum Dis **70**(8): 1375-1381.
Petty, R. E., T. R. Southwood, P. Manners, J. Baum, D. N. Glass, J. Goldenberg, X. He, J. Maldonado-Cocco, J. Orozco-Alcala, A. M. Prieur, M. E. Suarez-Almazor and P. Woo (2004). "International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001." J Rheumatol **31**(2): 390-392.
Pflanz, S., J. C. Timans, J. Cheung, R. Rosales, H. Kanzler, J. Gilbert, L. Hibbert, T. Churakova, M. Travis, E. Vaisberg, W. M. Blumenschein, J. D. Mattson, J. L. Wagner, W. To, S. Zurawski, T. K. McClanahan, D. M. Gorman, J. F. Bazan, R. de Waal Malefyt, D. Rennick and R. A. Kastelein (2002). "IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells." Immunity **16**(6): 779-790.
Pharoah, D. S., H. Varsani, R. W. Tatham, K. R. Newton, W. de Jager, B. J. Prakken, N. Klein and L. R. Wedderburn (2006). "Expression of the inflammatory chemokines CCL5, CCL3 and CXCL10 in juvenile idiopathic arthritis, and demonstration of CCL5 production by an atypical subset of CD8+ T cells." Arthritis Res Ther **8**(2): R50.
Pinzone, J. J., B. M. Hall, N. K. Thudi, M. Vonau, Y. W. Qiang, T. J. Rosol and J. D. Shaughnessy, Jr. (2009). "The role of Dickkopf-1 in bone development, homeostasis, and disease." Blood **113**(3): 517-525.
Piper, C., A. M. Pesenacker, D. Bending, B. Thirugnanabalan, H. Varsani, L. R. Wedderburn and K. Nistala (2014). "T cell expression of granulocyte-macrophage colony-stimulating factor in juvenile arthritis is contingent upon Th17 plasticity." Arthritis Rheumatol **66**(7): 1955-1960.
Pitha, P. M. (2004). "Unexpected similarities in cellular responses to bacterial and viral invasion." Proc Natl Acad Sci U S A **101**(3): 695-696.
Plater-Zyberk, C., L. A. Joosten, M. M. Helsen, J. Hepp, P. A. Baeuerle and W. B. van den Berg (2007). "GM-CSF neutralisation suppresses inflammation and protects cartilage in acute streptococcal cell wall arthritis of mice." Ann Rheum Dis **66**(4): 452-457.

Poddubnyy, D., K. G. Hermann, J. Callhoff, J. Listing and J. Sieper (2014). "Ustekinumab for the treatment of patients with active ankylosing spondylitis: results of a 28-week, prospective, open-label, proof-of-concept study (TOPAS)." Ann Rheum Dis **73**(5): 817-823.
Poddubnyy, D., J. Listing and J. Sieper (2016). "Course of active inflammatory and fatty lesions in patients with early axial spondyloarthritis treated with infliximab plus naproxen as compared to naproxen alone: Results from the INFAST study." Arthritis Rheumatol.
Poddubnyy, D., M. Protopopov, H. Haibel, J. Braun, M. Rudwaleit and J. Sieper (2016). "High disease activity according to the Ankylosing Spondylitis Disease Activity Score is associated with accelerated radiographic spinal progression in patients with early axial spondyloarthritis: results from the GErman SPondyloarthritis Inception Cohort." Ann Rheum Dis **75**(12): 2114-2118.
Poddubnyy, D., M. Rudwaleit, H. Haibel, J. Listing, E. Marker-Hermann, H. Zeidler, J. Braun and J. Sieper (2011). "Rates and predictors of radiographic sacroiliitis progression over 2 years in patients with axial spondyloarthritis." Ann Rheum Dis **70**(8): 1369-1374.
Poddubnyy, D. and J. Sieper (2017). "What is the best treatment target in axial spondyloarthritis: tumour necrosis factor alpha, interleukin 17, or both?" Rheumatology (Oxford).
Prieto, G. A. and Y. Rosenstein (2006). "Oestradiol potentiates the suppressive function of human CD4 CD25 regulatory T cells by promoting their proliferation." Immunology **118**(1): 58-65.
Radstake, T. R., M. F. Roelofs, Y. M. Jenniskens, B. Oppers-Walgreen, P. L. van Riel, P. Barrera, L. A. Joosten and W. B. van den Berg (2004). "Expression of toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflammatory cytokines interleukin-12 and interleukin-18 via interferon-gamma." Arthritis Rheum **50**(12): 3856-3865.
Rajaiah, R., M. Puttabyatappa, S. K. Polumuri and K. D. Moudgil (2011). "Interleukin-27 and interferon-gamma are involved in regulation of autoimmune arthritis." J Biol Chem **286**(4): 2817-2825.
Ramanan, A. V., A. D. Dick, A. P. Jones, A. McKay, P. R. Williamson, S. Compeyrot-Lacassagne, B. Hardwick, H. Hickey, D. Hughes, P. Woo, D. Benton, C. Edelsten, M. W. Beresford and S. S. Group (2017). "Adalimumab plus Methotrexate for Uveitis in Juvenile Idiopathic Arthritis." N Engl J Med **376**(17): 1637-1646.
Ramanathan, A., H. Srinivasalu and R. A. Colbert (2013). "Update on juvenile spondyloarthritis." Rheum Dis Clin North Am **39**(4): 767-788.
Ramiro, S., R. Landewe, A. van Tubergen, A. Boonen, C. Stolwijk, M. Dougados, F. van den Bosch and D. van der Heijde (2015). "Lifestyle factors may modify the effect of disease activity on radiographic progression in patients with ankylosing spondylitis: a longitudinal analysis." RMD Open **1**(1): e000153.
Ramiro, S., D. van der Heijde, A. van Tubergen, C. Stolwijk, M. Dougados, F. van den Bosch and R. Landewe (2014). "Higher disease activity leads to more structural damage in the spine in ankylosing spondylitis: 12-year longitudinal data from the OASIS cohort." Ann Rheum Dis **73**(8): 1455-1461.
Rashid, T. and A. Ebringer (2007). "Ankylosing spondylitis is linked to Klebsiella--the evidence." Clin Rheumatol **26**(6): 858-864.
Raychaudhuri, S. K. and S. P. Raychaudhuri (2017). "Janus kinase/signal transducer and activator of transcription pathways in spondyloarthritis." Curr Opin Rheumatol **29**(4): 311-316.
Rees, A. J. (2010). "Monocyte and macrophage biology: an overview." Semin Nephrol **30**(3): 216-233.
Reinhardt, A., T. Yevsa, T. Worbs, S. Lienenklaus, I. Sandrock, L. Oberdorfer, T. Korn, S. Weiss, R. Forster and I. Prinz (2016). "Interleukin-23-Dependent gamma/delta T Cells Produce Interleukin-17 and Accumulate in the Enthesis, Aortic Valve, and Ciliary Body in Mice." Arthritis Rheumatol **68**(10): 2476-2486.
Reveille, J. D. (2015). "Biomarkers for diagnosis, monitoring of progression, and treatment responses in ankylosing spondylitis and axial spondyloarthritis." Clin Rheumatol **34**(6): 1009-1018.
Reveille, J. D., R. Hirsch, C. F. Dillon, M. D. Carroll and M. H. Weisman (2012). "The prevalence of HLA-B27 in the US: data from the US National Health and Nutrition Examination Survey, 2009." Arthritis Rheum **64**(5): 1407-1411.

Revu, S., J. Wu, M. Henkel, N. Rittenhouse, A. Menk, G. M. Delgoffe, A. C. Poholek and M. J. McGeachy (2018). "IL-23 and IL-1beta Drive Human Th17 Cell Differentiation and Metabolic Reprogramming in Absence of CD28 Costimulation." Cell Rep **22**(10): 2642-2653.
Ribechini, E., J. A. Hutchinson, S. Hergovits, M. Heuer, J. Lucas, U. Schleicher, A. L. Jordan Garrote, S. J. Potter, P. Riquelme, H. Brackmann, N. Muller, H. Raifer, I. Berberich, M. Huber, A. Beilhack, M. Lohoff, C. Bogdan, M. Eyrich, H. M. Hermanns, E. K. Geissler and M. B. Lutz (2017). "Novel GM-CSF signals via IFN-gammaR/IRF-1 and AKT/mTOR license monocytes for suppressor function." Blood Adv **1**(14): 947-960.
Robbins, S. H., T. Walzer, D. Dembele, C. Thibault, A. Defays, G. Bessou, H. Xu, E. Vivier, M. Sellars, P. Pierre, F. R. Sharp, S. Chan, P. Kastner and M. Dalod (2008). "Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling." Genome Biol **9**(1): R17.
Rock, K. L., E. Reits and J. Neefjes (2016). "Present Yourself! By MHC Class I and MHC Class II Molecules." Trends Immunol **37**(11): 724-737.
Romagnani, S. (1994). "Lymphokine production by human T cells in disease states." Annu Rev Immunol **12**: 227-257.
Romero-Sanchez, C., D. A. Jaimes, J. Londono, J. De Avila, J. E. Castellanos, J. M. Bello, W. Bautista and R. Valle-Onate (2011). "Association between Th-17 cytokine profile and clinical features in patients with spondyloarthritis." Clin Exp Rheumatol **29**(5): 828-834.
Romero-Sanchez, C., W. H. Robinson, B. H. Tomooka, J. Londono, R. Valle-Onate, F. Huang, X. Deng, L. Zhang, C. Yang and D. T. Yu (2008). "Identification of acute phase reactants and cytokines useful for monitoring infliximab therapy in ankylosing spondylitis." Clin Rheumatol **27**(11): 1429-1435.
Rosenberg, A. M. and R. E. Petty (1982). "A syndrome of seronegative enthesopathy and arthropathy in children." Arthritis Rheum **25**(9): 1041-1047.
Rosser, E. C., H. Lom, D. Bending, C. L. Duurland, M. Bajaj-Elliott and L. R. Wedderburn (2019). "Innate Lymphoid Cells and T Cells Contribute to the Interleukin-17A Signature Detected in the Synovial Fluid of Patients With Juvenile Idiopathic Arthritis." Arthritis Rheumatol **71**(3): 460-467.
Roszer, T. (2015). "Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms." Mediators Inflamm **2015**: 816460.
Round, J. L. and S. K. Mazmanian (2009). "The gut microbiota shapes intestinal immune responses during health and disease." Nat Rev Immunol **9**(5): 313-323.
Rudwaleit, M., D. van der Heijde, R. Landewe, N. Akkoc, J. Brandt, C. T. Chou, M. Dougados, F. Huang, J. Gu, Y. Kirazli, F. Van den Bosch, I. Olivieri, E. Roussou, S. Scarpato, I. J. Sorensen, R. Valle-Onate, U. Weber, J. Wei and J. Sieper (2011). "The Assessment of SpondyloArthritis International Society classification criteria for peripheral spondyloarthritis and for spondyloarthritis in general." Ann Rheum Dis **70**(1): 25-31.
Rumsey, D. G., J. Guzman, A. M. Rosenberg, A. M. Huber, R. Scuccimarri, N. J. Shiff, A. Bruns, B. M. Feldman, D. T. Eurich and I. Research in Arthritis in Canadian Children Emphasizing Outcomes (2018). "Characteristics and Course of Enthesitis in a Juvenile Idiopathic Arthritis Inception Cohort." Arthritis Care Res (Hoboken) **70**(2): 303-308.
Ruof, J. and G. Stucki (1999). "Validity aspects of erythrocyte sedimentation rate and C-reactive protein in ankylosing spondylitis: a literature review." J Rheumatol **26**(4): 966-970.
Ruscitti, P., P. Cipriani, F. Carubbi, V. Liakouli, F. Zazzeroni, P. Di Benedetto, O. Berardicurti, E. Alesse and R. Giacomelli (2015). "The role of IL-1beta in the bone loss during rheumatic diseases." Mediators Inflamm **2015**: 782382.
Rusman, T., R. E. van Bentum and I. E. van der Horst-Bruinsma (2020). "Sex and gender differences in axial spondyloarthritis: myths and truths." Rheumatology (Oxford) **59**(Suppl4): iv38-iv46.
Rusman, T., R. F. van Vollenhoven and I. E. van der Horst-Bruinsma (2018). "Gender Differences in Axial Spondyloarthritis: Women Are Not So Lucky." Curr Rheumatol Rep **20**(6): 35.

Ruuska, M., A. S. Sahlberg, R. A. Colbert, K. Granfors and M. A. Penttinen (2012). "Enhanced phosphorylation of STAT-1 is dependent on double-stranded RNA-dependent protein kinase signaling in HLA-B27-expressing U937 monocytic cells." Arthritis Rheum **64**(3): 772-777.
Saad, C. G., A. C. Ribeiro, J. C. Moraes, L. Takayama, C. R. Goncalves, M. B. Rodrigues, R. M. de Oliveira, C. A. Silva, E. Bonfa and R. M. Pereira (2012). "Low sclerostin levels: a predictive marker of persistent inflammation in ankylosing spondylitis during anti-tumor necrosis factor therapy?" Arthritis Res Ther **14**(5): R216.
Sabbah, A., T. H. Chang, R. Harnack, V. Frohlich, K. Tominaga, P. H. Dube, Y. Xiang and S. Bose (2009). "Activation of innate immune antiviral responses by Nod2." Nat Immunol **10**(10): 1073-1080.
Sahlberg, A. S., M. A. Penttinen, K. M. Heiskanen, R. A. Colbert, L. Sistonen and K. Granfors (2007). "Evidence that the p38 MAP kinase pathway is dysregulated in HLA-B27-expressing human monocytic cells: correlation with HLA-B27 misfolding." Arthritis Rheum **56**(8): 2652-2662.
Sallusto, F., C. E. Zielinski and A. Lanzavecchia (2012). "Human Th17 subsets." Eur J Immunol **42**(9): 2215-2220.
Salmi, M., D. P. Andrew, E. C. Butcher and S. Jalkanen (1995). "Dual binding capacity of mucosal immunoblasts to mucosal and synovial endothelium in humans: dissection of the molecular mechanisms." J Exp Med **181**(1): 137-149.
Sarkar, S., L. A. Cooney, P. White, D. B. Dunlop, J. Endres, J. M. Jorns, M. J. Wasco and D. A. Fox (2009). "Regulation of pathogenic IL-17 responses in collagen-induced arthritis: roles of endogenous interferon-gamma and IL-4." Arthritis Res Ther **11**(5): R158.
Satpute, S. R., R. Rajaiah, S. K. Polumuri and K. D. Moudgil (2009). "Tolerization with Hsp65 induces protection against adjuvant-induced arthritis by modulating the antigen-directed interferon-gamma, interleukin-17, and antibody responses." Arthritis Rheum **60**(1): 103-113.
Savage, L., M. Goodfield, L. Horton, A. Watad, E. Hensor, P. Emery, R. Wakefield, M. Wittmann and D. McGonagle (2019). "Regression of Peripheral Subclinical Enthesopathy in Therapy-Naive Patients Treated With Ustekinumab for Moderate-to-Severe Chronic Plaque Psoriasis: A Fifty-Two-Week, Prospective, Open-Label Feasibility Study." Arthritis Rheumatol **71**(4): 626-631.
Saxena, N., A. Aggarwal and R. Misra (2005). "Elevated concentrations of monocyte derived cytokines in synovial fluid of children with enthesitis related arthritis and polyarticular types of juvenile idiopathic arthritis." J Rheumatol **32**(7): 1349-1353.
Schneider, M., A. G. Zimmermann, R. A. Roberts, L. Zhang, K. V. Swanson, H. Wen, B. K. Davis, I. C. Allen, E. K. Holl, Z. Ye, A. H. Rahman, B. J. Conti, T. K. Eitas, B. H. Koller and J. P. Ting (2012). "The innate immune sensor NLRC3 attenuates Toll-like receptor signaling via modification of the signaling adaptor TRAF6 and transcription factor NF-kappaB." Nat Immunol **13**(9): 823-831.
Seipp, M. T., M. Erali, R. L. Wies and C. Wittwer (2005). "HLA-B27 typing: evaluation of an allele-specific PCR melting assay and two flow cytometric antigen assays." Cytometry B Clin Cytom **63**(1): 10-15.
Sen, E. S. and A. V. Ramanan (2017). "Juvenile idiopathic arthritis-associated uveitis." Best Pract Res Clin Rheumatol **31**(4): 517-534.
Seror, R., S. Boudaoud, S. Pavy, G. Nocturne, T. Schaeverbeke, A. Saraux, P. Chanson, J. E. Gottenberg, V. Devauchelle-Pensec, G. J. Tobon, X. Mariette and C. Miceli-Richard (2016). "Increased Dickkopf-1 in Recent-onset Rheumatoid Arthritis is a New Biomarker of Structural Severity. Data from the ESPOIR Cohort." Sci Rep **6**: 18421.
Shah, M., A. Maroof, P. Gikas, G. Mittal, R. Keen, D. Baeten, S. Shaw and S. J. Roberts (2020). "Dual neutralisation of IL-17F and IL-17A with bimekizumab blocks inflammation-driven osteogenic differentiation of human periosteal cells." RMD Open **6**(2).
Shen, F., A. H. Verma, A. Volk, B. Jones, B. M. Coleman, M. J. Loza, R. Malaviya, B. Moore, D. Weinstock, M. M. Elloso, S. L. Gaffen and T. Ort (2019). "Combined Blockade of TNF-alpha and IL-17A Alleviates Progression of Collagen-Induced Arthritis without Causing Serious Infections in Mice." J Immunol **202**(7): 2017-2026.

Shen, H., J. C. Goodall and J. S. Gaston (2010). "Frequency and phenotype of T helper 17 cells in peripheral blood and synovial fluid of patients with reactive arthritis." J Rheumatol **37**(10): 2096-2099.
Shen, P., T. Roch, V. Lampropoulou, R. A. O'Connor, U. Stervbo, E. Hilgenberg, S. Ries, V. D. Dang, Y. Jaimes, C. Daridon, R. Li, L. Jouneau, P. Boudinot, S. Wilantri, I. Sakwa, Y. Miyazaki, M. D. Leech, R. C. McPherson, S. Wirtz, M. Neurath, K. Hoehlig, E. Meinl, A. Grutzkau, J. R. Grun, K. Horn, A. A. Kuhl, T. Dorner, A. Bar-Or, S. H. E. Kaufmann, S. M. Anderton and S. Fillatreau (2014). "IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases." Nature **507**(7492): 366-370.
Shenoy, S. and A. Aggarwal (2016). "Sonologic enthesitis in children with enthesitis-related arthritis." Clin Exp Rheumatol **34**(1): 143-147.
Sherlock, J. P., B. Joyce-Shaikh, S. P. Turner, C. C. Chao, M. Sathe, J. Grein, D. M. Gorman, E. P. Bowman, T. K. McClanahan, J. H. Yearley, G. Eberl, C. D. Buckley, R. A. Kastelein, R. H. Pierce, D. M. Laface and D. J. Cua (2012). "IL-23 induces spondyloarthropathy by acting on ROR-gammat+ CD3+CD4-CD8- entheseal resident T cells." Nat Med **18**(7): 1069-1076.
Shi, J. H. and S. C. Sun (2018). "Tumor Necrosis Factor Receptor-Associated Factor Regulation of Nuclear Factor kappaB and Mitogen-Activated Protein Kinase Pathways." Front Immunol **9**: 1849.
Shifrin, N., D. H. Raulet and M. Ardolino (2014). "NK cell self tolerance, responsiveness and missing self recognition." Semin Immunol **26**(2): 138-144.
Shiomi, A. and T. Usui (2015). "Pivotal roles of GM-CSF in autoimmunity and inflammation." Mediators Inflamm **2015**: 568543.
Shiomi, A., T. Usui, Y. Ishikawa, M. Shimizu, K. Murakami and T. Mimori (2014). "GM-CSF but not IL-17 is critical for the development of severe interstitial lung disease in SKG mice." J Immunol **193**(2): 849-859.
Shuai, K., G. R. Stark, I. M. Kerr and J. E. Darnell, Jr. (1993). "A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma." Science **261**(5129): 1744-1746.
Sica, A., M. Erreni, P. Allavena and C. Porta (2015). "Macrophage polarization in pathology." Cell Mol Life Sci **72**(21): 4111-4126.
Sieper, J. (2016). "New treatment targets for axial spondyloarthritis." Rheumatology (Oxford) **55**(suppl 2): ii38-ii42.
Sieper, J., T. Klopsch, M. Richter, A. Kapelle, M. Rudwaleit, S. Schwank, E. Regourd and M. May (2008). "Comparison of two different dosages of celecoxib with diclofenac for the treatment of active ankylosing spondylitis: results of a 12-week randomised, double-blind, controlled study." Ann Rheum Dis **67**(3): 323-329.
Sieper, J., J. Lenaerts, J. Wollenhaupt, M. Rudwaleit, V. I. Mazurov, L. Myasoutova, S. Park, Y. Song, R. Yao, D. Chitkara and N. Vastesaeger (2014). "Efficacy and safety of infliximab plus naproxen versus naproxen alone in patients with early, active axial spondyloarthritis: results from the double-blind, placebo-controlled INFAST study, Part 1." Ann Rheum Dis **73**(1): 101-107.
Sieper, J., J. Lenaerts, J. Wollenhaupt, M. Rudwaleit, V. I. Mazurov, L. Myasoutova, S. Park, Y. Song, R. Yao, D. Chitkara and N. Vastesaeger (2014). "Maintenance of biologic-free remission with naproxen or no treatment in patients with early, active axial spondyloarthritis: results from a 6-month, randomised, open-label follow-up study, INFAST Part 2." Ann Rheum Dis **73**(1): 108-113.
Sieper, J., J. Listing, D. Poddubnyy, I. H. Song, K. G. Hermann, J. Callhoff, U. Syrbe, J. Braun and M. Rudwaleit (2016). "Effect of continuous versus on-demand treatment of ankylosing spondylitis with diclofenac over 2 years on radiographic progression of the spine: results from a randomised multicentre trial (ENRADAS)." Ann Rheum Dis **75**(8): 1438-1443.
Sieper, J. and D. Poddubnyy (2016). "New evidence on the management of spondyloarthritis." Nat Rev Rheumatol **12**(5): 282-295.
Sieper, J., B. Porter-Brown, L. Thompson, O. Harari and M. Dougados (2014). "Assessment of short-term symptomatic efficacy of tocilizumab in ankylosing spondylitis: results of randomised, placebo-controlled trials." Ann Rheum Dis **73**(1): 95-100.

Sieper, J., D. van der Heijde, M. Dougados, W. P. Maksymowych, B. B. Scott, J. A. Boice, Y. Berd, G. Bergman, S. Curtis, A. Tzontcheva, S. Huyck and H. H. Weng (2015). "A randomized, double-blind, placebo-controlled, sixteen-week study of subcutaneous golimumab in patients with active nonradiographic axial spondyloarthritis." Arthritis Rheumatol **67**(10): 2702-2712.
Sims, A. M., B. P. Wordsworth and M. A. Brown (2004). "Genetic susceptibility to ankylosing spondylitis." Curr Mol Med **4**(1): 13-20.
Singh, R., A. Aggarwal and R. Misra (2007). "Th1/Th17 cytokine profiles in patients with reactive arthritis/undifferentiated spondyloarthropathy." J Rheumatol **34**(11): 2285-2290.
Singh, Y. P., A. K. Singh, A. Aggarwal and R. Misra (2011). "Evidence of cellular immune response to outer membrane protein of Salmonella typhimurium in patients with enthesitis-related arthritis subtype of juvenile idiopathic arthritis." J Rheumatol **38**(1): 161-166.
Smith, J. A. (2015). "Update on ankylosing spondylitis: current concepts in pathogenesis." Curr Allergy Asthma Rep **15**(1): 489.
Smith, J. A. (2016). "The Bench-to-Bedside Story of IL-17 and the Therapeutic Efficacy of its Targeting in Spondyloarthritis." Curr Rheumatol Rep **18**(6): 33.
Smith, J. A. (2018). "Regulation of Cytokine Production by the Unfolded Protein Response; Implications for Infection and Autoimmunity." Front Immunol **9**: 422.
Smith, J. A., M. D. Barnes, D. Hong, M. L. DeLay, R. D. Inman and R. A. Colbert (2008). "Gene expression analysis of macrophages derived from ankylosing spondylitis patients reveals interferon-gamma dysregulation." Arthritis Rheum **58**(6): 1640-1649.
Smith, J. A. and R. A. Colbert (2014). "Review: The interleukin-23/interleukin-17 axis in spondyloarthritis pathogenesis: Th17 and beyond." Arthritis Rheumatol **66**(2): 231-241.
Smolen, J. S., P. Mease, H. Tahir, H. Schulze-Koops, I. de la Torre, L. Li, M. Hojnik, C. Sapin, M. Okada, R. Caporali, J. Gratacos, P. Goupille, S. Liu Leage, S. Pillai and P. Nash (2020). "Multicentre, randomised, open-label, parallel-group study evaluating the efficacy and safety of ixekizumab versus adalimumab in patients with psoriatic arthritis naive to biological disease-modifying antirheumatic drug: final results by week 52." Ann Rheum Dis **79**(10): 1310-1319.
Soliman, E., W. Labib, G. el-Tantawi, A. Hamimy, A. Alhadidy and A. Aldawoudy (2012). "Role of matrix metalloproteinase-3 (MMP-3) and magnetic resonance imaging of sacroiliitis in assessing disease activity in ankylosing spondylitis." Rheumatol Int **32**(6): 1711-1720.
Song, I. H., C. E. Althoff, H. Haibel, K. G. Hermann, D. Poddubnyy, J. Listing, A. Weiss, S. Djacenko, G. R. Burmester, M. Bohl-Buhler, B. Freundlich, M. Rudwaleit and J. Sieper (2012). "Frequency and duration of drug-free remission after 1 year of treatment with etanercept versus sulfasalazine in early axial spondyloarthritis: 2 year data of the ESTHER trial." Ann Rheum Dis **71**(7): 1212-1215.
Song, I. H., F. Heldmann, M. Rudwaleit, H. Haibel, A. Weiss, J. Braun and J. Sieper (2011). "Treatment of active ankylosing spondylitis with abatacept: an open-label, 24-week pilot study." Ann Rheum Dis **70**(6): 1108-1110.
Song, I. H., F. Heldmann, M. Rudwaleit, J. Listing, H. Appel, J. Braun and J. Sieper (2010). "Different response to rituximab in tumor necrosis factor blocker-naive patients with active ankylosing spondylitis and in patients in whom tumor necrosis factor blockers have failed: a twenty-four-week clinical trial." Arthritis Rheum **62**(5): 1290-1297.
Song, I. H., D. A. Poddubnyy, M. Rudwaleit and J. Sieper (2008). "Benefits and risks of ankylosing spondylitis treatment with nonsteroidal antiinflammatory drugs." Arthritis Rheum **58**(4): 929-938.
Spits, H., D. Artis, M. Colonna, A. Diefenbach, J. P. Di Santo, G. Eberl, S. Koyasu, R. M. Locksley, A. N. McKenzie, R. E. Mebius, F. Powrie and E. Vivier (2013). "Innate lymphoid cells--a proposal for uniform nomenclature." Nat Rev Immunol **13**(2): 145-149.
Stanevicha, V., J. Eglite, D. Zavadska, A. Sochnevs, A. Lazareva, D. Guseinova, R. Shantere and D. Gardovska (2010). "HLA B27 allele types in homogeneous groups of juvenile idiopathic arthritis patients in Latvia." Pediatr Rheumatol Online J **8**: 26.
Stanley, E. R., K. L. Berg, D. B. Einstein, P. S. Lee, F. J. Pixley, Y. Wang and Y. G. Yeung (1997). "Biology and action of colony--stimulating factor-1." Mol Reprod Dev **46**(1): 4-10.

Stein, M., S. Keshav, N. Harris and S. Gordon (1992). "Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation." J Exp Med **176**(1): 287-292.
Steinman, R. M. (2012). "Decisions about dendritic cells: past, present, and future." Annu Rev Immunol **30**: 1-22.
Stoll, M. L., R. Bhore, M. Dempsey-Robertson and M. Punaro (2010). "Spondyloarthritis in a pediatric population: risk factors for sacroiliitis." J Rheumatol **37**(11): 2402-2408.
Stoll, M. L., R. Kumar, E. J. Lefkowitz, R. Q. Cron, C. D. Morrow and S. Barnes (2016). "Fecal metabolomics in pediatric spondyloarthritis implicate decreased metabolic diversity and altered tryptophan metabolism as pathogenic factors." Genes Immun **17**(7): 400-405.
Stoll, M. L., P. F. Weiss, J. E. Weiss, P. A. Nigrovic, B. S. Edelheit, S. L. Bridges, Jr., M. I. Danila, C. H. Spencer, M. G. Punaro, K. Schikler, A. Reiff, R. Kumar, R. Q. Cron, C. D. Morrow and E. J. Lefkowitz (2018). "Age and fecal microbial strain-specific differences in patients with spondyloarthritis." Arthritis Res Ther **20**(1): 14.
Stolwijk, C., M. van Onna, A. Boonen and A. van Tubergen (2016). "Global Prevalence of Spondyloarthritis: A Systematic Review and Meta-Regression Analysis." Arthritis Care Res (Hoboken) **68**(9): 1320-1331.
Straub, R. H. (2007). "The complex role of estrogens in inflammation." Endocr Rev **28**(5): 521-574.
Straub, R. H., S. Struharova, J. Scholmerich and P. Harle (2002). "No alterations of serum levels of adrenal and gonadal hormones in patients with ankylosing spondylitis." Clin Exp Rheumatol **20**(6 Suppl 28): S52-59.
Stumhofer, J. S., A. Laurence, E. H. Wilson, E. Huang, C. M. Tato, L. M. Johnson, A. V. Villarino, Q. Huang, A. Yoshimura, D. Sehy, C. J. Saris, J. J. O'Shea, L. Hennighausen, M. Ernst and C. A. Hunter (2006). "Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system." Nat Immunol **7**(9): 937-945.
Sun, S. C. (2012). "The noncanonical NF-kappaB pathway." Immunol Rev **246**(1): 125-140.
Sun, S. C. (2017). "The non-canonical NF-kappaB pathway in immunity and inflammation." Nat Rev Immunol **17**(9): 545-558.
Suri, A., S. B. Lovitch and E. R. Unanue (2006). "The wide diversity and complexity of peptides bound to class II MHC molecules." Curr Opin Immunol **18**(1): 70-77.
Sveaas, S. H., I. J. Berg, S. A. Provan, A. G. Semb, I. C. Olsen, T. Ueland, P. Aukrust, N. Vollestad, K. B. Hagen, T. K. Kvien and H. Dagfinrud (2015). "Circulating levels of inflammatory cytokines and cytokine receptors in patients with ankylosing spondylitis: a cross-sectional comparative study." Scand J Rheumatol **44**(2): 118-124.
Szekanecz, Z., A. Vegvari, Z. Szabo and A. E. Koch (2010). "Chemokines and chemokine receptors in arthritis." Front Biosci (Schol Ed) **2**: 153-167.
Tahir, H. (2018). "Therapies in ankylosing spondylitis-from clinical trials to clinical practice." Rheumatology (Oxford) **57**(suppl_6): vi23-vi28.
Takatsuki, A. and G. Tamura (1971). "Tunicamycin, a new antibiotic. 3. Reversal of the antiviral activity of tunicamycin by aminosugars and their derivatives." J Antibiot (Tokyo) **24**(4): 232-238.
Tamura, T., H. Yanai, D. Savitsky and T. Taniguchi (2008). "The IRF family transcription factors in immunity and oncogenesis." Annu Rev Immunol **26**: 535-584.
Tan, F. K. and K. Farheen (2011). "The potential importance of Toll-like receptors in ankylosing spondylitis." Int J Clin Rheumtol **6**(6): 649-654.
Taurog, J. D., M. L. Dorris, N. Satumtira, T. M. Tran, R. Sharma, R. Dressel, J. van den Brandt and H. M. Reichardt (2009). "Spondylarthritis in HLA-B27/human beta2-microglobulin-transgenic rats is not prevented by lack of CD8." Arthritis Rheum **60**(7): 1977-1984.
Taurog, J. D., S. D. Maika, N. Satumtira, M. L. Dorris, I. L. McLean, H. Yanagisawa, A. Sayad, A. J. Stagg, G. M. Fox, A. Le O'Brien, M. Rehman, M. Zhou, A. L. Weiner, J. B. Splawski, J. A. Richardson and R. E. Hammer (1999). "Inflammatory disease in HLA-B27 transgenic rats." Immunol Rev **169**: 209-223.

Taurog, J. D., S. D. Maika, W. A. Simmons, M. Breban and R. E. Hammer (1993). "Susceptibility to inflammatory disease in HLA-B27 transgenic rat lines correlates with the level of B27 expression." J Immunol **150**(9): 4168-4178.
Taurog, J. D., J. A. Richardson, J. T. Croft, W. A. Simmons, M. Zhou, J. L. Fernandez-Sueiro, E. Balish and R. E. Hammer (1994). "The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats." J Exp Med **180**(6): 2359-2364.
Taxter, A. J., E. P. Wileyto, E. M. Behrens and P. F. Weiss (2015). "Patient-reported Outcomes across Categories of Juvenile Idiopathic Arthritis." J Rheumatol **42**(10): 1914-1921.
Tejesvi, M. V., M. Arvonen, S. M. Kangas, P. L. Keskitalo, A. M. Pirttila, T. J. Karttunen and P. Vahasalo (2016). "Faecal microbiome in new-onset juvenile idiopathic arthritis." Eur J Clin Microbiol Infect Dis **35**(3): 363-370.
Terenzi, R., S. Monti, G. Tesei and L. Carli (2018). "One year in review 2017: spondyloarthritis." Clin Exp Rheumatol **36**(1): 1-14.
Thomson, W., J. H. Barrett, R. Donn, L. Pepper, L. J. Kennedy, W. E. Ollier, A. J. Silman, P. Woo and T. Southwood (2002). "Juvenile idiopathic arthritis classified by the ILAR criteria: HLA associations in UK patients." Rheumatology (Oxford) **41**(10): 1183-1189.
Ting, J. P., R. C. Lovering, E. S. Alnemri, J. Bertin, J. M. Boss, B. K. Davis, R. A. Flavell, S. E. Girardin, A. Godzik, J. A. Harton, H. M. Hoffman, J. P. Hugot, N. Inohara, A. Mackenzie, L. J. Maltais, G. Nunez, Y. Ogura, L. A. Otten, D. Philpott, J. C. Reed, W. Reith, S. Schreiber, V. Steimle and P. A. Ward (2008). "The NLR gene family: a standard nomenclature." Immunity **28**(3): 285-287.
Toshchakov, V., B. W. Jones, P. Y. Perera, K. Thomas, M. J. Cody, S. Zhang, B. R. Williams, J. Major, T. A. Hamilton, M. J. Fenton and S. N. Vogel (2002). "TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages." Nat Immunol **3**(4): 392-398.
Tournadre, A., B. Pereira, A. Lhoste, J. J. Dubost, J. M. Ristori, P. Claudepierre, M. Dougados and M. Soubrier (2013). "Differences between women and men with recent-onset axial spondyloarthritis: results from a prospective multicenter French cohort." Arthritis Care Res (Hoboken) **65**(9): 1482-1489.
Tran, T. M., M. L. Dorris, N. Satumtira, J. A. Richardson, R. E. Hammer, J. Shang and J. D. Taurog (2006). "Additional human beta2-microglobulin curbs HLA-B27 misfolding and promotes arthritis and spondylitis without colitis in male HLA-B27-transgenic rats." Arthritis Rheum **54**(4): 1317-1327.
Tran, T. M., S. Hong, J. H. Edwan and R. A. Colbert (2016). "ERAP1 reduces accumulation of aberrant and disulfide-linked forms of HLA-B27 on the cell surface." Mol Immunol **74**: 10-17.
Travassos, L. H., L. A. Carneiro, M. Ramjeet, S. Hussey, Y. G. Kim, J. G. Magalhaes, L. Yuan, F. Soares, E. Chea, L. Le Bourhis, I. G. Boneca, A. Allaoui, N. L. Jones, G. Nunez, S. E. Girardin and D. J. Philpott (2010). "Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry." Nat Immunol **11**(1): 55-62.
Tseng, H. W., M. E. Pitt, T. T. Glant, A. F. McRae, T. J. Kenna, M. A. Brown, A. R. Pettit and G. P. Thomas (2016). "Inflammation-driven bone formation in a mouse model of ankylosing spondylitis: sequential not parallel processes." Arthritis Res Ther **18**: 35.
Tsui, H. W., R. D. Inman, A. D. Paterson, J. D. Reveille and F. W. Tsui (2005). "ANKH variants associated with ankylosing spondylitis: gender differences." Arthritis Res Ther **7**(3): R513-525.
Tsui, H. W., R. D. Inman, J. D. Reveille and F. W. Tsui (2007). "Association of a TNAP haplotype with ankylosing spondylitis." Arthritis Rheum **56**(1): 234-243.
Turina, M. C., J. Sieper, N. Yeremenko, K. Conrad, H. Haibel, M. Rudwaleit, D. Baeten and D. Poddubnyy (2014). "Calprotectin serum level is an independent marker for radiographic spinal progression in axial spondyloarthritis." Ann Rheum Dis **73**(9): 1746-1748.
Turina, M. C., N. Yeremenko, J. E. Paramarta, L. De Rycke and D. Baeten (2014). "Calprotectin (S100A8/9) as serum biomarker for clinical response in proof-of-concept trials in axial and peripheral spondyloarthritis." Arthritis Res Ther **16**(4): 413.
Turina, M. C., N. Yeremenko, F. van Gaalen, M. van Oosterhout, I. J. Berg, R. Ramonda, C. M. Lebre, R. Landewe and D. Baeten (2017). "Serum inflammatory biomarkers fail to identify early axial

spondyloarthritis: results from the SpondyloArthritis Caught Early (SPACE) cohort." RMD Open **3**(1): e000319.
Turner, M. J., M. L. Delay, S. Bai, E. Klenk and R. A. Colbert (2007). "HLA-B27 up-regulation causes accumulation of misfolded heavy chains and correlates with the magnitude of the unfolded protein response in transgenic rats: Implications for the pathogenesis of spondylarthritis-like disease." Arthritis Rheum **56**(1): 215-223.
Turner, M. J., D. P. Sowders, M. L. DeLay, R. Mohapatra, S. Bai, J. A. Smith, J. R. Brandewie, J. D. Taurog and R. A. Colbert (2005). "HLA-B27 misfolding in transgenic rats is associated with activation of the unfolded protein response." J Immunol **175**(4): 2438-2448.
Turvey, S. E. and D. H. Broide (2010). "Innate immunity." J Allergy Clin Immunol **125**(2 Suppl 2): S24-32.
Uderhardt, S., D. Diarra, J. Katzenbeisser, J. P. David, J. Zwerina, W. Richards, G. Kronke and G. Schett (2010). "Blockade of Dickkopf (DKK)-1 induces fusion of sacroiliac joints." Ann Rheum Dis **69**(3): 592-597.
Ugrinovic, S., A. Mertz, P. Wu, J. Braun and J. Sieper (1997). "A single nonamer from the Yersinia 60-kDa heat shock protein is the target of HLA-B27-restricted CTL response in Yersinia-induced reactive arthritis." J Immunol **159**(11): 5715-5723.
Ugur, M., N. K. Baygutalp, M. A. Melikoglu, F. Baygutalp, E. U. Altas and B. Seferoglu (2015). "Elevated serum interleukin-23 levels in ankylosing spondylitis patients and the relationship with disease activity." Nagoya J Med Sci **77**(4): 621-627.
Ushach, I. and A. Zlotnik (2016). "Biological role of granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) on cells of the myeloid lineage." J Leukoc Biol **100**(3): 481-489.
Vaile, J. H., J. B. Meddings, B. R. Yacyshyn, A. S. Russell and W. P. Maksymowych (1999). "Bowel permeability and CD45RO expression on circulating CD20+ B cells in patients with ankylosing spondylitis and their relatives." J Rheumatol **26**(1): 128-135.
van der Heijde, D., X. Baraliakos, L. S. Gensler, W. P. Maksymowych, V. Tseluyko, O. Nadashkevich, W. Abi-Saab, C. Tasset, L. Meuleners, R. Besuyen, T. Hendrikx, N. Mozaffarian, K. Liu, J. M. Greer, A. Deodhar and R. Landewe (2018). "Efficacy and safety of filgotinib, a selective Janus kinase 1 inhibitor, in patients with active ankylosing spondylitis (TORTUGA): results from a randomised, placebo-controlled, phase 2 trial." Lancet **392**(10162): 2378-2387.
van der Heijde, D., A. Deodhar, J. Braun, M. Mack, B. Hsu, T. A. Gathany, R. D. Inman, C. Han and G.-R. investigators (2014). "The effect of golimumab therapy on disease activity and health-related quality of life in patients with ankylosing spondylitis: 2-year results of the GO-RAISE trial." J Rheumatol **41**(6): 1095-1103.
van der Heijde, D., A. Deodhar, J. C. Wei, E. Drescher, D. Fleishaker, T. Hendrikx, D. Li, S. Menon and K. S. Kanik (2017). "Tofacitinib in patients with ankylosing spondylitis: a phase II, 16-week, randomised, placebo-controlled, dose-ranging study." Ann Rheum Dis **76**(8): 1340-1347.
van der Heijde, D., B. Dijkmans, P. Geusens, J. Sieper, K. DeWoody, P. Williamson, J. Braun and G. Ankylosing Spondylitis Study for the Evaluation of Recombinant Infliximab Therapy Study (2005). "Efficacy and safety of infliximab in patients with ankylosing spondylitis: results of a randomized, placebo-controlled trial (ASSERT)." Arthritis Rheum **52**(2): 582-591.
van der Heijde, D., A. Kivitz, M. H. Schiff, J. Sieper, B. A. Dijkmans, J. Braun, M. Dougados, J. D. Reveille, R. L. Wong, H. Kupper, J. C. Davis, Jr. and A. S. Group (2006). "Efficacy and safety of adalimumab in patients with ankylosing spondylitis: results of a multicenter, randomized, double-blind, placebo-controlled trial." Arthritis Rheum **54**(7): 2136-2146.
van der Heijde, D. and R. Landewe (2005). "Selection of a method for scoring radiographs for ankylosing spondylitis clinical trials, by the Assessment in Ankylosing Spondylitis Working Group and OMERACT." J Rheumatol **32**(10): 2048-2049.

van der Heijde, D., R. Landewe, X. Baraliakos, H. Houben, A. van Tubergen, P. Williamson, W. Xu, D. Baker, N. Goldstein and J. Braun (2008). "Radiographic findings following two years of infliximab therapy in patients with ankylosing spondylitis." Arthritis Rheum **58**(10): 3063-3070.
van der Heijde, D., R. Landewe, S. Einstein, P. Ory, D. Vosse, L. Ni, S. L. Lin, W. Tsuji and J. C. Davis, Jr. (2008). "Radiographic progression of ankylosing spondylitis after up to two years of treatment with etanercept." Arthritis Rheum **58**(5): 1324-1331.
van der Heijde, D., E. Lie, T. K. Kvien, J. Sieper, F. Van den Bosch, J. Listing, J. Braun and R. Landewe (2009). "ASDAS, a highly discriminatory ASAS-endorsed disease activity score in patients with ankylosing spondylitis." Ann Rheum Dis **68**(12): 1811-1818.
van der Heijde, D., S. Ramiro, R. Landewe, X. Baraliakos, F. Van den Bosch, A. Sepriano, A. Regel, A. Ciurea, H. Dagfinrud, M. Dougados, F. van Gaalen, P. Geher, I. van der Horst-Bruinsma, R. D. Inman, M. Jongkees, U. Kiltz, T. K. Kvien, P. M. Machado, H. Marzo-Ortega, A. Molto, V. Navarro-Compan, S. Ozgocmen, F. M. Pimentel-Santos, J. Reveille, M. Rudwaleit, J. Sieper, P. Sampaio-Barros, D. Wiek and J. Braun (2017). "2016 update of the ASAS-EULAR management recommendations for axial spondyloarthritis." Ann Rheum Dis **76**(6): 978-991.
van der Heijde, D., D. Salonen, B. N. Weissman, R. Landewe, W. P. Maksymowych, H. Kupper, S. Ballal, E. Gibson, R. Wong, g. Canadian study and A. s. group (2009). "Assessment of radiographic progression in the spines of patients with ankylosing spondylitis treated with adalimumab for up to 2 years." Arthritis Res Ther **11**(4): R127.
van der Heijde, D. M., D. A. Revicki, K. L. Gooch, R. L. Wong, H. Kupper, N. Harnam, C. Thompson, J. Sieper and A. S. Group (2009). "Physical function, disease activity, and health-related quality-of-life outcomes after 3 years of adalimumab treatment in patients with ankylosing spondylitis." Arthritis Res Ther **11**(4): R124.
van der Horst-Bruinsma, I. E. and M. T. Nurmohamed (2012). "Management and evaluation of extra-articular manifestations in spondyloarthritis." Ther Adv Musculoskelet Dis **4**(6): 413-422.
van der Horst-Bruinsma, I. E., D. J. Zack, A. Szumski and A. S. Koenig (2013). "Female patients with ankylosing spondylitis: analysis of the impact of gender across treatment studies." Ann Rheum Dis **72**(7): 1221-1224.
van der Linden, S., H. A. Valkenburg and A. Cats (1984). "Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria." Arthritis Rheum **27**(4): 361-368.
van der Linden, S. M. and M. A. Khan (1984). "The risk of ankylosing spondylitis in HLA-B27 positive individuals: a reappraisal." J Rheumatol **11**(6): 727-728.
van Nieuwenhuijze, A. E., F. A. van de Loo, B. Walgreen, M. Bennink, M. Helsen, L. van den Bersselaar, I. P. Wicks, W. B. van den Berg and M. I. Koenders (2015). "Complementary action of granulocyte macrophage colony-stimulating factor and interleukin-17A induces interleukin-23, receptor activator of nuclear factor-kappaB ligand, and matrix metalloproteinases and drives bone and cartilage pathology in experimental arthritis: rationale for combination therapy in rheumatoid arthritis." Arthritis Res Ther **17**: 163.
Van Praet, L., L. Jans, P. Carron, P. Jacques, E. Glorieus, R. Colman, H. Cypers, H. Mielants, M. De Vos, C. Cuvelier, F. Van den Bosch and D. Elewaut (2014). "Degree of bone marrow oedema in sacroiliac joints of patients with axial spondyloarthritis is linked to gut inflammation and male sex: results from the GIANT cohort." Ann Rheum Dis **73**(6): 1186-1189.
Van Praet, L., F. E. Van den Bosch, P. Jacques, P. Carron, L. Jans, R. Colman, E. Glorieus, H. Peeters, H. Mielants, M. De Vos, C. Cuvelier and D. Elewaut (2013). "Microscopic gut inflammation in axial spondyloarthritis: a multiparametric predictive model." Ann Rheum Dis **72**(3): 414-417.
van Tok, M. N., S. Na, C. R. Lao, M. Alvi, D. Pots, M. G. H. van de Sande, J. D. Taurog, J. D. Sedgwick, D. L. Baeten and L. M. van Duivenvoorde (2018). "The Initiation, but Not the Persistence, of Experimental Spondyloarthritis Is Dependent on Interleukin-23 Signaling." Front Immunol **9**: 1550.
van Tok, M. N., L. M. van Duivenvoorde, I. Kramer, P. Ingold, S. Pfister, L. Roth, I. C. Blijdorp, M. G. H. van de Sande, J. D. Taurog, F. Kolbinger and D. L. Baeten (2019). "Interleukin-17A Inhibition

Diminishes Inflammation and New Bone Formation in Experimental Spondyloarthritis." Arthritis Rheumatol **71**(4): 612-625.
van Tubergen, A., S. Ramiro, D. van der Heijde, M. Dougados, H. Mielants and R. Landewe (2012). "Development of new syndesmophytes and bridges in ankylosing spondylitis and their predictors: a longitudinal study." Ann Rheum Dis **71**(4): 518-523.
Vanden Eijnden, S., S. Goriely, D. De Wit, F. Willems and M. Goldman (2005). "IL-23 up-regulates IL-10 and induces IL-17 synthesis by polyclonally activated naive T cells in human." Eur J Immunol **35**(2): 469-475.
Vendhan, K., D. Sen, C. Fisher, Y. Ioannou and M. A. Hall-Craggs (2014). "Inflammatory changes of the lumbar spine in children and adolescents with enthesitis-related arthritis: magnetic resonance imaging findings." Arthritis Care Res (Hoboken) **66**(1): 40-46.
Venken, K., P. Jacques, C. Mortier, M. E. Labadia, T. Decruy, J. Coudenys, K. Hoyt, A. L. Wayne, R. Hughes, M. Turner, S. Van Gassen, L. Martens, D. Smith, C. Harcken, J. Wahle, C. T. Wang, E. Verheugen, N. Schryvers, G. Varkas, H. Cypers, R. Wittoek, Y. Piette, L. Gyselbrecht, S. Van Calenbergh, F. Van den Bosch, Y. Saeys, G. Nabozny and D. Elewaut (2019). "RORgammat inhibition selectively targets IL-17 producing iNKT and gammadelta-T cells enriched in Spondyloarthritis patients." Nat Commun **10**(1): 9.
Vermeire, K., H. Heremans, M. Vandeputte, S. Huang, A. Billiau and P. Matthys (1997). "Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice." J Immunol **158**(11): 5507-5513.
Verreck, F. A., T. de Boer, D. M. Langenberg, M. A. Hoeve, M. Kramer, E. Vaisberg, R. Kastelein, A. Kolk, R. de Waal-Malefyt and T. H. Ottenhoff (2004). "Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria." Proc Natl Acad Sci U S A **101**(13): 4560-4565.
Veys, E. M., C. J. Menkes and P. Emery (1997). "A randomized, double-blind study comparing twenty-four-week treatment with recombinant interferon-gamma versus placebo in the treatment of rheumatoid arthritis." Arthritis Rheum **40**(1): 62-68.
Vieira-Sousa, E., L. M. van Duivenvoorde, J. E. Fonseca, R. J. Lories and D. L. Baeten (2015). "Review: animal models as a tool to dissect pivotal pathways driving spondyloarthritis." Arthritis Rheumatol **67**(11): 2813-2827.
Vignali, D. A. and V. K. Kuchroo (2012). "IL-12 family cytokines: immunological playmakers." Nat Immunol **13**(8): 722-728.
Viswanath, V., A. Myles, R. Dayal and A. Aggarwal (2011). "Levels of serum matrix metalloproteinase-3 correlate with disease activity in the enthesitis-related arthritis category of juvenile idiopathic arthritis." J Rheumatol **38**(11): 2482-2487.
Vleeming, A., M. D. Schuenke, A. T. Masi, J. E. Carreiro, L. Danneels and F. H. Willard (2012). "The sacroiliac joint: an overview of its anatomy, function and potential clinical implications." J Anat **221**(6): 537-567.
Vogl, T., K. Tenbrock, S. Ludwig, N. Leukert, C. Ehrhardt, M. A. van Zoelen, W. Nacken, D. Foell, T. van der Poll, C. Sorg and J. Roth (2007). "Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock." Nat Med **13**(9): 1042-1049.
Vosse, D., R. Landewe, P. Garnero, D. van der Heijde, S. van der Linden and P. Geusens (2008). "Association of markers of bone- and cartilage-degradation with radiological changes at baseline and after 2 years follow-up in patients with ankylosing spondylitis." Rheumatology (Oxford) **47**(8): 1219-1222.
Wanders, A., D. Heijde, R. Landewe, J. M. Behier, A. Calin, I. Olivieri, H. Zeidler and M. Dougados (2005). "Nonsteroidal antiinflammatory drugs reduce radiographic progression in patients with ankylosing spondylitis: a randomized clinical trial." Arthritis Rheum **52**(6): 1756-1765.
Wang, H., D. Zhong, H. Chen, J. Jin, Q. Liu and G. Li (2019). "NLRP3 inflammasome activates interleukin-23/interleukin-17 axis during ischaemia-reperfusion injury in cerebral ischaemia in mice." Life Sci **227**: 101-113.

Wang, S. Y., Y. Y. Liu, H. Ye, J. P. Guo, R. Li, X. Liu and Z. G. Li (2011). "Circulating Dickkopf-1 is correlated with bone erosion and inflammation in rheumatoid arthritis." J Rheumatol **38**(5): 821-827.
Wang, Y., S. Gao, S. Gao, N. Li, B. Xie and X. Shen (2021). "Blocking the interaction between interleukin-17A and endoplasmic reticulum stress in macrophage attenuates retinal neovascularization in oxygen-induced retinopathy." Cell Biosci **11**(1): 82.
Wang, Y., J. Kim, A. Chan, C. Whyne and D. Nam (2018). "A two phase regulation of bone regeneration: IL-17F mediates osteoblastogenesis via C/EBP-beta in vitro." Bone **116**: 47-57.
Wang, Z., Y. Han, Z. Zhang, C. Jia, Q. Zhao, W. Song, T. Chen, Y. Zhang and X. Wang (2018). "Identification of genes and signaling pathways associated with the pathogenesis of juvenile spondyloarthritis." Mol Med Rep **18**(2): 1263-1270.
Ward, M. M., J. D. Reveille, T. J. Learch, J. C. Davis, Jr. and M. H. Weisman (2008). "Occupational physical activities and long-term functional and radiographic outcomes in patients with ankylosing spondylitis." Arthritis Rheum **59**(6): 822-832.
Watad, A., C. Bridgewood, T. Russell, H. Marzo-Ortega, R. Cuthbert and D. McGonagle (2018). "The Early Phases of Ankylosing Spondylitis: Emerging Insights From Clinical and Basic Science." Front Immunol **9**: 2668.
Watad, A., R. J. Cuthbert, H. Amital and D. McGonagle (2018). "Enthesitis: Much More Than Focal Insertion Point Inflammation." Curr Rheumatol Rep **20**(7): 41.
Weber, U., A. G. Jurik, A. Zejden, E. Larsen, S. H. Jorgensen, K. Rufibach, C. Schioldan and S. Schmidt-Olsen (2018). "Frequency and Anatomic Distribution of Magnetic Resonance Imaging Features in the Sacroiliac Joints of Young Athletes: Exploring "Background Noise" Toward a Data-Driven Definition of Sacroiliitis in Early Spondyloarthritis." Arthritis Rheumatol **70**(5): 736-745.
Webers, C., I. Essers, S. Ramiro, C. Stolwijk, R. Landewe, D. van der Heijde, F. van den Bosch, M. Dougados and A. van Tubergen (2016). "Gender-attributable differences in outcome of ankylosing spondylitis: long-term results from the Outcome in Ankylosing Spondylitis International Study." Rheumatology (Oxford) **55**(3): 419-428.
Weinreich, S., F. Eulderink, J. Capkova, M. Pla, K. Gaede, J. Heesemann, L. van Alphen, C. Zurcher, B. Hoebe-Hewryk, F. Kievits and et al. (1995). "HLA-B27 as a relative risk factor in ankylosing enthesopathy in transgenic mice." Hum Immunol **42**(2): 103-115.
Weiss, A., K. Minden, J. Listing, I. Foeldvari, J. Sieper and M. Rudwaleit (2017). "Course of patients with juvenile spondyloarthritis during 4 years of observation, juvenile part of GESPIC." RMD Open **3**(1): e000366.
Weiss, P. F., T. G. Brandon, J. Bohnsack, M. Heshin-Bekenstein, M. L. Francavilla, J. L. Jaremko, L. Liao, A. McHugh, E. J. Oberle, D. Rumsey, H. Srinivasalu, M. L. Stoll and N. A. Chauvin (2020). "Variability in magnetic resonance imaging interpretation of the pediatric sacroiliac joint." Arthritis Care Res (Hoboken).
Weiss, P. F., R. A. Colbert, R. Xiao, C. Feudtner, T. Beukelman, E. M. DeWitt, I. Pagnini, T. B. Wright and C. A. Wallace (2014). "Development and retrospective validation of the juvenile spondyloarthritis disease activity index." Arthritis Care Res (Hoboken) **66**(12): 1775-1782.
Weiss, P. F., A. J. Klink, E. M. Behrens, D. D. Sherry, T. H. Finkel, C. Feudtner and R. Keren (2011). "Enthesitis in an inception cohort of enthesitis-related arthritis." Arthritis Care Res (Hoboken) **63**(9): 1307-1312.
Weiss, P. F., W. P. Maksymowych, R. G. Lambert, J. L. Jaremko, D. M. Biko, J. Paschke, T. G. Brandon, R. Xiao and N. A. Chauvin (2018). "Feasibility and reliability of the Spondyloarthritis Research Consortium of Canada sacroiliac joint inflammation score in children." Arthritis Res Ther **20**(1): 56.
Weiss, P. F. and J. Roth (2020). "Juvenile-Versus Adult-Onset Spondyloarthritis: Similar, but Different." Rheum Dis Clin North Am **46**(2): 241-257.
Weiss, P. F., R. Xiao, D. M. Biko and N. A. Chauvin (2016). "Assessment of Sacroiliitis at Diagnosis of Juvenile Spondyloarthritis by Radiography, Magnetic Resonance Imaging, and Clinical Examination." Arthritis Care Res (Hoboken) **68**(2): 187-194.

Weiss, P. F., R. Xiao, T. G. Brandon, D. M. Biko, W. P. Maksymowych, R. G. Lambert, J. L. Jaremko and N. A. Chauvin (2018). "Radiographs in screening for sacroiliitis in children: what is the value?" Arthritis Res Ther **20**(1): 141.
Weiss, P. F., R. Xiao, T. G. Brandon, I. Pagnini, T. B. Wright, T. Beukelman, E. Morgan-DeWitt and C. Feudtner (2018). "Comparative Effectiveness of Tumor Necrosis Factor Agents and Disease-modifying Antirheumatic Therapy in Children with Enthesitis-related Arthritis: The First Year after Diagnosis." J Rheumatol **45**(1): 107-114.
Wellcome Trust Case Control, C., C. Australo-Anglo-American Spondylitis, P. R. Burton, D. G. Clayton, L. R. Cardon, N. Craddock, P. Deloukas, A. Duncanson, D. P. Kwiatkowski, M. I. McCarthy, W. H. Ouwehand, N. J. Samani, J. A. Todd, P. Donnelly, J. C. Barrett, D. Davison, D. Easton, D. M. Evans, H. T. Leung, J. L. Marchini, A. P. Morris, C. C. Spencer, M. D. Tobin, A. P. Attwood, J. P. Boorman, B. Cant, U. Everson, J. M. Hussey, J. D. Jolley, A. S. Knight, K. Koch, E. Meech, S. Nutland, C. V. Prowse, H. E. Stevens, N. C. Taylor, G. R. Walters, N. M. Walker, N. A. Watkins, T. Winzer, R. W. Jones, W. L. McArdle, S. M. Ring, D. P. Strachan, M. Pembrey, G. Breen, D. St Clair, S. Caesar, K. Gordon-Smith, L. Jones, C. Fraser, E. K. Green, D. Grozeva, M. L. Hamshere, P. A. Holmans, I. R. Jones, G. Kirov, V. Moskivina, I. Nikolov, M. C. O'Donovan, M. J. Owen, D. A. Collier, A. Elkin, A. Farmer, R. Williamson, P. McGuffin, A. H. Young, I. N. Ferrier, S. G. Ball, A. J. Balmforth, J. H. Barrett, T. D. Bishop, M. M. Iles, A. Maqbool, N. Yuldasheva, A. S. Hall, P. S. Braund, R. J. Dixon, M. Mangino, S. Stevens, J. R. Thompson, F. Bredin, M. Tremelling, M. Parkes, H. Drummond, C. W. Lees, E. R. Nimmo, J. Satsangi, S. A. Fisher, A. Forbes, C. M. Lewis, C. M. Onnie, N. J. Prescott, J. Sanderson, C. G. Matthew, J. Barbour, M. K. Mohiuddin, C. E. Todhunter, J. C. Mansfield, T. Ahmad, F. R. Cummings, D. P. Jewell, J. Webster, M. J. Brown, M. G. Lathrop, J. Connell, A. Dominiczak, C. A. Marcano, B. Burke, R. Dobson, J. Gungadoo, K. L. Lee, P. B. Munroe, S. J. Newhouse, A. Onipinla, C. Wallace, M. Xue, M. Caulfield, M. Farrall, A. Barton, R. A. G. Biologics in, C. Genomics Study Syndicate Steering, I. N. Bruce, H. Donovan, S. Eyre, P. D. Gilbert, S. L. Hilder, A. M. Hinks, S. L. John, C. Potter, A. J. Silman, D. P. Symmons, W. Thomson, J. Worthington, D. B. Dunger, B. Widmer, T. M. Frayling, R. M. Freathy, H. Lango, J. R. Perry, B. M. Shields, M. N. Weedon, A. T. Hattersley, G. A. Hitman, M. Walker, K. S. Elliott, C. J. Groves, C. M. Lindgren, N. W. Rayner, N. J. Timpson, E. Zeggini, M. Newport, G. Sirugo, E. Lyons, F. Vannberg, A. V. Hill, L. A. Bradbury, C. Farrar, J. J. Pointon, P. Wordsworth, M. A. Brown, J. A. Franklyn, J. M. Heward, M. J. Simmonds, S. C. Gough, S. Seal, C. Breast Cancer Susceptibility, M. R. Stratton, N. Rahman, M. Ban, A. Goris, S. J. Sawcer, A. Compston, D. Conway, M. Jallow, M. Newport, G. Sirugo, K. A. Rockett, S. J. Bumpstead, A. Chaney, K. Downes, M. J. Ghori, R. Gwilliam, S. E. Hunt, M. Inouye, A. Keniry, E. King, R. McGinnis, S. Potter, R. Ravindrarajah, P. Whittaker, C. Widden, D. Withers, N. J. Cardin, D. Davison, T. Ferreira, J. Pereira-Gale, I. B. Hallgrimsdo'ttir, B. N. Howie, Z. Su, Y. Y. Teo, D. Vukcevic, D. Bentley, M. A. Brown, A. Compston, M. Farrall, A. S. Hall, A. T. Hattersley, A. V. Hill, M. Parkes, M. Pembrey, M. R. Stratton, S. L. Mitchell, P. R. Newby, O. J. Brand, J. Carr-Smith, S. H. Pearce, R. McGinnis, A. Keniry, P. Deloukas, J. D. Reveille, X. Zhou, A. M. Sims, A. Dowling, J. Taylor, T. Doan, J. C. Davis, L. Savage, M. M. Ward, T. L. Learch, M. H. Weisman and M. Brown (2007). "Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants." Nat Genet **39**(11): 1329-1337.
Wendling, D. (2016). "The gut in spondyloarthritis." Joint Bone Spine **83**(4): 401-405.
Wendling, D., J. P. Cedoz and E. Racadot (2008). "Serum levels of MMP-3 and cathepsin K in patients with ankylosing spondylitis: effect of TNFalpha antagonist therapy." Joint Bone Spine **75**(5): 559-562.
Wendling, D., J. P. Cedoz, E. Racadot and G. Dumoulin (2007). "Serum IL-17, BMP-7, and bone turnover markers in patients with ankylosing spondylitis." Joint Bone Spine **74**(3): 304-305.
Wendling, D. and P. Claudepierre (2013). "New bone formation in axial spondyloarthritis." Joint Bone Spine **80**(5): 454-458.
Wicks, I. P. and A. W. Roberts (2016). "Targeting GM-CSF in inflammatory diseases." Nat Rev Rheumatol **12**(1): 37-48.

Wieczorek, M., E. T. Abualrous, J. Sticht, M. Alvaro-Benito, S. Stolzenberg, F. Noe and C. Freund (2017). "Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation." Front Immunol **8**: 292.
Wilson, N. J., K. Boniface, J. R. Chan, B. S. McKenzie, W. M. Blumenschein, J. D. Mattson, B. Basham, K. Smith, T. Chen, F. Morel, J. C. Lecron, R. A. Kastelein, D. J. Cua, T. K. McClanahan, E. P. Bowman and R. de Waal Malefyt (2007). "Development, cytokine profile and function of human interleukin 17-producing helper T cells." Nat Immunol **8**(9): 950-957.
Wong-Baeza, I., A. Ridley, J. Shaw, H. Hatano, O. Rysnik, K. McHugh, C. Piper, S. Brackenbridge, R. Fernandes, A. Chan, P. Bowness and S. Kollnberger (2013). "KIR3DL2 binds to HLA-B27 dimers and free H chains more strongly than other HLA class I and promotes the expansion of T cells in ankylosing spondylitis." J Immunol **190**(7): 3216-3224.
Wu, B. and Y. Wan (2020). "Molecular control of pathogenic Th17 cells in autoimmune diseases." Int Immunopharmacol **80**: 106187.
Wulffraat, N. (2017). "The value of old drugs for juvenile idiopathic arthritis." Lancet **389**(10072): 883-884.
Wurfel, M. M., W. Y. Park, F. Radella, J. Ruzinski, A. Sandstrom, J. Strout, R. E. Bumgarner and T. R. Martin (2005). "Identification of high and low responders to lipopolysaccharide in normal subjects: an unbiased approach to identify modulators of innate immunity." J Immunol **175**(4): 2570-2578.
Xu, W. D., G. S. Firestein, R. Taetle, K. Kaushansky and N. J. Zvaifler (1989). "Cytokines in chronic inflammatory arthritis. II. Granulocyte-macrophage colony-stimulating factor in rheumatoid synovial effusions." J Clin Invest **83**(3): 876-882.
Yago, T., Y. Nanke, M. Kawamoto, T. Furuya, T. Kobashigawa, N. Kamatani and S. Kotake (2007). "IL-23 induces human osteoclastogenesis via IL-17 in vitro, and anti-IL-23 antibody attenuates collagen-induced arthritis in rats." Arthritis Res Ther **9**(5): R96.
Yamamoto, M., S. Sato, H. Hemmi, H. Sanjo, S. Uematsu, T. Kaisho, K. Hoshino, O. Takeuchi, M. Kobayashi, T. Fujita, K. Takeda and S. Akira (2002). "Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4." Nature **420**(6913): 324-329.
Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda and S. Akira (2003). "TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway." Nat Immunol **4**(11): 1144-1150.
Yamamoto, M., S. Sato, K. Mori, K. Hoshino, O. Takeuchi, K. Takeda and S. Akira (2002). "Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling." J Immunol **169**(12): 6668-6672.
Yang, J., Y. Lin, Z. Guo, J. Cheng, J. Huang, L. Deng, W. Liao, Z. Chen, Z. Liu and B. Su (2001). "The essential role of MEKK3 in TNF-induced NF-kappaB activation." Nat Immunol **2**(7): 620-624.
Yang, Z., Q. Liu, H. Shi, X. Jiang, S. Wang, Y. Lu, J. Zhang, X. Huang and A. Yu (2018). "Interleukin 17A exacerbates ER-stress-mediated inflammation of macrophages following ICH." Mol Immunol **101**: 38-45.
Yang, Z. X., Y. Liang, Y. Zhu, C. Li, L. Z. Zhang, X. M. Zeng and R. Q. Zhong (2007). "Increased expression of Toll-like receptor 4 in peripheral blood leucocytes and serum levels of some cytokines in patients with ankylosing spondylitis." Clin Exp Immunol **149**(1): 48-55.
Yao, J., T. W. Kim, J. Qin, Z. Jiang, Y. Qian, H. Xiao, Y. Lu, W. Qian, M. F. Gulen, N. Sizemore, J. DiDonato, S. Sato, S. Akira, B. Su and X. Li (2007). "Interleukin-1 (IL-1)-induced TAK1-dependent Versus MEKK3-dependent NFkappaB activation pathways bifurcate at IL-1 receptor-associated kinase modification." J Biol Chem **282**(9): 6075-6089.
Yatsunenko, T., F. E. Rey, M. J. Manary, I. Trehan, M. G. Dominguez-Bello, M. Contreras, M. Magris, G. Hidalgo, R. N. Baldassano, A. P. Anokhin, A. C. Heath, B. Warner, J. Reeder, J. Kuczynski, J. G. Caporaso, C. A. Lozupone, C. Lauber, J. C. Clemente, D. Knights, R. Knight and J. I. Gordon (2012). "Human gut microbiome viewed across age and geography." Nature **486**(7402): 222-227.

Yeremenko, N., J. E. Paramarta and D. Baeten (2014). "The interleukin-23/interleukin-17 immune axis as a promising new target in the treatment of spondyloarthritis." Curr Opin Rheumatol **26**(4): 361-370.
Yeremenko, N., K. Zwerina, G. Rigter, D. Pots, J. E. Fonseca, J. Zwerina, G. Schett and D. Baeten (2015). "Tumor necrosis factor and interleukin-6 differentially regulate Dkk-1 in the inflamed arthritic joint." Arthritis Rheumatol **67**(8): 2071-2075.
Ying, R., S. W. Li, J. Y. Chen, H. F. Zhang, Y. Yang, Z. J. Gu, Y. X. Chen and J. F. Wang (2018). "Endoplasmic reticulum stress in perivascular adipose tissue promotes destabilization of atherosclerotic plaque by regulating GM-CSF paracrine." J Transl Med **16**(1): 105.
Yoshida, H., M. Oku, M. Suzuki and K. Mori (2006). "pXBP1(U) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response." J Cell Biol **172**(4): 565-575.
Yu, L., L. Wang and S. Chen (2010). "Endogenous toll-like receptor ligands and their biological significance." J Cell Mol Med **14**(11): 2592-2603.
Yucong, Z., L. Lu, L. Shengfa, Y. Yongliang, S. Ruguo and L. Yikai (2014). "Serum functional dickkopf-1 levels are inversely correlated with radiographic severity of ankylosing spondylitis." Clin Lab **60**(9): 1527-1531.
Yurkovetskiy, L., M. Burrows, A. A. Khan, L. Graham, P. Volchkov, L. Becker, D. Antonopoulos, Y. Umesaki and A. V. Chervonsky (2013). "Gender bias in autoimmunity is influenced by microbiota." Immunity **39**(2): 400-412.
Zakharova, M. and H. K. Ziegler (2005). "Paradoxical anti-inflammatory actions of TNF-alpha: inhibition of IL-12 and IL-23 via TNF receptor 1 in macrophages and dendritic cells." J Immunol **175**(8): 5024-5033.
Zanwar, A., S. Phatak and A. Aggarwal (2018). "Prospective validation of the Juvenile Spondyloarthritis Disease Activity Index in children with enthesitis-related arthritis." Rheumatology (Oxford) **57**(12): 2167-2171.
Zeng, L., M. J. Lindstrom and J. A. Smith (2011). "Ankylosing spondylitis macrophage production of higher levels of interleukin-23 in response to lipopolysaccharide without induction of a significant unfolded protein response." Arthritis Rheum **63**(12): 3807-3817.
Zhang, M. A., D. Rego, M. Moshkova, H. Kebir, A. Chruscinski, H. Nguyen, R. Akkermann, F. Z. Stanczyk, A. Prat, L. Steinman and S. E. Dunn (2012). "Peroxisome proliferator-activated receptor (PPAR)alpha and -gamma regulate IFNgamma and IL-17A production by human T cells in a sex-specific way." Proc Natl Acad Sci U S A **109**(24): 9505-9510.
Zhong, Y., A. Kinio and M. Saleh (2013). "Functions of NOD-Like Receptors in Human Diseases." Front Immunol **4**: 333.
Zuniga, L. A., R. Jain, C. Haines and D. J. Cua (2013). "Th17 cell development: from the cradle to the grave." Immunol Rev **252**(1): 78-88.
Zwerina, J., K. Redlich, K. Polzer, L. Joosten, G. Kronke, J. Distler, A. Hess, N. Pundt, T. Pap, O. Hoffmann, J. Gasser, C. Scheinecker, J. S. Smolen, W. van den Berg and G. Schett (2007). "TNF-induced structural joint damage is mediated by IL-1." Proc Natl Acad Sci U S A **104**(28): 11742-11747.