The Role of Interleukin 10 in children with Juvenile Idiopathic Arthritis

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Dedication

This thesis is dedicated to my mother who chose to have a family and children rather than fulfil her dreams of becoming a doctor. I hope this thesis will make her proud and validate her decision made so many years ago.
Abstract

This thesis investigates the function of the IL-10 5' flanking region haplotypes and the association of these haplotypes in children with Juvenile Idiopathic Arthritis (JIA).

We have shown that children with extended oligoarticular JIA are less likely to have a genotype containing the ATA haplotype than children with oligoarticular JIA (p<0.05, OR = 1.9, 95% CI: 1 to 3.5). The ATA haplotype is associated with lower reporter gene expression in transfection studies (Crawley, 1999c). The ATA/ATA genotype was shown to be associated with lower IL-10 production using whole blood culture in controls (p<0.02). This is consistent with the observation that mean IL-10 production was lower in the parents of those with extended oligoarticular disease when compared to the parents of children with oligoarticular disease (p=0.03, 95% CI 88 to 2016).

A similar effect was seen in asthma where those with severe asthma were less likely to have ATA containing genotypes than controls (p=0.04, OR1.57, CI: 1.01 to 2). We did not show a difference in genotype distribution between patients with SLE and controls.

The transmission disequilibrium test (TDT) demonstrated an increase in transmission of the ATA haplotype to patients with oligoarticular onset JIA (p=0.05). There was also increased transmission of both the ACC and the ATA haplotype to patients with uveitis (p=0.014 for each transmission).

Strong linkage disequilibrium was demonstrated (p<0.0005) between these haplotypes and a microsatellite at -1000 in the IL-10 5' flanking region which has previously been shown to be associated with rheumatoid arthritis and systemic lupus erythematosus.

Treatment of patients with methylprednisolone was associated with large changes in ex vivo stimulated IL-10 production which was strongly correlated with the change in ESR (correlation coefficient 0.922, p<0.01).

To investigate whether the IL-10 5’ flanking region haplotypes affected transcription, THP-1 cells were transfected with IL-10 luciferase DNA reporter constructs. Overall, reporter gene expression was higher with the GCC than the ATA construct when the THP-1 cells were transfected using Effectene Liposomes (median fold difference 1.71, range 1.12 to 5.49).
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Thanks are also due to my family and friends, especially Helen, Jeanette, Jo, Tim, Maria and Robin who never stopped believing I could complete this work and to my mother and Sean who proof read large sections of this thesis. I am also very grateful to Rupert who gave me the space, facilities and support to print this thesis.

Whilst doing this PhD I supervised Pritash Patel and Sam Kon’s BSc projects and some of the fruits of their labour are presented in this thesis. Pritash Patel was involved with the whole blood culture work and steroid studies. Sam Kon contributed to the TDT results presented here. Both worked incredibly hard and were enthusiastic and fun members of the laboratory.

Several collaborators have been involved over the years (see section 2.2) and thanks are due to all of them but especially Richard Kay who provided the IL-10 Luciferase DNA reporter constructs as well as some great brain storming sessions.

This thesis would never have been finished without Sean who has picked me up more times than I care to remember and never let me give up.
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Pro-inflammatory cytokines in arthritis

Anti-inflammatory cytokines in arthritis

Cytokine association in Juvenile Idiopathic Arthritis

Oligoarticular JIA

Polyarticular, Rheumatoid factor negative JIA

Polyarticular, Rheumatoid factor positive JIA

Systemic JIA

Psoriatic JIA

Spondyloarthropathies

Uveitis

TNFα in uveitis

IL-10 in uveitis

Interleukin-10

Function of IL-10

Inhibition of pro-inflammatory cytokine production

IL-10 can decrease pro-inflammatory cytokine production indirectly

Antagonism of pro-inflammatory cytokine production

IL-10 inhibits macrophage production of nitric oxide (NO)

Down regulation of antigen presenting cell function

IL-10 inhibits macrophage proliferation

IL-10 prevents activation of dendritic cells

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<tr>
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<th>Description</th>
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<tr>
<td>λs</td>
<td>Sibling relative risk</td>
</tr>
<tr>
<td>ACR</td>
<td>American College Rheumatology</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention deficit hyperactive disorder</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>ANA</td>
<td>Anti Nuclear Antibody</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1 complex</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ARC</td>
<td>Arthritis Rheumatism Campaign</td>
</tr>
<tr>
<td>ASP</td>
<td>Affected sib pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP (adenosine monophosphate)</td>
</tr>
<tr>
<td>CDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic GMP (guanosine monophosphate)</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>COS cells</td>
<td>Monkey cell system derived from transformed simian kidney DV-1 cells</td>
</tr>
<tr>
<td>CRP</td>
<td>C Reactive Protein</td>
</tr>
<tr>
<td>Cy</td>
<td>Cytochrome</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>Dibutyladenosine 3':5' – cyclic monophosphate</td>
</tr>
<tr>
<td>DCChol</td>
<td>3b-[N-(N,N-dimethylaminoethane)carbamoyl] cholesterol</td>
</tr>
<tr>
<td>DOPE</td>
<td>Dioleyl L-1-phosphatidylethanolamine</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreital</td>
</tr>
<tr>
<td>DXM</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>EIU</td>
<td>Experimental Immune Uveitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte Sedimentation Rate</td>
</tr>
<tr>
<td>ETDT</td>
<td>Extended TDT</td>
</tr>
<tr>
<td>EULAR</td>
<td>European League Against Rheumatism</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorter</td>
</tr>
<tr>
<td>FBC</td>
<td>Full blood count</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft Versus Host Disease</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cell line derived from Helen Lane</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>Hu-IL-10R</td>
<td>Human IL-10 Receptor chain</td>
</tr>
<tr>
<td>IBD</td>
<td>Identity-By-Descent</td>
</tr>
<tr>
<td>IBS</td>
<td>Identity-By-State</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular Adhesion Molecule-1</td>
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<tr>
<td>IDDM</td>
<td>Insulin Dependant Diabetes Mellitus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>IL-1 receptor antagonist</td>
</tr>
<tr>
<td>ILAR</td>
<td>International League Against Rheumatism</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus tyrosine Kinases</td>
</tr>
<tr>
<td>JIA</td>
<td>Juvenile Idiopathic Arthritis</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccaride</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
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<td>------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Monocyte Inflammatory Protein</td>
</tr>
<tr>
<td>MP</td>
<td>Methylprednisolone</td>
</tr>
<tr>
<td>MPC</td>
<td>Monocyte Chemotactic Protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor κB</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non-Insulin Dependant Diabetes Mellitus</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterases</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinosital</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis (adult onset)</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid Factor</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>SAA</td>
<td>Serum Amyloid A</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immune deficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulphate</td>
</tr>
<tr>
<td>SDW</td>
<td>Sterile distilled water</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SFMC</td>
<td>Synovial fluid monocytes</td>
</tr>
<tr>
<td>SLAM index</td>
<td>Systemic lupus activity measures</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SSOP</td>
<td>Sequence specific oligonucleotide probing</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing (TAP)</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDT</td>
<td>Transmission disequilibrium test</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th1 or Th2</td>
<td>T helper cells 1 or 2</td>
</tr>
<tr>
<td>THP-1</td>
<td>Human monocyctic cell line from a child with acute monocyctic leukaemia</td>
</tr>
<tr>
<td>(Tsuchiya, 1980)</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>Tyk</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
</tr>
</tbody>
</table>
Publications arising from this work


The first mark of intelligence, to be sure, is not to start things; the second mark of intelligence is to pursue to the end what you have started.

*Panchatantra (c. 5th c.)*
CHAPTER 1: INTRODUCTION
1.1 Juvenile Idiopathic Arthritis

Juvenile idiopathic arthritis (JIA) is defined as arthritis in a child under the age of 16 years affecting one or more joints, lasting for at least 6 weeks and currently having no other cause (Petty, 1998).

1.1.1 Incidence and prevalence of JIA

The prevalence of arthritis in children varies between 1 and 10 per 1000 (Oen and Cheang 1996). The incidence rate varies between 1 and 20 per 100,000 (Oen and Cheang 1996). This prevalence and incidence rates vary between different studies depending on the source population (whether a population survey or based on referral to tertiary level clinics) and geographical differences between populations. The geographical differences could be environmental or due to ethnic/genetic differences and the factors involved have not yet been defined.

A difference in prevalence has not been shown to be due to a difference in classification system used (ACR Vs EULAR). Ethnic origin does not affect overall incidence or prevalence but it does appear to affect subgroup diagnosis. For example in Caucasian populations, oligoarticular arthritis accounts for more than half of the patients (58%, CI: 56-60), and the proportion of polyarticular onset arthritis accounts for 27% (CI: 25-28). This is contrast to the higher proportion of children with polyarticular arthritis in African (35-50%), East Indian (41-87%), Japanese (53%) and Thai (77%) children who have a correspondingly lower proportion of children with oligoarticular arthritis (Oen and Cheang 1996).

1.1.2 Morbidity associated with arthritis in children

Arthritis in children can have both systemic and local consequences. Systemic effects include general growth retardation and osteoporosis (Woo 1994), malaise and loss of appetite and an increased risk of amyloidosis. In addition to joint destruction, local inflammation results in accelerated growth of the epiphyses in the affected joints.

Overall, an estimated 49% of children end up with severe functional limitation (class III and IV, Steinbroker classification) because of JIA (Wallace and Levinson 1991). This is due to persistent arthritis, the consequences of arthritis in the form of permanent joint damage or uveitis (15 to 30 % of children with uveitis are ultimately blind (Smiley 1974)).

In addition to the physical effects of JIA there are important (but more difficult to measure) emotional and social consequences. For example, in a 10 year follow up,
young people who had JIA as a child were significantly more likely to be unemployed than their siblings (29.9% Vs 11.4% respectively) (Packham and Hall 1999).

JIA is therefore an important disease in children in terms of the number of children who have a poor long term outcome.

### 1.2 Classification of JIA

In 1959, Ansell and Bywaters proposed the first clinical classification for children with arthritis (Ansell and Bywaters 1959). This classification was according to mode of onset which is the form adopted first by the American Rheumatism Association in 1977 (Brewer, 1972) and then the World Health Organisation European League against Rheumatism (WHO EULAR) in 1978 (Wood 1978). Although similar, these classifications were not identical and in 1994 the Paediatric Standing Committee of the International League of Associations for Rheumatology (ILAR) bravely met to develop a classification system which was universally acceptable. The classification was based on the clinical experience of Paediatric Rheumatologists from each of the four regional leagues of ILAR. The proposals from this meeting were published in 1995 (Fink 1998) and were subsequently revised in Durban in 1997 (Hochberg 1995; Malleson 1996). The primary aim was to develop criteria that would enable the identification of homogenous groups of children to facilitate research and communication between physicians and scientists (Petty, 1998). The committee has acknowledged that homogeneity was more important than inclusivity and children were therefore excluded if they did not strictly fulfil criteria or if they fitted into more than one category (Petty, 1998). Even members of the committee agree that this classification is based on some “entirely arbitrary factors” but as no other factors have superseded these in terms of providing helpful prognostic information, the classification has remained intact. It is this classification that is used in this thesis.

A summary of some of the important features of the ILAR classification which are relevant for this thesis are given here – for full details please see appendix B.

#### 1.2.1 Oligoarticular JIA

This is when the child has arthritis affecting 1-4 joints during the first 6 months of disease. If the child never has more than 4 joints involved they are considered to have persistent oligoarthritis. However if they “extend” and have more than 4 joints involved after the first 6 months of disease, they are considered to have extended oligoarticular JIA. One of the problems with this classification is that if a child extends before 6
months, they are classified as having polyarticular JIA and it is not always easy to know when a child extends.

1.2.2 Polyarticular JIA
When a child has polyarticular JIA they have arthritis affecting 5 or more joints during the first 6 months of the disease. In children, tests for rheumatoid factor are usually negative and it is termed polyarticular JIA – rheumatoid factor negative. In a small percentage of children, the tests for rheumatoid factor are positive. If the disease is associated with positive tests for rheumatoid factor on at least 2 occasions at least 3 months apart then it is called polyarticular JIA – rheumatoid factor positive JIA.

1.2.3 Enthesitis related arthritis
Enthesitis related arthritis is defined as arthritis occurring with enthesitis, or arthritis or enthesitis with at least two of the following:
- Sacroiliac joint tenderness and/or Inflammatory spinal pain
- Presence of HLA-B27
- Family history in at least one first or second degree relative of medically confirmed HLA-B287 associated disease
- Anterior uveitis that is usually associated with pain, redness or photophobia
- Onset of arthritis in a boy > 8 yrs of age

1.2.4 Systemic onset JIA
Clinically this is a very different disease as the arthritis is often not a predominant feature. The arthritis occurs with or is preceded by a daily fever of at least 2 weeks duration. The fever is classically quotidian. An evanescent, non-fixed, erythematous rash is common as are generalised lymph node enlargement, hepatomegaly or splenomegaly and serositis.

1.2.5 How helpful is the classification of JIA in genetic studies?
A good clinical classification system is clearly helpful in terms of prognosis and clinical management if the patients within each group have a homogenous disease course. However there are several problems with the classification of JIA which may hamper genetic studies.

1. Genetic associations may not be associated with a clinically defined disease but a clinical or laboratory disease parameter. This means that they would have an effect across different disease groups. This was demonstrated in a recent study examining
the association of HLA alleles with adult patients with a polyarthritis where an association was demonstrated between inflammatory polyarthritis and the presence of any shared epitope allele (Thomson, 1999). The strongest association was with HLA-DRB1*0404, which gave a three to four fold increase in risk. The odds ratio (OR) was increased in individuals homozygous for DRB1*04 in whom at least 1 of the alleles was DRB1*0404 suggesting a gene dosage effect. There was no difference in genotypic data when the patients were stratified by RA status although the associations were stronger in those who were rheumatoid factor positive. In other words the genetic risk factor was for polyarthritis not for rheumatoid factor positive arthritis or for any other disease.

An example of a disease parameter which can occur in any subtype of JIA is anterior uveitis and this is discussed in further detail in section 1.3.

2. The classification system loses data on disease characteristics that are continuous traits. For example, subdividing patients into more or less than 4 joints does not use all the available information. If data is available on continuous traits then the results can be regressed onto the number of disease alleles (Waldman, 1998). Other examples of continuous traits are degree of growth arrest or high inflammatory markers. Genetic factors may affect quantitative traits with a gene dosage effect. For example, consider gene X with alleles A and a. Allele A increases the risk of joint disease and allele a is protective. Analysis using data on total number of joints may find a correlation between number of joints involved and either 0, 1 or 2 A alleles and this important affect may be missed if clinical data is analysed only as a binary characteristic.

3. Genetic factors may determine disease severity across several different disease groups affecting many different parameters. For example, several risk alleles could interact to produce high inflammatory markers, anorexia, growth retardation and multiple joint involvement in systemic JIA, polyarticular onset JIA or extended oligoarticular onset JIA. In other words, each disease group could have a different onset pattern because of environmental factors or other disease onset genetic loci, but the same outcome because of genetic severity loci. Analysis of each of these groups by disease onset may miss these severity risk factors. One possible method of analysis would be to enrich for severity in each disease group and analyse for association with genetic factors.
4. All classification systems are open to miss-classification but in JIA this is particularly difficult. For example, there is the more or less than 4 joints key classification between the oligoarticular and extended oligoarticular disease. The timing of arthritis affecting more than 4 joints (before or after 6 months) determines whether the child is classified as having extended oligoarticular or polyarticular onset disease. Recall of number of joints involved or date of onset is notoriously bad and considerable inter-observer variability has been demonstrated even between very experienced Paediatric Rheumatologists (Giannini and Lovell 1994).

Ultimately the question that plagues the study of JIA and adult onset RA is whether the phenotypic heterogeneity observed within JIA (highlighted by the multiple attempts to make homogenous subgroups) represents genetic heterogeneity (Silman 1997). For further discussion see section 4.1.1.2 to 4.1.1.5.

1.3 Anterior Uveitis

An important physical cause of morbidity in children with JIA is anterior uveitis. The first description of uveitis and band keratopathy in a child with arthritis was by Ohm in 1910 (Ohm 1910). In a population based study, in Finland, 16% of children with JIA developed uveitis. Diagnosis was made at a mean age of 6.8 years with a mean interval from diagnosis of JIA to detection of uveitis of 2.9 years (Kotaniemi, 1999). Despite nearly 90 years of research we understand little of the pathophysiology of the uveitis associated with JIA.

1.3.1 Clinical features

1.3.1.1 Children at risk

Although all groups of JIA can develop uveitis, the highest risk group are young girls with an oligoarthritis who are ANA positive. In fact ~ 95% of children from this group who present under the age of 2, will develop uveitis (Chylack, 1979).

1.3.1.2 Clinical symptoms

The onset of uveitis in children with JIA is usually asymptomatic although up to 50% may have symptoms such as pain, redness, headache and photophobia later in the onset of disease. Less then 5% develop uveitis before the onset of arthritis and most children develop their uveitis within 7 years of arthritis onset (Cassidy and Petty 199; Kotaniemi, 1999). The activity of arthritis does not parallel the course or severity of uveitis (Rosenberg and Oen 1986). Both eyes are involved in 65% of children with uveitis.
1.3.1.3 **Prognosis**

Although both the management and prognosis of children with uveitis has improved, blindness is still relatively common with estimates of 15 to 30% (Smiley 1974). Other studies from the mid 1970s report that 38% of children had decreased visual acuity to 20/200 with band keratopathy occurring in 49% (Key and Kimura 1975). Kanski’s study on Taplow patients showed that although 57% of affected eyes had a visual acuity equal to or better than 6/9, 63% had active inflammation with band keratopathy occurring in 41%, cataracts in 42% and secondary glaucoma in 19% (Kanski 1977). Uveitis is not a benign disorder.

1.3.1.4 **Laboratory markers**

Positive ANA titres is the most common laboratory marker and occurs in 65 to 88% of children who develop uveitis. It is usually present in relatively low titre and has unknown specificity (Kanski 1977).

1.3.2 **Pathophysiology of uveitis in children with JIA**

The uveitis in JIA is predominantly a chronic non-granulomatous inflammation affecting the iris and ciliary body (Cassidy and Petty 1987) (the anterior uveal tract). Under slit lamp examination a cellular exudate of inflammatory cells can be seen in the anterior chamber of the eye. A punctate keratitic precipitate develops later on the posterior corneal surface. Progressive damage can lead to the development of posterior synechiae that are caused by adherence between the iris and the anterior surface of the lens. This results in an irregular or poorly reactive pupil. Other late manifestations include band keratopathy (a degenerative lesion), secondary cataracts, glaucoma and phthisis bulbi.

There is an intense immune reaction within the ocular fluid in these children (Petty, 1987) and the aqueous humour has increased immunoglobulin levels and ANA (Kanski 1976; Rahi, 1977; Person, 1981; Kaplan, 1982). In some children there is immunity to retinal antigens (Petty, 1987). The presence of high levels of ANA (IgG) has led some authors to postulate that there is an abnormal blood-ocular barrier or local antibody synthesis (Person, 1981). This is consistent with the findings that 90% of vitreous lymphocytes in one adult with uveitis and JIA were B lymphocytes (Kaplan, 1982).
1.4 Is there a genetic component to JIA?

Moroldo et al have shown a higher than expected degree of concordance for subtypes of JIA with different clinical onset patterns between 71 affected sib pairs (ASP) (Moroldo, 1997). This increase in concordance was greater than that predicted from the known incidence of different disease types and suggests a genetic component which determines the subtypes of JIA.

One way in which the extent of a genetic component to a disease can be quantified is as the sibling relative risk of disease denoted as $\lambda_s$. This is the disease prevalence in siblings of affected individuals divided by the disease prevalence in the general population. $\lambda_s$ is therefore usually small for common diseases even if there is a significant genetic component and larger for rare diseases even if there is a small genetic component because of the difference in the size of the denominator. The $\lambda_s$ for JIA calculated from 300 sib pairs, is 15 (Glass and Giannini 1999). This is higher than the $\lambda_s$ for RA which has been calculated as 4.9 (John, 1997). Although this means it is likely that there is a genetic component to JIA, the children studied are likely to have had common environmental exposure which can also act to increase sibling relative risk. This value for $\lambda_s$ is only the relative risk for developing JIA suggesting that a genetic component plays a role in determining susceptibility. The data from Moroldo et al suggest that a genetic component may also determine disease course suggesting that genetic factors are important in determining severity as well as susceptibility.

To date, no major gene effect has been demonstrated although associations with several genes have been described (Donn, 1994; Fishman, 1998; Crawley, 1999c; Miller, 1985; Fraser, 1990; Van Kerckhove, 1990). It seems likely therefore that the genetic influence in JIA is polygenic i.e. that a large number of genes each exert a relatively small effect (see also section 1.6.1).

1.4.1 Environment versus genetic

A seasonal onset of systemic onset JIA in the prairie region of Canada has been described, with significant peaks in the autumn and early spring (Feldmann, 1996). Although this suggests an environmental trigger such as a virus, the incidence of systemic JIA did not correlate with viral incidence in the same region. In Manitoba however onset of all types of JRA (now classified as JIA) correlated significantly with Mycoplasma Pneumoniae infections detected in the province suggesting a possible environmental link (Oen, 1995). Several studies have reported associations between raised antibody titres against environmental pathogens and the development of JIA.
Pritchard et al describe a clustering of children born in 1963 who developed late onset (ages 13 to 19) severe erosive polyarticular JIA (5 RF positive and 36 RF negative) (Pritchard, 1988). They believed this clustering was due to antigen exposure in utero to *Influenza A H_{2}N_{2}* with subsequent re-exposure in later life (Pritchard, 1988). These children had higher antibody titres to this virus but comparable antibody titres to control viruses such as rubella when compared with healthy controls. However this group of children also had a “surprisingly” high parental incidence of seropositive rheumatoid disease suggesting either additional environmental effects or genetic susceptibility. A retrospective study on children presenting in Massachusetts with joint symptoms and associated constitutional symptoms found 22 out of 104 had serological evidence of recent parvovirus infection (Nocton, 1993). Although the duration of symptoms was usually brief, 3 children had arthritis which lasted for 3 months and 8 children continued to have symptoms 2 to 13 months after disease onset.

The picture is complicated by the inclusion of children within these studies with spondyloarthropathies. Several studies have suggested that infection plays a role in the development of adult onset spondyloarthropathies (Tani, 1997; Tiwana, 1997). Indeed, childhood onset spondyloarthropathies appear to be a different disease from other forms of JIA (Burgos Vargas and Vazquez Mellado 1995) and it is possible that the aetiology is different. However, there is no clear evidence that infection plays a role in childhood onset spondyloarthropathy but there is definite evidence that genetic factors are important (Malleson and Petty 1997).

Although environmental triggers appear to play a role, there is no unifying trigger for JIA or indeed for any subgroup of JIA. It may be that it is the interaction between different environmental triggers with a susceptible genetic background that determines the development of JIA (Seldin, 1999; Brown and Wordsworth 1998).

### 1.4.2 Susceptibility versus severity

An emerging feature of polygenic diseases is that genetic effects may play a role in defining severity rather than susceptibility. This paradigm is important for diseases such as attention deficit disorder (Waldman, 1998) and adult onset RA (Weyand, 1992; de Jongh, 1984). In attention deficit hyperactive disorder (ADHD) a dose dependant association has been described between the dopamine transporter gene and severity of hyperactive-impulsive symptoms but not for onset of ADHD (Waldman, 1998). This is similar to adult onset RA where some HLA haplotypes are only associated with patients
with severe disease and in some studies a gene dosage effect correlates with disease severity (Weyand, 1992; de Jongh, 1984).

1.4.3 Disease characteristics versus disease subgroups

Alternatively, genetic factors could determine a disease characteristic rather than define a JIA subgroup. An example of this is uveitis. In 1873, Jonathan Hutchinson observed that the parents of children with iritis usually had arthritis. Anecdotally, uveitis in JIA is described as a heritable condition although there is little evidence for this (Jacobs 19; Cassidy and Petty 199). Although several studies report genetic associations with both HLA alleles and IL-1α (see sections 1.6.1.3 and 1.6.1.4), no genotype has been consistently shown to present a high risk factor for the development of uveitis.
1.5 Methods for studying genes associated with disease

1.5.1 Genome wide scan versus candidate gene approach

Genome wide scanning can be performed either using either linkage based methods or association methods. Both methods use multiplexed fluor-conjugated microsatellite markers which allow a rapid and large throughput of both samples and markers (Hsieh, 1997). The European Consortium on Rheumatoid Arthritis Families have recently published a genome wide screen for RA susceptibility genes and describe 14 regions which were compatible with linkage in addition to the HLA region (Cornelis, 1998). Other groups are using this approach to look for susceptibility loci in this country (Hardwick, 1997). The main drawback to genome wide scans is the large numbers of individuals required to determine linkage (Risch and Merikangas, 1996). The multiple testing that is intrinsic to genome wide scans requires correction so the acceptable p value is very low. If alleles only have a modest effect, multiple testing using candidate genes probably has greater power to detect an effect than linkage analysis on a genome wide scan (Risch and Merikangas, 1996) although this is controversial (Scott, 1997). This is the case for loci outside the HLA region in RA, where the λs is likely to be less than 2, and therefore a candidate gene rather than a genome wide approach has been used by some groups (John, 1997).

At the moment, most genome wide scans are performed using microsatellite maps, with an interval between markers of 10 cM (Dib, 1996). This means that the markers used can be up to 5 cM from the disease gene. The next step is to refine the position of the disease susceptibility locus, identify candidate genes and then confirm or refute association with association studies. Refining the position of the disease susceptibility locus requires the construction of a transcript map (Cox, 1997) and the identification of candidate genes. Identifying candidate genes results in smaller disease-marker distances and therefore greater power for linkage or association studies such as the TDT.

In a sense, genome wide screening generates hypothesis, which can then be tested using association tests. An alternative, if there are insufficient families for a genome wide screening as in JIA, is to go straight to the hypothesis testing using candidate genes.
1.5.1.1 Genetic marker maps: microsatellites versus Single Nucleotide Polymorphisms (SNPs)

Microsatellites and SNPs are used in both genome wide and candidate gene approaches for association and family based studies. There are advantages and disadvantages to each.

The most widely used maps at the moment are microsatellite maps, which mark stretches of dinucleotide repeats. Over 5000 of these markers have been defined at intervals of <1 cM (Dib, 1996). One of the main advantages of microsatellites is that they are very polymorphic and therefore most individuals are heterozygous making transmission within families easier to determine. The main advantage of SNP maps is that SNPs exist every 1000bp. Current SNP maps have an average density of ~2-3 per cM, i.e. two to three times denser than microsatellite maps. However SNPs are less polymorphic than microsatellites leading to some loss of power although this can be overcome using flanking markers or pooling data from several adjacent SNPs which both help determine transmission patterns and can restore heterozygosity (Brown and Wordsworth 1998; Zhao, 1998).

1.5.2 Association studies

Association studies (or case-control or cohort studies) have been widely used to investigate the association of putative disease genes or markers with disease. They have been used both to identify allele association from candidate genes and to narrow candidate regions identified through genome searches. Association studies test the null hypothesis that there is no difference in the proportion of patients carrying the putative disease gene or marker and controls carrying the putative disease gene or marker. This clearly requires that both the control and the disease population have the same genetic background. The main problem of association studies is that of stratification, which means that allele frequencies are compared between two genetically distinct populations. Differences described can therefore be due to population diversity and not due to disease associations. In reality this may not be such a huge problem as few spurious type 1 errors have ever been reported as being due to stratification. One

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1 In this thesis, as in the literature, association studies refer to case control or cohort studies where allele frequency is compared between a disease group and a control group. The nomenclature is confusing as many family-based tests such as the TDT, test association (as well as linkage) but for the purposes of this thesis they are not described as association studies.
example of such a case is the association between variants in the immunoglobulin genes Gm and non-insulin-dependent diabetes mellitus in American Indians (Knowler, 1988). These problems with population stratification can be overcome by using family studies.

1.5.2.1 Problems with controls

Association studies require that controls are matched ethnically and geographically (our best surrogates for genetic matching) otherwise stratification may occur. This is difficult to achieve and therefore few studies have matched control groups. Blood donors are frequently used because they are an easy and willing source of blood and ethnic origin can be identified when the sample is taken. They can also be matched to the patient population geographically. However, few studies report on whether donors are first time donors or recalled donors. This is important, as blood banks will tend to recall particular blood groups which may skew results in terms of genetic analysis. Geographical matching may be almost impossible in association studies if the disease is rare and the patients identified from tertiary referral units with a wide geographical basis for referral. It is because of these problems that alternative methods have been developed.

1.5.3 Family based studies

The principle of all family based studies is that all the samples come from within the same family and therefore have the same genetic background. The choice of method depends on the demographics of the disease population and the number of affected members within families. The Transmission Disequilibrium Test (TDT) requires genotyping of the affected child and their parents and is therefore ideal for a population such as JIA where the parents are available and willing to donate samples. Clearly, this is not ideal for diseases with an older age of onset where the parents may not be available. For these diseases, one can use either affected or non-affected sib pair tests which are described and discussed in more detail below. It is possible to increase the power of the TDT using siblings and this is discussed below however the ethical considerations rarely make this an option in children.

1.5.3.1 Sib pair studies

1.5.3.1.1 Affected sib pairs (ASP)

Studies using affected sib pairs measure sharing of haplotypes or alleles between the pair. The null hypothesis is that sharing should be 0.5 if there is no linkage between the gene (or marker) studied and disease. ASP studies can measure either “identity-by-descent” (IBD) haplotypes or sharing of “identity-by-state” (IBS) haplotypes between
the pair. For the former method, knowledge of parental genotypes is required to determine which haplotype came from which parent. Of the two, this method is more powerful but is not always possible in adult onset diseases where the parents may not be available or alive. Alternatively, the probability that alleles that are IBS are also IBD can be computed using allele frequencies. Although this method can be more sensitive than straightforward measurement of IBD (because parental genotypes are not required), it is not sensitive for markers with low heterozygosity or highly skewed allele frequencies (Marlow, 1997). An alternative is simulation based methods which allow the IBD method to be used with a “best guess” when the IBD status is unavailable (Weeks, 1997). This method has recently been used to analyse the association of the candidate gene NRAMP1 with RA patients (Shaw, 1997).

ASP analysis has several advantages as it does not depend on the existence of linkage disequilibrium between the markers being tested and the disease genes. This means that markers can be a considerable distance away and still be informative. Another advantage is that no particular assumptions need to be made about mode of inheritance (Brown and Wordsworth 1998). In JIA however, there are insufficient ASP to provide sufficient power to make the test worthwhile. For genes with a risk ratio \( \lambda \) of \( \leq 2.5 \), these studies require thousands or tens of thousands of sibling pairs (Risch and Merikangas 1996). Although the \( \lambda \) for JIA is 15, this is the total risk ratio (Glass and Giannini 1999). The \( \lambda \) for individual genes will be smaller and is likely to be \( \leq 2.5 \) because it is a polygenic disease.

Comparison of the ASP test and the TDT (McGinnis 1998) has suggested that the TDT can be more powerful (i.e. there is a lower type 2 error) and therefore can be the test of choice when parental genotypes are available. If there is no parental data, a variety of methods have been developed (including extensions of the TDT) using both affected and unaffected siblings (Monks, 1997).

1.5.3 1.2 Unaffected sib pairs

Unaffected siblings can be used as controls in an adaptation of the TDT if the parental genotype is not known. Two methods have been described. Curtis et al (Curtis 1997) described the discordant-sibship test where allele frequencies are compared between discordant pairs. In this method the affected sibling is chosen (at random) and alleles are compared with an unaffected sibling (control) whose genotype is maximally different from that of the case. This avoids the introduction of correlation arising from using multiple sibs but may result in loss of information. Horvath and Laird extended
this using what is essentially a non-parametric sign test allowing all the affected and unaffected siblings to be used (Horvath and Laird 1998). These tests can be used to determine association but not linkage because of correlation between siblings (Horvath and Laird 1998). Although this method has less power then association studies, it avoids the problems of stratification and is therefore useful for late onset diseases (Curtis 1997). There is no increase in power in using unaffected siblings if the parents have been genotyped (Whittaker and Lewis 1998) unless the disease prevalence is high (Horvath and Laird 1998) and this is not the case in JIA. Another disadvantage of using unaffected sib pairs is that assumptions on penetrance must be made in order to define a sibling as being "unaffected" and therefore a suitable control. The TDT however makes no assumptions on penetrance.

The TDT was described in 1993 by Spielman et al as a test for association and linkage disequilibrium (Spielman, 1993; Spielman, 1994; Spielman and Ewens 1996). The principles of the TDT are depicted in figure 1.1. The test is based on the concept that a heterozygous parent has an equal chance of transmitting either allele in a biallelic system to an affected child. If the marker gene is in linkage disequilibrium with the disease gene, and the disease gene is associated with disease, there will be increased transmission of the disease allele to the affected child. In the TDT, the "case" is the disease allele and the "control" is the untransmitted allele. The results for each family trio are summarised into a contingency table of transmitted and non-transmitted alleles and tested for departures from random transmission using McNemar’s test to produce a chi-square statistic with one degree of freedom.

The TDT has already been used to confirm genetic associations defined in case cohort studies investigating HLA alleles in children with JIA (Moroldo, 1998).

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**Figure 1.1 Principles of the transmission disequilibrium test (TDT)**

Marker locus with 2 alleles A and a. If there is no linkage or association with disease then there is a 50% chance of transmission to child. If there is both linkage and association there will be increased transmission of the disease gene to the child. In this example the heterozygous father transmitted the a allele to his daughter.
1.5.3.2 Power considerations

Because only heterozygous parents can be used in the TDT, maximal information is obtained with an allele of 50% frequency because this allows for the maximum number of heterozygous parents. Addition of affected siblings improves power but the addition of non-affected siblings does not improve power if the parental genotype is known (Whittaker and Lewis 1998).

1.5.3.2.1 Power advantages

When the marker is extremely close to the disease locus or is the disease locus itself, tests such as the TDT can be far more powerful than conventional linkage tests. For example, when a candidate gene is present, the TDT is at least an order of magnitude more efficient than sib-pair tests (Allison 1997; Risch and Merikangas 1996). This is the case for the studies described in this thesis because the SNPs investigated are within candidate genes.

1.5.3.2.2 Power disadvantages

The disadvantages of the TDT are as follows:

1. As the TDT relies on transmission of alleles from a heterozygous parent then information is “lost” from families where both parents are homozygous for the disease allele. This problem is increased in cases when the disease allele is rare because this increases the number of homozygous parents for the normal allele.

2. If there is only information from one parent it is often possible to work out the genotype from the missing parent using the child’s genotype but this is subject to bias in a biallelic system as only certain genotypes can be ascribed this way (Curtis and Sham 1995).

3. As the test was initially created for a biallelic system, multiallelic markers and haplotypes cannot be investigated using the standard TDT.

Although it is possible to convert multiallelic loci or haplotypes to a biallelic system, this produces loss of data as it does not allow discrimination for different levels of effect for the different alleles. For example, suppose there are 3 alleles M1, M2 and M3. M1 is associated with disease, M3 is protective and M2 neutral. To investigate the association of M1 with disease, it would be possible to do the TDT test as transmission of M1 against transmission of M2+M3. However, this would mean that the data for the protective effect of M3 would be lost. These problems can be overcome to a certain extent by using the extended TDT or one of its adaptations (Sham and Curtis 1995).
1.5.3.3 Adaptations of the TDT

1.5.3.3.2 Multiple alleles

Adaptations to allow the TDT to be used for multiallelic markers such as microsatellites include logistic regression analysis (Sham and Curtis 1995), the likelihood based test of linkage disequilibrium (Terwilliger 1995) and the randomisation test (Morris, 1997) or the Monte Carlo simulation methodology. These methods are preferable to the alternative which is to form a contingency table for each allele versus all other alleles pooled and perform the original TDT within each table. This requires a Bonferonni correction for multiple testing and the unavoidable loss of power. The randomisation test has an additional advantage in that it determines the direction of each association i.e. it defines not only the power or significance of the association but whether the allele is protective against disease or associated with it (Morris, 1997). The power of these tests is determined in part by mode of inheritance (Morris, 1997).

1.5.3.3.3 Multiple loci

Recently methods of using the extended-TDT (ETDT) to examine the effect of more then one disease loci or two marker loci around one disease loci have been described (Morris and Whittaker 1999; Wilson 1997). Morris and Whittaker (Morris and Whittaker 1999) describe a method which allows two unlinked disease loci to be examined by simultaneously examining both loci using models where the markers are either linked or unlinked to the disease loci and allowing for epistasis (interaction among alleles at different loci). This method appears to be more powerful when tested than previous methods. If there is a genetic component to severity, then the power is increased further if only individuals with the severe form of the disease are examined.

Although this method suffers from the drawback of multiple testing it has an advantage in that a greater proportion of parents will be “heterozygous” for the pair of markers and therefore informative for linkage.

1.5.3.3.4 Quantitative traits

Quantitative trait analysis is important because of the burden of disease where quantitative traits are expressed. Allison recently developed 5 TDT-type tests for use with quantitative traits (Allison 1997). These tests use either random sampling or extreme sampling. In each case he makes the following assumptions; the family trio has one parent who is heterozygous (except for the 5th method) with one affected child, the genetic markers are the trait locus and the locus is biallelic. This method has since been extended to allow examination of markers that are not the trait locus in families where
both parents are heterozygous and more than one child is affected (Xiong, 1998). Other methods to improve power have since been described. For example, the TDT can be used to compare transmission of an allele in individuals with an extreme trait value at either end of the spectrum. This increases power such that if the most extreme 20% of the phenotypic distribution is selected, quantitative trait loci that explain as little as 5% of the phenotypic variation can be detected with an alpha value (risk of type 1 error) of 0.0001 with less than 300 observations (Allison, 1998). Alternatively, symptoms can be stratified and the TDT can be used within each stratification (Waldman, 1998). One can also compare the trait values for individuals to whom one allele has been passed versus another allele. The most powerful statistic however is to regress the trait value on the genotype of the affected individuals while including dummy variables for the parental mating types (Allison, 1998). This regression methodology can also be extended to allow for environmental variables.

1.5.3.3.5 Multiple affected siblings

If there is more than one sibling affected, then the TDT test can be used independently for both children. In this situation however, the TDT is only valid as a test of linkage and not of association as the transmission of alleles between children is not independent. As affected sib pairs are rare in JIA this is unlikely to be a frequent problem.

1.5.4 Linkage disequilibrium

Linkage disequilibrium occurs where alleles at two genetic loci are not transmitted independently because they have rarely been separated by recombination's. This results in the frequency of the haplotypes between the two alleles differing from that predicted by the population frequencies of the alleles from each loci. To determine whether alleles are in linkage disequilibrium the allele frequency at each loci must be determined e.g. as R and R'. Expected haplotype frequency (E) is therefore R x R'. Actual haplotype frequency is then observed (O) by counting haplotypes in the population. Chi$^2$ ($X^2$) for each haplotype is therefore (O-E)$^2$/O+E. The total $X^2$ for linkage disequilibrium is the sum $X^2$ for all haplotypes. A p value can then be calculated using statistical tables.
1.6 Potential genes important in JIA

The number of genes that could be involved in either susceptibility or severity of JIA is huge. Virtually any gene encoding any molecule involved in inflammation such as immune recognition, cell interaction, intracellular signalling or cytokine pathways could be proposed (John, 1997).

Some of the components are illustrated in this simplified diagram. Other components include immune recognition molecules, adhesion molecules and molecules associated with apoptosis.

Figure 1.2 Potential pathways to look for genetic associations in JIA
1.6.1 Genetic associations described in JIA

The sibling relative risk of disease for JIA is 15 suggesting that there is a genetic component to developing JIA (see section 1.4) (Glass and Giannini 1999). In addition, sib pair studies suggest that there is a higher than expected degree of concordance for subtypes with different clinical onset patterns suggesting that a genetic component determines disease course. In addition, there is some evidence that certain disease characteristics such as uveitis are influenced by genotype. To date however, although many genetic associations have been described they do not fully account for the calculated relative risk.

Genetic associations described include associations with an allele at the serum amyloid A locus, TAP genes, HLA associations and cytokine gene associations.

1.6.1.1 Serum amyloid A (SAA) locus

Inducible members of the serum amyloid A (SAA) protein family are secreted into the serum in response to inflammation as part of the acute phase response. Prolonged inflammation can lead to deposition of SAA in a variety of tissues in reactive amyloidosis. Patients with systemic JIA who develop amyloidosis are more likely to have the SAA<sub>2,2</sub> allele than patients with systemic JIA who have not developed amyloidosis (Faulkes and Woo 1997).

1.6.1.2 TAP gene locus

An association has also been described between the TAP2B gene and patients with early onset pauciarticular arthritis (now defined as oligoarticular arthritis) (OR 2.1, 95% CI 0.9-4.7) (Donn, 1994). The TAP genes lie within the major histocompatibility complex (MHC). The products of the TAP genes are thought to be involved in the transferral of processed cytoplasmic peptide fragments to the endoplasmic reticulum playing a role in the class I endogenous peptide processing pathway. As they lie within the MHC and are in linkage disequilibrium with HLA-DR1 and DR4 (Donn, 1994), they may explain some of the multiple HLA associations with JIA.

1.6.1.3 HLA association with JIA

The MHC is important in the presentation of peptide Ag that is recognised by T cell receptors (TCR) of particular disease-promoting T cells. This then leads to proliferation of lymphocytes with receptor specificity for this antigen-MHC complex resulting in the release of inflammatory cytokines and proteolytic enzymes and the recruitment of other
cells including macrophages and B cells. The association of HLA haplotypes with disease would therefore suggest that T cells were triggered by a specific Ag.

Multiple HLA associations have been described in JIA for all disease groups and for patients with uveitis and these are described in detail in a review by Donn and Ollier (Donn and Ollier 1996). Specific HLA alleles also appear to be associated with disease severity in that they are associated with children that progress from oligoarticular to extended oligoarticular disease (Grom, 1994).

The TDT has recently been used to confirm some of these described HLA associations in an American Caucasian population of 101 children with pauciarticular (oligoarticular by ILAR classification) JIA (Moroldo, 1998). In this study there was increased transmission of HLA class I alleles A2, B27 and B35 and class II alleles, DR5 and DR8 to affected children. Transmission of these alleles to unaffected siblings was not increased.

The situation is complicated in that recent work has suggested that certain HLA alleles are strongly associated with susceptibility or increased risk in some ages but provide protection at other ages (Murray, 1999). For example the susceptibility produced by HLA DR5 and HLA DR8 appears early in life and then tails off rapidly in that it is not associated with increased risk beyond the age of 10 years (Murray, 1999). Whereas HLA-B27 is protective early in life for the development of oligoarticular JIA but is associated with increased risk later in childhood. In addition, the HLA extended haplotypic associations are different in childhood and adult onset disease for polyarticular rheumatoid arthritis (Fraser, 1990; Nepom, 1986) and the association of HLA-DR4 is only true for childhood onset systemic JIA not for adult onset systemic JIA (Miller, 1985).

To complicate matters further, it appears that several loci interact in a polygenic model to increase the risk of what is now called oligoarticular JIA. Grom et al describe how HLA genes from 4 loci which are not in linkage disequilibrium are associated with oligoarticular JIA. There is an increasing cumulative Odds Ratio (OR) for these factors suggesting independent cumulative effects in a polygenic model (Grom, 1994). There is also evidence that there is an increased risk of developing eye disease in children who are heterozygous for HLA-DR5/DR8 with independent risk factors from an HLA-DP gene and HLA-A2 (Hall, 1986).

A complication of all HLA association studies is that many HLA haplotypes are in linkage disequilibrium with genes that could be involved in autoimmune diseases.
These include genes in the class III region such as complement proteins, heat shock protein 70 family and TNFα (Hsieh, 1997; Jacob, 1990). TNFα production is associated with particular HLA genotypes e.g. low TNFα is associated with HLA-DR2/DQw1 (Jacob, 1990). As low TNFα is associated with lupus nephritis, the association of HLA-DR2/DQw1 with lupus nephritis may actually be due to linkage with the TNFα gene (Jacob, 1990).

However unlike adult RA, there does not appear to be a shared epitope (DNA sequence common to the different HLA haplotypes) that predicts disease in JIA (Gregersen, 1987; Jawaheer, 1994). With multiple associations, each with a relatively low odds ratio, it is hard to conclude that there is a specific Ag involved in determining susceptibility in JIA or indeed that the MHC complex explains all of the genetic susceptibility in JIA. It is likely that other genetic factors are involved.

### 1.6.1.4 Cytokine 5' flanking region polymorphisms in children with JIA

To date, two cytokine 5’ flanking region polymorphism associations have been described within the JIA population. The first was an association between an IL-1α 5’ flanking allele and patients with early onset pauciarticular JRA in white Norwegian patients (p=0.01, OR=2.1) (McDowell, 1995). The association was increased in those patients in whom anterior uveitis developed (p=0.002, OR=6.2). However, this association was not confirmed in a UK population (Donn, 1998).

The second is an association between an allele in the IL-6 5’ flanking region and children with systemic JIA (Fishman, 1998). Children with systemic JIA have higher IL-6 levels in peripheral blood than controls (Mangge, 1995; Rooney, 1995; De Benedetti, 1997) and IL-6 has been proposed as a pathogenic cytokine in this disease see section 1.8.4. Fishman et al (Fishman, 1998) from this lab investigated whether this could be due to polymorphisms in the 5’ flanking region of the IL-6 gene. A G/C polymorphism was detected at position –174 and transient transfection studies showed that the C allele was associated with lower expression than the G alleles both constitutively and when stimulated by LPS or IL-1. Plasma levels in 102 controls also suggested that the C allele was associated with significantly lower levels of plasma IL-6. The association in systemic JIA is shown in table 1.3.
Table 1.3 The IL-6 5' flanking region genotype in patients with systemic onset JIA

<table>
<thead>
<tr>
<th>Genotype</th>
<th>All patients (n=92)</th>
<th>Onset ≤5 yrs (n=56)</th>
<th>Onset ≥ 6 yrs (n=36)</th>
<th>Controls (n=383)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>28 (0.3)</td>
<td>16 (0.29)</td>
<td>12 (0.33)</td>
<td>144 (0.38)</td>
</tr>
<tr>
<td>GC</td>
<td>54 (0.59)</td>
<td>36 (0.64)</td>
<td>18 (0.5)</td>
<td>169 (0.44)</td>
</tr>
<tr>
<td>CC</td>
<td>10 (0.11)*</td>
<td>4 (0.07)**</td>
<td>6 (0.17)</td>
<td>70 (0.18)</td>
</tr>
</tbody>
</table>

**P=0.01, * P=0.03

These results suggest that the CC genotype is protective with lower IL-6 production and is therefore seen in reduced frequency in children with disease and especially those with young onset.
1.7 Cytokines in JIA

1.7.1 Th1 versus Th2

Human CD4+ T-helper cells (Th) cells can be divided into three different subsets defined by their cytokine profile (Mosmann and Coffman 1989; Abbas, 1996). Th1 cells produce IL-2, IFNγ and TNFβ whilst Th2 cells produce IL-4, IL-5, IL-6, IL-10 (in mice) and IL-13. Th0 cells can produce both types of cytokines. The concept however is more than just a division based on cytokine production but also on T cell function. For example, Th1 cells increase macrophage activation and the production of complement-fixing and opsonising antibodies. In contrast, Th2 cells stimulate antibody production by B cells and upregulate humoral or allergic responses.

The pattern of cytokine production in different disease states is therefore often described as a Th1 or Th2 response based on the cytokines produced. Although helpful, the Th1/Th2 paradigm has limitations in terms of the classification of cytokines in that cytokines such as IL-6 and IL-10 are synthesised mainly by monocytes in arthritis rather than Th1 or Th2 cells. In addition, the effect cytokines have on inducing a Th1 or Th2 response depends on the activation state of the cell and the presence of other cytokines (for example see section 1.9.1.11).

A more helpful paradigm therefore may be the classification of cytokines according to whether they up regulate (pro-inflammatory) or down regulate (anti-inflammatory) the inflammatory cascade or response.

1.7.2 The balance of cytokines: pro-inflammatory versus anti-inflammatory

Both pro-inflammatory and anti-inflammatory cytokines can be found in synovial tissue and fluid in the joints of patients with both adult and juvenile onset arthritis (see table 1.1)(Schlaak, 1996; Maini, 1994; Miller, 1993; Lettesjo, 1999; Maini, 1994; Miller, 1993). Pro-inflammatory cytokines such as interleukin (IL)-1β, IL-1α, TNFα, IL-6, IL-12 and IFNγ promote inflammatory changes leading to bone and cartilage degradation (van den Berg, 1993; van den Berg, 1991; van der Loo, 1995; Chomarat, 1995b; van den Berg, 1991; van der Loo, 1995; Chomarat, 1995b; Thorbecke, 1992). This destruction can be inhibited or controlled by receptor antagonists, soluble receptors and anti-inflammatory cytokines such as IL-10 and IL-4 (Hart, 1995; Chomarat, 1995b; van Roon, 1996; Joosten, 1997; Walmsley, 1996; Persson, 1996; Yin, 1997b; Chomarat, 1995b; van Roon, 1996; Joosten, 1997; Walmsley, 1996; Persson, 1996;
In chronic inflammatory arthritis, a paradigm has been described between pro- and anti-inflammatory cytokines such that the ratio of pro-inflammatory/anti-inflammatory cytokines within the joints could determine outcome (Feldmann, 1994). Imbalances have since been described for juvenile chronic arthritis, Lyme disease, rheumatoid arthritis and reactive arthritis (Rooney, 1994; Miller, 1993; Chomarat, 1995b; Yin, 1997b; Miller, 1993; Chomarat, 1995b; Yin, 1997b). For example, in Lyme disease a prevalent pro-inflammatory pattern of cytokine release has been demonstrated when synovial fluid mononuclear cells are stimulated with *Borrelia burgdorferi* (Bb) with high levels of IFNγ and TNFα but low levels of IL-4 (Yin, 1997a). This pro-inflammatory response can be down regulated by exogenous IL-10 suggesting that insufficient IL-10 was produced *in vivo*. When cytokine production was related to outcome, patients with a high IL-1ra/IL-1β ratio in the synovial fluid at the start of Lyme arthritis had a better outcome with faster resolution than patients with a high IL-1β/IL-1ra (Miller, 1993).

In JIA, a longitudinal study over two years demonstrated significant correlation between CRP, serum Iron and haemoglobin with serum levels of soluble IL-2R, IL-6 and soluble TNF receptor (Mangge, 1995). This suggests a predominantly pro-inflammatory response. Platelet levels correlated with IL-6. Interestingly these observations were made across all subtypes of JIA suggesting an association between inflammation *per se* and cytokines rather than with particular disease groups. In the same study, normalisation of these values accompanied clinical improvement after treatment was started.

**Table 1.1 Examples of pro and anti-inflammatory cytokines**

<table>
<thead>
<tr>
<th>Pro-inflammatory cytokines</th>
<th>Anti-inflammatory cytokines/ cytokine inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon gamma (IFNγ)</td>
<td>Interleukin 1 receptor antagonist (IL-1ra)</td>
</tr>
<tr>
<td>Interleukin 1 (IL-1)</td>
<td>Interleukin 4 (IL-4)</td>
</tr>
<tr>
<td>TNF alpha (TNFα)</td>
<td>Interleukin 10 (IL-10)</td>
</tr>
<tr>
<td>Interleukin 6 (IL-6)</td>
<td>Interleukin 13 (IL-13)</td>
</tr>
<tr>
<td></td>
<td>Transforming growth factor beta (TGFβ)</td>
</tr>
<tr>
<td></td>
<td>Soluble TNF receptors</td>
</tr>
</tbody>
</table>

### 1.7.3 Pro-inflammatory cytokines in arthritis

In the murine collagen induced arthritis model, high levels of the pro-inflammatory cytokines TNFα and IL-1β were detected at the onset of disease (Mauri, 1996).
**Anti-cytokine treatment in arthritis**

Anti-TNF is the most well known anti-cytokine treatment. Eternacept (trade name Enbrel) is a recombinant human TNF receptor Fc fusion protein consisting of a dimer of the extracellular portion of two p75 receptors fused to the Fc portion of human IgG1. In adult RA, just over 50% of those treated and 11% placebo achieved an ACR response of 20%. In children with JIA there is a much better response in those with polyarticular onset and extended oligoarticular JIA but a similar response rate for those with systemic onset JIA (ie only approximately 50% respond). In those with polyarticular JIA there is an increased median time to disease flare when compared to placebo and 74% met the definition of improvement in all measures of disease activity.

Reference:

Transgenic mice expressing high levels of TNFα spontaneously develop a severe erosive arthritis suggesting the importance of TNFα in initiating disease (Butler, 1997). Feldman and Maini showed from in vitro experiments that in rheumatoid arthritis, TNFα stimulates a cascade of pro-inflammatory cytokines. Furthermore, blockade of TNFα by chimeric monoclonal antibodies to TNF (cA2) not only inhibits the release of pro-inflammatory cytokines but also results in improvement in adult rheumatoid arthritis (Williams, 1992; Elliott, 1994; Elliott, 1994). When cA2 was used to treat a teenager with systemic onset JIA, there was rapid and complete control of fever for five days after treatment and improvement in anorexia, chest and abdominal pain suggesting that TNFα is also important pathologically in systemic onset JIA (Elliott, 1997). However the fever recurred and the arthritis was unaffected throughout therefore it is unlikely to be the only cytokine that is relevant in this disease. Many pro-inflammatory cytokines have been found in serum and synovial fluid of patients with JIA. Serum levels of the IL-2 receptor (IL-2R), IL-6 and the p55 soluble TNF receptor (p55-TNFR) have been shown to correlate with inflammatory markers in children with JIA (Mangge, 1995). The soluble p55-TNFR and IL-2R correlate closely with disease severity and are more sensitive parameters than CRP (Mangge, 1995). A further consequence of pro-inflammatory cytokines in vivo is the up-regulation of the acute phase protein, serum amyloid A (Betts, 1993; Uhlar, 1997; Edbrooke, 1991; Uhlar, 1997; Edbrooke, 1991). This protein polymerises to produce amyloid fibres resulting in systemic amyloidosis in susceptible individuals.

1.7.4 Anti-inflammatory cytokines in arthritis

The cytokines that have a predominantly anti-inflammatory effect in arthritis are IL-13, IL-4, TGFβ and IL-10. There are also cytokine antagonists such as the IL-1 receptor antagonist (IL-1ra), which binds both IL-1 receptors but does not transduce any signals (Arend 1991).

IL-13 is present in the synovial fluid of patients with rheumatoid arthritis and IL-13 mRNA can be detected in synovial fluid monocytes (SFMC) (Isomäki, 1996). Addition of exogenous IL-13 in these patients down regulates IL-1β and TNFα production by SFMC but not as efficiently as the addition of IL-10. Inhibition of endogenous IL-13 by SFMC had no effect on IL-1β or TNFα production suggesting that it may not be a potent inhibitor of pro-inflammatory cytokine activity in vivo (Isomäki, 1996).
IL-4 also inhibits the production of IL-1β and TNFα (Chomarat, 1995b; van Roon, 1996; Lubberts, 1998) and appears to stimulate the production of cytokine inhibitors such as IL-1ra and soluble TNF receptor thereby reducing inflammation and cartilage degradation in arthritis (Chomarat, 1995b) (Hart, 1995). Several in vitro studies have suggested that IL-10 is more effective than IL-4 in decreasing pro-inflammatory cytokine production by mononuclear cells from synovial fluid and cells and peripheral blood of patients with rheumatoid arthritis (Hart, 1995) (van Roon, 1996; van Roon, 1996). Furthermore, IL-10 appears to be more effective than IL-4 in ameliorating inflammation in the collagen induced arthritis animal model and reversing the cartilage degradation by mononuclear cells from patients with rheumatoid arthritis in tissue culture (van Roon, 1996) (Jorgensen, 1998; Lubberts, 1998). In the murine model of collagen induced arthritis, IL-10 production increases as inflammation decreases (during the remission phase) and levels correlate with decreased production of pro-inflammatory cytokines TNFα and IL-1β (Mauri, 1996). A similar picture is seen in other inflammatory models (such as autoimmune encephalomyelitis) where an increase in IL-10 production correlates with recovery (Kennedy, 1992).

Although IL-10 has stimulatory as well as anti-inflammatory effects, IL-10 acts mainly as an anti-inflammatory cytokine in arthritis as it inhibits the synthesis of and antagonises the effects of pro-inflammatory cytokines (Chernoff, 1995), 22, 74 (Katsikis, 1994; Hart, 1995; Cassatella, 1993). IL-10 also down regulates antigen presenting cell function of monocytes (Spittler, 1995; De Waal Malefyt, 1991) and prevents activation of dendritic cells (de Saint-Vis, 1998; Allavena, 1998; Kawakami, 1997; Kawakami, 1997). For full details of IL-10 action see section 1.9.1.

TGFβ has similar anti-inflammatory actions to IL-10 but in vitro, IL-10 decreases TNFα production 25 fold more than TGFβ (Bogdan, 1992).
1.8 Cytokine association in Juvenile Idiopathic Arthritis

Fewer studies have investigated cytokines in children with arthritis than in adults with RA. There does however appear to be some evidence that cytokine production correlates with disease and inflammatory markers across all disease groups (Mangge, 1995). The involvement of cytokines varies with each disease subtype as discussed below.

1.8.1 Oligoarticular JIA

Synovial fluid T cell clones from oligoarticular JIA have mainly a Th1/Th0-type pattern of cytokine secretion (Gattorno, 1997). This is consistent with a recent report describing increased IFNγ levels in serum and synovial fluid of patients with both oligoarticular and polyarticular disease when compared to controls (Yetgin, 1999). Children with oligoarticular JIA appear to have more IL-4 mRNA in synovial tissue biopsies and less TNFα than children with polyarticular onset JIA or adults with rheumatoid arthritis (RA) (Grom, 1996; Murray, 1998). The combination of IL-4 and IL-10 mRNA is also found more frequently in non-erosive disease when compared to erosive disease (Murray, 1998). IL-4 mRNA is also detected more often in patients who remain oligoarticular than those who extend (Murray, 1998). This suggests that the presence of anti-inflammatory cytokines such as IL-4 and IL-10 may be protective in preventing the development of severe disease.

Differences have been described in serum cytokine levels between children who have an early onset of pauciarticular JIA (now termed oligoarticular JIA) with raised IL-1β, sIL-2R, TNFα and soluble p55-TNFR when compared with those with a late onset pauciarticular JIA (raised sIL-2R and soluble p55-TNFR) (Mangge, 1995). The late onset pauciarticular JIA will include patients who would now be classified as having a spondlyarthropathy. The soluble p55-TNFR inhibits the action of TNFα. As the receptor is increased in late onset oligoarticular JIA without an increase in TNFα, this suggests a relative decrease in TNFα/TNFR in patients with late onset disease with less biologically effective TNFα. This is consistent with what is observed clinically in that those with late onset disease frequently have less destructive disease clinically than those with early onset disease.
1.8.2 Polyarticular, Rheumatoid factor negative JIA

In contrast to oligoarticular JIA, polyarticular JIA is frequently associated with joint damage which can be erosive. This laboratory has shown that serum TNFα levels are higher in patients with polyarticular JIA than in those with oligoarticular disease or spondyloarthropathies. Furthermore, the balance of TNFα with soluble TNF receptors I and II (or p55 and p75) in synovial fluid is skewed towards high TNFα/soluble TNFα receptors in children with polyarticular onset disease (Rooney, 1999). As these soluble receptors function as TNFα antagonists, this means there is increased biologically active TNFα in this subgroup of patients which may partly explain the increased morbidity associated with this subtype of disease. Other cytokines such as IL-6 and IL-2R appear to be elevated in active disease in different onset types (Madson, 1994).

1.8.3 Polyarticular, Rheumatoid factor positive JIA

The clinical picture of early onset polyarticular, rheumatoid factor positive JIA is identical to that seen in adults and it is likely that the pathology is similar. Cells isolated from the synovium of adult patients with rheumatoid arthritis express higher levels of IL-6 and IL-6 like cytokine mRNA, and spontaneously produce greater quantities of these proteins into culture than cells isolated from the synovium of patients with osteoarthritis (Okamoto, 1997). Leistad et al (Leistad, 1998) have compared mRNA expression in the synovium of patients with rheumatoid and osteoarthritis. Patients with rheumatoid arthritis expressed IL-6, IL-6R IL-7 IL-8, IL-10 and IL-12 whilst patients with osteoarthritis only expressed IL-8 and IL-10. This suggests a pro-inflammatory response within the synovium of patients with rheumatoid arthritis but not in those with osteoarthritis. In patients with rheumatoid arthritis, exogenous IL-10 decreases the production of pro-inflammatory cytokines (TNFα, IL-1α and IFNγ) and neutralising anti-IL-10 monoclonal antibodies increases the production of these cytokines from stimulated synovial and peripheral blood mononuclear cells (Isomake, 1996))(Katsikis, 1994; van Roon, 1996; Hart, 1995). These results suggest that the IL-10 is functionally relevant but may not be produced in sufficient quantities to suppress the production of pro-inflammatory cytokines.

1.8.4 Systemic JIA

The first description of cytokine abnormalities in children with systemic JIA was described by Prieur et al in 1987 (Prieur, 1987). In this study they showed that IL-1 activity, as measured by stimulatory activity on prostaglandin E2 (PGE2), was low in serum and urine samples when patients with systemic JIA were febrile and normal or
high when patients were afebrile (Prieur, 1987). This inhibitory factor at the time of fever is likely to be IL-1ra which has since been shown to mirror the rise and fall of body temperature (Rooney, 1995). In this later study, IL-1β was not detected during the course of the fever (probably because IL-1ra was in excess by 1000 fold) but became detectable as the temperature fell. IL-1β has been shown to be significantly elevated in patients with systemic JIA compared to healthy controls (Mangge, 1995).

IL-1α is undetected in patients with systemic JIA unlike those with RA or pauciarticular JIA (De Benedetti, 1997). IL-6 and TNFα levels are increased in peripheral blood and synovial fluid of children with systemic JCA even when compared to children with polyarticular JIA (Rooney, 1995; Mangge, 1995; De Benedetti, 1997). IL-6 levels correlate strongly with ESR and when IL-6 levels were measured during febrile episodes, IL-6 closely mirrored body temperature. In contrast, TNFα levels rise and fall out of phase with the temperature with the peak being 5 hours after the peak of fever (Rooney, 1995).

The IL-6 agonist soluble IL-6 receptor (sIL-6R) is elevated in patients with systemic JIA (Keul, 1998) and de Benedetti et al shown increased IL-6-sIL-6R complexes at the height of fever (De Benedetti, 1994). As IL-6 bioactivity is increased when complexed to the IL-6R, there may be an imbalance in systemic patients with increased production of IL-6 and its soluble receptor and reduced production of IL-10 resulting in the persistence of inflammation.

Whether IL-10 is increased (Raziuddin, 1998) or decreased (Muller, 1998) in systemic patients with JIA is controversial. This may reflect the complexities of analysing cytokine production in children on a variety of treatment and a range of endogenous stimulation due to the disease.

1.8.5 Psoriatic JIA

The synovitis of psoriatic arthritis is similar but not identical to other forms of arthritis with less synovial lining cell hyperplasia and increased vascularity (Veale, 1998). Measurement of cytokine production in psoriatic synovial membranes has shown increased production of IL-10, IL-1β, IL-2, IFNγ and TNFα (Ritchlin, 1998). The production of IL-1β, and IFNγ was strongly correlated with IL-10. The cytokine levels were higher in patients with psoriatic arthritis despite higher histopathological scores in patients with rheumatoid arthritis (Ritchlin, 1998). The balance of pro- versus anti-inflammatory cytokines has not been defined.
1.8.6 Spondyloarthropathies

Little is known about cytokines in children with spondyloarthropathies. Rooney et al showed that patients with spondyloarthropathies had higher synovial TNF receptors I and II/TNFα than patients with polyarticular disease. This higher level of anti-inflammatory cytokines is consistent with the marked delay of erosive disease seen in patients with spondyloarthropathy when compared to those with polyarticular disease (Rooney, 1999). Serum IL-10 levels have been shown to correlate with disease activity in adult patients with a spondyloarthropathy (Claudepierre, 1997). This data however is difficult to interpret because the increased disease activity would be expected to increase both pro- and anti-inflammatory cytokines. When pro- and anti-inflammatory cytokines were measured in patients with spondyloarthropathy, RA and osteoarthritis, higher levels of IL-4, IL-10 and TGFβ were found in those with RA and spondyloarthopathies. These increased levels of anti-inflammatory cytokines were not associated with lower levels of pro-inflammatory cytokines. This suggests that the immunosuppressive activity of IL-4, IL-10 and TGFβ is not sufficient to control pro-inflammatory cytokine production in vivo.

1.8.7 Uveitis

1.8.7.1 TNFα in uveitis

Several experimental models of uveitis have been developed. Depending on the stimulus used and the animal, the uveitis is mainly anterior or posterior. A common model used to investigate anterior uveitis is endotoxin-induced uveitis where sublethal doses of endotoxin are given systemically to rodents. In rats this causes bilateral disruption of the blood-aqueous barrier 4 hours after injection with maximal uveitis detected 18 to 24 hours after injection. Cytokine mRNA for IL-1α, IL-1β and TNFα increases dramatically to a peak within the iris/ciliary body only 1 hour after endotoxin injection (Planck, 1994). It was therefore proposed that TNFα might be one of the early pro-inflammatory cytokines in the development of uveitis. This was consistent with other models such as subcutaneous inoculation of melanin associated antigen isolated from bovine iris/ciliary body and choroid into Lewis rats who develop severe inflammation in the anterior segment of the eye (Woon, 1999). Analysis of the kinetics of cytokine mRNA from both the target organ (iris/ciliary) and associated lymph nodes have demonstrated increased levels of TNFα which paralleled disease within the iris/ciliary body and increased IFNγ in the popliteal lymph node which also paralleled
disease activity (Woon, 1999). IL-10 also increased modestly within the popliteal lymph node at the peak of disease. However further examination of the role of TNFα reveals a paradoxical effect. Although anti TNFα suppresses the induction of experimental autoimmune uveoretinitis in mice (Sartani, 1996), in this model the uveitis affects the posterior segment of the eye, which is unlike uveitis in children with JIA. In EIU in mice (anterior uveitis), anti-TNFα monoclonal antibody exacerbates the uveitis (Kasner, 1993). The uveitis is also exacerbated by repeated TNF injections prior to induction of TNFα resistance. Ocular inflammation in this study was graded on the number of inflammatory cells in the anterior chamber and the posterior vitreous. Indeed, in some animal models anti-TNF antiserum exacerbates the intraocular inflammation as does the administration of pentoxifylline which is known to inhibit LPS induced TNF synthesis (de Vos, 1995). This is consistent with EIU in the rat (de Vos, 1995). In this experiment, either anti-TNFα monoclonal antibody (mAb) or pentoxifylline given prior to induction with LPS significantly reduced serum levels of TNFα however intraocular TNFα levels remained unchanged. In both groups the uveitis as assessed by hyperaemia, cell infiltrate, flare and meiosis worsened. These models are confusing however as pentoxifylline also increases IL-10 levels (Platzer, 1995). Neutralising TNFα therefore worsens uveitis even though it can protect against the systemic affects of LPS (Kasner, 1993). This is analogous to the clinical situation anecdotally described in JIA where it appears that children with mild arthritis are more likely to have severe uveitis (Cassidy and Petty 199; Sartani, 1996). It has been hypothesised that there is compartmentalisation of the inflammatory response such that the LPS stimulates the production of circulating cytokines such as TNFα or IL-6 stimulating the inflammatory response in the eye. The presence of TNFα systemically then activates the production of anti-inflammatory cytokines such as IL-10 which reduce inflammation in the eye. It may be that this second stage of modulation does not occur if systemic levels of TNFα are low, whilst the compartmentalisation in the eye ensures that inflammation persists.

1.8.7.2 IL-10 in uveitis

The pleiotropic effects of IL-10 in reducing inflammation suggest that it should be protective in anterior uveitis. IL-10 mRNA levels increase in the spinal cord of mice during the recovery phase in animal models of uveitis (Kennedy, 1992) and intravitreal injection of IL-10 has been shown to reduce inflammation after endotoxin injection in mice and rabbits. However intraperitonel IL-10 worsened the uveitis in mice in a dose
dependent manner (Roenbaum and Angell 1995). The role of TNFα and IL-10 in EAU is therefore not clear at present.
1.9 Interleukin 10

1.9.1 Function of IL-10

IL-10 exerts its anti-inflammatory effect by a variety of mechanisms.

1.9.1.1 Inhibition of pro-inflammatory cytokine production

IL-10 directly inhibits the synthesis of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, IL-12, and TNFα in macrophages stimulated with LPS, or IFNα (Chernoff, 1995; Chomarat, 1995a; D’Andrea, 1993; Katsikis, 1994; De Waal Malefyt, 1991; Hart, 1995; de Waal, 1991; Fiorentino, 1991a). IL-10 also inhibits the production of TNF, IL-1β and IL-8 from human polymorphonuclear leucocytes (Cassatella, 1993) and IL-8 from eosinophils (Wang, 1994a). In monocytes stimulated by either TNFα or IFNγ, the addition of anti IL-10 -mAb increases cytokine production even further suggesting that endogenous IL-10 plays a role in regulating cytokine production during monocyte stimulation (De Waal Malefyt, 1991). IL-10 also reduces IL-6 production by synovial tissue and synovial monocytes (Chomarat, 1995a).

This down regulation of cytokine production by IL-10 is paralleled by a decrease in mRNA levels suggesting that IL-10 acts may at the transcriptional level (Bogdan, 1992; Wang, 1994b; Wang, 1994a).

Some studies have suggested that IL-10 acts to decrease transcription of pro-inflammatory cytokine genes and may require de novo protein synthesis as the suppression is nearly abrogated in the presence of cycloheximide (Wang, 1994b; Aste-Amezaga, 1998). However, not all studies have confirmed this (Takeshita, 1996; Bogdan, 1992). IL-10 also appears to be able to increase mRNA degradation in some systems (Takeshita, 1996; Bogdan, 1992; Wang, 1994a; Wang, 1994b; Brown, 1996). This is seen when actinomycin is added to cell cultures stimulated with LPS to block transcription allowing the demonstration that the T 1/2 life of cytokine mRNA is reduced in the presence of IL-10 (Takeshita, 1996; Wang, 1994a; Wang, 1994b).

1.9.1.2 IL-10 can decrease pro-inflammatory cytokine production indirectly

IL-10 also decreases IFNγ production by T cells (D’Andrea, 1993; Macatonia, 1993) but this appears to be indirect as it only occurs in the presence of monocytes. It appears that the inhibition of IFNγ production by IL-10 in PBMCs is via inhibition of IL-12 and

2 Unless specified, all references to IL-10 are human IL-10.
probably IL-1β from monocytes and other accessory cells (D'Andrea, 1993). This probably explains how dendritic and monocytic-stimulated IFNγ production by Th1 cells is down regulated by IL-10 (Macatonia, 1993).

1.9.1.3 Antagonism of pro-inflammatory cytokines

In addition to down regulation of TNFα production (see above), IL-10 also antagonises the effect of TNFα. There appear to be several mechanisms by which IL-10 achieves this. IL-10 both down regulates the expression of TNFα cell surface receptor in monocytes in a dose dependant manner (decreasing the biological activity of TNFα) and increases the release of soluble TNF p75 receptor from monocytes, which blocks its biological activity (Joyce, 1994; Dickensheets, 1997). The increase in soluble TNF p75 protein correlated with an increase in mRNA. In addition, IL-10 upregulates LPS induced TNF p75 receptor protein production in transient transfection studies suggesting that it upregulates transcription. IL-10 does not affect mRNA degradation of TNF p75 receptor as mRNA levels were not altered in the presence of actinomycin D (Dickensheets, 1997). Addition of actinomycin D at the time of stimulation rather than afterwards abrogated the effect of IL-10. This is consistent with IL-10 upregulating transcription in this system (Dickensheets, 1997).

IL-10 antagonises the effect of TNFα by other routes, for example by inhibiting TNFα induced synthesis of COX-2 which mediates production of PGE2 (Alaaeddine, 1999). This reduces TNFα stimulated PGE2 (a major inflammatory mediator) release from synovial fibroblasts (Alaaeddine, 1999). Studies on dendritic cells have suggested that IL-10 exerts some of its effect by decreasing TNFα stimulated tyrosine phosphorylation of intracellular Mitogen-activated protein (MAPKs) (Allavena, 1998). Indeed in this system, it appears that IL-10 downregulates the TNFα stimulated phosphorylation of Extracellular Signal-Regulated Kinase 2 (ERK2), SAPK/ c-Jun N-terminal kinase (JNK) and p38 MAPK.

IL-10 antagonises the action of other pro-inflammatory cytokines such as IL-1α and IL-1β by increasing the release of soluble IL-1 receptor antagonist (IL-1ra) (Hart, 1995; Marie, 1996; Cassatella, 1994; Marie, 1996; Cassatella, 1994). The increase in IL-1ra protein release correlates with an increase in mRNA production and this appears to be due to increased mRNA stability rather than increased transcription (Cassatella, 1994).
IL-10 inhibits NO production from murine inflammatory macrophages (Oswald, 1992). This is associated with decreased macrophage schistosomal killing and is synergistic with IL-4.

**Down regulation of antigen presenting cell function**

Inhibition of Th1 cytokine release by IL-10 requires the presence of monocytes as antigen presenting cells in both mice and men (Fiorentino, 1991b; de Waal, 1991). IL-10 also inhibits the antigen specific proliferation of T cells in a dose dependent manner when monocytes are used as antigen presenting cells (de Waal, 1991). This inhibition is associated with a reduction in the expression of constitutive and IFNγ or IL-4 driven HLA-DR, HLA-DP and HLA-DQ expression on human monocytes (Spittler, 1995; Allavena, 1998; de Waal, 1991) and dendritic cells (Mitra, 1995). In fact the inhibition in class II MHC expression on human monocytes has a dose dependant effect on inhibited IFNγ and IL-4 production in Th1 cell clones (de Waal, 1991). This is a direct effect of IL-10 and does not require the presence of other cytokines (de Waal, 1991). IL-10 also suppresses concanavalin A stimulated proliferation of resting T cells and this effect appears to be primarily secondary to antagonism of IL-2 (Ding and Shevach, 1992).

Activation of monocytes with LPS reduces constitutive HLA-DR/DP. This down-regulation of MHC class II expression is reduced in the presence of anti-IL-10 -mAb suggesting that endogenous IL-10 is involved (De Waal Malefyt, 1991). These effects of IL-10 are not seen on other antigen presenting cells (APC) such as B cells (de Waal, 1991).

IL-10 inhibits up-regulation of murine monocyte B7 expression by either IFNγ or by LPS (Ding, 1993). B7 expression is important in the costimulation of T cells and therefore this is another immuno-suppressive effect of IL-10. More specifically, IL-10 down regulates B7-2 and moderately up-regulates B7-1 isoforms on monocytes which have differential effects in the development of either a Th-1 or Th-2 response (Creery, 1996). IL-10 does not affect the expression of these molecules on B cells. These actions of IL-10 on monocytes may explain how IL-10 inhibits monocyte induced T cell proliferation (Macatonia, 1993).

**IL-10 inhibits macrophage proliferation**

As well as inhibiting macrophage activation, IL-10 has recently been shown to inhibit macrophage proliferation (O'Farrell, 1998). This is not due to an increase in apoptosis.
but appears to be due to cell cycle arrest. The inhibition is reversible using anti-IL-10 mAb and can be shown to occur in monocytic cell lines (J774) and in murine macrophages (O'Farrell, 1998).

1.9.1.7 **IL-10 prevents activation of dendritic cells**

IL-10 inhibits TNFα stimulated cell surface expression of activation markers CD86, HLA-DR and CD83 suggesting that it prevents TNFα induced activation of dendritic cells as well as suppressing dendritic cells ability to stimulate T cell proliferation (Macatonia, 1993; Allavena, 1998).

1.9.1.8 **IL-10 stimulates monocyte cytotoxic and phagocytic activity**

In contrast to the down regulation of MHC class II Ag expression on monocytes, IL-10 stimulates FcγRI/CD64 expression on human monocytes (Te Velde, 1992; Spittler, 1995). This correlates with an increased capacity of monocytes to lyse anti-D-coated human rhesus-positive erythrocytes (Te Velde, 1992). The increase in FcγR1 also results in increased IFNγ stimulated FcγR1 mediated phagocytosis by monocytes (Spittler, 1995). IL-10 also down regulates the expression of ICAM-1 on monocytes which is the counter receptor for leukocyte function associated antigen-1 (LFA-1) on Ag-presenting cells thereby reducing co-stimulation of resting T cells (Spittler, 1995).

1.9.1.9 **IL-10 affects chemotaxis**

IL-10 increases spontaneous migration of T lymphocytes but not monocytes or neutrophils (Jinquan, 1993). This chemotactic response appears to be specific for CD8+ cells and in fact IL-10 appears to inhibit the chemotactic effect of IL-8 on CD4+ cells (Jinquan, 1993). This suggests that the production of IL-10 could increase the ratio of CD8/CD4 cells at the site of inflammation. IL-10 does not increase the spontaneous migration of monocytes (Jinquan, 1993; Sozzani, 1998) but does increase the chemotactic response of monocytes to human recombinant monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1β. This is probably via upregulation of the monocyte chemokine receptors CCR1, 2, and 5 by IL-10 (Houle, 1999; Sozzani, 1998). However, IL-10 also suppresses ICAM-1/CD54 expression by synovial cells and monocytes (Kawakami, 1997; Spittler, 1995; van Roon, 1996). This decrease in the expression of ICAM-1 but not VCAM-1 by synovial cells is associated with decreased mononuclear cell recruitment in human rheumatoid synovium engrafted onto SCID mice in vivo and with chondroprotection (Jorgensen, 1998).
Rheumatoid factor (RF) is the collective name for autoantibodies directed against the Fc portion of IgG. In patients with positive RF rheumatoid arthritis, terminally differentiated B cells secrete high levels of RF in PBMC culture. This requires the presence of IL-10 as 80% of RF secretion by B cells is inhibited with anti-IL-10-mAb or if the cells are cultured in serum free media (Perez, 1995). This is similar to the increased secretion of IgG and anti-DNA autoantibodies by B cells in patients with SLE which is also dependent on IL-10 (Llorente, 1995). It therefore appears that IL-10 is involved in the induction of autoreactive B cells in patients with RA and SLE. Whether this is important in other diseases or in healthy controls is unknown.

**IL-10 suppresses Th1 development**

In addition to inhibiting the production of Th1 cytokines, IL-10 suppresses the development of Th1 cells in vitro. This is probably via inhibition of macrophage IL-12 production which is known to induce Th1 development during an immune response (Hsieh, 1993).

**Generation of Tr1 cells**

The presence of IL-10 during chronic activation of both human and murine CD4+ T cells gives rise to CD4+ T cell clones with specific characteristics (Groux, 1997). These include, low proliferative capacity, production of high levels of IL-10 and low levels of IL-2 and IL-4. These Tr1 cells have been shown to suppress the proliferation of CD4+ cells in response to antigen. They seem to be functional in vivo as transfer of as few as 2 x 10^3 Tr1 cells into a SCID mouse prevents inflammatory bowel disease induced by pathogenic CD4+CD45RB^hi splenic T cells. The Tr1 cells are only effective after stimulation. The nature of these cells suggests that they may be involved in vivo in controlling inflammation.

**IL-10 regulates T cell maturation**

IL-10 transgenic mice have a defect in T cell maturation with thymic aplasia that occurs soon after birth (Rouleau, 1999). This defect occurs at the pre-T stage and is reverted using anti-IL-10 mAbs. These initial findings suggest that dysregulation of IL-10 can lead to T cell immunodeficiency.
### Table 1.2. Summary of IL-10 function in vitro

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<th>IL-10 function</th>
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<tr>
<td>Inhibition of pro-inflammatory cytokines from stimulated macrophages,</td>
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<td>Decreases pro-inflammatory cytokine production indirectly</td>
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<td>Antagonism of pro-inflammatory cytokines</td>
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<td>Inhibition of macrophage NO production</td>
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<td>Inhibition of macrophage proliferation</td>
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<td>Prevention of dendritic cell activation</td>
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<td>Stimulation of monocyte cytotoxic and phagocytic activity</td>
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<td>Increases migration of CD8+ cells</td>
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<td>Increases chemotactic response of monocytes to MIP-1</td>
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<td>Decreases ICAM-1/CD54 expression by synovial cells and monocytes</td>
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<td>Induction of antoantibodies</td>
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#### 1.9.2 In vivo effects of IL-10

All of the *in vitro* effects of IL-10 described have been demonstrated to be important in a variety of *in vivo* animal models.

IL-10 knock out mice kept under specific pathogen free conditions develop chronic enterocolitis by 2 to 3 months of age (Rennick, 1997). This appears to be due to excessive pro-inflammatory cytokine production in response to normal enteric antigens. Interestingly the T cells are not abnormally skewed towards a Th1 phenotype but there is no regulation of Th1 cytokine production (Rennick, 1997). This is consistent with the fact that IL-10 knock out mice succumb to an avirulent strain of *Toxoplasma gondii*. Once again, this is due to an overwhelming pro-inflammatory cytokine response from both macrophages and CD4+ T cells rather than increased parasite proliferation (Gazzinelli, 1996). IL-10 knock out mice also develop chronic enterocolitis (after stimulation with normal enteric antigens) which is mediated by an "overzealous" pro-inflammatory response from Th1 T cells (Rennick, 1997).

Transgenic mice, which express high levels of human IL-10 only by MHC class II-positive cells, are highly susceptible to infection with intracellular pathogens such as *Listeria Monocytogenes* or *Leishmania major* (Groux, 1999). This increased susceptibility is completely reversed by treatment with anti-IL-10 monoclonal antibody.
and is thought to be due to the fact that the transgenic mice are unable to induce a Th1 response necessary for parasite killing.

Models using a T cell receptor transgenic mouse have demonstrated in vivo that IL-10 inhibits Th1 development by blocking IL-12 production from monocytes (Hsieh, 1993).

In lupus prone mice (NZB/W), administration of anti-IL-10 delays the onset of autoimmunity and increases survival at 9 months from 10 to 80% (Ishida, 1994). This protection appears to be due to increased levels of TNFα as treatment with anti-TNFα antibodies abrogates the protective effect. Treatment of the mice with IL-10 accelerates the onset of autoimmunity. IL-10 treatment however does not cause development of lupus in normal BALB/c mice (Ishida, 1994). The genetic background of the mice therefore determines the effect IL-10 treatment will have.

1.9.3 In vivo models of arthritis

Treatment of mice with established collagen-induced arthritis with recombinant murine IL-10 significantly inhibited paw swelling, disease progression and cartilage destruction compared to untreated controls (Walmsley, 1996). However, the mice required daily treatment with subcutaneous IL-10 (or in another study, twice daily intraperitoneal injections (Joosten, 1997)) which may be unacceptable to patients. A solution to this may be gene transfer.

Systemic administration of IL-10 using adenovirus encoding viral IL-10 (vIL-10) in mice delayed the onset of disease and reduced the severity of arthritis (Ma, 1998). This was associated with decreased pro-inflammatory cytokine (IL-2 and IL-1β) mRNA levels. However an effect was only seen if treatment was given before disease onset of collagen induced arthritis. Treatment of established disease was ineffective (Ma, 1998). In arthritis, local rather than systemic delivery is possible and may have benefits for patients with less side effects. In collagen induced arthritis, intra-articular adenoviral vIL-10 injected into the knee joints did not affect knee inflammation but inhibited the development of arthritis in the paws (Ma, 1998). Both the IL-10 treated and the buffer treated knees developed severe inflammation which was worse in the adenoviral vIL-10 injected knee, presumably as a reaction to the virus. The beneficial effect of treatment on the paws was not due to viral leakage as infection remained localised but was probably due to IL-10 entering the circulation. In a similar study, adenovirus containing a homologue of viral IL-10 (vIL-10) suppressed arthritis when injected into the periaricular tissue surrounding the ankle joints of mice with collagen induced arthritis. This single injection also suppressed development of disease in other
non-injected paws (Whalen, 1999). In another animal model, adenoviral gene transfer of IL-10 into rabbit knees with Ag-induced arthritis decreased leukocytosis, cartilage degradation and endogenous TNFα within the joint (Lechman, 1999). An anti-arthritic effect also occurred in the contralateral knee joints which appeared to be due to trafficking of adenoviral infected cells to the other knee.

However, IL-10 does not appear to have a beneficial effect in all animal models. TNFα transgenic mice develop spontaneous inflammatory arthritis. Treatment of these mice with engrafted xenogeneic transfected Chinese hamster ovary fibroblasts expressing human IL-10 decreased expression of the TNFα transgene, endogenous mouse TNFα and IL-1 mRNA but did not attenuate the arthritis (Bessis, 1998).

To try and assess the effect of IL-10 on human tissue, human rheumatoid synovial tissue has been engrafted into SCID mice. In this model, systemic administration of adenoviral vectors containing the vIL-10 gene resulted in inhibition of cartilage invasion by rheumatoid synovial tissue (Jorgensen, 1999). When a retroviral delivery system was used to insert IL-10 into human RA synovial fibroblasts which were implanted into SCID mice with co-implanted human cartilage and prerichondrocytic cartilage, there was reduced invasion of co-implanted cartilage (Muller-Ladner, 1999). IL-10 appears to be more effective than IL-4 in ameliorating inflammation in the collagen induced arthritis animal model (Thorbecke, 1992). However, murine recombinant IL-4 and IL-10 given intraperitoneally twice daily from the onset of arthritis act synergistically to ameliorate collagen induced arthritis even when treatment is delayed to a week after the onset of inflammation (Joosten, 1997). In addition to reducing the macroscopic signs of inflammation, IL-10 and IL-4 together reduce cartilage damage and proteoglycan deposition (Joosten, 1997). Treatment of these mice with anti-IL10 -mAb increases the severity of disease as well as the number of mice affected. This suggests that endogenous IL-10 is important in this model for controlling disease expression (unlike IL-4 where inhibition of endogenous IL-4 had no effect on the arthritis) (Joosten, 1997).

1.9.4 Treatment with IL-10

When healthy volunteers were given intravenous IL-10 (1, 10 or 25 μg/kg) there was a transient neutrophilia and monocytosis at 6 hours whilst lymphocytes counts were decreased. In addition, ex vivo LPS stimulated TNFα and IL-1β production in whole blood culture was decreased after IL-10 administration (Chernoff, 1995).
In a study investigating the use of IL-10 in patients with RA, recombinant human IL-10 was given as daily subcutaneous injection for 4 weeks to patients with RA as part of a multicenter randomised double blind placebo controlled trial (Maini, 1997). In the patient group there was a trend towards improvement in RA disease activity was observed at 4 weeks with the 5μg/kg dose with less use of disease modifying drugs during the 8 weeks after completing treatment. There was an increase in TNF receptor levels (p55 and p75) and an increase in IL-1 receptor antagonist (IL-1-ra). There was a decrease in IL-1β and TNFα production \textit{ex vivo} after treatment but this was not significantly different.

1.9.5 IL-10 in disease

1.9.5.1 JIA

There is now considerable evidence that IL-10 acts as an anti-inflammatory cytokine by inhibiting the production of pro-inflammatory cytokines and suppressing major histocompatibility complex (MHC) expression and Ag presentation. The \textit{in vitro} and \textit{in vivo} work described suggest that in arthritis, insufficient IL-10 in the presence of inflammation could be one of the mechanisms allowing joint inflammation to continue unchecked. In children with arthritis, the relative expression of IL-10 may be one of the factors that determines outcome of disease in terms of both joint involvement and eye disease. A relatively low level of IL-10 production on stimulation could increase joint severity and decrease the risk of eye disease as discussed in section 1.8.7.

1.9.5.2 Asthma

In addition to the \textit{in vitro} and \textit{in vivo} effects already described, IL-10 has additional beneficial effects in asthma. IL-10 appears to play a role in IgE production by decreasing IL-4 induced IgE class switching by peripheral blood mononuclear cells (PBMC) \textit{in vitro} (Jeannin, 1998). IL-10 also increases the proportion of monocytes which have a phenotype consistent with T cell suppressive activity \textit{in vitro}. These are reduced in patients with asthma which could be due to a relative deficiency of IL-10 (Tormey, 1998).

In severe asthma, the net cytokine activity in bronchial lavage fluids is pro-inflammatory (Tillie-Leblond, 1999). This is mainly due to the presence of IL-1β and TNFα. In patients with mild to moderate asthma, constitutive and stimulated IL-10 production is reduced from alveolar macrophages and PBMC of patients when
compared to controls in some studies (Borish, 1996; John, 1998) and increased in others (Magnan, 1998). These differences may be due to differences in asthma severity between studies as well as the different stimulation used ex vivo. The biological activity of a cytokine is difficult to compare between ex vivo studies when the in vivo situation is so variable. It is probably more biologically relevant to measure net cytokine activity using a functional assay (Tillie-Leblond, 1999).

Relatively low IL-10 levels could contribute to the pro-inflammatory milieu found in patients with severe asthma.

### 1.9.5.3 **Systemic Lupus Erythematosus**

Serum titres (and PBMC production) of IL-10 is higher in patients with Systemic Lupus Erythematosus (SLE) when compared to controls (Lacki, 1997a; Lacki, 1997b; Yang, 1997; Viallard, 1999; Llorente, 1994). This increase is mainly due to an increase in IL-10 production by monocytes, a subset of B cells and possibly CD4+CD45 RO+ memory T cells (al Janadi, 1996). Serum titres of IL-10 are positively correlated with anti-dsDNA antibody titres in patients with SLE and with the SLE disease activity index and negatively correlated with complement C3 levels (Houssiau, 1995; Park, 1998). Production of IL-10/IFNγ from stimulated whole blood culture is inversely correlated with indices of disease activity such as the Systemic lupus activity measures (SLAM) index (p<0.001) (Swaak, 1996). Low IL-12 production is impaired in patients with SLE (Liu and Jones 1998). This appears to be due to the increased levels of IL-10 as anti-IL-10 neutralising antibody reverses the IL-12 deficiency in monocytes and PBMCs from patients with SLE but not from normal PBMC (Liu and Jones 1998).

In murine models of SLE, lowering IL-10 levels was associated with a rapid decrease in anti-dsDNA and anti-ssDNA IgGs and a decrease in the percentage of mice developing proteinuria (Kalechman, 1997). Treatment of lupus prone NZB/W mice with anti-IL-10 delays the onset of autoimmunity and increases survival at 9 months from 10% to 80% (Ishida, 1994). This is thought to be due to increased TNFα as the protective effect of anti-IL-10 is abrogated in the presence of anti-TNFα antibody. Treatment of the same strain with IL-10 accelerates the development of autoimmunity but this does not occur in non-lupus prone mice (Ishida, 1994). It has been postulated that the pregnancy induced flare seen in patients with SLE could be due to increased production of IL-10 which occurs in pregnancy (Huizinga, 1999).

In multiplex families, IL-10 production by PBMC is increased in healthy relatives, which suggests that the increase in IL-10 production seen in patients may be genetically
controlled (Llorente, 1997). The genetic regulation of IL-10 in patients with SLE has therefore been investigated by looking at the IL-10 5’ flanking region haplotypes at both the microsatellites and the SNPs section 1.14.

1.10 Regulation of IL-10 protein production

1.10.1 Stimulation of IL-10 production

IL-10 production is stimulated by LPS, TNFα and IL-1 as well as immune complexes and via triggering of the CD23 antigen.

1.10.1.1 Triggering of the CD23 antigen

CD23 antigen is implicated in the initiation and the development of parasitic and allergic responses (Dugas, 1996). CD23 engagement induces the accumulation of adenosine 3’:5’ – cyclic monophosphate (cAMP) and cGMP thereby initiating the generation of pro-inflammatory cytokines TNFα and IL-6 as well as stimulating the production of nitric oxide (NO) via inducing the inducible form of NO synthase (Paul-Eugene, 1995). Incubation of monocytes derived from PBMCs with IL-4 results in the induction of CD23. Culture of these CD23+ macrophages in the presence of anti-CD23-mAb results in the upregulation of IL-10 mRNA 2 hours later (Dugas, 1996). This is potentiated by inhibition of the NO pathway, which is also stimulated by engagement of the CD23 ligand. The effect of CD23 ligation on IL-10 is inhibited by an inhibitor of the cAMP pathway, Rp diastereoisomer of adenosine 3’5’-phosphorothioate (Rp-cAMP). It therefore seems likely that this effect of CD23 ligation on IL-10 production is mediated by the cAMP pathway such that ligation of the CD23 ligand induces cAMP which upregulates IL-10 production antagonising the effect of NO (Dugas, 1996).

1.10.1.2 Immune complex induced IL-10 production

Immune complexes formed of tetanus toxoid and polyclonal anti-tetanus toxoid antiserum induce the release of IL-10 (and IL-6) in a dose and antigen:antibody ratio dependent manner (Berger, 1996). Neither tetanus toxoid nor anti-tetanus toxoid antiserum upregulates IL-10 production alone. This upregulation of IL-10 production is reduced in the presence of indomethacin which inhibits prostaglandin (PG) production however the production of IL-10 cannot be suppressed completely by indomethacin suggesting that there are both PG dependant and independent pathways (Berger, 1996).

1.10.1.3 Lipopolysaccaride (LPS)

LPS is an amphipathic molecule with a hydrophobic domain (lipid A) which forms the outer leaflet of the outer membrane of gram-negative organisms. The core

LPS induces IL-10 release in vitro and in vivo (van der Poll, 1994). Although several cell surface proteins have been shown to bind to LPS (Ulevitch and Tobias 1994), CD14 is the best characterised (Weinstein, 1993). CD14 is a glycopophatidlyinositol anchored protein and is expressed on the surface on monocytes and macrophages. When LPS binds to CD14, there is activation of the CD14 associated protein tyrosine kinases (PTK) p53/56^lyn and simultaneous activation of PTKs p58/64^hck and p59^c-fgr (Stefanova, 1993). This response is rapid (Weinstein, 1992). The increase in tyrosine phosphorylation of p53/56^lyn and p58/64^hck is detectable at 1 minute after stimulation and p59^c-fgr is involved after 15 minutes of treatment with LPS. TNFα and IL-1β secretion (and TNFα mRNA) in LPS stimulated monocytes are dependent on both PTK and protein kinase C (PKC) activity as inhibitors of both PTK and PKC can independently completely inhibit LPS stimulated TNFα and IL-1β production (Shapira, 1994). Inhibition of PTK is also necessary for both early and late LPS induction of IL-10 (Meisel, 1996).

Pre-incubation of monocytes with an anti-CD14 -mAb did not prevent LPS induced PKC induction suggesting that another receptor apart from CD14 was involved (Shapira, 1994). This could be the CD11c/CD18 transmembrane signalling receptor which is capable of initiating signal transduction in response to LPS binding (Ingalls and Golenbock 1995) and appears to be important in endotoxin signalling in sepsis (Ertel, 1993).

1.10.1.4 LPS induction of nuclear transcription factors

1.10.1.4.1 Nuclear factor -κB (NFκB)

Stimulation of monocytes with LPS results in activation of the transcription factor NFκB which is known to be involved in the expression of a variety of genes such as TNFα, IL-1 and IL-6 (O'Connell, 1998). However inhibition of NFκB using over expression of IκBα (which inhibits nuclear translocation of NFκB) by adenoviral gene transfer in human monocytes, does not inhibit IL-10 production except at very high adenoviral infection (Bondeson, 1999). This is probably because there are no NFκB binding sites on the IL-10 5' flanking region suggesting that this mechanism is not important in LPS stimulation of IL-10 from monocytes.
1.10.1.4.2 Activator protein-1 complex (AP-1)

LPS stimulates rapid AP-1 transactivation in RAW 264.7 cells (a murine monocytic cell line via CD14 (Hambleton, 1996). Stimulation of AP-1 transactivation appears to be via the c-Jun N-terminal kinase (JNK) pathway with activation of pre-existing c-Jun. Both JNK isoforms are activated (Hambleton, 1996). Activation of JNK by LPS is rapid, occurring by 5 minutes of stimulation. This pathway is also important in the human monocytic cell line THP-1 cells with similar kinetics and dose response (Hambleton, 1996). This is interesting in terms of the IL-10 5' flanking region where there is a theoretical AP-1 binding site at -2423 to -2429 (Kube, 1995).

1.10.1.5 LPS and TNFα in IL-10 stimulation

The induction of IL-10 by LPS is partially attenuated by simultaneous anti-TNF -mAb. This has been shown to occur in vivo in chimpanzees, and in vitro in PBMC and purified monocytes (Platzer, 1995; van der Poll, 1994; van der Poll, 1996; Wanidworanun and Strober 1993; Foey, 1998). In chimpanzees this attenuating affect of anti-TNFα does not occur if the antibody is administered 30 minutes after the LPS despite the fact that production of TNFα is completely abolished (van der Poll, 1994). These results suggest that endogenous TNFα is involved in the early stages of LPS stimulation of IL-10.

Stimulated T cells consistently increase LPS stimulated IL-10 production from monocytes and this induction is abrogated when cell to cell contact is prevented by a porous membrane insert separating the two cell populations (Parry, 1997). LPS stimulated IL-10 production was inhibited when T cell membrane bound TNFα was inhibited with prior incubation of excess neutralising anti-TNFα -mAb Fab fragments prior to fixation. This shows that in addition to soluble TNFα, membrane bound TNFα is important in modulating LPS stimulated monocyte IL-10 production. In the absence of LPS however, cytokine stimulated T cells are not required for monocyte production of IL-10 (although they do stimulate monocyte production of TNFα).

Endogenous IL-1 is also required for LPS stimulation of IL-10 production in human monocytes, isolated after elutriation from PBMCs (Foey, 1998). However inhibition of TNFα and IL-1 with anti-TNF -mAb and IL-1ra does not completely inhibit LPS stimulated IL-10 production suggesting the presence of TNFα and IL-1 independent mechanisms. In addition, the combination of anti-TNFα -mAb and IL-1ra did not markedly increase the inhibition caused by each alone, suggesting that IL-1 and TNFα regulate IL-10 production by a common pathway.
To elucidate the intracellular pathways involved in LPS stimulation of IL-10, Foey et al used a variety of specific inhibitors (Foey, 1998). PD98059 inhibits mitogen-activated protein kinase kinase (MAPKK) which is the kinase that activates p42/44 MAPK via phosphorylation on both threonin and tyrosine residues. SB203580 is a bicyclic imidazole which inhibits LPS induced p38 MAPK activity down to background levels. Pre-treating monocytes with PD98059 prior to LPS stimulation, inhibited TNFα production but not IL-10 production (Foey, 1998). Treatment with SB203580 however, inhibited LPS induced IL-10 production in a dose dependent manner (Foey, 1998). This suggests that the p38 MAPK pathway and not the p42/p44 MAPK pathway is involved in LPS stimulation of IL-10.

As well as augmenting LPS stimulated IL-10 production, TNFα induces IL-10 mRNA in vitro and in vivo (and protein in vivo) without LPS stimulation (van der Poll, 1994; Platzer, 1995). This is mainly via a dose dependant upregulation of de novo IL-10 mRNA synthesis in the monocyte fraction of PBMC (Wanidworanun and Strober 1993). Given the role that TNFα plays in LPS stimulated IL-10 production, it is paradoxical that cAMP elevating drugs (such as iloprost and pentoxifylline) that inhibit TNFα production, augment LPS stimulated IL-10 production (Platzer, 1995). This augmentation of IL-10 protein correlates with an increase in IL-10 mRNA suggesting that the effect of increased cAMP occurs at the level of transcription (Platzer, 1995). Prostaglandin E2 also increases IL-10 production in vitro via inducing cAMP (van der Pouw Kraan, 1995). Interestingly, there is a theoretical serum response element at -330 in the IL-10 5' flanking region (Kube, 1995). cAMP is hydrolysed by cyclic nucleotide phosphodiesterases (PDE)s. Inhibition of PDE IV (by Rolipram) in LPS stimulated macrophages augments IL-10 production and suppresses TNFα production in a dose dependent manner (Kambayashi, 1995). This effect is only seen in the presence of LPS. Anti-IL-10 -mAb reduces (but does not completely abolish) the inhibitory effect of rolipram on TNFα suggesting that it is partly the upregulation of IL-10 (IL-10 mRNA appears earlier in rolipram treated cells) that inhibits TNFα production (Kambayashi, 1995).

1.10.1.6 Augmentation of LPS stimulated IL-10 production by adrenaline

A three hour exposure to adrenaline augments LPS induced IL-10 production in vivo and in vitro (van der Poll, 1996) but a 24 hour exposure does not augment IL-10 production. Adrenaline suppresses TNFα production at after 3 and 24 hour exposure and this is partially abolished in the presence of anti-IL-10 -mAb but only by 10% or
less. This suggests that the majority of the inhibitory effect of adrenaline on TNFα production is IL-10 independent.

1.10.1.7 IL-12 stimulated IL-10 production

The effect of IL-12 on stimulated IL-10 from T cells is complicated as it appears to be determined by both the activation state of the T cell and the presence of other cytokines. For example, IL-12 synergises with anti-CD3 -mAb or con A in inducing IL-10 production by human T cell lines (Jeannin, 1996; Peng, 1997). In Th0, Th1 and Th2 T cell lines this increase in secreted IL-10 was associated with an increase in IL-10 mRNA. The synergy was partly mediated by endogenous IL-2. IL-12 also induced IL-10 in unstimulated T cell lines (Jeannin, 1996) but only minimally although IL-10 production was increased in the presence of IL-2. IL-12 does not up-regulate IL-10 production from stimulated purified human T cells unless IL-2 is present (Jeannin, 1996). IL-12 also induces IL-10 production in T cell clones and in freshly isolated T cells stimulated by CD2 in the presence or absence of accessory cells (Meyaard, 1996). Once again, the increase in IL-10 secretion is paralleled by an increase in IL-10 mRNA. In certain circumstances however, IL-12 decreases IL-10 production and this is discussed below.

1.10.2 Inhibition of IL-10 production

1.10.2.1 IL-12

Although IL-12 increases IL-10 production in T cell lines and primary purified T cells, it has a very different action in CD4+ T cells from allergic donors stimulated with antigen. In these memory T cells, IL-12 inhibits IL-10 and IL-4 synthesis and up regulates IFNγ and IL-2 (Marshall, 1995). This effect of IL-12 is independent of IFNγ and is only seen in resting memory T cells (no expression of class II molecules). It therefore appears that IL-12 can either up regulate or down regulate IL-10 production depending on the activation state of the cell and the presence of other cytokines such as IL-2.

1.10.2.2 IFNγ

IFNγ suppresses LPS and TNFα induced IL-10 production in human monocytes in a dose dependant manner (Donnelly, 1995; Wanidworanun and Strober 1993). This effect is seen at both the mRNA and protein level and correlates inversely with TNFα levels. This suggests that the IFNγ upregulation of TNFα depends in part on suppression of IL-10 and may partly explain the priming phenomena observed in terms
of cytokine production when cells are pre-incubated with IFNγ prior to LPS stimulation (Donnelly, 1995; Ma, 1996). The mechanism by which IFNγ down regulates IL-10 appears to be through induction of IL-12 as the addition of neutralising anti-IL-12 -mAb abrogated the ability of UFNγ to down regulate IL-10 (Libraty, 1997). IL-4 has also been shown to reverse TNFα or LPS induction of IL-10 mRNA synthesis in human monocytes (Wanidworanun and Strober 1993; De Waal Malefyt, 1991). Intracellular adhesion molecule-1 (ICAM-1) co-stimulation with superantigen inhibits IL-10 production in human CD4+ cells (Labuda, 1998). IL-10 also has an autoregulatory role in that exogenous IL-10 decreases endogenous IL-10 mRNA levels in LPS stimulated monocytes (De Waal Malefyt, 1991).

### 1.10.3 Which cells produce IL-10?

IL-10 is produced by monocytes and lymphocytes (activated T helper cells and B cells), keratinocytes and dendritic cells (Coze 1994; Cohen, 1995; Mosmann 1994; Stordeur, 1995; Dubois, 1999; de Saint-Vis, 1998; Enk and Katz 1992; Cohen, 1995; Novina and Roy 1996; Mosmann 1994). In the monocyte population, IL-10 is only detectable in CD14++ cells not in CD14+/CD16+ cells (which account for approximately 18% of the monocyte population) (Frankenberger, 1996). This is in contrast to pro-inflammatory cytokines such as TNFα where both subsets of monocytes produce equivalent quantities of cytokine (Frankenberger, 1996). Although murine IL-10 is produced by Th2 cells, human IL-10 is produced by stimulated Th1, Th2 and Th0 like T cell clones (Yssel, 1992). Stimulated CD4+CD45- T cells produce 20 fold higher levels of IL-10 than CD4+CD45RA+ cells. IL-10 is also produced by activated CD8+ cells (Te Velde, 1992) and by eosinophils if incubated for at least 3 hours in the presence of IL-5 (Nakajima, 1996).

As mice with severe combined immune deficiency (SCID) challenged with LPS produce the same amounts of IL-10 as control mice, it seems likely that monocytes are primarily responsible for LPS stimulated IL-10 production (Bourrie, 1995). This is consistent with the fact that when human B cells, T cells and monocytes are positively selected by flow cytometry and then stimulated with LPS for 24 hours, production of IL-10 was only detectable in monocyte cultures (Visser, 1998). LPS stimulated IL-10 production increases in vitro if the monocytes are adherent and therefore differentiated (Mandrekar, 1996). In addition to intracellular IL-10, surface IL-10 has been described on the cell surface of macrophage cell lines and appears to be biologically active (Fleming and Campbell 1996).
Which cells produce IL-10, however, depends on the age of the patient and whether they are healthy or not. In elderly women, IL-10 is produced by CD8+CD3+ T cells as well as monocytes whilst in young female controls, IL-10 expression occurred exclusively in monocytes (Llorente, 1997). In patients with RA and SLE, IL-10 is produced by B cells as well as monocytes but the monocytes produced the higher mean level of IL-10 (Llorente, 1994).

1.10.4 Drug and environmental effects

1.10.4.1 Ethanol

Total serum IL-10 is increased in alcoholics when compared to healthy controls (Gonzalez-Quintela, 1999). The up-regulation of IL-10 by ethanol is also seen in animal models and appears to be increased in the presence of infection (Zisman, 1998). IL-10 is stimulated by ethanol in human monocytes selected by adherence (Mandrekar, 1996; Girouard, 1999). The effect appears to have a dose response between 25mM (which is approximately equivalent to blood ethanol levels after one unit) and 100mM. The same doses of ethanol also increase IL-10 production in LPS stimulated monocytes at 18 hours (but not 10 hours) and this is paralleled by mRNA levels suggesting that ethanol exerts its effect at the transcriptional level (Mandrekar, 1996). This increase in IL-10 appears to partially account for the reduced TNFα production by monocytes after exposure to ethanol (Mandrekar, 1996) and may explain some of the immunosuppressive effects of acute ethanol ingestion. An independent mechanism by which ethanol inhibits TNFα production appears to be via inhibition of LPS-induced activation of NFκB (Mandrekar, 1999).

1.10.4.2 Glucocorticoids

Glucocorticoids (GC) can either upregulate, down regulate or have no effect on IL-10 production depending on the cell type, dose (and type) of glucocorticoid and time course investigated.

In vitro, constitutive IL-10 production by PBMC from healthy controls is up regulated by Methylprednisolone (MP) at 48 hours (Gayo, 1998). This up regulation was reversed by the glucocorticoid receptor antagonist RU486 and therefore appears to be steroid specific. It is controversial whether MP up or down regulates IL-10 in cells stimulated with LPS. In some studies IL-10 production is down regulated (Gayo, 1998) and in some it is up regulated (Marchant, 1996; Hodge, 1999). This difference could be due to a difference in MP dose. In a recent study, published during the course of this thesis, low dose MP (10⁻⁹ M and 10⁻¹¹ M) was shown to up regulate LPS stimulated
monocyte IL-10 production in whole blood culture (Hodge, 1999). In the same study, high dose MP (10^5 M) down regulated IL-10 production in monocytes. All concentrations of MP decreased IL-10 production in T cells and NK cells (Hodge, 1999). This is in contrast to an animal model of murine endotoxaemia where low doses of MP (2 to 10 mg/kg) did not affect serum levels of IL-10 but high MP doses (50 mg/kg) increased LPS induced IL-10 levels (Marchant, 1996).

Another glucocorticoid, dexamethasone (DXM) appears to have yet a different effect on IL-10 production. DXM added to the whole blood culture, suppressed LPS stimulated IL-10 production (mean of 13 donors) at 10^-6 mol/L but not at lower doses (Visser, 1998). This was an average effect on IL-10 production whilst individual donors either upregulated or down regulated IL-10 production reflected by changes in mRNA levels after addition of DXM to the culture. This is in contrast to suppression of TNFα and IL-12 at all doses in all patients in a dose dependent manner (Visser, 1998). The GC receptor antagonist RU486 inhibited IL-10 production suggesting that endogenous cortisol may be inhibiting IL-10 production (Visser, 1998). It is therefore possible that the inconsistent effect of DXM in different donors may be due to variable occupancy of the GC receptor by endogenous cortisol.

Glucocorticoids also have variable effects on cytokine production by T cells depending on the stage of T cell activation. If CD4+45RO- T cells are primed in the presence of beclomethasone (0.1 μM) and then re-stimulated in the absence of beclomethasone, IL-10 production is increased 5 fold (Brinkmann and Kristofic 1995). At higher doses of beclomethasone (10 μM) the T cells could not be primed and IL-10 production was increased 2 fold. When the beclomethasone was present during re-stimulation, but not during priming, there was no significant effect on IL-10 production (Brinkmann and Kristofic 1995).

Several studies have now been performed ex vivo after glucocorticoids have been given in the clinical setting. Serum IL-10 levels were unchanged 60 minutes after 30 μg/kg of methylprednisolone (compared to placebo) given as a bolus during cardiac surgery (Kawamura, 1999) although they did increase after declamping of the aorta compared to pre-operative levels. In patients with multiple sclerosis, one gram of intravenous MP for four days increased IL-10 mRNA in PBMC and IL-10 concentration in serum (Gayo, 1998). This was in contrast to pro-inflammatory cytokines TNFα and IFNγ which decreased following steroid therapy in the same experiment (Gayo, 1998). Ex vivo experiments on PBMC from RA patients before and after dexamethasone pulse
therapy (3 doses of 200mg of DXM) showed that constitutive IL-10 production was higher after dexamethasone treatment and this was sustained for 6 weeks (Verhoef, 1999). In addition, the IL-4/IFN\(\gamma\) ratio increased suggesting a shift from a Th1 to a Th2 response. In healthy volunteers however, three doses of DXM of 1.5 mg 8 hours apart did not change either constitutive or stimulated IL-10 production (Bleeker, 1997). GCs may have an effect on IL-10 production when administered by other routes in addition to IV. Inhaled GCs taken for 3 months by patients with asthma have been shown to increase stimulated IL-10 mRNA and protein release from alveolar macrophages (John, 1998). An additional mechanism by which GCs could alter the Th1/Th2 ratio is by differential regulation of IL-10 and IL-12 as IL-12 is crucial in the development of a Th1 response (Segal, 1998; Meynard, 1996; Hsieh, 1993).

### 1.10.4.3 Methotrexate (MTX)

Methotrexate is frequently used to treat JIA and is especially effective for children with extended oligoarticular JIA. Although no studies have investigated the effect of MTX on IL-10 production, the effect of MTX on IL-10 production has been investigated in other diseases. Serum IL-10 is increased in adult patients with rheumatoid arthritis treated with MTX than those treated with non-steroidal anti-inflammatory drugs (Lacki, 1995). *In vitro*, MTX increases IL-10 production when PBMC are stimulated with phytohemagglutinin (PHA) (Constantin, 1998). When SLE was induced in BALB/c female mice by injection of monoclonal anti-DNA antibody, treatment with MTX reverses the levels of cytokines to those seen in control mice. For IL-10 this meant a reduction to control levels (Dayan, 1997; Segal, 1995).

### 1.10.4.4 Adrenaline

Short term exposure of mononuclear cells to adrenaline augments LPS or endotoxin stimulated IL-10 release whereas exposure to adrenaline over 24 hours does not augment IL-10 release (van der Poll, 1996). Both norepinephrine and epinephrine (noradrenaline and adrenaline) increase IL-10 in a dose dependant manner in whole blood culture (Elenkov, 1996).

### 1.10.5 Environment versus genetic influences in the regulation of the IL-10 gene

Despite the considerable wealth of data on how the environment alters IL-10 production the majority of the variability in stimulated IL-10 production is genetically regulated. The evidence for this comes from family studies where the correlation for IL-10 production (stimulated with LPS) is 0.75 for monozygotic twins and 0.33 for siblings
(Westendorp, 1997). This suggests that the genetic contribution to variability in IL-10 production is 84%. When IL-10 production is compared in spouses, there is no correlation between IL-10 production and the time they lived together (Westendorp, 1997) suggesting that shared environment contributes little to the production of IL-10. This is consistent with the fact that variation in LPS stimulated IL-10 production is not due to other factors such as variation in TNFα levels (Platzer, 1995).
1.11 The IL-10 receptor

IL-10 binds as a non-covalently linked homodimer to the IL-10 receptor (Tan, 1993). The human IL-10 receptor binding chain (Hu-IL-10R1) was cloned and characterised in 1994 (Liu, 1994). Two IL-10 dimers bind to 4 Hu-IL-10 receptor chains (Hoover, 1999). The Hu-IL-10R1 is a cell surface receptor with a single transmembrane domain and is a member of the class II cytokine receptor family in that it is structurally related to the interferon γ receptor (Ho, 1993).

1.11.1 The second chain of the IL-10 receptor

The predicted amino acid sequence of the Hu-IL-10R1 is 60% identical to the mouse IL-10 receptor (Ho, 1993). Despite this homology however, mouse IL-10 does not bind to the Hu-IL-10R1, although human IL-10 can bind to the mouse IL-10 receptor. The calculated molecular mass of the Hu-IL-10R1 according to the amino acid sequence is 61 kDa but the observed size of the receptor was 90-110 kDa. This suggested that either the receptor binding chain was glycosylated or there was association with another component. Viral IL-10 (which overlaps functionally with human IL-10) does not compete with the human IL-10 in terms of binding to the human IL-10 receptor in transfected cell lines (Liu, 1994). This also suggests the existence of additional components of the IL-10 receptor on those cells allowing viral IL-10 to activate the cells (human B cells and activated macrophages). A second chain was also suspected because of the structural similarity to the interferon γ receptor. IFNγ signals through a receptor complex that consists of two different chains, the IFNγ receptor binding subunit and a transmembrane accessory factor which is necessary for signal transduction (Kotenko, 1995).

Kotenko et al identified the second chain of the human IL-10 receptor (Hu-IL-10R2) in 1997 and characterised it using a variety of transfected receptors and chimeric receptors (Kotenko, 1997). The second chain was originally identified as an orphan receptor CRFB4 encoded on chromosome 21 (Hu-IL-10-R1 is encoded on chromosome 11). The intracellular domain of the Hu-IL-10-R2 associates with a member of the Janus kinase family, Tyk 2 (Kotenko, 1997). IL-10 induces tyrosine phosphorylation of the tyrosine kinases Janus kinase 1 (JAK 1) and Tyk 2 (Ho, 1995). Tyk 2 is necessary for IL-10 signal transduction in a variety but not all cell lines. Co-transfection of both IL-10 receptor chains into COS cells (monkey cell system derived from transformed simian kidney DV-1 cells) treated with IL-10 resulted in the formation of the following
STAT (Signal transducers and Activators of Transcription) complexes; STAT1α homodimers, STAT1: STAT3 heterodimer and STAT 3 homodimer (Kotenko, 1997). STATs are transcription factors that are normally resident in the cytoplasm in monomeric inactive forms (Sadowski, 1993). They are activated by phosphorylation by a member of the Janus kinase family (Jak or Tyk) after which they dimerize and translocate to the nucleus where they activate target genes.

STAT activation was not seen in IL-10 treated COS cells transfected with only the Hu-IL-10R2 chain but could be observed in COS cells transfected with the R1 chain after longer exposure to IL-10. In hamster cells however there was no STAT activation with transfection of only the Hu-IL-10R1 chain even with prolonged IL-10 stimulation however there was STAT activation when both chains were transfected. This and earlier results suggests that Hu-IL-10R2 is expressed at low levels in some cells allowing some activation by IL-10 through just the Hu-IL-10 R1 chain (Kotenko, 1997; Liu, 1994). Kotenko et al therefore made chimeric receptors to allow detection of IL-10 induced activities in cells where IL-10 was not expressed. This demonstrated that both chains were necessary for signalling to occur with IL-10 treatment (Kotenko, 1997). Using a chimeric receptor containing extracellular Hu-IL-10-R2 and intracellular IFNγR2, they also showed that another member of the Janus Kinase family, Jak2 can functionally substitute for Tyk2 in IL-10 signalling. They therefore concluded that the main function of the Hu-IL-10-R2 was to bring an additional tyrosine kinase activity to the IL-10 receptor complex when IL-10 binds to the Hu-IL-10-R1 thereby initiating signal transduction. This is a similar mechanism of signal transduction as the IFNγ receptor complex (Kotenko, 1995).

The IL-10R1 chain is co-precipitated with anti CRFB4 antibodies in PBMCs treated with IL-10 but not from untreated cells suggesting that the IL-10R2 chain, the IL-10R1 chain and IL-10 form a complex (Kotenko, 1997). It therefore seems likely that the IL-10 receptor complex is as shown in figure 1.3 below with the IL-10R2 creating an active intracellular receptor complex necessary for signal transduction.
Figure 1.3 *The IL-10 receptor*

The IL-10 receptor consists of 4 chains, 2 each of chain 1 and chain 2. Tyk 2 associates with chain 2 and Jak 1 with chain 1. Activation of the receptor by IL-10 results in recruitment and activation of STAT3.
1.12 IL-10 intracellular signalling

1.12.1 Jak/STAT pathway

The Hu-IL-10R1 cytoplasmic domain contains two sequence motifs (Tyrosine residues 427 and 477) that are known to be required for activating STAT 3 in the cytoplasmic domain of the Hu-IL-10R1, (Riley, 1999). Studies using deletion mutants have shown that deletion of the distal sequence motif (Δ 433-559) resulted in a loss of gene induction and reduced STAT3 activation. A more proximal deletion mutant (Δ 402) eliminated all signalling activity (Lai, 1996). Studies using chimeric receptors that consist of extracellular and transmembrane human IFNγ chain and intracellular murine IL-10 receptor domain expressed in murine macrophages have recently been used to investigate the function of the IL-10 receptor (Riley, 1999). These studies showed that the tyrosine residues play a redundant role in activating STAT3. These tyrosine residues are preserved in the human IL-10 receptor. Mutation at either residue did not alter STAT3 activation but mutation of both eliminated STAT3 activation. Deletion mutants of these chimeric receptors showed that receptors lacking anything more than the most distal 15 residues were unable to mediate the dose dependent inhibition of TNFα. These deletion mutants did not affect STAT3 activation. An additional residue is therefore required in the carboxy terminal of the IL-10 receptor for complete inhibition of TNFα by IL-10.

STAT1 is also activated by IL-10 in macrophages (see below) however a truncated receptor lacking these tyrosine domains can still activate STAT1 DNA binding activity (O'Farrell, 1998).

STATs are transcription factors that are normally resident in the cytoplasm in monomeric inactive forms (Sadowski, 1993). Activation requires phosphorylation by a member of the Janus kinase family (Jak or Tyk). Immunoprecipitation with Abs directed against the four known Jak kinases showed that IL-10 induced the tyrosine phosphorylation of Tyk 2 and Jak 1 and not Jak 2 or Jak 3 in both T cells and monocytes (Finbloom and Winestock 1995). Tyk 2 is associated with the intracellular domain of the second chain (Ho, 1995) whilst JAK1 is associated with the intracellular domain of the first chain. When members of the STAT family are activated, they dimerize and translocate to the nucleus where they activate target genes.
1.12.1.1 Can the Jak/STAT pathway provide cell specificity?

Finbloom and Winestock (Finbloom and Winestock 1995) showed that IL-10 activation of both T cells and monocytes induced the assembly of a Interferon γ response region (GRR) binding complex as does IFNγ (Larner, ). This complex contained phosphorylated STAT1α and STAT3 identified using anti STAT1α and anti STAT3 antibodies (Finbloom and Winestock 1995). This work was consistent with previous studies (Lehmann, 1994; Larner, ). Supershift experiments suggested that the complex in T cells was more likely to contain STAT3 homodimers compared to monocytes where the complex appeared to consist mostly of STAT1α/STAT3 heterodimers. They therefore suggested that cell specificity could be due to differential homo and heterodimer formation allowing a limited group of factors to have cell specific effects. IL-10 also appears to recruit STAT 5 in a transfected pre-B cell line as well as STAT 1 and STAT 3 and in this cell line there is differential recruitment of STATs to sites of specific promoters (Wehinger, 1996). However the role of STAT 5 is controversial as STAT 5 was not induced by IL-10 in B cells derived from a patient with chronic lymphoblastic leukaemia (Lai, 1996).

1.12.1.2 Is the Jak/STAT pathway involved in all IL-10 intracellular pathways?

Mutations of the two HU-IL-10-R1 cytoplamic tyrosine residues (see above) known to be involved in recruitment and activation of STAT3, prevented IL-10 inhibiting macrophage proliferation (O'Farrell, 1998). Generation of a truncated STAT3 lacking the transactivation domain and therefore acting as a dominant negative had a similar effect (O'Farrell, 1998). Generation of a STAT3 mutant (STAT3-Gyr B) which could be inducibly dimerized by Courmermycin acted as a positive control in that addition of Courmermycin activated the STAT3 mutant in the absence of IL-10. Activation of the STAT3-GyrB mutant by Courmermycin inhibited macrophage proliferation demonstrating that STAT3 was both necessary and sufficient to inhibit macrophage proliferation (O'Farrell, 1998).

Experiments using the same mutants suggested that STAT3 is used but is not the sole pathway in upregulation of FcγR, (the receptor for IgG) and does not play a role in inhibiting LPS induced TNFα and IL-1β production. Further experiments suggested that the ability of IL-10 to reduce CD86 (B7-2) required the Tyrosine residues on the Hu-IL-10-R1 but not STAT3. Inhibition of NO by IL-10 also did not appear to require STAT3 (O'Farrell, 1998).
Riley et al used murine macrophages which were STAT3 deficient to investigate the role of STAT 3 in IL-10 induced TNFα inhibition (Riley, 1999). IL-10 treatment of wild type macrophages resulted in a dose dependent inhibition of LPS induced TNFα whilst the STAT3 deficient macrophages were unable to inhibit LPS induced TNFα at any dose of IL-10. A similar result was seen with JAK1/- macrophages which were unable to respond to IL-10 whilst STAT1/- macrophages were identical to wild type macrophages. These results suggest that STAT3 and Jak 1 are required for IL-10 induced inhibition of LPS stimulated TNFα but not STAT1. As IL-6 induced similar levels of Jak1 and STAT3 in macrophages they concluded that STAT3 was required but not sufficient for inhibition of LPS stimulated TNFα (Riley, 1999). These results are in conflict with those of O'Farrell et al described above using the STAT3 dominant mutant. This could be because endogenous STAT3 is still present in addition to the dominant mutant and therefore able to exert an effect. Alternatively, the difference could be explained by the fact that O’Farrell et al (O'Farrell, 1998) were investigating the human receptor whilst Riley et al (Riley, 1999) were using the murine receptor.

Mutation of both of the tyrosine residues at 427 and 477 on the intracellular component of the IL-10 receptor prevented STAT3 activation and prevented proliferative responses to IL-10 and reduced the inhibition of LPS stimulated TNFα (Riley, 1999).

It therefore appears that STAT 3 is involved but to different degrees in both IL-10 induced inhibition of proliferation as well as inhibition of LPS induced TNFα production. The JAK/STAT pathway is important but does not explain all intracellular effects of IL-10. This is consistent with the fact that IL-10 has unique biological actions that are not expressed by other cytokines that activate similar patterns of Jaks and STATs.

1.12.2 Phophatidylinositol (PI) 3 Kinase and p70 S6 Kinase stimulation

A distinct pathway that appears to be involved in IL-10 intracellular action is the PI 3 kinase/S6 pathway. IL-10 increases the kinase activity of the p85α subunit of the PI 3 kinase and the p70 S6 kinase in a mast cell line and in monocytes. Although the proliferative effect of IL-10 (on mast cells) appears to be mediated by the p85 subunit of phosphatidylinositol 3-kinase and p70 S6 kinase, neither of these kinases are involved in IL-10 suppression of LPS induced TNFα production (Crawley, 1996). Once again, this suggests that multiple pathways are necessary to account for the pleiotropic actions of IL-10.
1.12.3 MAPKinases
Foey et al have shown that the MAPK p42/p44 pathway is not involved in IL-10 induced inhibition of LPS stimulated TNFα or the IL-10 induced release of sTNFR in human monocytes (Foey, 1998). In addition, IL-10 did not induce MAP/ERK phosphorylation and therefore did not activate p42 MAPK. This is in disagreement with work by Geng et al (Geng, 1994) who showed that IL-10 inhibited LPS induced tyrosine phosphorylation of p56\textsuperscript{lyk} and p56\textsuperscript{c} with a decrease in Ras and p42 MAPK activation. This inconsistency is difficult to explain but could be due to the different dose of LPS used, different methods of monocyte purification or different analysis. Geng et al measured a decrease in phosphorylation in the presence of IL-10 whilst Foey et al inhibited the p42/p44 pathway and showed that IL-10 ability to regulate LPS stimulated TNFα production was not decreased. It is possible that whilst the p42/p44 path was inhibited, IL-10 inhibited TNFα expression using another pathway. Foey et al also showed that IL-10 did not inhibit p38 MAPK activity which is involved in LPS stimulated TNFα production. This is in contrast to recent work on dendritic cells (which may explain the different results) where the addition of IL-10 was shown to antagonise TNFα induced tyrosine phosphorylation of extracellular signal-regulated kinase 2 (ERK2), Stress-activated protein kinase/c-Jun N-terminal kinase (SPK/JNK) and p38mapk (Sato, 1999).

1.12.4 Inhibition of NFκB translocation
In monocytes stimulated with LPS or TNFα, IL-10 added 5 hours prior to stimulation inhibits nuclear localisation of NFκB (Wang, 1995). As the gene promoters of IL-1β, IL-6, IL-8 and TNFα contain NFκB binding motifs, inhibition of nuclear localisation of NFκB could prevent transcription of these genes. However complete inhibition of NFκB nuclear localisation required 1-10ng/ml of IL-10 which is 10 to 100 fold greater than that required to inhibit TNFα or IL-1β expression (Wang, 1995). This suggests that this is not an important pathway in inhibiting pro-inflammatory cytokine production.
1.13 The interleukin 10 gene

Twin studies and family studies have suggested that approximately 75% of the variation in IL-10 production is genetically determined (Westendorp, 1997). Study of the IL-10 gene may therefore give us clues on the regulation of IL-10 production.

1.13.1 Genetic regulation of protein production

1.13.1.1 5' flanking region

Structurally, the 5' flanking region of a gene is the region 5' to the coding sequence. It is important functionally because it contains sequence motifs that allow binding of transcription factors which control transcription. Functional polymorphisms have been described in the 5' flanking region of several genes including TNFα, IL-1α, IL-6, Apolipoprotein A-1 and cytochrome P45011E1 (Bailly, 1993a; Fishman, 1998; Smith, 1992; Hayashi, 1991). Both in vitro and association studies have been used to define the effect of these polymorphisms (Hayashi, 1991; McDowell, 1995; Donn, 1998; Fishman, 1998; Francis, 1999).

1.13.1.2 Regions outside the 5' flanking region

The 5' flanking region is not the only part of a gene that regulates transcription. For example, there is a variable number of the 46 bp tandem repeat (VNTR) in intron 6 of the IL-1α gene with seven alleles ranging from 5 to 18 repeats (Bailly, 1993a; Bailly, 1993b). Each repeat contains several consensus binding sites for transcriptional factors (Furutani, 1986). Transient transfection experiments using the human monocytic cell line Mono Mac 6 have shown that the expression of a reporter gene was decreased by increasing numbers of repeats in a copy number dependent manner (Bailly, 1996a).

Electrophoresis mobility shift assay confirmed the suggestion from sequence data that several proteins can bind to the 47 bp repeat (Bailly, 1996b). In IL-1β, three nuclear factor binding sites have been identified by DNase 1 footprinting and electromobility shift assay (EMSA) between +448 and +548 (within intron 1 and exon 2) (Zhang and Duff 1998). This element appears to be important in maximising IL-1β gene expression and may function as a second transcription initiation element although the region does not function as such without the upstream 5' flanking region.

1.13.1.3 5' versus 3' mechanisms (e.g. TNF (Goldfeld, 1991))

Many cytokine mRNAs have an adenosine-uridine (AU)-rich motif in the 3’ untranslated region of the gene. When this motif is spliced into the 3’ UTR of the rabbit β globin gene it causes the β globin mRNA to decay rapidly with a half-life of about 15 minutes.
although it does not alter the rate of gene transcription (Shaw and Kamen 1986). This AU rich motif therefore appears to function as a destabilising element. This is consistent with the fact that alleles where the AU sequence is disrupted with insertion mutations in either the TNFα or the IL-10 genes are associated with decreased cytokine production in animal models (Jacob and Tashman 1993). The protein synthesis inhibitor cyclohexamide superinduced levels of TNFα mRNA by 50 fold in TPA activated monocytes whereas actinomycin D (which blocks transcription) decreased the amount of TNFα transcripts by 75% (Sariban, 1988). Cyclohexamide was also shown to prolong the half life of TNFα mRNA in cells where actinomycin A was used to block transcription. This suggests that cyclohexamide prevented the degradation of TNFα mRNA by inhibiting the synthesis of a labile protein or that translation of the TNFα mRNA is required for its degradation. The IL-10 mRNA has AU instability elements and IL-10 mRNAs become unstable after LPS stimulation if monocytes are treated for several hours with IL-10 (Brown, 1996).

As well as providing a mechanism for increased mRNA degradation, interaction between the 5' and the 3' untranslated regions can have a multiplicative effect on transcription and on the quantity of protein produced (Han, 1991; Han, 1990). For example, LPS stimulation of macrophages increases TNFα transcription 3 fold, TNFα mRNA 50 to 100 fold and TNFα protein secretion by a factor of ~10,000 fold (Beutler and Cerami 1989; Han, 1990).

1.13.2 Chromosomal localisation and IL-10 gene structure
The human IL-10 gene is located on chromosome 1 (Kim, 1992). The IL-10 cDNA encodes a 178 amino acid protein which is a member of the four α-helix bundle family of cytokines (Moore, 1993).

1.13.2.1 Is IL-10 production controlled at the transcriptional level?
There is now some evidence that IL-10 production is controlled by regulation of transcription (Bienvenu, 1995). In vitro studies on monocytes show that LPS stimulated IL-10 production correlates with mRNA production in a dose dependant manner (van der Poll, 1994; Platzer, 1995). Augmentation of LPS stimulated IL-10 production with either TNFα or cAMP also results in increased IL-10 levels paralleled by an increase in IL-10 mRNA (van der Poll, 1994; Platzer, 1995). Stimulation of T cells with IL-12 in the presence of anti-CD3 results in an increase in IL-10 which is also paralleled by an increase in mRNA (Jeannin, 1996). Suppression of IL-10 production
by IFNγ occurs at both the mRNA and protein levels (Donnelly, 1995). *In vivo* IL-10 mRNA levels are strongly correlated with *in vitro* IL-10 protein production (Llorente, 1994) in both patients with RA or SLE and controls. Studies have also shown that SNPs and microsatellites within the IL-10 5′ flanking region are both functional and associated with disease (see below).

It must not be forgotten however that even though regulation of IL-10 production via the 5′ flanking region is important, other mechanisms are also involved. These include regulation of mRNA stability via adenosine-uridine instability elements in the 3′ untranslated region of IL-10 mRNA which destabilises after several hours treatment with IL-10 (Brown, 1996).

### 1.13.3 The IL-10 5′ flanking region

The function of the IL-10 5′ flanking region was investigated by Kube *et al* in 1995 using transfection studies in Burkitt lymphoma cells (Kube, 1995). In these experiments they made the following observations. Transcription could be induced with a construct −1318/+27 (initiation site at +1 and TATA-box at position −77) from the coding sequence. The minimal DNA necessary to direct luciferase activity above background levels was between −141 to +27 which contains the TATA-box. Deletion of basepairs −1000/−800 in the construct resulted in a decrease in reporter gene assay suggesting that there is a positive control element present whilst removal of the construct region −750/−350 resulted in an increase in reporter gene expression suggesting that a negative control element is present. There are two microsatellites between −4000 and −1200 (Eskdale, 1997) and three single base pair substitutions at positions −1082, −819 and −592 respectively (see figure 1.4)(Turner, 1997; Lazarus, 1997; Lazarus, 1997).
The Ets transcription factor and a putative Stat 3 binding sites are shown on the diagram. Other putative transcription factor binding sites are shown in table 1.4 below. All numbers indicate base pairs from start codon.

Table 1.4 *Putative transcription factor binding sites in the IL-10 5' flanking region*

<table>
<thead>
<tr>
<th>Position (1st bp)</th>
<th>Transcription Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1260</td>
<td>STAT 3</td>
</tr>
<tr>
<td>-1085</td>
<td>Sp-1 (Ets family)</td>
</tr>
<tr>
<td>-1019</td>
<td>AP-1</td>
</tr>
<tr>
<td>-926</td>
<td>SRF</td>
</tr>
<tr>
<td>926</td>
<td>CBF-B</td>
</tr>
<tr>
<td>-804</td>
<td>C/EBP</td>
</tr>
<tr>
<td>-755</td>
<td>AP-1</td>
</tr>
<tr>
<td>-737</td>
<td>CEBP_</td>
</tr>
<tr>
<td>-707</td>
<td>CEBP_</td>
</tr>
<tr>
<td>-647</td>
<td>C/EBP</td>
</tr>
<tr>
<td>-620</td>
<td>STAT3</td>
</tr>
<tr>
<td>-601</td>
<td>Sp1</td>
</tr>
<tr>
<td>-421</td>
<td>CBP</td>
</tr>
<tr>
<td>-374</td>
<td>CAMP RE</td>
</tr>
</tbody>
</table>

1.13.3.1 *IL-10 5' flanking region microsatellites*

There are two microsatellites in the IL-10 5' flanking region. The first microsatellite described is a CA repeat at approximately -1064 upstream from the TATA box.
Eskdale et al first looked for polymorphisms in 102 individuals in Scotland and described eleven alleles with allele number 9 being the most common (Eskdale and Gallagher 1995). A second microsatellite has been defined approximately 4 Kb from the TATA box (Eskdale, 1997). This microsatellite has 5 alleles with R2 being the most common (Eskdale, 1997; Eskdale, 1998). Eskdale et al describe these two satellite regions as G (∼-1000kb) and R (∼-4000kb). Whole blood culture suggests the presence of high and low IL-10 producing haplotypes after stimulation with LPS with R3G7 being the lowest IL-10 producing haplotype and R2G14 having the highest IL-10 production. Haplotypes were identified by considering inheritance of alleles from parents to children. Comparison of haplotypes within each locus suggests that G7 and R3 are associated with low IL-10 production after stimulation with LPS and G14 with high IL-10 production. However although the whole blood culture method described in this paper is virtually identical to that described in this thesis, one major difference is that blood samples were not “corrected” for lymphocyte and monocyte count. In this paper, blood samples were diluted in an equal volume of RPMI and 1 ml of the diluted blood was cultured with LPS (1 µg/ml) for 24 hours. As the method is by volume and not number of cells it is unclear what the cellular production of IL-10 is.

Associations have been described between alleles at both microsatellites and SLE and RA (see section 1.14.1 for more detail). At the moment it is not clear whether these microsatellites are themselves functionally important or whether they are markers for functionally relevant alleles such as the SNPs described below as they are only 100 base pairs apart.

To date, linkage has not been described between the microsatellites and the SNPs even though the microsatellite at ∼1000kb is less than 200bps from the first SNP. Jorde et al have shown that over 50-500kb, there is generally a good correlation between linkage disequilibrium and physical distance (Jorde, 1994). Associations between microsatellites and SNPs have been described in other 5’ flanking regions in relation to disease (McGinnis and Spielman 1994). McGinnis et al treated allele length in a microsatellite in the 5’ flanking region of the insulin gene as a quasi-continuous variable in relation to allelic variation at a nearby SNP (McGinnis and Spielman 1994). This allowed them to identify clustering of the SNP with microsatellites of different allelic lengths that were distributed within a bimodel distribution. The SNP was associated primarily with microsatellite alleles whose lengths represent consecutive numbers of tandem repeats. This could be explained by “replication slippage” so that a SNP which
is originally associated with one microsatellite allele may ultimately be re-coupled to slightly larger or smaller alleles. This concept is important when linkage disequilibrium between microsatellites and SNPs is investigated to allow relevant associations to be described. Alternatively, grouping strategies with multiallelic markers can be used to allow comparison with biallelic SNPs and this is described by Cox et al in a comparison of three methods for grouping alleles at multiallelic marker loci (Cox, 1998).

1.13.3.2 IL-10 5’ flanking region Single Nucleotide Polymorphisms

3 base pair substitutions have been described at -1082 (G to A), -819 (C to T) and -592 (C to A). The G to A nucleotide polymorphism occurs within a putative Ets transcription factor-binding site. The Ets family includes a variety of DNA binding proteins with a conserved DNA binding region which are involved in the transcriptional regulation of a variety of genes (John, 1996; Boccia, 1996; Chen, 1995; Jacob, 1995; Groupp and Donovan Peluso 1996; Nimer, 1996; Thomas, 1995; Logan, 1996). The C to T nucleotide polymorphism lies within a putative positive regulatory region (Kube, 1995) and the C to A polymorphism lies within a putative STAT 3 binding site and a negative regulatory region (Kube, 1995). There is linkage disequilibrium between the alleles with the two downstream polymorphisms occurring in tandem, and only three out of four possible haplotypes described in Caucasian populations: GCC, ACC and ATA.

Initial in vitro work on PBMC cultures has suggested that the GCC/GCC genotype is associated with higher IL-10 production than other genotypes although preliminary studies using the whole blood culture method appear to contradict this (Turner, 1997).
1.14 Genetic regulation of IL-10 production in disease

1.14.1 Microsatellites

In adult RA, an association between the IL-10.R2 allele and RA has been described at the expense of R3 (relatively low IL-10 production) in two UK populations (Eskdale, 1998).

In patients with SLE there appears to be a reduction in the microsatellite IL-10.G9 allele and an increase in the IL-10G13 allele using Monte Carlo simulation methodology (Eskdale, 1997). An association between alleles and patients in different disease subgroups was also undertaken, and the allele distribution is described as being different between patients who were anti-DNA positive anti-ENA negative and other patients. However the numbers in the disease subgroups are small and although the Monte Carlo simulation method allows analysis of multiple alleles without a Bonferoni correction, it does not allow for multiple analysis of disease subgroups without a Bonferoni correction. In addition, the G13 allele, which is increased in patients with SLE, does not appear to be the allele associated with higher IL-10 production. The IL-10 microsatellite at -1Kb has also been investigated in Mexican Americans with SLE in a relatively large study involving 158 patients and 222 ethnically matched controls (Mehrian, 1998). In this study, microsatellites were also typed for the bcl-2, Fas-L and CTLA-4 genes as these genes are thought to be involved in dysfunctional programmed cell death. The most common IL-10 microsatellite allele in Mexican Americans is the 125 bp allele which is probably equivalent to the IL-10.G9 allele (also the most common allele with a similar population frequency of 0.5) described by Eskdale et al.

In this study, associations were investigated using logistic regression analysis which showed a decrease in the 125 bp allele in patients with SLE consistent with the results from Eskdale et al. In addition, they describe an increase in the 127bp allele which was not seen in the Glaswegian population. They also describe a synergistic effect between this high risk allele and the high risk bcl-2 allele and showed significant interaction with an Odds Ratio for both alleles of 40. As the bcl-2 and IL-10 genes in the human reside on different chromosomes, this synergism is not due to linkage disequilibrium.

Alleles of the microsatellite at \( \approx 1000 \) kb (G) that possess greater number of dinucleotide repeats have been described as being associated with more severe graft versus host disease (GVHD) (Middleton, 1999). In this study both TNF\( \alpha \) and IL-10
microsatellite alleles were investigated with different grades of GVHD. The results however were not corrected for multiple testing which would have made them non-significant.

1.14.2 SNPs

Associations between different IL-10 5' flanking region haplotypes and patients with systemic lupus erythematosi (SLE) and rheumatoid arthritis have been reported. In those with SLE, an association has been reported between patients with anti-Ro antibodies and the GCC or ACC haplotype (Lazarus, 1997) although the numbers studied were small and these results need confirmation from larger population association studies. An association has also been described in a southern Chinese population between patients with SLE nephritis and the ATA haplotype. However the distribution of haplotypes in the Chinese population are so different from those described in white Caucasian populations it is not clear whether this is due to ethnic differences or the different methodology used (Mok, 1998). An association has also been described between patients with SLE and neuropsychiatric symptoms and the ATA haplotype (Rood, 1999). Whether this association is biologically valid however is difficult to know as the pathological mechanisms producing neuropsychiatric symptoms are extremely varied and it is unlikely that IL-10 production contributes to all of them. The authors attributed the increased risk of symptoms to high IL-10 production but it is generally accepted now that the ATA haplotype is associated with low IL-10 production.

Although the SNP haplotypes do not appear to be associated with susceptibility to RA (Keijzers, 1997; Coakley, 1998), there is a report of increased frequency of the ACC and ATA haplotypes in patients who are IgA RF+/IgG RF- (Hajeer, 1998). A retrospective study of 291 patients and controls who were homozygous for the G at -1082 showed that they had their first erosion earlier (2.5 ± 3.3 Vs 3.4 ± 5.2 years) and had more erosions in the first three years (5.3 ± 7.4 Vs 3.4 ± 5.2) compared to patients with other genotypes (Keijzers, 1998). However neither study corrected for multiple testing and the confidence intervals are wide making interpretation of these results difficult.

Other associations that are not directly related to autoimmunity include associations between the ATA haplotype and increased susceptibility to Epstein-Barr virus infection (Helminen, 1999) as well as response to IFNα in patients with chronic hepatitis C (Edwards-Smith, 1999). Patients with the ATA haplotype appear to have an increased
1.15 Do the genetic associations described account for the full genetic component of JIA?

Although many genetic associations have been described with JIA (see section 1.6.1), the cumulative relative risk does not account for the sibling risk factor of 15 (Glass and Giannini 1999) for developing JIA or the increased concordance in disease type described by Moroldo et al (Moroldo, 1997). Therefore the search for other genetic factors that determine susceptibility and outcome in JIA continues.
1.16 Hypothesis
There is a genetic component to JIA which is not fully explained by genetic associations described to date. One candidate gene in terms of control of inflammation is IL-10. Relatively low levels of IL-10 production could increase disease severity. Relatively low IL-10 production could be due to polymorphisms in the 5' flanking region resulting in lower IL-10 transcription in the presence of inflammation.

Our hypothesis is that children with extended oligoarticular JIA would be more likely to have a low IL-10 producing genotype than children with oligoarticular JIA. We hypothesised a similar association for children who develop uveitis and for other inflammatory diseases. In children or adults with SLE we hypothesised an association with a high IL-10 producing genotype.

1.17 Aims of this thesis

1.17.1 Genotype association and disease
The first aim of this thesis was to determine if the IL-10 5' flanking SNPs were associated with either disease severity or with eye disease in children with JIA. Our aim was to investigate this initially using association studies whilst collecting parental samples for TDT analysis. The disease groups of particular interest were those with oligoarticular JIA who then extended and children with eye disease.

In addition we decided to investigate whether the same SNPs could explain the relatively high levels of IL-10 production in patients with SLE.

To investigate whether associations could be found in other inflammatory diseases we investigated genotype distribution in patients with asthma.

1.17.2 Are children with severe joint disease or eye disease predisposed genetically to low or high IL-10 production?
Because most children with severe disease and some children with eye disease are on treatment such as glucocorticoids or MTX which alter cytokine production, we measured IL-10 production in the parents using the whole blood culture method.

1.17.3 Does the IL-10 5' flanking region genotype affect protein production in healthy controls?
To find out whether the IL-10 5' flanking region SNPs were relevant functionally we measured IL-10 protein production in response to LPS in healthy controls using the whole blood culture method.
1.17.4 How do the IL-10 5' flanking region SNPs affect transcription?

During the course of this thesis, our collaborator, Richard Kay, demonstrated that the GCC haplotype was associated with low IL-10 production and the ATA haplotype associated with high IL-10 production in U937 cells stimulated with a cAMP analogue dbcAMP. His results are described and discussed in our joint paper (Crawley, 1999c).

We wanted to see if these results would extend to other cell lines. We also wanted to investigate transcriptional regulation using LPS as this was the stimulant used in the whole blood culture method. We therefore used transient transfection to investigate transcriptional regulation of each haplotype in both HeLa cells and THP-1 cells using a variety of transfection methods.
CHAPTER 2: MATERIALS AND METHODS
### 2.1 Reagents

#### 2.1.1 Reagents for PCR and SSOP

<table>
<thead>
<tr>
<th>Table 2.1. Reagents for polymerase chain reaction (PCR) and sequence specific oligonucleotide probing (SSOP)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FOR PCR</strong></td>
</tr>
<tr>
<td>TAE X50</td>
</tr>
<tr>
<td>Glacial acetic acid (BDH, Merck)</td>
</tr>
<tr>
<td>EDTA (0.5M, pH 8)</td>
</tr>
<tr>
<td>PCR buffer (x10)</td>
</tr>
<tr>
<td>KCL</td>
</tr>
<tr>
<td>Triton (x 100) (Sigma)</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
</tr>
<tr>
<td>Betaine (Sigma)</td>
</tr>
<tr>
<td>dNTPs</td>
</tr>
</tbody>
</table>

| **For SSOP** | **Ingredient** | **Quantity** | **Final concentration** |
| SSC (x20) | NaCl | 175.2g/l | 3M |
| Na Citrate (BDH, Merck) | 88.2g/l | 3M |
| SDS (x10) | sodium dodecylsulphate (BDH, Merck) | 100g/100mls | 10% |
| Denaturing Solution | NaOH (BDH, Merck) | 20g/l | 0.5M |
| NaCl (BDH, Merck) | 87.5g/l | 1.5M |
| Neutralising Solution | NaCl | 87.7g/l | 1.5M |
| Tris | 60.6g/l | 0.5M, pH to 7.5 |
| Hybridising buffer | SSC (x20) | 62.5 mls | 30% |
| Marvel milk powder | 1.25 g | 0.5% |
| 0.1% laurylsarcosine | 0.25 g | 0.1% |
| SDS | 0.25 mls | 0.02% |
| Buffer 1 | NaCl | 8.7g/l | 0.15M, pH to 7.5 |
| Tris | 12.1g/l | 0.1M |
| Buffer 2 | NaCl | 0.4M, pH to 7.5 |
| Tris | 0.1M |
| Blocking solution | Buffer 1 | 200mls | 0.5% |
| Milk powder (Marvel) | 1g |
### 2.1.2 Oligonucleotides for PCR/SSOP

#### Table 2.2 Oligonucleotides (Genosys) for PCR and SSOP

<table>
<thead>
<tr>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR primers -1120 to -533</td>
<td><strong>Position</strong></td>
</tr>
<tr>
<td></td>
<td>Upstream</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
</tr>
<tr>
<td>Probes for SSOP(position)</td>
<td>1a (-1082)</td>
</tr>
<tr>
<td></td>
<td>1b (-1082)</td>
</tr>
<tr>
<td></td>
<td>2a (-819)</td>
</tr>
<tr>
<td></td>
<td>2b (-819)</td>
</tr>
<tr>
<td></td>
<td>3a (-592)</td>
</tr>
<tr>
<td></td>
<td>3b (-592)</td>
</tr>
</tbody>
</table>

### 2.1.3 Oligonucleotides for RT-PCR

#### Table 2.3. Oligonucleotides for RT-PCR

<table>
<thead>
<tr>
<th>Fragment size (bp)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA unless stated</td>
<td></td>
</tr>
<tr>
<td>Actin-5’</td>
<td>132 (DNA=448) ATGGATGATGATATCGCCGC</td>
</tr>
<tr>
<td>Actin-3’</td>
<td>AAGGCCAACCGCGAGAAGAT</td>
</tr>
<tr>
<td>TNFα-5’</td>
<td>702 ATGAGCAGATGAGATAGGCGCCGC</td>
</tr>
<tr>
<td>TNFα-3’</td>
<td>ATGAGCAGCTAAAGCTATGATGTC</td>
</tr>
<tr>
<td>IL-1α-5’</td>
<td>816 ATGGCCAAAGTTCGAGACTG</td>
</tr>
<tr>
<td>IL-1α-3’</td>
<td>CTACGCCTGCTTTCCAGTATCTGAAAGTCG</td>
</tr>
<tr>
<td>IL-6-5’</td>
<td>639 ATGAAGTCTCCCTCCACAGGC</td>
</tr>
<tr>
<td>IL-6-3’</td>
<td>CTACATTTGCGGAAGAGCCCTGAGCGGCTGGACTG</td>
</tr>
<tr>
<td>IL-10-5’</td>
<td>314 TGAGAACCAAGACCCAGAC</td>
</tr>
<tr>
<td>IL-10-3’</td>
<td>GGGAACACTCTTTGGAATAAC</td>
</tr>
</tbody>
</table>

Oligonucleotide sequence for Actin cDNA is derived from genbank accession number M10277 (Nakajima-Iijima, 1985). Note the fragment crosses an intron allowing genomic DNA to be identified as a contaminant. The IL-10 sequence is derived from genbank accession number M57627. All remaining sequences derived from (Brenner, 1991).
2.1.4 Reagents for whole blood culture work

Table 2.4. Reagents for whole blood culture

<table>
<thead>
<tr>
<th>Stock solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylprednisolone 40mg/ml sterile water</td>
</tr>
<tr>
<td>Ethanol (1M) 2.3g in 50mls sterile water</td>
</tr>
</tbody>
</table>

2.2 Collaborating Centres

1. Richard Kay, Dept. of Molecular and Cellular Pathology, Ninewells Medical School, Dundee DD1 9SY.

Richard Kay generously donated IL-10 5’ flanking region luciferase DNA reporter constructs used in the transfection studies. Our collaborative work is reported in (Crawley, 1999c).

2. Ian Hutchinson, Immunology research group, University of Manchester, Manchester M13 9PT, UK.

Ian Hutchinson’s team developed the SSOP method for the IL-10 5’ flanking region SNPs.

3. David Isenberg, Centre for Rheumatology, Department of Medicine, University College London, LONDON W1P 6DB

David Isenberg provided the patients and clinical details of the SLE patients. Our collaborative work is described in (Crawley, 1999a).

4. Rachelle Donn, ARC Epidemiology Research Unit (UK Paediatric Rheumatology HLA database).

Rachelle Donn collaborated on the linkage analysis data with the IL-10 5’ flanking region microsatellites. The ARC epidemiology Research unit provided patient genomic DNA for analysis.

5. Sam Lim, The Royal Brompton Hospital, London.

Sam Lim provided the genomic samples and the clinical details on the patients with asthma. Our collaborative work is described in (Lim, 1998).
2.3 General Methods

2.3.1 Sources of genomic DNA

DNA was obtained from 3 sources. The first source was a bank of DNA that had been extracted from blood samples from patients with JIA from Northwick Park hospital. Review of these samples however suggested that there were insufficient numbers for analysis. Therefore new samples were obtained from left over EDTA samples from patients attending Great Ormond Street Hospital and the Middlesex hospital outpatient departments. The third source of genomic DNA samples was the ARC Epidemiology Research Unit (UK Paediatric Rheumatology HLA database).

2.3.2 DNA extraction

The original Northwick Park genomic DNA was extracted as previously described (Kunkel, 1977) (see Appendix C for details). (Miller, 1988)

The new samples were prepared using the BACC 2 kit (Nucleon, Coatbridge, Lanarkshire, UK). In brief, blood was collected into EDTA tubes. 300μl of blood was then added to 1.2 mls of Reagent A (10mM Tris-HCL; 320 mM sucrose; 5mM MgCl2; 1% Triton X-100. pH = 8.0) for cell lysis. This was then shaken for 4 minutes at room temperature then centrifuged for 5 minutes at 1300g. The supernatant was discarded. 350μl of reagent B was then added to the pellet (400mM Tris-HCL; 60mM EDTA; 150mM NaCl; 1% SDS . pH 8.0) which was vortexed to resuspend the pellet. 100μl of sodium perchlorate (5M) was then added and the solution was mixed by inverting at least 7 times by hand. 600μl of chloroform was added and mixed by inverting 7 times to emulsify the phases after which 150μl of nucleon resin was added and without remixing the phases the entire solution was centrifuged at 350g for 2 minutes. The upper phase (approximately 450μl) was then added to a new eppendorf tube. The sample was centrifuged briefly at top speed and the suspension removed once more to ensure that there was no resin carried over. 900μl of cold (-20°C) 100% ethanol was then added and the sample mixed by inverting before being centrifuged again. The supernatant was discarded and the pellet of DNA was air dried at room temperature. The DNA was re-dissolved into 200 μl of sterile water and stored at 4°C.

This method enabled us to produce genomic DNA from 300μl of EDTA anticoagulated blood (final concentration approximately 60μg/ml). We used the remainder of samples that had been used to measure full blood counts or erythrocyte sedimentation rates and then stored in the Haematology Department at either the Great Ormond Street Hospital.
for Children or the Middlesex Hospital, London. Using the proposed ILAR criteria, children were classified as having oligoarticular, extended oligoarticular, polyarticular rheumatoid factor negative, polyarticular rheumatoid factor positive, systemic, enthesitis associated or psoriatic associated arthritis.

2.3.3 Polymerase Chain Reaction for IL-10 5’ flanking region
The IL-10 5’ flanking region was amplified between -1122 bp and -533 bp by PCR. PCR reactions were carried out either in a Biometra Trio-Block PCR machine (Biometra, Germany) or in a GeneAmp PCR system 9700, (PE applied Biosystem). The choice of machine depended on the number of samples that were being PCR amplified. PCR reactions were in a total volume of 30μl and contained 2μl of sample genomic DNA (approximately 0.6μg). Final concentrations were 10mM Tris-HCL pH 8.8, 50mM KCL, 1% Triton, dNTPs each at 0.2 mM, 2.5 mM MgCl2 and 1M Betaine, 25 mM of both 5’ and 3’ primers (see table 7 for sequence) with 1 unit Taq (Promega, Madison, WI) per reaction. The following cycles were used: 95°C for 4 minutes, 30 cycles of 95°C, 55.5°C and 72°C for 1 minute each followed by 72°C for 4 minutes. To confirm whether PCR was successful, samples were run on a horizontal 1.5% agarose gel (Biorad, UK) in TAE and visualised under UV light. The size of the product was estimated by comparison against a 100 base ladder (Promega, UK).

2.3.4 mRNA extraction and cDNA isolation
RNAzol (Cinna/Biotecx, Texas) was added to the cells using 0.2 mls per 1 x 10⁶ cells. 0.4 mls of chloroform was added to each sample and shaken vigorously for 15 seconds and then put on ice for 5 minutes. The suspension was then centrifuged at 12,000g (4°C) for 15 minutes. The colourless upper aqueous phase was transferred to a fresh eppendorf tube and an equal volume of isopropranol was added before incubation for 15 minutes at 4°C. The samples were centrifuged at 12000g (4°C) for 15 minutes and the supernatant removed. The samples were then washed once with 70% ethanol spun at 7500g for 8 minutes and allowed to air dry. After extraction, the RNA pellets were each dissolved in 40μl of RNAse free water (Promega) in an eppendorf tube and put on ice for 20 minutes followed by a 30 minute incubation at 55°C and then spun at 14000 rpm for 5 minutes. For first strand synthesis, 2.5 μl (200ng) RNA supernatant was added to 8.5μl of RNAse free water and 0.5μl of random primer (hexamer) (0.5μg/ml) (Promega). The mixture was heated to 70°C for 10 minutes followed by 5 seconds on ice. The contents of the tube were spun down. 0.5μl of RNasin (40U/μl, recombinant ribonuclease inhibitor, Promega) was added to
each sample with 4 µl of first stand buffer (x5)(Life Technologies, Paisley, UK), 2µl of
dithiothreitol (DTT) (0.1M) (Life Technologies, Paisley, UK) and 1µl of dNTP mix
(10mM) and mixed by gently pipetting the sample. 1µl of Superscript II (200 units)(Life
Technologies, Paisley, UK) was added and the sample was incubated at room
temperature for 10 minutes, followed by an incubation at 42° for 50 minutes. The
sample was inactivated by heating to 70° for 15 minutes before being spun at 1400 rpm
for 5 minutes and then frozen at -70°C.

2.3.4.1  **PCR of Actin cDNA**
Actin was amplified using the PCR reaction as follows: 3µl of cDNA was added to 5 µl
of 10 x PCR buffer (Promega), 2µl of MgCl$_2$ (Promega), 1µl (25mM) of 5’ and 3’ actin
primer (table 2.3), 24.3 µl of sterile distilled water (SDW) and 25 µl (25mM) of dNTPS.
For each reaction, 0.2 µl of Taq (Gibco BRL, 5U/µl) was added to 8.5 µl of SDW and
then added to the reaction. The following cycles were used: 95°C for 5 minutes, 35
cycles of 94°C, 55°C for 1 minute and 72°C for 2 minutes followed by 72°C for 5
minutes.

2.3.4.2  **PCR of IL-6 cDNA**
IL-6 was amplified using the PCR reaction as follows: 2µl of cDNA was added to 5 µl
of 10 x PCR buffer (Promega), 2µl of MgCl$_2$ (1 mM) (Promega), 1µl each of IL-6
primers (25 mM) (table 2.3) 24.3 µl of SDW and 5µl(25mM) of dNTPS. For each
reaction, 0.2µl of Taq (Gibco BRL, 5U/µl) was added to 8.5µl of SDW and then added
to the reaction mixture. The following temperatures were used: 95°C for 5 minutes, 35
cycles of 94°C, 60°C for 1 minute and 72°C for 2 minutes followed by 72°C for 5
minutes.

2.3.4.3  **PCR of IL-10 cDNA**
IL-10 was amplified using the PCR reaction as follows: 2µl of cDNA was added to 5 µl
of 10 x PCR buffer (Promega), 2µl of MgCl$_2$ (1 mM)(Promega), 5µl each of IL-10
primers (4mM) (table 2.3) 16.3 µl of SDW and 5µl (25mM) of dNTPS. For each
reaction, 0.2µl of Taq (Gibco BRL, 5U/µl) was added to 8.5µl of SDW and then added
to the reaction mixture. The following temperatures were used: 95°C for 5 minutes, 35
cycles of 94°C, 58°C for 1 minute and 72°C for 2 minutes followed by 72°C for 5
minutes.
2.3.5 Sequence Specific Oligonucleotide Probing (SSOP).
The IL-10 5' flanking region was amplified from genomic DNA by PCR. 2μl of PCR product was then dotted onto each of 6 nylon transfer membranes (Hybond-N+, Amersham). The membranes were air dried before being placed in Denaturing solution for 5 minutes and then Neutralising solution for 1 minute (see table 2.1 for solution recipes) on a shaker at room temperature. The membranes were then baked for 10 minutes at 80°C. Ultra violet light was used to crosslink the DNA to the membranes using a UV stratalinker 2400 (Stratagene). Each membrane was then placed into Hybridising solution at 42°C and rotated in a hybridising oven (hybridiser HB-ID, Techne). The oligonucleotides were each added to a final concentration of 20ng/ml after one hour of prehybridisation and were left to hybridise for two hours. The membranes were then washed using 5 x SSC/0.1% SDS for 5 minutes at room temperature twice before a stringency wash using 1 x SCC/0.1% SDS for 15 minutes twice at 49°C for probes 1 and 3 and 59°C for probes 2. After the washes they were placed into buffer 1 for one minute and then blocked using Blocking solution for 30 minutes on a shaker at room temperature. 35μl of Streptavidin-horseradish peroxidase conjugate (Amersham) was then added to 300 mls of buffer 1 and the membranes were shaken for 30 minutes at room temperature. The membranes were then soaked in buffer 2 for ten minutes twice before detection using a chemiluminescent detection kit (Kirkegaard & Perry, Gaithersburg, MD) according to the manufacturer’s guidelines. All samples were numbered before analysis to prevent bias in the interpretation of SSOP results.

2.3.6 Peripheral blood mononuclear cell (PBMC) culture
Blood was collected into 20 ml universal tubes (Sterilin) with preservative-free heparin and diluted an equal volume with plain RPMI 1640 (Sigma) (with glutamine, penicillin and streptomycin but no foetal calf serum). 20 mls of this diluted blood was cushioned above 5 mls of Histopaque (Sigma, St Louise, MO) and centrifuged at 1200 rpm, 20°C for 30 minutes. The cells at the interface were recovered, washed twice with plain RPMI for 10 minutes at 1200 rpm and then a third time with RPMI with 10% foetal calf serum (FCS). The cells were then counted in a haemocytometer and resuspended to 1.5 x 10^6/ml in RPMI/10% FCS.

2.3.7 Whole blood culture
The whole blood culture system was optimised to produce maximum IL-10 when stimulated by LPS (Serratia marcescens, Sigma). Blood was collected into 20 ml
universal tubes with preservative free heparin. Heparin was used rather than EDTA because EDTA has been reported to inhibit cell function in bioassays and to inhibit the production of TNF (Cannon, 1993). The blood was diluted 1:1 with plain RPMI (no additives). LPS was added at 1μg/ml to stimulated samples. Analysis was performed at 0 and 24 hours when the samples were spun down for 5 minutes and the supernatant was frozen at -20°C before being analysed at a later stage by enzyme linked immuno sorbent assay (ELISA). The differential white cell count was performed on the day blood was obtained.

2.3.8 Enzyme-linked immunosorbent assay (ELISA) for IL-10
ELISA plates (Costar, Cambridge, MA) were coated with 50μl per well of anti-human IL-10 monoclonal antibody (mAb) (Pharmingen, San Diego, CA) in PBS (1μg/ml) and left to incubate overnight at 4°C. After washing twice with PBS/tween 0.05%, the plates were blocked by adding 200μl per well of PBS/3% bovine serum albumin and incubated for one hour at room temperature. Identical standards made using recombinant IL-10 human protein (Pharmingen) at the following concentrations (5ng/ml, 1ng/ml, 500pg/ml, 100pg/ml, 50 pg/ml) were used to plot a standard curve for each plate. All samples were loaded in triplicate. After 2 washes, the plates were then incubated for 45 minutes with 50μl of biotinylated anti-human IL-10 -mAb (1μg/ml, Pharmingen, San Diego, CA), followed by a 30 minute incubation with Avidin-peroxidase (0.05mg/ml, Sigma). Hydrogen peroxide (30%, Sigma)(10μl) added to O-phenyldiaminediHCL (10mg) dissolved in 50mM NaP04 (25mls) was used as the substrate for the colour reaction which was stopped by adding 100μl of 1M H2SO4 to each well. The plates were read at OD450nm. The lower limit for detection was 50 pg/ml. Variability between triplicate wells was less then 5%.

2.3.9 ELISA for IL-1α, IL1β, IL-4, IL-6, IL-12, IFNγ and TNFα
ELISA kits for IL-α, IL-1β, IL-4, IL-6, IFNγ and TNFα (EASIA, Biosource) and IL-12 (R & D Systems) were used as per the manufacturer’s instructions. All standards and samples were loaded in duplicate.
2.4 FACS analysis

2.4.1 Reagents used

Table 2.5. Reagents used for FACS analysis

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Media</th>
<th>FACS buffer</th>
<th>Fixing buffer (1)</th>
<th>Fixing buffer (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamine</td>
<td></td>
<td>FCS (Insight Biotech)</td>
<td>NaCl</td>
<td>FCS</td>
</tr>
<tr>
<td>penicillin/streptomycin (Sigma)</td>
<td>2mM</td>
<td>1%</td>
<td>2 M</td>
<td>1%</td>
</tr>
<tr>
<td>RPMI (Sigma)</td>
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<td>Azide</td>
<td>KCL</td>
<td>Azide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PBS (x 1) (Sigma)</td>
<td>Na₂HPO₄</td>
<td>FCS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>KH₂PO₄</td>
<td>1%</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pH to 7.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.4.2 Antibody titration

Titration of antibody was performed using PBMC (see section 2.3.6 for method to prepare PBMC). The antibody was diluted in FACS buffer to a total volume of 25μl at the following concentrations: 1:10, 1:20, 1:30, 1:40, 1:50, 1:100. Cells were resuspended to 1 x 10⁶ cells and 100μl of cell suspension was placed per well in a 100 well microtitre plate. The plate was spun at 1500 rpm for 4 minutes and the supernatant discarded before the diluted anti-CD14 antibody was added in the concentrations listed above. The cells were incubated for 30 minutes in the dark on ice before being washed in FACS buffer (100μl per well) at 1200rpm for 10 minutes. This step was then repeated once before fixing with fixing buffer 2 (see table 2.5) was added. The cells were kept at 4°C prior to analysis.

2.4.3 Cell surface and intracellular staining

Whole blood was diluted 1:1 with RPMI and additives into a polystyrene tube. Surface markers were added at the required concentration (see table 2.6 below) and left in the
dark at room temperature for 30 minutes. 2 mls of Lysis solution (Optilyse C, Becton Dickinson) was then added to each tube and the samples were incubated for a further 10 minutes at room temperature. The samples were then spun at 1500 rpm for 5 minutes. If the samples were only stained with cell surface markers, they were washed once with FACS buffer (see table 2.5 above) and then resuspended in 250μl of FACS buffer or 0.5% paraformaldehyde/0.1% azide. If intracellular staining was required, the supernatant was discarded and 500μl of permeabilising buffer (Becton Dickinson) was added to each tube. Samples were incubated for 10 minutes in the dark at room temperature. If the intracellular antibody CD68 was used, it was added with the permeabilising buffer. 2 mls of FACS buffer was added to each tube and the samples were spun at 1500 rpm for 5 minutes, the supernatant was removed and the samples were vortexed. Intracellular antibodies were then added at the required concentration and incubated in the dark at room temperature for 30 minutes. After this the samples were washed with 2 mls of FACS buffer, spun at 1000 rpm for 5 minutes and the supernatant was thrown away. 200μl of fixing buffer (see table 2.5 above) was added to each tube and they were then kept for analysis later.

Table 2.6 Antibodies used in FACS staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conjugated to</th>
<th>Conc.</th>
<th>Binds to</th>
<th>Used for</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3</td>
<td>Chyochrome</td>
<td>1/40</td>
<td>Surface antigen</td>
<td>T cells</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-CD64</td>
<td>Phycoerythrin (PE)</td>
<td>1/20</td>
<td>Cell surface Antigen FcR1</td>
<td>Monocytes +/- neutrophils</td>
<td>Serotec</td>
</tr>
<tr>
<td>Anti-CD66b</td>
<td>Fluorescein isothiocyanate (FITC)</td>
<td>1/100</td>
<td>GP1 anchored molecule expressed on blood polymorphonuclear cells</td>
<td>Neutrophils</td>
<td>Serotec</td>
</tr>
<tr>
<td>Anti-CD68</td>
<td>FITC</td>
<td>1/50</td>
<td>CD68: transmembrane glycoprotein 111kDa</td>
<td>Macrophage and myeloid precursors</td>
<td>DAKO</td>
</tr>
<tr>
<td>Anti-IL-10</td>
<td>PE</td>
<td>1/20</td>
<td>Human IL-10</td>
<td></td>
<td>Serotec</td>
</tr>
</tbody>
</table>

2.4.4 Stimulation of whole blood culture prior to FACS analysis

Monensin (Sigma) was used for intracellular staining of IL-10 as a protein transport inhibitor. Monensin (60 μM) was added with the stimulant to a final concentration of 3 μM.

The following stimulants were used:
Table 2.7 Stimulants used in FACS analysis

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>0.15ng/ml</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>6ng/ml</td>
</tr>
<tr>
<td>LPS</td>
<td>1μg/ml</td>
</tr>
</tbody>
</table>

2.4.5 Acquisition and analysis of flow cytometry data

Samples were analysed using the FAScan FACS machine (BD) using Cellquest software. 5000 events per sample was acquired and analysed after setting for compensation using single stained tubes. Analysis gates were set on lymphocytes and monocytes according to forward and side scatter properties. Results are expressed as the percentage cells expressing either the cell surface marker or the intracellular cytokine.

2.5 Transfection Studies

2.5.1 Cell lines used

HeLa cells and THP-1 cells were obtained from the European Collection of Cell Culture (ECACC).

2.5.2 Bacterial growth reagents

Table 2.8 Bacterial growth reagents

<table>
<thead>
<tr>
<th>Bacterial growth reagents</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB Broth</td>
<td>EZMix LB Broth Base (Sigma)</td>
</tr>
<tr>
<td></td>
<td>Ampicillin (Sigma)</td>
</tr>
<tr>
<td>Agar Plates</td>
<td>Bacto-agar (Difco, Michigan)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Glycerol</td>
</tr>
<tr>
<td></td>
<td>50mg/ml</td>
</tr>
<tr>
<td></td>
<td>3g/150ml</td>
</tr>
<tr>
<td></td>
<td>20%</td>
</tr>
</tbody>
</table>

2.5.3 Luciferase vectors

The pGL3 Luciferase reporter vectors (Promega) were used because of increase in reporter gene expression over the pGL2 (Promega) vector allowing luciferase expression in cell types that are generally considered difficult to transfect. Each plasmid vector contains a high copy number prokaryotic origin of replication for maintenance in E Coli and an ampicillin resistance gene for selection. Restriction sites for insertion of DNA are located upstream and downstream of the luciferase gene (see figure 2.1).
Figure 2.1  *pGL3- promoter vector map (adapted from Promega catalogue)*

The pGL3-Promoter vector contains an SV40 promoter upstream of the luciferase gene. The IL-10 5' flanking region fragment is inserted as shown. The Luc+ is cDNA encoding the modified firefly luciferase. Amp' is the gene conferring ampicillin resistance in *E. coli*. F1 Ori is the origin of replication derived from filamentous phage whilst ori is the origin of plasmid replication in *E. Coli*. Arrows indicate the direction of transcription.

The vectors used in this study were the pGL3-Control and the pGL3-Basic vectors (Promega, Maddison, USA). The pGL3-control contains the SV40 promoter and
enhancer sequences so that there is strong luciferase expression. It is therefore used to monitor transfection efficiency as an internal standard. The pGL3-Basic does not have a promoter or enhancer sequence and can therefore be used for assessing the promoter activity of a ligated sequence. Expression of luciferase activity depends on insertion and proper orientation of the 5' flanking region insert upstream of the luciferase gene.

2.5.4 IL-10 Luciferase DNA reporter constructs

The IL-10 luciferase DNA reporter constructs were made by our collaborator Dr Richard Kay using the following method. The human IL-10 gene from base -1137 to +25 with respect to the initiation start site, was amplified by PCR from patients who were homozygous for GCC, ACC or ATA haplotypes using the primers AGATGAAGATCTCAACAGGCAGTGCTGAGCTGT and TTATTCGGGCGAAGACAACACTACTAAGGCTTC. The 50μl reaction mixture contained 500ng genomic DNA, 200μM dNTPs, 1.4 mM MgCl₂ and 25 pMoles of each primer. The reaction was hot started with 1U Bio-X-act polymerase (Bioline) and underwent 35 cycles of amplification with denaturation at 95°C for 30s, annealing at 60°C for 30s and extension at 72°C for 1 min with a final extension period of 5 min at 72°C.

Amplified products were cut with the restriction enzymes Xba I and Bgl II and cloned into the similarly restricted polylinker of the pGL3.basic firefly luciferase vector (Promega). The sequence of each construct was checked by MWG-Biotech and is shown in figure 2.2.
Figure 2.2 IL-10 haplotype sequences inserted in pGL.3 basic luciferase vector.

Each haplotype is shown. Biallelic polymorphism in red. PvuII restriction site in blue. Start codon indicated in green. TATAA box in yellow.
2.5.5 Transformation
The plasmid DNA was prepared from clones kindly donated by our collaborator Richard Kay (see above) transformed into E Coli JM109 (see Appendix A.)

2.5.6 RSVβ-gal transfection control plasmid
The Rous sarcoma virus (RSV) β-gal vector was used to measure transfection efficiency. The vector is based on pKS containing the β-galactosidase gene (lacZ) under the control of the RSV promoter. A full circular map of the vector is not available as it was originally the gift from another lab (Dr G. Nabel, University of Michigan Medical Centre, Ann Arbor, USA).

2.5.7 Bacterial culture
The Glycerol stocks of the clones were streaked onto Agar plates containing ampicillin (80mg/ml) and grown up over night at 37°C. A single colony was picked and inoculated into 5 mls of broth and grown up over 8 hours. The 5 mls was put into 400 mls of broth with ampicillin and grown up over 16 hours at 37°C in a shaker.

2.5.8 Glycerol Stocks
Glycerol stocks were made from transformed bacteria after three passages through media containing antibiotics by adding 0.8mls of bacterial culture to 0.2 mls 100% glycerol and freezing immediately at -70°C.

2.5.9 Maxiprep Methodology
The plasmids were then isolated using the Endofree Plasmid Maxikit (Qiagen) as described below. The DNA concentration was calculated by measuring the optical density using a spectrophotometer (Hitachi, U-1100). Readings were taken at OD of 260nm and 280nm. The constructs were used if the final concentration was greater than 1mg/ml and the ratio of 280 to 260 was less then 0.7.

2.5.10 Isolation of plasmids
2.5.10.1 Small scale plasmid preparation
Small quantities of plasmid DNA were prepared using the Wizard Miniprep DNA purification system (Promega, UK) according to the manufacturer’s protocol. 2.5 mls of bacterial culture was pelleted by centrifugation at 10,000 g in a microcentrifuge for 3 minutes. The cells were resuspended in 200μl of resuspension solution containing 50mM Tris-HCL, pH7.5, 10mM EDTA and 100μg/ml RNase A. The cells were lysed by adding 200μl of Lysis solution (0.2M NaOH and 1% SDS) and inverting the tube 4
times. 200μl of Neutralising solution was then added (1.32M potassium acetate, pH 4.8) and the tube was inverted 4 times. The solution was centrifuged at 10,000 g for 5 minutes. The cleared lysate was then mixed with 1ml of Wizard DNA purification resins (containing 7M Guanidine HSL) and drawn into a minicolumn by vacuum. The resin, which was bound to the plasmid, bound to the column. This was then washed to remove debris using 2 mls of Column wash solution (190mM potassium acetate, 20mM Tris-HCL, pH7.5 and 1mM EDTA in 55% Ethanol). An additional step was added to improve purity and this was an additional wash with 70% ethanol. After drying, the plasmid was eluted in 50μl of SDW and stored at -20°C until required.

2.5.10.2  **Large scale plasmid preparation**

The Endofree Plasmid Maxikit (Qiagen) was used for large scale plasmid preparation. The principles of the method are outlined below. This method of plasmid purification is based on a modified alkaline lysis procedure. The Qiagen tip has anion-exchange resin. RNA and proteins are removed by a medium salt wash. The DNA is eluted with a high-salt buffer and then desalted using the isopropranol precipitation step.

**Table 2.9 Buffers used in Endofree Plasmid Maxikit**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer P1</td>
<td>50mM Tris-Cl. pH 8; 10mM EDTA; 100μg/ml Rnase</td>
<td></td>
</tr>
<tr>
<td>Buffer P2</td>
<td>Lysis buffer: 200mM NaOH, 1% SDS</td>
<td>Buffer P2 is NaOH-SDS. The SDS causes lysis of the cells and release of the cell contents. The NaOH denatures the chromosomal and plasmid DNAs and proteins.</td>
</tr>
<tr>
<td>Buffer P3</td>
<td>Neutralisation buffer: 3M potassium acetate(pH 5.5)</td>
<td>This neutralises Buffer P3. The high salt concentration causes the SDS to precipitate and the denatured proteins, chromosomal DNA and cellular debris becomes trapped in salt-detergent complexes. The plasmid DNA however remains in solution and is separated from the debris when it is passed through the Qiagen cartridge</td>
</tr>
<tr>
<td>Buffer QC</td>
<td>Wash buffer: 1 M NaCl, 50 mM MOPS, pH 7; 15% isopropranol</td>
<td>Medium salt buffer</td>
</tr>
<tr>
<td>Buffer QN</td>
<td>Elution buffer: 1.6M NaCl; 10mM MOPS, pH 7, 15% isopropranol</td>
<td>High salt buffer.</td>
</tr>
</tbody>
</table>
Centrifugation of bacterial cells at 6000 x g for 10 minutes using a Sorvall SLA300 rotor

Resuspend pellet in 10 mls of buffer P1

Add 10 mls of Buffer P2, invert 4 to 6 times

Add 10 mls of chilled Buffer P3, invert X 4 to 6

Incubate 10 minutes in cartridge

Filter cell lysate through cartridge

Add 2.5 mls of Buffer ER, incubate on ice for 30

Apply lysate to Qiagen-tip, resin enters by gravity

Wash Qiagen-tip with Buffer QC

Elute DNA with Buffer QN

DNA precipitated with 10.5 mls of isopropanol and centrifuged at 15000 x g for 30 minutes

DNA washed twice with endotoxin free 70% ethanol

Dry pellet at room temperature

Redissolve in 200 μls TE

Figure 2.3 Endofree Plasmid Maxikit (Quigen) - outline of the method.
2.5.11 Reporter gene assay

200μl of reporter Lysis buffer (x 1, Promega) was added to each well before freezing the plates at -70°C. This lysis buffer is suitable for β-galactosidase and luciferase assay. After thawing, the lysate was scraped from the plates, placed into eppendorf tubes and spun at 14000 rpm for 5 minutes prior to assay.

2.5.11.1 Luciferase assay

The luciferase assay system relies on a reaction catalysed by the firefly luciferase which is the oxidation of beetle luciferin with concomitant production of a photon using coenzyme A (CoA) as a substrate. As CoA is used, there is almost constant emission of light for up to 60 minutes which eliminates the need for rapid mixing protocols making each assay more reproducible.

20μl of supernatant each was mixed with 100μl of room temperature Luciferase Assay Reagent (Promega, UK) and placed into a Luminometer (Bioorbit 1253, Finland). The luminescence was measured for 15 seconds twice and the average taken. Background luminescence was subtracted from the readings by the luminometer.

2.5.11.2 RSV β-galactosidase assay

The results were standardised for β-galactosidase using the Galacto-light Plus reporter gene assay (Tropix, USA). Briefly, 20μl of supernatant was mixed with 200μl of β-galactosidase reaction buffer and left to incubate for at least one hour at room temperature at which point 300μl of room temperature light emission accelerator was added. The luminescence was measured for 5 seconds and repeated twice. The average of the three readings was used.

2.5.12 Protein assay

440μl of CuSO₄ (4%) was added to 22 mls of Bicinchoninic acid solution (Sigma) to make the reagent. 200μl of reagent was added to each well on a 100 well plate. The standards were made by diluting bovine serum albumin (BSA) to a stock solution (1mg/ml) with lysis buffer and then diluted to the following concentrations using sterile distilled water: 0.1 mg/ml, 0.2 mg/ml, 0.5 mg/ml, 0.8 mg/ml and 1 mg/ml. 10μl of sample was then added to the reagent. Samples were either added neat or diluted 1:5 with sterile distilled water. Each sample was done in triplicate. The OD was measured at 562nm after a 30 minute incubation at 37°C.
2.5.13 Transfection of HeLa cells using Calcium Phosphate method

2.5.13.1 Reagents used for transfection of HeLa cells

Table 2.10 Reagents used for transfection of HeLa cells

<table>
<thead>
<tr>
<th>Media</th>
<th>Additives</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbeccos modified Eagles Medium (Gibco BRL)</td>
<td>L-Glutamine (Sigma)</td>
<td>2mM</td>
</tr>
<tr>
<td></td>
<td>Non essential amino acids</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>Penicillin and streptomycin (Sigma)</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>FCS (Insight Biotech)</td>
<td>10%</td>
</tr>
<tr>
<td>Hepes buffered Saline (x 10)</td>
<td>NaCl</td>
<td>8.18%</td>
</tr>
<tr>
<td></td>
<td>Hepes (Sigma)</td>
<td>5.94%</td>
</tr>
<tr>
<td></td>
<td>Na$_2$HPO$_4$</td>
<td>0.2%</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>CaCl$_2$</td>
<td>2M</td>
</tr>
<tr>
<td>TE</td>
<td>Tris</td>
<td>1M</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>0.5 M</td>
</tr>
</tbody>
</table>

All stock solutions were sterilised by filtration through a 0.2μm nitrocellulose membrane (Gelman).

2.5.13.2 Culture of HeLa cells

HeLa cells were cultured in Dulbeccos modified Eagles Medium with additives as above at 5% CO$_2$ and 37°C.

72 hours prior to transfection, the medium was removed and 1 ml of filtered trypsin was added to each flask for 5 seconds to wash out the Ca$^{2+}$ and Mg$^{2+}$. 2.5 mls of trypsin was then added to each flask for 3 to 4 minutes. When the cells had detached from the flask, 10 mls of medium was added and the cells were spun for 5 minutes at 1000rpm. The medium was aspirated and a fresh 10 mls added. The cells were then counted on a haemocytometer and seeded into 6 well plates at a density of 0.5 x 10$^6$ cells per well.

2.5.13.3 Transfection of HeLa cells

All transfections were carried out when the HeLa cells were in log phase by changing the media 24 hours previously. On the day of transfection fresh Hepes buffered saline (x 2) (see table 2.10) was adjusted to pH 7.12 +/- 0.05 using 2 M NaOH. DNA (10μg of
construct and 3µg of RSVβ galactosidase) was added to 25µl of filtered CaCl₂ (2M) and
to a total volume of 200µl for each experiment. The DNA mixture was then added
to 200µl of the prepared Hepes Buffered Saline (pH 7.12) by dropping the DNA down
the side of the polypropylene tubes. After 30 minutes incubation at room temperature,
the mixture was then re-suspended and dropped onto the cells in each well. The cells
were then incubated for between 4 and 16 hours, washed with medium and stimulated.
When the cells were ready to harvest, the media was removed and 200µl of reporter
lysis buffer (Promega) was added and the samples were immediately frozen to -70°C.
2.5.14 Transfection of THP-1 cells

2.5.14.1 Reagents for Transfection of THP-1 cells

Table 2.11 Reagents for culture of THP-1 cells

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Ingredients</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture media</td>
<td>RPMI 1640 (hybrimax grade)</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>Penicillin/Streptomycin (Sigma)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-Glutamine (Sigma)</td>
<td>2mM</td>
</tr>
<tr>
<td></td>
<td>Non essential amino acids</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>Mercaptopurine</td>
<td>10^{-5} mM</td>
</tr>
<tr>
<td></td>
<td>FCS (Insight Biotech)</td>
<td>20% or 10%</td>
</tr>
<tr>
<td></td>
<td>50μg/ml ethanol</td>
<td>0.05 μg/ml</td>
</tr>
<tr>
<td>1α, 25-dihydroxycholecalciferol (Calbiochem)</td>
<td>Stock solution=1mg/ml RPMI-1640</td>
<td>1μg/ml</td>
</tr>
<tr>
<td>Lipopolysaccharide (Sigma)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.12 Reagents for transfection using DEAE Dextran method

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Stock solution</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>1M, pH 7.3</td>
<td>50mM</td>
</tr>
<tr>
<td>DEAE-Dextran (Sigma)</td>
<td>100mg/ml (5M)</td>
<td>1.5U/ml</td>
</tr>
<tr>
<td>Heparin (CP Pharmaceuticals)</td>
<td></td>
<td>1.2%</td>
</tr>
<tr>
<td>DMSO (Sigma)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.5.14.2 Culture of THP-1 cells

THP-1 cells were cultured in RPMI 1640 (Sigma) supplemented with penicillin and streptomycin, L-glutamine, non essential amino acids and 10% foetal calf serum (or 20% foetal calf serum for DEAE dextran method) at 4% CO_2 and 37°C. Cell density was maintained at approximately 2 x 10^5 cells per ml. The THP-1 cells were fed weekly by dilution 1:5 with fresh media.

2.5.14.3 Differentiation of THP-1 cells

The THP-1 cells were seeded at a concentration of 4 x 10^5 cells per well to a total final volume of 1.5 mls per well. The THP-1 cells were differentiated by adding 1,25-dihydroxycholecalciferol (Calbiochem) at a final concentration of 0.05 μg/ml when the cells were seeded. 1,25-dihydroxycholecalciferol was also added at 48 hours after seeding to a final concentration of 0.05 μg/ml to differentiate the THP-1 cells which
then become adherent (Miyaura, 1981). Differentiation was checked by measuring CD14 expression (see section 2.4.2). The cells were incubated for 72 hours or until adherence of cells such that 50 to 80\% confluence was achieved (maximum incubation time 92 hours). All transfections were carried out when the cells were in log phase by changing the media 24 hours prior to transfection.

### 2.5.15 Transfection of THP-1 cells using liposomal preparations

The methods described are those recommended by the manufacturers. These have then been optimised for transfection with THP-1 cells. For details on optimisation see sections 3.6.4.3 to 3.6.4.5.

#### 2.5.15.1 Transfection with Fugene 6 (Boeringer Mannheim)

3\(\mu\)l of Fugene 6 reagent was added to 97\(\mu\)l of RPMI (serum free) taking care that the reagent did not touch the sides of the tube. The Fugene mixture was added drop wise to 2\(\mu\)g of DNA (1.5\(\mu\)g construct), the contents were tapped gently and then left to incubate for 15 minutes at room temperature. The DNA/Fugene mixture was then added drop wise to the cells which were then incubated for 24 hours.

#### 2.5.15.2 Transfection with DCChol:DOPE liposomes

##### 2.5.15.2.1 Liposome formulation

The liposomes were prepared by our collaborator Andrew Miller, Department of Chemistry Imperial college by using the following standard formulation.

6\(\mu\)mol of 3b-[N-(N,N-dimethylaminoethane) carbamoyl] cholesterol (DCChol) and 4\(\mu\)mol of dioleoyl L-a-phosphatidylethanolamine (DOPE) (Sigma, Poole, UK) was added to freshly distilled CH\(_2\)Cl\(_2\) (5ml) under nitrogen. 20mM N-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonic acid) (HEPES; Sigma) buffer pH 7.8 (5ml) was then added and the mixture sonicated for 3 min. Organic solvents were removed under reduced pressure and the resulting liposome suspension was then sonicated for a further 3 min. The liposomes (1.2mg/ml) were stored at 4\(^\circ\)C before use.

10\(\mu\)g of DNA (7.5\(\mu\)g of construct and 2.5 \(\mu\)g of RSV \(\beta\)galactosidase construct) was added to the liposomal preparation in a polystyrene tube (Falcon 2052) and incubated for 5 minutes. The THP-1 cells were washed twice with serum free RPMI and the liposomal/DNA mixture was added to the cells and left to incubate for an hour. The liposomal mixture was then gently aspirated and replaced with RPMI and serum and incubated for 24 hours at 37\(^\circ\)C and 4\% CO\(_2\).
2.5.15.3 Transfection with Superfect (Qiagen)

2 μg of DNA (of which 1.5 μg was the construct) was dissolved in plain RPMI to a total volume of 100 μl. 10 μl of Superfect Transfection Reagent was added to the DNA and the mixture was pipetted up and down 5 times. The samples were incubated for 5 minutes at room temperature to allow complex formation. During this incubation the medium was aspirated from each well and the THP-1 cells were washed with complete medium. 600 μl of RPMI (with FCS, antibiotics and Vitamin D) was then added to each reaction tube and the total volume was added to each well. The final concentration of vitamin D was 0.05 μg/ml. The cells were then incubated in complete RPMI with vitamin D at a final concentration of 0.05 μg/ml for 24 hours.

2.5.15.4 Transfection with Transfast (Promega)

1.25 μg to 3.75 μg of DNA was added to 1 ml of RPMI (serum free). The Transfast reagent was then added to the mixture in either a 1:1 or a 2:1 ratio with the DNA. The Transfast reagent/DNA mixture was then incubated for 10 minutes at room temperature. The RPMI was removed from the cells before adding DNA mixture to the THP-1 cells which were incubated for either 24 or 48 hours.

2.5.15.5 Transfection with Lipotaxi (Stratagene)

10 μg of DNA (7.5 μg construct) was added to 300 μl of RPMI with 12 μl of Lipotaxi transfection reagent. The mixture was incubated for 15 minutes at room temperature. The media was removed from the cells. 700 μl of RPMI was added to the DNA/Lipotaxi mixture and the entire mixture was added to the THP-1 cells dropwise whilst swirling the dish which was then incubated for 4-6 hours at 37°C and 5% CO2. The RPMI was then replaced with RPMI with 20% FCS and incubated for 14 hours when the RPMI was replaced again with RPMI/10% FCS.

2.5.15.6 Transfection with Effectene (Qiagen)

THP-1 cells were plated out at 2 x 10^5/ml with 1 ml per well. Half a ml of RPMI with vitamin D was added giving a final concentration of vitamin D of 0.05 μg/ml. On day 2, a further 0.5 mls of RPMI with vitamin D was added to increase adherence (final concentration 0.05 μg/ml). For each transfection, 0.4 μg of DNA (0.3 μg of construct and 0.1 μg of βgal) was added to 100 μl of buffer EC to which 3.2 μl of Enhancer was added and then vortexed for 1 second before an incubation of 5 minutes at room temperature. 4 μl of effectene was then added and mixed before an incubation of 5 to 10 minutes at room temperature to allow complex formation. The medium was aspirated
from the cells and the cells were washed once with RPMI before adding 1.6 mls of fresh RPMI (with vitamin D (final concentration 0.05μg/ml) to each well. The transfection mixture was added dropwise to each well before incubation at 37°C and 5% CO₂.

2.5.16 mRNA extraction and cDNA isolation from THP-1 cells
THP-1 cells were resuspended at 2 x 10⁵ cells per ml in a total volume of 100mls. Vitamin D was added to each flask (final concentration 0.05μg/ml). Two out of four 100 ml flasks were transfected using Effectene. 24 hours after transfection, the cells were harvested by removing the medium from all 4 flasks. 10 mls of ice cold RPMI was added to each flask and the cells scraped off. The supernatant was spun at 1500rpm for 5 minutes, the media was removed and 4 mls of RNAzol (Cinna/Biotecx) was added to each pellet. The mRNA was then extracted as described above (section 2.3.4).

2.5.17 Stimulation with IL-1β, TNFα and IL-6
THP-1 cells were transfected using Effectene and stimulated with IL-1β at the following concentrations: 20pg/ml, 40pg/ml 400pg/ml 800 pg/ml. TNF at the following concentrations: 1ng/ml, 2ng/ml 5ng/ml 10ng/ml and IL-6 at the following concentrations: 0.125 U/ml, 0.25U/ml, 0.75 U/ml. The IL-1β was diluted in RPMI and additives to a final concentration of 40pg/μl prior to stimulation and the TNFα and IL-6 were diluted respectively to 1ng/μl and 125U/ml prior to stimulation.

2.5.18 Transfection of THP-1 cells using DEAE dextran method
THP-1 cells (passage less then 30) were resuspended to 1 x 10⁶/ml in RPMI/20% FCS and 50mM Tris (pH 7.3). 40μg of plasmid DNA (32 μg construct and 8 μg of RSV-βgal) and 50μl of DEAE-dextran (100mg/ml) were then added to 20 mls of the resuspended cells for each condition and then incubated for 1 hour at 37°C/5% CO₂. The cells and media were then spun for 8 minutes at 1000rpm and washed once with 20 mls of RPMI and 100mM (1.5U/ml) heparin followed by two washes of RPMI/20% FCS. The cells were then resuspended to 1 x 10⁶/ml in RPMI/10% FCS and vitamin D (0.05μg/ml) to a total of 10 mls in a T-25 flask (Nunc). The suspensions were incubated for 18 hours at 37°C and 5% CO₂ before being treated with 1.2% DMSO for 16 hours after which they were stimulated with LPS at 1μg/ml. After 8 hours of stimulation the cells were scraped from the base of the flask and the solution was spun at 1500rpm for 5 minutes, the media removed and the cells lysed using reporter lysis buffer (Promega).
2.6 Statistical methodology

2.6.1 Genotype and haplotype association studies
To test our hypothesis that disease severity is associated with an IL-10 5' flanking region haplotype that has lower transcriptional activity, the genotypes defined by SSOP were expressed as 0, 1 and 2 ATA haplotypes. Comparison was then made between different groups using the Chi-square test.

Power size calculations estimated that 90 patients were needed in each patient group for the test to have an 80% chance of finding a 10% difference between groups.

2.6.2 TDT analysis
The TDT was performed by analysing transmission of alleles as if it were a biallelic system. I.e. ATA versus non-ATA or GCC versus non-GCC. Children were diagnosed as having oligoarthritis or extended oligoarthritis and eye disease or no eye disease using the current ILAR definitions. Children who had oligoarthritis with no eye disease were considered to be controls.

Cumulative results were then analysed using the McNemar test.

2.6.3 Linkage analysis
Linkage analysis was performed on informative genotypes (i.e. where either the microsatellite or the SNPs were homozygous using SPSS version 8 statistical software. Exact Chi square analysis was performed using Monte Carlo simulations (100000).

2.6.4 Whole blood culture results
2.6.4.1 Comparison of PBMC and whole blood culture
The Mann-Witney U test was used to compare PBMC and whole blood culture results.

2.6.4.2 Association of IL-10 production with genotype
The Shapiro-Wilk statistic was used to test for normality. The Kruskel-Wallis test was used to analyse groups with different genotypes. If groups were significantly different then the Mann-Witney U test was used to test individual groups.

2.6.4.3 Comparison of IL-10 production in different parents
If results from both parents were available, the result between the mother and the father was averaged. If only one parent was available the result from one parent was used.
Parents of children with oligoarthritis and no eye disease were considered to be controls. The Shapira-Wilk statistic determined that the data had a normal distribution.
Comparisons both groups and the control group were therefore made using the Student’s T test.

2.6.4.4 Effect of ethanol and methylprednisolone on IL-10 production ex-vivo

Each sample group was tested for normality using the Kolmogorov-Smirnov test. If the data did not have a normal distribution it was logged and then retested. Results were analysed using either paired T test on logged data or the Wilcoxon rank test if the data could not be normalised.

2.6.5 Transfection results

For the transient transfection experiments, variance between groups was analysed non-parametrically using the Kruskal-Wallis test. If groups were significantly different using this test, individual groups were compared using the Wilcoxon rank sum test.
CHAPTER 3: RESULTS
3.1 Genotype analysis

3.1.1 Rationale for genotype analysis

Previous work by Turner et al (Turner, 1997) had already suggested that healthy controls which were GCC homozygous had higher IL-10 production than other genotypes. Transient transfection studies by our collaborator, Richard Kay, had demonstrated that reporter gene assay expression was higher using constructs with the GCC haplotype than the ATA haplotype in U937 cells stimulated with dbcAMP (Crawley, 1999c).

We therefore hypothesised that the ATA haplotype would be associated with more severe arthritis because of the lower capability to produce the anti-inflammatory cytokine IL-10. We were particularly interested in the children with oligoarticular onset because we hypothesised that a genotype predisposing to a pro-inflammatory response may determine the subgroup of children that later developed extended oligoarticular JIA. We therefore compared the genotype distribution between children with oligoarticular JIA, extended oligoarticular JIA and healthy controls.

Association studies were used as the first stage to investigate haplotype and genotype distribution between controls and patients with JIA because at the time the study was undertaken there were insufficient parental samples to make the TDT sufficiently powerful statistically. The studies are now being repeated using the TDT and preliminary results are described.

Genotypes were defined by SSOP of amplified PCR products. The advantage of SSOP is that it is fast and allows rapid genotype determination from many samples simultaneously. Since starting this thesis it has been validated and used in several published studies (Hajeer, 1998; Lazarus, 1997; Coakley, 1998; Turner, 1997). The methods used are described in section 2.3.5.

An example of typical PCR products is shown in figure 3.1. SSOP films were always read independently by two investigators using numbered samples. An example of a typical film is given in figure 3.2. In common with other investigators we were able to assign haplotypes in every case when the alleles were identified through SSOP (Hajeer, 1998; Lazarus, 1997; Coakley, 1998; Turner, 1997). This is because the alleles are in linkage disequilibrium and only 3 haplotypes have been observed in Caucasian populations.
Figure 3.1 *IL-10 PCR products*

PCR from genomic DNA run on an agarose gel in lanes A to D. Lane E = negative control Ladder = 1 kb (Promega).
Figure 3.2. *Radiograph of sequence specific oligonucleotide products.*

□ = GCC/GCC, □ = GCC/ATA, □ = ATA/ATA

### 3.1.2 Genotype and haplotype distribution in controls

#### 3.1.2.1 Controls used for genotype analysis

All controls were Anglo-Saxon Caucasians from the UK and were obtained from several sources. For the association study analysis, two groups were used, one group of controls were laboratory personnel from central and north London (N=127). The other consisted of male patients from two general medical practices in north London (N=147).

There was no significant difference in haplotype or genotype distribution between the controls obtained from laboratory personnel from central and north London (N=127) or the male patients from two general medical practises in north London (N=147)
There was no difference in haplotype distribution between female and male controls \((P=0.98)\). The results were also comparable to previous studies (Turner, 1997).

The controls that were obtained for linkage analysis (see section 3.1.7.2) were not included in the association studies described here because they were from a different population geographically. Comparison of genotype frequency for this population with the controls used showed no significant difference between these control groups (controls 2 and 3) and the controls used for the association studies (results pooled as control 1) as shown in the table 3.1.

**Table 3.1 Genotype frequency in different control groups**

<table>
<thead>
<tr>
<th>Control</th>
<th>GCC/GCC</th>
<th>GCC/ACC</th>
<th>GCC/ATA</th>
<th>ACC/ACC</th>
<th>ACC/ATA</th>
<th>ATA/ATA</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70(25.5%)</td>
<td>70(25.5%)</td>
<td>54(19.7%)</td>
<td>24(8.8%)</td>
<td>38(13.9%)</td>
<td>18(6.6%)</td>
<td>274</td>
</tr>
<tr>
<td>2</td>
<td>19(24.4)</td>
<td>19(24.4)</td>
<td>21(26.9)</td>
<td>7(8.97)</td>
<td>9(11.5)</td>
<td>3(3.8)</td>
<td>78</td>
</tr>
<tr>
<td>3</td>
<td>27(32.5)</td>
<td>31(37.3)</td>
<td>14(16.9)</td>
<td>3(3.6)</td>
<td>6(7.2)</td>
<td>2(2.4)</td>
<td>83</td>
</tr>
</tbody>
</table>

Control 1: those used for haplotype and genotype analysis in patients with JIA. Control 2 and control 3: controls used for linkage disequilibrium analysis (see section 3.1.7.2)

**3.1.3 Juvenile Idiopathic Arthritis**

All patients were Anglo-Saxon Caucasians from the UK and were obtained either from Paediatric Rheumatology clinics held at Great Ormond Street Hospital for Sick Children, the Middlesex Hospital or from the United Kingdom paediatric Rheumatology HLA database (ARC Epidemiology Unit, Manchester). Patients were clinically classified according to the ILAR classification (see Appendix B).

SSOP was carried out on 274 Caucasian controls and 444 Caucasian children with JIA. Samples were collected consecutively from children attending clinic and were numbered to allow the analysis to be blind. This means that genotypic data was collected on all children attending clinic and not just those with oligoarticular onset.

**3.1.3.1 IL-10 5' flanking region genotypes in patients with JIA**

The distribution of genotypes is shown in table 3.2. Although there appears to be fewer patients possessing the ATA/ATA homozygous genotype in the subgroups with oligoarticular or systemic JIA, the numbers in each subgroup according to genotype are too small for statistical analysis. There was no change in the results if the patients were stratified by age. Those with psoriatic arthritis appear to be more likely to possess the GCC homozygous genotype but this is not statistically significant. Those with polyarticular
rheumatoid factor negative arthritis are also more likely to possess a GCC/GCC genotype (p=0.05). However, as this was an observation outside the initial aims of our investigation it must be treated with caution and should undergo a Bonferroni correction (Bland and Altman 1995). The Bonferroni correction is used to correct for multiple tests where there is an increased risk of a type 1 error (false positive). It is not necessary when one hypothesis is being tested but if we investigated an association with all disease subgroups (7 in total) the p value would be 7 x 0.05 i.e. 0.35 which would make the result non-significant (Perneger 1998). The association between psoriatic arthritis and the GCC/GCC genotype is an interesting observation however and to test whether the association is true one could use an alternative method such as the TDT or examine another group of patients.

Table 3.2  *IL-10 genotypes in controls and children with JIA*

<table>
<thead>
<tr>
<th></th>
<th>GCC/GCC</th>
<th>GCC/ACC</th>
<th>GCC/ATA</th>
<th>ACC/ACC</th>
<th>ACC/ATA</th>
<th>ATA/ATA</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>70(25.5)</td>
<td>70(25.5)</td>
<td>54(19.7)</td>
<td>24(8.8)</td>
<td>38(13.9)</td>
<td>18(6.6)</td>
<td>274</td>
</tr>
<tr>
<td>Oligoarthritis</td>
<td>22(25.6)</td>
<td>23(26.7)</td>
<td>20(23.3)</td>
<td>9(10.5)</td>
<td>9(10.5)</td>
<td>3(3.5)</td>
<td>86</td>
</tr>
<tr>
<td>Ext. Oligo</td>
<td>16(20.5)</td>
<td>16(20.5)</td>
<td>24(30.8)</td>
<td>5(6.4)</td>
<td>11(14.1)</td>
<td>6(7.7)</td>
<td>78</td>
</tr>
<tr>
<td>Poly. RF-</td>
<td>24(37.5)</td>
<td>13(20.3)</td>
<td>12(18.8)</td>
<td>3(4.7)</td>
<td>7(10.9)</td>
<td>5(7.8)</td>
<td>64</td>
</tr>
<tr>
<td>Poly. RF+</td>
<td>5(29.4)</td>
<td>3(17.6)</td>
<td>4(23.5)</td>
<td>2(11.8)</td>
<td>2(11.8)</td>
<td>1(5.9)</td>
<td>17</td>
</tr>
<tr>
<td>Systemic JIA</td>
<td>22(21.2)</td>
<td>28(26.9)</td>
<td>24(23.1)</td>
<td>9(8.7)</td>
<td>17(16.3)</td>
<td>4(3.8)</td>
<td>104</td>
</tr>
<tr>
<td>Enthesitis associated</td>
<td>17(30.4)</td>
<td>11(19.6)</td>
<td>8(14.3)</td>
<td>4(7.1)</td>
<td>12(21.4)</td>
<td>4(7.1)</td>
<td>56</td>
</tr>
<tr>
<td>Psoriatic arthritis</td>
<td>12(40)</td>
<td>8(26.7)</td>
<td>3(10)</td>
<td>2(6.7)</td>
<td>3(10)</td>
<td>2(6.7)</td>
<td>30</td>
</tr>
</tbody>
</table>

Genotype frequency in patients with JIA and controls. Actual number of patients with each genotype given with (%)

3.1.3.2  *ATA genotypes in patients with oligoarticular or extended oligoarticular JIA*

If a haplotype is physiologically important, we would expect to see a gene dosage effect with disease. This means that the effect of 2 genes (or haplotypes) would be greater than 1 gene (or haplotype) which would be greater than none. The exception to this would be if the effect of one gene acts dominantly rather than recessively and this is unlikely in disease which is polygenic. We therefore examined whether there was a gene dosage effect for the ATA haplotype in children with arthritis.

The gene dosage of ATA haplotypes in controls and patients with JIA is shown in table 3.3. Children with oligoarthritis are more likely to have 0 ATA containing genotypes (i.e. GCC/GCC or GCC/ACC or ACC/ACC genotypes) than patients with extended...
oligoarticular JIA and less likely to have a genotype containing 2 ATA haplotypes (p<0.05. OR = 1.9, 96% CI: 1 – 3.5).

Table 3.3  IL-10 ATA haplotypes in controls and children with JIA

<table>
<thead>
<tr>
<th></th>
<th>0 ATA</th>
<th>1 ATA</th>
<th>2 ATA</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>164(59.9)</td>
<td>92(33.6)</td>
<td>18(6.6)</td>
<td>274</td>
</tr>
<tr>
<td>Oligoarthritis</td>
<td>54(62.8)</td>
<td>29(33.7)</td>
<td>3(3.5)</td>
<td>86</td>
</tr>
<tr>
<td>Extended oligoarthritis</td>
<td>37(47.4)‡</td>
<td>35(44.9)</td>
<td>6(7.7)</td>
<td>78</td>
</tr>
<tr>
<td>Polyarticular, RF-</td>
<td>40(62.5)</td>
<td>19(29.7)</td>
<td>5(7.8)</td>
<td>64</td>
</tr>
<tr>
<td>Polyarticular, RF+</td>
<td>10(58.8)</td>
<td>6(35.3)</td>
<td>1(5.9)</td>
<td>17</td>
</tr>
<tr>
<td>Systemic arthritis</td>
<td>59(56.7)</td>
<td>41(39.4)</td>
<td>4(3.8)</td>
<td>104</td>
</tr>
<tr>
<td>Enthesitis associated</td>
<td>32(57.1)</td>
<td>20(35.7)</td>
<td>4(7.1)</td>
<td>56</td>
</tr>
<tr>
<td>Psoriatic arthritis</td>
<td>22(73.3)</td>
<td>6(20)</td>
<td>2(6.7)</td>
<td>30</td>
</tr>
</tbody>
</table>

Genotype according to number of ATA haplotypes. 0ATA = GCC/GCC, GCC/ACC and ACC/ACC. 1 ATA = GCC/ATA and ACC/ATA. 2 ATA = ATA/ATA. Number of patients with each genotype is given with (5). ‡ Patients with extended oligoarthritis are less likely to have 0 ATA haplotypes than patients with oligoarthritis (p<0.05. OR = 1.9, 96% CI: 1 – 3.5)).

This is consistent with our hypothesis in that there may be a protective effect of high IL-10 producing haplotypes. Although there is a concomitant increase in the number of genotypes containing 1 ATA and 2 ATA haplotypes in patients with extended oligoarthritis, this result is not significant.

3.1.3.3  ATA containing genotypes in children with uveitis

As IL-10 has been implicated in animal models of uveitis, the data was then analysed to look at the association of genotypes containing the ATA haplotypes in patients with uveitis. As uveitis is identical pathologically in each disease subtype, patients were included irrespective of whether they had oligoarticular or extended oligoarticular JIA. Children with no uveitis had all been examined by an ophthalmologist at the time the blood sample was collected for genotype analysis.

As can be seen in table 3.4, there is an increase in ATA containing genotypes in patients with uveitis when compared to those without uveitis however this was not statistically significant. However, this could be a type 2 error (false negative) as there are only 41 patients in the disease group. Power calculations have suggested that to have an 80% chance of detecting a 10% difference in genotype frequency, 90 patients would need to
be studied in each group. At this point in time therefore, there is insufficient power to have a reasonable chance to detect an association.

Table 3.4  **IL-10 ATA haplotypes in JIA patients with uveitis**

<table>
<thead>
<tr>
<th></th>
<th>0 ATA</th>
<th>1 ATA</th>
<th>2 ATA</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uveitis</td>
<td>10(55.6)</td>
<td>7(38.9%)</td>
<td>1(5.6)</td>
<td>18</td>
</tr>
<tr>
<td>extended oligoarthritis</td>
<td>9(39)</td>
<td>10(43.5)</td>
<td>4(17.4)</td>
<td>23</td>
</tr>
<tr>
<td>total</td>
<td>19(46.3)</td>
<td>17(41.5)</td>
<td>5(12.2)</td>
<td>41</td>
</tr>
<tr>
<td>No uveitis</td>
<td>28(68.3)</td>
<td>12(29.3)</td>
<td>1(2.4)</td>
<td>41</td>
</tr>
<tr>
<td>oligoarthritis</td>
<td>22(48.8)</td>
<td>21(46.7)</td>
<td>2(4.4)</td>
<td>45</td>
</tr>
<tr>
<td>extended oligoarthritis</td>
<td>30(58.1)</td>
<td>33(48.4)</td>
<td>3(3.5)</td>
<td>86</td>
</tr>
</tbody>
</table>

Genotype according to ATA haplotypes. 0 ATA = GCC/GCC, GCC/ACC and ACC/ACC. 1 ATA = GCC/ATA or ACC/ATA. 2 ATA = ATA/ATA. Numbers for each genotype given with (%).

### 3.1.4 Systemic Lupus Erythematosus (SLE)

#### 3.1.4.1 Rationale

IL-10 production is higher in both serum and PBMC of patients with SLE when compared to controls (Lacki, 1997a; Lacki, 1997b; Yang, 1997; Llorente, 1994). There is some evidence that IL-10 production in these patients may be genetically controlled (Llorente, 1997). Associations with microsatellite alleles and patients with SLE have been described (Eskdale, 1997; Mehrian, 1998). However, these associations are likely to be microsatellite alleles in linkage disequilibrium with a functional gene such as SNPs in the 5' flanking region which alter IL-10 transcription. We therefore decided to investigate haplotype and genotype distribution in patients with SLE. In addition to investigating susceptibility to SLE we investigated characteristics of SLE that are associated with high IL-10 production. We also decided to repeat previous investigations where associations with the IL-10 SNP haplotypes had previously been described but using a larger patient group.

Subgroup analysis therefore included patients with renal disease, positive dsDNA antibodies and anti Ro antibodies. Renal disease was studied because decreasing IL-10 results in a decreased risk of developing proteinuria in an animal model of SLE (Kalechman, 1997). An association has also been described between the ATA haplotype and Chinese patients with SLE who develop nephritis (Mok, 1998). dsDNA antibodies could be expected to be associated with high IL-10 producing haplotypes.
because dsDNA titres are related to IL-10 levels in patients with SLE (Houssiau, 1995; Jouvenne, 1998). An association between anti-Ro antibodies and GCC/ACC haplotypes has been described in patients with SLE and therefore this analysis was repeated in our study (Lazarus, 1997).

### 3.1.4.2 SLE patients

All patients (n=120) were UK Anglo-Saxon Caucasians from the same geographical area and met four or more of the revised ACR criteria for the disease. Renal disease was assessed histologically (n=50). A patient was considered to be anti-dsDNA positive if at least three ELISA measurements were twice the recommended upper limit of normal (50IU/l). In addition, virtually all of these patients were positive by Crithidia. There was no difference in genotype distribution between patients with SLE and controls. There was no difference in genotype distribution between any of the disease phenotypes shown. There was no association between any of the three haplotypes and renal disease or any laboratory marker including anti Ro antibodies. In agreement with other studies (Lazarus, 1997; Mok, 1998) we did not show an association between the haplotypes and susceptibility to SLE. We were also unable to reproduce previous work showing associations between patients with renal disease or anti-ro antibodies and IL-10 5' flanking region haplotypes.

#### Table 3.5 IL-10 5' flanking region SNP haplotypes in patients with SLE and controls.

<table>
<thead>
<tr>
<th></th>
<th>GCC/GCC</th>
<th>GCC/ACC</th>
<th>GCC/ATA</th>
<th>ACC/ACC</th>
<th>ACC/ATA</th>
<th>ATA/ATA</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>70 (25.5)</td>
<td>70 (25.5)</td>
<td>54 (19.7)</td>
<td>24 (8.8)</td>
<td>38 (13.9)</td>
<td>18 (6.6)</td>
<td>274</td>
</tr>
<tr>
<td>SLE</td>
<td>27 (22.5)</td>
<td>40 (33.7)</td>
<td>25 (20.8)</td>
<td>10 (8.3)</td>
<td>8 (6.7)</td>
<td>10 (8.3)</td>
<td>120</td>
</tr>
<tr>
<td>Renal disease</td>
<td>13 (26)</td>
<td>16 (32)</td>
<td>9 (18)</td>
<td>4 (8)</td>
<td>4 (8)</td>
<td>4 (8)</td>
<td>50</td>
</tr>
<tr>
<td>No renal disease</td>
<td>14 (20)</td>
<td>24 (34.3)</td>
<td>16 (22.9)</td>
<td>6 (8.6)</td>
<td>4 (5.7)</td>
<td>6 (8.6)</td>
<td>70</td>
</tr>
<tr>
<td>Anti ds-DNA</td>
<td>18 (24)</td>
<td>25 (33.3)</td>
<td>20 (26.7)</td>
<td>4 (5.3)</td>
<td>5 (6.7)</td>
<td>3 (4)</td>
<td>75</td>
</tr>
<tr>
<td>No anti ds-DNA</td>
<td>8 (22.2)</td>
<td>12 (33.3)</td>
<td>6 (16.7)</td>
<td>3 (8.3)</td>
<td>2 (5.5)</td>
<td>5 (13.9)</td>
<td>36</td>
</tr>
<tr>
<td>Anti Ro-Ab</td>
<td>6 (20)</td>
<td>10 (33.3)</td>
<td>8 (26.7)</td>
<td>2 (6.7)</td>
<td>2 (6.7)</td>
<td>2 (6.7)</td>
<td>30</td>
</tr>
<tr>
<td>No anti Ro</td>
<td>19 (26)</td>
<td>26 (35.6)</td>
<td>14 (19.2)</td>
<td>4 (5.5)</td>
<td>4 (5.5)</td>
<td>6 (8.2)</td>
<td>73</td>
</tr>
</tbody>
</table>

Numbers indicate total number of patients or controls with a particular genotype with (%). There is no difference in genotype distribution between any of the disease phenotypes shown or between patients with SLE and controls.
3.1.5 Asthma

3.1.5.1 Rationale

IL-10 would be predicted to have a beneficial effect in preventing the development of severe asthma. This is because, in addition to decreasing pro-inflammatory cytokine production and APC MHC class II expression, IL-10 also decreases IL-4 induced IgE class switching by PBMC (Jeannin, 1998). We therefore decided to look at haplotype and genotype distribution in patients with mild and severe asthma compared to controls.

3.1.5.2 Asthma patients

200 white Caucasian asthmatic patients were recruited from asthma clinics held at the Royal Brompton Hospital, London. The patients were determined as having mild or severe asthma, on the basis of lung function and the use of corticosteroids for adequate control of their disease. Patients with mild asthma had no regular asthma symptoms, required no maintenance corticosteroids and had normal lung function (FEV₁: 90.1 ± 0.95% predicted). Severe patients (N=113) had daily asthma symptoms, an ongoing regular need for β₂ agonist therapy, high dose inhaled (≥ 800μg/day BDP, or equivalent) or oral corticosteroids and impaired lung function (mean FEV₁: 64.4 ± 2.06% predicted). Demographic details are given in table 3.6 below.

Table 3.6 Demographic details of asthma patients

<table>
<thead>
<tr>
<th></th>
<th>Mild Asthma</th>
<th>Severe Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>N =</td>
<td>82</td>
<td>113</td>
</tr>
<tr>
<td>Mean Age</td>
<td>27.6 ± 0.85 (SE)</td>
<td>44.9±1.39 (SE)</td>
</tr>
<tr>
<td>F:M</td>
<td>35:47</td>
<td>58:55</td>
</tr>
</tbody>
</table>

SSOP was used to genotype 82 patients with mild asthma and 113 patients with severe asthma. The initial analysis included haplotype and genotype distribution which is how these results have been reported (Lim, 1998). For the purpose of this thesis however a gene dosage effect for both the ATA and the GCC genotype is also described.

The distribution of haplotypes in those with mild asthma was no different to controls (p=0.5) (table 3.7). However, the distribution of haplotypes in those with severe asthma was different to controls (p=0.04). This is because of a decrease in the frequency of the putative high IL-10 producing haplotype GCC (p=0.02, OR 1.5, CI 1.1 –2)) and an increase in the frequency of the putative low IL-10 producing haplotype, ATA (p=0.04, OR=1.4, CI 1-2).
Table 3.7 Haplotype distribution in patients with asthma when compared to controls

<table>
<thead>
<tr>
<th></th>
<th>GCC</th>
<th>ACC</th>
<th>ATA</th>
<th>N (x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>264 (48.2)</td>
<td>156 (28.5)</td>
<td>128(23.4)</td>
<td>274(548)</td>
</tr>
<tr>
<td>Mild asthma</td>
<td>71(43.3)*</td>
<td>50(30.5)</td>
<td>43(26.2)†</td>
<td>82(164)</td>
</tr>
<tr>
<td>Severe asthma</td>
<td>88(38.9)*</td>
<td>69 (30.5)</td>
<td>69 (30.5)†</td>
<td>113 (226)</td>
</tr>
</tbody>
</table>

Total number of haplotypes within each group are given with (%). Where N = numbers of patients studied and x = no. of haplotypes (2 x N).
* There is a decrease in the frequency of the GCC haplotype and an increase in the frequency of the ATA haplotype † in patients with extended oligoarticular JIA when compared to those with oligoarticular JIA.

Table 3.8 Genotype distribution in patients with asthma

<table>
<thead>
<tr>
<th></th>
<th>GCC/GCC</th>
<th>GCC/ACC</th>
<th>GCC/ATA</th>
<th>ACC/ACC</th>
<th>ACC/ATA</th>
<th>ATA/ATA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70(25.5.)</td>
<td>70(25.5)</td>
<td>54(19.7)</td>
<td>24(8.8)</td>
<td>38(13.9)</td>
<td>28(6.6)</td>
</tr>
<tr>
<td>Mild asthma</td>
<td>16(19.5)</td>
<td>18(22)</td>
<td>21(25.6)</td>
<td>11(13.4)</td>
<td>10(12.2)</td>
<td>6(7.3)</td>
</tr>
<tr>
<td>Severe asthma</td>
<td>21(18.6)</td>
<td>26(23)</td>
<td>20(17.7)</td>
<td>8(7.1)</td>
<td>27(23.9)</td>
<td>11(9.7)</td>
</tr>
</tbody>
</table>

Numbers indicate total number of patients with a particular genotype with (%). There is no difference in genotype distribution between the two groups and controls.

The genotype distribution is shown in table 3.8. If genotypes are analysed according to gene dosage of the ATA and GCC haplotypes, there is an increase in genotypes containing an ATA haplotype in patients with severe asthma when compared to controls, table 3.9 (p=0.04, OR 1.57, CI 1.01-2). There is also an increase in those with no GCC haplotype table 3.10 (p=0.028, OR 1.66, CI 1.02-2.69) when compared to controls. It therefore appears that the GCC haplotype may be protective in preventing the development of severe asthma.

Table 3.9 Genotype distribution according to ATA gene dosage

<table>
<thead>
<tr>
<th></th>
<th>0 ATA</th>
<th>1 ATA</th>
<th>2 ATA</th>
<th>N =.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>164(59.9)†</td>
<td>92(33.6)</td>
<td>18(6.6)</td>
<td>274</td>
</tr>
<tr>
<td>Mild Asthma</td>
<td>45(54.9)</td>
<td>31(37.8)</td>
<td>6(7.3)</td>
<td>82</td>
</tr>
<tr>
<td>Severe Asthma</td>
<td>55(48.7)†</td>
<td>47(41.6)</td>
<td>11(9.7)</td>
<td>113</td>
</tr>
</tbody>
</table>

Genotype according to ATA haplotypes. 0 ATA = GCC/GCC, GCC/ACC or ACC/ACC. 1 ATA = GCC/ATA or ACC/ATA. 2ATA = ATA/ATA. Total numbers of each genotype shown with (%).† fewer patients with severe asthma have a genotype without an ATA haplotype.
Table 3.10 Genotype distribution according to GCC gene dosage

<table>
<thead>
<tr>
<th></th>
<th>0 GCC</th>
<th>1 GCC</th>
<th>2 GCC</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>80(29.2)*</td>
<td>124(45.3)</td>
<td>70(25.5)</td>
<td>274</td>
</tr>
<tr>
<td>Mild Asthma</td>
<td>27(32.9)</td>
<td>39(47.6)</td>
<td>16(19.5)</td>
<td>82</td>
</tr>
<tr>
<td>Severe Asthma</td>
<td>46(40.7)*</td>
<td>46(40.7)</td>
<td>21(18.6)</td>
<td>113</td>
</tr>
</tbody>
</table>

Genotype according to GCC haplotypes. 0 GCC = ACC/ACC, ACC/ATA or ATA/ATA. 1 GCC = ACC/GCC or GCC/ATA. 2 GCC = GCC/GCC. Numbers with each genotype given with (%). *Patients with severe asthma are more likely to have a genotype with 0 GCC haplotypes than controls.

A decrease in 0 ATA containing genotypes has therefore been described in patients at the severe end of the spectrum two different inflammatory conditions; asthma and children with extended oligoarticular disease suggesting that these polymorphisms are important in controlling inflammation.

3.1.6 Other paediatric rheumatological diseases

3.1.6.1 Rationale

Because the initial association study was performed blind on all genomic DNA that we had access to, children with other paediatric rheumatological diseases were also studied. Within this group there were 13 patients with dermatomyositis, 1 patient with Familial Mediterranean Fever and 6 patients with vasculitis. The numbers however were too small in each subgroup to analyse statistically. There were also 40 children who were seen in the out patient department with either muscular pain or chronic pain syndrome that did not have a rheumatological problem. The genotype distribution in this heterogeneous group of patients was no different to controls (results not shown).

3.1.7 Linkage Analysis with IL-10 5’ flanking region microsatellites

3.1.7.1 Rationale

There are two microsatellites in the IL-10 5’ flanking region at ~1000kb (G) and ~4000kb (R). Whole blood culture suggests the presence of high and low IL-10 producing haplotypes and association studies have described association of alleles between SLE and RA (Eskdale, 1998; Eskdale, 1997). It is not clear however whether these associations are due to a functional effect of the microsatellites or due to linkage disequilibrium with other genes (such as the 5’ flanking SNPs) that regulate IL-10 production.

There is currently no published data on linkage between the IL-10 5’ flanking region microsatellites and the SNPs. In a collaboration with Dr Rachelle Donn (ARC
Epidemiology research unit in Manchester) we investigated linkage disequilibrium between the microsatellite at $\sim$-1000kb (G) in controls and in patients with JIA.

### 3.1.7.2 Controls used for linkage between IL-10 5' flanking region SNPs and microsatellites

For this analysis, DNA from healthy controls was collected by the ARC Epidemiology unit in Manchester. All of these controls were white Caucasians. The DNA was collected from two sources, Controls 1 and Controls 2. Controls 1 were collected from donors to the blood transfusion service in the Oxford region. The donors were not necessarily first time donors. Controls 2 were collected from GP practices in the Norfolk region as controls for an early arthritis study (Symmons, 1997). They were collected as a match for each case in the study and were selected from the Family Health Service Authority Register of the Norwich Health Authority. For each case, 5 potential controls (based on age and sex) were identified and one was chosen at random to participate. The sex distribution is shown in table 3.11. There is no significant difference in genotype distribution when compared to controls used in the association studies.

Microsatellite analysis was carried out by the ARC Epidemiology unit.

**Table 3.11 Epidemiology of controls used for linkage analysis**

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>Unknown</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls 1</td>
<td>34</td>
<td>38</td>
<td>20</td>
<td>92</td>
</tr>
<tr>
<td>Controls 2</td>
<td>34</td>
<td>58</td>
<td>0</td>
<td>92</td>
</tr>
</tbody>
</table>

The microsatellite at $\sim$-1000kb (G) typed for this assay is shown in figure 3.3 below.

**Figure 3.3 IL-10 5' flanking region G microsatellite.**

The microsatellite is shown in red, the primers used in blue and the closest SNP (-1082) in green.

SNP genotypes were identified by SSOP as described. Haplotypes were assigned if the genotypes were informative which means the genotype was homozygous at either the
microsatellite or the SNPs. For the controls, the analysis was carried out by using SPSS version 8 software and the output data is shown in table 3.12, below.

**Table 3.12 Results of linkage analysis between G microsatellite and SNP haplotypes**

<table>
<thead>
<tr>
<th>Microsatellite</th>
<th>SNP haplotype</th>
<th>Expected Frequency</th>
<th>Observed Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6</td>
<td>GCC</td>
<td>0.001322</td>
<td>0.003333</td>
</tr>
<tr>
<td></td>
<td>ACC</td>
<td>0.000689</td>
<td>0.000000</td>
</tr>
<tr>
<td></td>
<td>ATA</td>
<td>0.001322</td>
<td>0.000000</td>
</tr>
<tr>
<td>G7</td>
<td>GCC</td>
<td>0.132222</td>
<td>0.008517</td>
</tr>
<tr>
<td></td>
<td>ACC</td>
<td>0.006889</td>
<td>0.024816</td>
</tr>
<tr>
<td></td>
<td>ATA</td>
<td>0.013222</td>
<td>0.000000</td>
</tr>
<tr>
<td>G8</td>
<td>GCC</td>
<td>0.046278</td>
<td>0.065455 *</td>
</tr>
<tr>
<td></td>
<td>ACC</td>
<td>0.024111</td>
<td>0.015350</td>
</tr>
<tr>
<td></td>
<td>ATA</td>
<td>0.046278</td>
<td>0.035861</td>
</tr>
<tr>
<td>G9</td>
<td>GCC</td>
<td>0.128256</td>
<td>0.132878</td>
</tr>
<tr>
<td></td>
<td>ACC</td>
<td>0.066822</td>
<td>0.83234</td>
</tr>
<tr>
<td></td>
<td>ATA</td>
<td>0.128256</td>
<td>0.107221</td>
</tr>
<tr>
<td>G10</td>
<td>GCC</td>
<td>0.035700</td>
<td>0.063322</td>
</tr>
<tr>
<td></td>
<td>ACC</td>
<td>0.018600</td>
<td>0.000011</td>
</tr>
<tr>
<td></td>
<td>ATA</td>
<td>0.035700</td>
<td>0.026667</td>
</tr>
<tr>
<td>G11</td>
<td>GCC</td>
<td>0.035700</td>
<td>0.037949</td>
</tr>
<tr>
<td></td>
<td>ACC</td>
<td>0.018600</td>
<td>0.014227</td>
</tr>
<tr>
<td></td>
<td>ATA</td>
<td>0.035700</td>
<td>0.037823</td>
</tr>
<tr>
<td>G12</td>
<td>GCC</td>
<td>0.034378</td>
<td>0.029373</td>
</tr>
<tr>
<td></td>
<td>ACC</td>
<td>0.017911</td>
<td>0.027293</td>
</tr>
<tr>
<td></td>
<td>ATA</td>
<td>0.034378</td>
<td>0.03</td>
</tr>
<tr>
<td>G13</td>
<td>GCC</td>
<td>0.056856</td>
<td>0.044723</td>
</tr>
<tr>
<td></td>
<td>ACC</td>
<td>0.029622</td>
<td>0.018788</td>
</tr>
<tr>
<td></td>
<td>ATA</td>
<td>0.056856</td>
<td>0.079822</td>
</tr>
<tr>
<td>G14</td>
<td>GCC</td>
<td>0.044956</td>
<td>0.011116</td>
</tr>
<tr>
<td></td>
<td>ACC</td>
<td>0.023422</td>
<td>0.022946</td>
</tr>
<tr>
<td></td>
<td>ATA</td>
<td>0.044956</td>
<td>0.079272</td>
</tr>
</tbody>
</table>

This output data shows observed and expected genotype. Overall there is strong linkage disequilibrium. *increased frequency of haplotype in observed over expected frequency.

In the controls, there is strong linkage disequilibrium between the microsatellite and the SNP haplotypes (p<0.0005). The strongest association was between the microsatellite allele G8 and GCC. The G8 allele is decreased in patients with either oligoarticular, extended oligoarticular or polyarticular JIA p ≤ 0.001, by Monte Carlo analysis, (Rachelle Donn personal communication). At the moment it is not possible to compare
the microsatellite allele frequency between children with extended and those with oligoarticular JIA as there are insufficient numbers for statistical analysis.

To see whether microsatellite and SNP alleles cosegregated with disease, the same analysis was undertaken on children with JIA. The analysis was performed using the following formulae (Thomson and Klitz 1987):

\[
X^2 = \frac{(O-E)^2}{O+E}
\]

Where \( X \) is Chi, \( E \) = expected haplotype frequency calculated from known allele frequency in the normal population and \( O \) is observed haplotype frequency in the population studied. Total \( \chi^2 \) is additive for each haplotype (degrees of freedom \( = n-1 \) where \( n \) = number of alleles) (Thomson and Klitz 1987). The \( p \) value was then calculated from statistical tables.

In this group of patients there was an unexpectedly high increase in the observed association between the ATA SNP haplotype and the G9 microsatellite allele (\( \chi^2 = 8.13, p<0.01 \)) and between G12 and ACC (\( \chi^2 = 5.26, p<0.05 \)). A decrease in the observed association between G14 and GCC was noted (\( \chi^2 = 8.3, p<0.01 \)). The association between G8 and GCC was not observed.

It is surprising that there is a difference between the controls and patients in terms of which microsatellites are associated with which SNP allele. This could due to any of the following reasons:

1. The association has occurred by chance in the patients population where the sample size is relatively small.

2. The patient group is from a different population genetically from the control group. The patients are from the whole of the UK with a significant percentage being from London whereas the controls are all from East Anglia.

3. There is a synergistic effect between the microsatellite alleles and the SNP alleles which increases the risk of developing JIA.

4. The same environmental pressure that has increased the observed frequency of the microsatellite alleles has increased the frequency of the SNP alleles.
3.1.8 Transmission disequilibrium test

3.1.8.1 Rationale

As our control group was not perfectly matched with any patient group geographically our association studies are vulnerable to stratification. We therefore used TDT studies to confirm or refute our results.

3.1.8.2 TDT families

Family trios (father, mother and patient) for TDT analysis came from two sources, the United Kingdom Paediatric Rheumatology HLA database (ARC Epidemiology Unit, Manchester) and the Outpatient Departments at both Great Ormond Street Hospital and the Middlesex hospitals. Parents gave informed consent. When samples were collected from the outpatient departments, the history was reviewed to confirm the diagnosis. Altogether 105 families were studied for the TDT however the majority of these families were either incomplete or non informative as shown in figure 3.4

![Figure 3.4 Composition of families studied for the TDT](image)

<table>
<thead>
<tr>
<th></th>
<th>Patient only</th>
<th>1 parent</th>
<th>Parents only</th>
<th>Trio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligoarticular</td>
<td>9</td>
<td>29</td>
<td>19</td>
<td>12</td>
</tr>
<tr>
<td>Ext. Oligo</td>
<td>2</td>
<td>30</td>
<td>8</td>
<td>16</td>
</tr>
</tbody>
</table>

Figure 3.4 Composition of families studied for the TDT

Genotypic data has been collected from 267 individuals but this includes only 28 trios. This is either because we do not have the genomic DNA on for example one of the parents or because SSOP was unsuccessful for one of the family members.

Data was analysed on haplotype transmissions from parents to children. There were a total of 28 trios, although 2 were uninformative because both parents were homozygous. In two trios, there was a question of non-paternity and they were excluded. The data was analysed using the Transmit software by Sham and Curtis. This software allows analysis of transmission from a system with more than two alleles. In addition to testing
the transmission of each haplotype, it also performs a global test using, in this case, two
degrees of freedom. It also estimates haplotype transmission by assigning parental
genotypes based on estimated haplotype probabilities. This allows us to use data from
families where we have only genotyped one parent.

The following results were obtained:

There was an increase in transmission of the ATA haplotype to patients with both
oligoarticular and extended oligoarticular JIA. This increase was statistically significant
\((p = 0.05)\) although would not be significant after a Bonferonni correction as the
transmission of three haplotypes was tested.

There was no increase or decrease in transmission of any haplotype when the groups
were subdivided according to disease (oligoarticular or extended oligoarticular arthritis).
When the TDT was performed on patients with uveitis there was a significant increase
in the transmission of both the ACC and the ATA haplotype to patients with uveitis
\((p=0.014\) for each transmission). Global Chi\(^2\) test on all three haplotypes with 2 degrees
of freedom was also statistically significant \((p=0.015)\). This is consistent with the
trends seen in the association study where patients with uveitis where less likely to
posses a genotype with 0 ATA haplotypes and more likely to possess a genotype with 2
ATA haplotypes than controls. These associations however were not statistically
significant unlike the TDT which is highly significant. The implications for this will be
discussed further (section 4.2.3.2).
3.2 Ex vivo IL-10 production

3.2.1 Rationale

Association of either haplotype or genotype with disease is only biologically meaningful if it can be shown to be functionally relevant. We decided to use ex vivo blood cell culture to investigate the association of IL-10 5' flanking region SNP genotype with stimulated IL-10 production. The method had to be reliable and reproducible. Peripheral blood mononuclear cell culture (PBMC) culture has been used in the past. More recently whole blood culture has been used to measure cytokine production because it is reproducible and felt to be more physiological because there are no separation steps and all blood components are present. We therefore initially compared PBMC culture with the whole blood culture method.

3.2.2 IL-10 secretion in PBMC culture

3.2.2.1 Time course

Initial time course experiments using PBMC culture showed that IL-10 production (measured by ELISA) reached a plateau between 20 and 24 hours following stimulation with LPS (figure 3.5). PBMC culture was then performed on 6 additional controls using only 4 time points: 0, 6, 24 and 48 hours as shown in figure 3.6. Maximum IL-10 production occurred at 24 hours. IL-10 production using PBMC culture was then compared to IL-10 production using the whole blood culture method.

![Figure 3.5](image)

**Figure 3.5** Time course of IL-10 production from PBMC culture

PBMC culture from person with GCC/GCC genotype stimulated with LPS 1µg/ml. Samples analysed in duplicate, mean values given with range shown as error bars. Unstimulated samples not done in duplicate therefore no range given.
3.2.3 IL-10 secretion in whole blood culture

3.2.3.1 Optimisation of method

At the time this work was carried out, there was no published method for measuring IL-10 production using whole blood culture. The following variables were therefore tested to optimise the culture method: timing of samples, RPMI dilution, LPS dose.

3.2.3.1.1 Time course

Limited time course studies were carried out for a range of RPMI dilutions and LPS doses (see below). In each case, maximum IL-10 production occurred at 24 hours. Further more detailed time courses confirmed this (figure 3.13, section 3.2.6).

3.2.3.1.2 RPMI dilution

The blood was diluted in RPMI as follows: 1:1 RPMI, 1:5 RPMI and 1:10 RPMI. The results are shown in figure 3.7. Maximum IL-10 production occurred using a RPMI dilution of 1:1. A dilution of 1:1 was therefore used in subsequent experiments. This ratio is also used by other groups looking at IL-10 production (Westendorp, 1997).
Figure 3.7 *IL-10 production in whole blood culture with different RPMI dilutions.*

Results are shown as mean with range shown as error bars. For each dilution, the culture was stimulated with LPS 1μg/ml.

### 3.2.3.1.3 LPS dose response

LPS doses (1μg/ml, 10ng/ml and 100pg/ml) were used with an RPMI dilution of 1:1. The results are shown in figure 3.8. Maximum IL-10 production occurred with a dose of 1μg/ml and this was therefore used in subsequent experiments. This is in accord with subsequent published methods (Westendorp, 1997; Foca, 1998). LPS at 1μg/ml is within the range expected during septic shock or close to an infectious focus (Foca, 1998). It can therefore be considered to be a physiological stimulus to IL-10 production during acute inflammation.

Figure 3.8 *LPS dose response in whole blood cell culture*

IL-10 production measured in whole blood culture using RPMI dilution of 1:1 over 48 hours and an LPS range of 100pg/ml to 1μg/ml. Highest IL-10 production seen with LPS dose 1μg/ml.
3.2.4 Comparison of whole blood culture and PBMC culture for IL-10 production

PBMC culture was performed on 15 healthy white Caucasians and whole blood culture on 45 healthy Caucasian volunteers living in North London. Subjects were excluded if there was a past history of arthritis or if they were suffering from a symptomatic viral infection. IL-10 production in the unstimulated samples was significantly higher in the PBMC cultures than in the whole blood cultures at 24 hours (P<0.001). This is consistent with the fact that spontaneous IL-10 can be detected in freshly isolated PBMC by FACS analysis (Inoges, 1999). This probably reflects stimulation of the cells during cell separation for PBMC culture, thus making true constitutive IL-10 production difficult to assess. Unstimulated IL-10 production was low using the whole blood culture method (266 to 401 pg/ml). The association of genotype with IL-10 production was therefore investigated using the whole blood culture method.

3.2.5 IL-10 production: relationship to genotype

IL-10 levels measured at 24 hours were corrected for the lymphocyte and monocyte count as measured on the day the culture was started. The results are shown in figure 3.9. There was a minimum of 5 subjects with each genotype. The production of IL-10 is lower in those with the ATA/ATA genotype and this is statistically significant (p<0.02). This result is consistent with previous studies (Turner, 1997). It is also consistent with the results from our collaborators showing that reporter gene assay expression was lowest in U937 stimulated with dbcAMP transfected with the ATA haplotype compared to the GCC haplotype (Crawley, 1999c).

![IL-10 Production According to Genotype](image)

**Figure 3.9** IL-10 production according to genotype

Genotype expressed as number of ATA haplotypes such that 0 ATA = GCC/GCC, GCC/ACC, ACC/ACC. 1 ATA = GCC/ATA and ACC/ATA. 2 ATA = ATA/ATA. All results are corrected for the monocyte and lymphocyte count. Median for each genotype is shown as red line.
3.2.6 Time course: IL-1α, IL-4, IL-6, IL-12, IFNγ, TNFα production: ATA/ATA versus GCC/GCC

Because we believe it is the balance of pro-and anti-inflammatory cytokines that influence inflammation, detailed time courses were also performed, using the whole blood culture method, for IL-1α, IL-4, IL-6, IL-12, IFNγ and TNFα as well as for IL-10. From these time courses we were therefore able to derive appropriate time points to use in whole blood culture experiments where the balance of cytokines was investigated. Blood was taken from two healthy volunteers with genotypes GCC/GCC and ATA/ATA. Results were corrected for total monocyte and lymphocyte count measured on the day the culture was started. The time courses were identical for both genotypes. For IL-1α, IL-β, IL-6, TNFα and IFNγ, the person with the ATA/ATA genotype produced more than the person with the GCC/GCC genotype (figures 3.10, 3.11, 3.12, 3.15 and 3.16 respectively). Although there was no significant difference in IL-10 levels between the two genotypes in this experiment, maximum levels of all the pro-inflammatory cytokines (IL-1α, IL-1β, IL-6 and TNFα) were lower from the person with the GCC/GCC genotype than in the ATA/ATA genotype (associated with lower IL-10 production, see section 3.2.5). This is consistent with the expected action of IL-10 as an anti-inflammatory cytokine. To look at differences in cytokine production between people with different genotypes, we would clearly need larger numbers of subjects in each group.

The time course characteristics for each cytokine are summarised in table 3.13 below:

Table 3.13 Cytokine time courses

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>First detectable</th>
<th>Maximum levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>6 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td>IL-1β</td>
<td>4 hours</td>
<td>18 hours</td>
</tr>
<tr>
<td>IL-4</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>IL-6</td>
<td>4 hours</td>
<td>18-24 hours</td>
</tr>
<tr>
<td>IL-10</td>
<td>6 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td>IL-12</td>
<td>8 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td>TNFα</td>
<td>2 hours</td>
<td>6-8 hours</td>
</tr>
<tr>
<td>IFNγ</td>
<td>6 hours</td>
<td>18-30 hours</td>
</tr>
</tbody>
</table>
Figure 3.10  *Time course of IL-1α production in whole blood culture*

IL-1α production over 30 hours from two controls with GCC/GCC and ATA/ATA genotype respectively. Results are corrected for monocyte and lymphocyte count. Experiments analysed in duplicate with range shown as error bars. Unstimulated samples not shown.

Figure 3.11  *Time course of IL-1β production in whole blood culture*

IL-1β production over 30 hours from two controls with GCC/GCC and ATA/ATA genotype respectively. Results are corrected for monocyte and lymphocyte count. Experiments analysed in duplicate with range shown as error bars. Unstimulated samples not shown.
**Figure 3.12** *IL-6 production in whole blood culture*

Time course over 30 hours from two controls with GCC/GCC and ATA/ATA genotype. Results are corrected for monocyte and lymphocyte count. Experiments analysed in duplicate. Error bars show range of results. Unstimulated samples not shown.

**Figure 3.13** *IL-10 production in whole blood culture*

Time course over 30 hours from two controls with GCC/GCC and ATA/ATA genotype. Results are corrected for monocyte and lymphocyte count. Experiments analysed in triplicate. Error bars show range of results. Unstimulated samples not shown.
Figure 3.14  *IL-12 production in whole blood culture*

Time course over 30 hours from laboratory control with GCC/GCC genotype. Results are corrected for monocyte and lymphocyte count. Experiments analysed in duplicate. Error bars show range of results. Unstimulated samples not shown.

Figure 3.15  *TNFα production in whole blood culture*

Time course over 30 hours from two controls with GCC/GCC and ATA/ATA genotype. Results are corrected for monocyte and lymphocyte count. Experiments analysed in duplicate. Error bars show range of results. Unstimulated samples not shown.
Figure 3.16  *IFNγ production in whole blood culture*

Time course over 30 hours from two controls with GCC/GCC and ATA/ATA genotype. Results are corrected for monocyte and lymphocyte count. Experiments analysed in duplicate. Error bars show range of results. Unstimulated samples not shown.

Clearly, this type of experiment measures accumulation of cytokine within the whole blood culture system and therefore reflects both production and degradation of the cytokine tested.

At the time these experiments were performed there was no published data on the relationship of IL-10 production to other cytokines in whole blood culture. However, these results are consistent with results from several studies measuring cytokine production using other methods of cell culture.

In elutriated human monocytes IL-10 production is first detected in supernatants at 7.5 hours but maximal production is at 20 to 48 hours (De Waal Malefyt, 1991). In the same study, IL-6 first appeared at 3.5 hours and was maximal 7.5 to 90 hours whilst TNFα was first seen at 3.5 hours and was maximal at 7.5 hours thereafter decreasing quickly.

In LPS stimulated PBMC (100ng/ml), TNFα appears at the protein level within 30 minutes whilst IL-1α, IL-1β and IL-6 peaked at 4 to 6 hours (Andersson, 1992). In this study, IL-10 was only produced by a small percentage of monocytes with the maximum percentage of cells producing IL-10 being 0.9%. Even though the LPS dose was only one tenth the dose used in our studies, the time course for IL-10 was similar with a peak in IL-10 production between 24 and 48 hours. IFNγ was detected maximally at 24 hours whilst IL-4 was undetectable which is consistent with our results (Andersson,
In another study using LPS (1μg/ml) stimulated PBMC, TNFα mRNA appears at 1 hour, whilst IL-12 p40 and p35 protein appeared at 4 hours which is slightly earlier than in our results (Aste-Amezaga, 1998).

In a study comparing cytokine production in PBMC and whole blood culture, IL-1β, IL-6 and TNFα were produced rapidly during the first 8 hours whilst IL-2 and IFNγ were produced maximally after 24 hours using both methods (De Groote, 1997).
3.3 Parental IL-10 production and association with disease phenotype

3.3.1 Rationale
If the association of a low IL-10 producing genotype with children with extended oligoarticular JIA and not with those with oligoarticular JIA is biologically important, we would expect that children with extended JIA produce less IL-10 during inflammation than children with oligoarticular JIA. IL-10 production cannot be measured in many children with arthritis because of the treatment they receive. As discussed in sections 1.10.4.2 and 1.10.4.3, both glucocorticoids and MTX can up-regulate IL-10 production \textit{in vitro} and \textit{in vivo}. Both of these are used in children with severe disease and therefore cytokine production cannot be measured in these children. Parental IL-10 production was therefore measured as a marker for IL-10 production in the children.

It has been estimated that 84% of IL-10 variability is genetically regulated (Westendorp, 1997). Measurement of parental IL-10 production is therefore likely to represent childhood IL-10 production. This method has been used to estimate childhood cytokine production in other studies (Westendorp, 1997).

3.3.2 Parental data
38 parents were enrolled in the study but there were 7 exclusions (see below, table 3.14) and 2 samples could not be used for technical problems. Therefore, blood was cultured from the parents of 26 children with JIA. 14 of these children had extended oligoarticular JIA and 12 had oligoarticular JIA. 7 children had uveitis whilst 12 had no uveitis at the time the sample was taken. The presence or absence of uveitis was not recorded in the remaining children. IL-10 production was analysed on both sets of parents from 2 children and from 24 when only one parent was available. If both parents were available the results mean IL-10 production was used in the analysis.

Parents were excluded if they were taking medication that may have interfered with \textit{in vitro} IL-10 production or if they were suffering from a viral infection at the time of the study (see table 3.14). In these cases blood was taken for genomic analysis and the parents and children were entered into the TDT study.
Parents were excluded from 5 children with extended oligoarticular JIA and one with oligoarticular JIA. In one case this was because the father had influenza but in the remainder it was because of concurrent treatment. * treatment not known

A full family history was taken at the time the sample was taken and in over 30% of cases, parents described autoimmune related diseases either as part of their medical history or in first and second degree relatives (table 3.15). It should be noted that these are only preliminary descriptions and further work needs to be done to define the disease, for example the cause of hypothyroidism.

**Table 3.15 Medical background and family history of parents donating blood**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Parent: diagnosis</th>
<th>1st and 2nd ° relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligoarticular</td>
<td>Father: asthma,</td>
<td></td>
</tr>
<tr>
<td>Oligoarticular</td>
<td>Mother: hypothyroid</td>
<td></td>
</tr>
<tr>
<td>Oligoarticular</td>
<td>Mother: hypothyroid, iritis</td>
<td></td>
</tr>
<tr>
<td>Oligoarticular</td>
<td>Father: asthma</td>
<td></td>
</tr>
<tr>
<td>Extended oligoarticular</td>
<td>Father: autoimmune alopecia</td>
<td>Sister: colitis</td>
</tr>
<tr>
<td>Extended oligoarticular</td>
<td>Mother: colitis, IDDM</td>
<td></td>
</tr>
<tr>
<td>Extended oligoarticular</td>
<td>Mother: NIDDM</td>
<td></td>
</tr>
<tr>
<td>Extended oligoarticular</td>
<td>Mother: IDDM</td>
<td></td>
</tr>
<tr>
<td>Extended oligoarticular</td>
<td>Mother: hypothyroid</td>
<td></td>
</tr>
<tr>
<td>Extended oligoarticular</td>
<td>Mother: asthma</td>
<td></td>
</tr>
<tr>
<td>Extended oligoarticular</td>
<td></td>
<td>RA</td>
</tr>
</tbody>
</table>

An autoimmune history or family history was obtained from the parents of 11 out of a total of 30 children. 7 of these children had extended oligoarticular JIA and 4 had oligoarticular JIA. The parent enrolled in the study and their diagnosis is given in the second column. Parents who were excluded because of treatment are highlighted. Any family history in first or second degree relatives is given in the third column.

The Shapira Wilk test was used as a test of normality and indicated that the data was distributed normally. A students T test was therefore used in the following calculations.
3.3.3 Extended versus oligoarticular JIA

Stimulated IL-10 production was compared between parents of children with extended oligoarticular JIA and parents of children with oligoarticular JIA. The results are shown in figure 3.17.

Figure 3.17 Average IL-10 production in parents of children with oligoarticular or extended oligoarticular JIA

Mean shown as ◆. Each point (●) represents average IL-10 production corrected for lymphocyte and monocyte count for one child with JIA. If results were only available from one parent then these are plotted as a single point. Mean IL-10 production is lower in the parents of those with extended disease (N=14, Mean = 2109.2, SE 251) than those with oligoarticular JIA (n=12, mean = 3161.7, SE=411) and the results are statistically significant (p=0.034, CI 88 to 2016). However the confidence intervals are wide.

Mean IL-10 production was lower in the parents of children with extended disease when compared with those with oligoarticular JIA and this was significant. Mean IL-10 production was lower in both groups than in laboratory volunteers (see section 3.2.5). The possible reasons for this are discussed in section 3.3.5.

Although there were more ATA containing genotypes in the parents of children with extended oligoarticular JIA than in those with oligoarticular JIA, the difference was not statistically significant. There was no relationship between IL-10 production and genotype according to number of ATA haplotypes although it was not possible to look at gene dosage as there was only one parent who was ATA homozygous (see table 3.16).
Table 3.16 Genotype frequency in parents of children with oligoarticular or extended oligoarticular JIA

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Oligoarticular</th>
<th>Extended Oligoarticular</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ATA</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>1 ATA</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>2 ATA</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

The genotype frequency is compared between parents of children with oligoarticular or extended oligoarticular disease in terms of ATA containing haplotypes. Although more parents have an ATA containing haplotype if their child has extended oligoarticular JIA than oligoarticular JIA. The result is not statistically significant.

Realistically however there are insufficient numbers to draw any conclusions from this in terms of genotype. The relationship of genotype and IL-10 production was investigated separately and the results are described in section 3.2.5. The important conclusion here is that IL-10 production is lower in the parents of children with severe disease when compared to those with mild disease. As most of the variability in IL-10 production is genetically controlled, this suggests that IL-10 production in children with severe disease is lower than in those with mild disease.

As there is some suggestion in the literature (see section 1.8.7.2) that uveitis may be associated with either high or low IL-10 production, these children may therefore confound the results. The results were therefore reanalysed only for children who we knew did not have eye disease. The mean IL-10 production in parents of children with extended oligoarticular JIA was nearly half that of parents of children with oligoarticular JIA. Although the numbers were low, there was still a statistically significant difference between the two groups (see table 3.17).

Table 3.17 Comparison of IL-10 production in parents of children with either Oligoarticular or Extended Oligoarticular JIA but without eye disease

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligoarticular no uveitis</td>
<td>7</td>
<td>3029.6</td>
<td>±534</td>
</tr>
<tr>
<td>Extended Oligoarticular no uveitis</td>
<td>5</td>
<td>1616.3</td>
<td>±334</td>
</tr>
</tbody>
</table>

Mean IL-10 production is significantly different lower in the parents of children with extended oligoarticular JIA when compared to those with oligoarticular JIA in children without uveitis (p=0.05).

3.3.4 Eye disease versus no eye disease

Stimulated IL-10 production was compared between parents of children with uveitis (N=7) and the parents of children without eye disease (N=12). These included children with either oligoarticular or extended oligoarticular JIA. There was no significant difference between the two groups.
3.3.5 IL-10 production in different “control” groups

During the course of this thesis, IL-10 production was measured in two different “control” groups because of two different experiments. The first was to analyse the relationship of genotype to IL-10 production and the second to look at IL-10 production in the parents of children with JIA. Both these groups were controls in that they did not have JIA or any history of arthritis but neither were controls in terms of representing the normal population. For the analyses of IL-10 production by genotype, samples were collected from laboratory volunteers. The mean age in this group was 25.19 years (95% CI 23.27-27.11) at the time the blood sample was taken. The second group was the parents of children with JIA. Examination of both of these groups using the Shapiro-Wilk statistic revealed that IL-10 production in the parents of children with JIA was normally distributed unlike IL-10 production in laboratory controls. This was unexpected as one might expect the parents of children with JIA to have skewed IL-10 production. The distribution of IL-10 production is more skewed in the laboratory volunteer group with an excess of high IL-10 producers than in the parents (table 3.8).

Table 3.18 Test of normality on whole blood culture data

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Median</th>
<th>Skewness (SE)</th>
<th>Kurtosis (SE)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab. Volunteers</td>
<td>49</td>
<td>7770</td>
<td>1.3 (0.34)</td>
<td>1.6 (0.67)</td>
<td>0.01</td>
</tr>
<tr>
<td>Parents</td>
<td>27</td>
<td>2441</td>
<td>0.541 (0.448)</td>
<td>-0.62 (0.87)</td>
<td>0.162</td>
</tr>
</tbody>
</table>

Characteristics of IL-10 production corrected for lymphocyte and monocyte count in whole blood culture samples. The p value is a test of whether the group tested is significantly different from theoretical values for a normal distribution. Skewness is a measure of asymmetry. Kurtosis measures the extent to which observations cluster around a central point. A Positive Kurtosis such as in the laboratory volunteers indicates that the observations cluster more and the histogram plot of samples has longer tails than those with a normal distribution. A negative Kurtosis such as in the parental samples, indicates that the observations cluster less with shorter tails.

In addition, the parents of children with JIA had lower median IL-10 values than the laboratory volunteers. This could be due to several factors. The parents of the children with JIA could have lower IL-10 production than the “normal” population or the laboratory volunteers could have higher IL-10 production because of age or environmental factors such as alcohol, exercise or stress increasing catecholamine levels. These environmental factors have all been shown to be important in determining cytokine production (Jacob, 1990; Mandrekar, 1996; Elenkov, 1996). As some of the volunteers anecdotally had a history of high alcohol consumption, the effect of ethanol on IL-10 production was tested both in vitro and ex vivo.
3.4 Environmental effects on IL-10 production: ethanol and methylprednisolone

3.4.1 Effect of ethanol on IL-10 production

3.4.1.1 Rationale:

It was noticed that some of the laboratory volunteers with high stimulated IL-10 production anecdotally were thought generally to have a high alcohol consumption. Review of the literature suggested that ethanol consumption augmented LPS stimulated IL-10 production (reviewed section 1.10.4.1). The effect of ethanol on IL-10 production was therefore tested \textit{in vitro} and \textit{ex vivo} to see if increased alcohol consumption could explain the skewed IL-10 production seen in the laboratory volunteers compared to the parents of children with JIA.

3.4.1.2 Ethanol: \textit{in vitro}

Ethanol concentrations were calculated to approximate to blood levels after alcohol ingestion of one moderate drink or more. One unit of ethanol on average results in a blood ethanol level of 0.1g/dl which approximates to 25mM \textit{in vitro}. Higher blood levels of 0.4g/dl (e.g. by a chronic alcoholic after an acute intake) approximate to a final concentration of 100mM \textit{in vitro} (Mandrekar, 1996). Initial experiments examined the effect of 25mM, 50 mM, 75 mM and 100mM of ethanol on IL-10 production over a 48 hour time course with time points at 5, 24, 28, 32 and 48 hours. Samples were stimulated with LPS (1\mu g/ml).

As can be seen from figure 3.18, all doses of ethanol increased IL-10 production at 24 hours. Ethanol however did not increase IL-10 production in unstimulated samples (figure 3.19). This means that ethanol was synergistic with LPS in increasing IL-10 production.
**Figure 3.18** Synergistic effect of ethanol on LPS (1 μg/ml) stimulated IL-10 production

Ethanol was added to a final concentration of 25 to 100 mM. All doses of ethanol appear to increase IL-10 production in stimulated samples at 24 hours. Results are in triplicate, mean shown with range in error bars.

**Figure 3.19** 50 mM of ethanol has no effect on IL-10 production in unstimulated cells

Ethanol was added to a final concentration of 50 mM. The samples were not stimulated. 50 mM of ethanol has no effect on IL-10 production. This is also true for all other doses, (results not shown). Results are in triplicate, mean results given with range shown as error bars.
Figure 3.20 Synergistic effect of ethanol and LPS on IL-10 production

Maximum increase in IL-10 production seen at 20 hours with ethanol at final concentration of 50mM which increases IL-10 production by 67.2% over LPS alone. Results are in triplicate. Mean shown with range in error bars.

These results were reproducible over a wider range of ethanol doses where a maximum increase of 67.2 % was seen at 20 hours with 50mM dose of ethanol and 66.9% and 50.7% at 28 hours with the 50mM and 75mM dose of ethanol respectively (figure 3.20). Even the 25mM dose however increased IL-10 production at 28 hours by 56.6% and this is equivalent to blood ethanol levels after just one moderate drink. The dose response is interesting with an increase in IL-10 production between 1-50mM of ethanol followed by a reduction in this increased production between 50 and 250mM.

Of course the in vivo blood ethanol level is temporary and the length of time that cells might be exposed to ethanol will depend on factors such as liver metabolism. The effect of alcohol on stimulated IL-10 production was therefore measured ex vivo.

3.4.1.3 Ethanol: ex-vivo

To investigate whether these results were relevant in vivo, alterations in IL-10 production were measured ex-vivo before and after acute alcohol intake. Laboratory volunteers and medical students were recruited into this study and gave informed consent. A questionnaire was filled out with details on ethanol ingestion over the preceding 24 hours. Volunteers were excluded if they were suffering from a concurrent viral infection or had a history of arthritis or other auto-immune disorder. An anonymous coded sample was sent to the biochemistry department in University...
College London for a blood ethanol level on all samples received whether ethanol ingestion was admitted or not. The sample was collected within twelve hours of ethanol ingestion.

Firstly, IL-10 production was measured using the whole blood culture method in 14 volunteers *in vitro* after addition of IL-10 to the whole blood culture. In 11 of these, IL-10 production increased after addition of 25mM of ethanol to the blood culture. This result was significant (see figure 3.21) \((p=0.05)\).

For the *ex vivo* investigation, complete sets of data were collected on 15 volunteers. Average alcohol consumption was 8.37 units (range 2.5 to 16). This is equivalent to 4 and a half pints of lager or 9 small glasses of wine. The data was log transformed to produce a normal distribution for statistical analysis using the paired students T test. 11 volunteers had increased IL-10 production after ethanol and 4 had decreased IL-10 production. This is shown in figure 3.22. There was no statistically significant difference in terms of stimulated IL-10 production before and after alcohol consumption. Whether stimulated IL-10 production increased or decreased after alcohol was not related to genotype in terms of either ATA or GCC haplotypes.

![Figure 3.21](image-url)\(_1\)

**Figure 3.21** *In vitro increase in IL-10 production in the presence of alcohol*

25mM final concentration of ethanol added to culture. All samples stimulated with LPS (1μg/ml). Each • represents one persons *in vitro* results with and without 25mM ethanol.
Figure 3.22  *IL-10 production before and after acute intake of alcohol*

*Ex vivo* effect of alcohol on IL-10 production stimulated by LPS (1µg/ml). IL-10 production is measured after 24 hours alcohol free and within 12 hours of alcohol ingestion. IL-10 production as measured at 24 hours is corrected for lymphocyte and monocyte count. Each • represents one person’s paired results.

### 3.4.2  Effect of methylprednisolone on IL-10 production

#### 3.4.2.1  Rationale

Whether MP increases or decreases IL-10 production is controversial (see section 1.10.4.2). However MP is a mainstay of treatment in JIA. If MP increases IL-10 production at the doses used in treating children with JIA, this could be one of its mechanisms of action. Therefore the effect of MP on IL-10 production was therefore investigated *in vitro* and *in vivo*.

#### 3.4.2.2  Methylprednisolone *in vitro*

The study was designed to examine the effect of pulsed MP on cytokine production. Children receive a pulse of 30mg/kg unless they are more than 15 kg when they receive a standard dose of 500 mg. The pulse is repeated three times at 24 hourly intervals to maintain a steady plasma level of MP over 72 hours. The maximum blood concentration is likely to be that obtained after the 30mg/kg dose in a smaller child and this was estimated by the Pharmacy Department at Great Ormond Street Hospital as being 35 mg/l in the blood. The MP concentrations used in this study were designed to be within this estimated range of the therapeutic blood concentration.

The initial experiment examined the effect of 0, 20, 40 and 120 mg/l of MP on IL-10 production at 0, 5, 24, 29, 45 and 51 hours. IL-10 production was investigated when the MP was added before, during and after LPS. The latter was thought to be closer to the *in vivo* situation where inflammation precedes steroid treatment. For the initial experiments, blood was obtained from a lab worker with genotype ACC/ATA.
As can be seen (figures 3.23 and 3.24), IL-10 production is not increased by steroid alone but is increased by 28% with 20µg/ml, and 46% with 40µg/ml of MP at 24 hours when the MP is added 1.5 hours before the addition of LPS. However between 29 and 53 hours, IL-10 production is suppressed by the addition of MP at all doses used. The increase in IL-10 production at 24 hours is reduced when the steroid is added with the LPS (figure 3.25). When MP is added 1.5 hours after LPS, IL-10 production is reduced at all doses used relative to IL-10 production with LPS only and no MP (figure 3.26). The experiment was repeated using lower doses of MP and earlier time points (figures 3.27-3.30). The blood for these experiments was from a lab worker with genotype GCC/ATA. As can be seen in figures 3.27 and 3.29, IL-10 production is increased between 5 and 24 hours at all doses used (1 to 40µg/ml). The greatest increase was seen with 1µg/ml which increased IL-10 production by 83.4% over samples which had been stimulated with LPS but no MP. The dose response at each time point suggested that lower doses were more effective than higher doses of MP in synergistically upregulating IL-10 production with LPS. A similar effect was seen if the steroid was added three hours after the LPS but the effect is attenuated (figures 3.28 and 3.30). As these results suggested that MP could indeed increase IL-10 production an ex vivo investigation was performed.

**Figure 3.23.** Methylprednisolone (MP) does not increase IL-10 production in unstimulated whole blood culture samples

There is no difference between samples with or without MP when the samples are not stimulated with LPS. Samples are done in triplicate. The mean is shown with range in error bars.
Figure 3.24  *MP added to whole blood culture 1.5 hours before LPS*

MP was added to whole blood culture to final concentration of either 20 or 40mg/l 1.5 hours prior to adding LPS (1μg/ml). Samples analysed in triplicate. The mean results are given with the range in error bars.

Figure 3.25  *IL-10 production when MP added with LPS*

MP is added to the whole blood culture at the same time as LPS (1μg/ml) to final concentration of either 20, 40 or 120 mg/l. The results are analysed in triplicate, means are shown with range shown as error bars.
Figure 3.26. *IL-10 production when MP added 1.5 hours after LPS*

Methylprednisolone added 1.5 hours after LPS (1μg/ml). Final concentration of MP is 20, 40 or 120 mg/l. Results are analysed in triplicate, mean shown with range shown as error bars.

Figure 3.27 *MP added to whole blood culture with LPS at doses 0, 1, 4, 10, 20, 40 mg/l*

All samples are stimulated with LPS, 1μg/ml. MP added at doses: 1, 4, 10, 20 and 40 mg/l). For samples with no MP, mean values from two experiments are given. All experiments analysed in triplicate and range is shown as error bars.
**Figure 3.28** *MP added to whole blood culture 3 hours after LPS at doses 0, 1, 4, 10, 20, 40 mg/l*

MP added in doses 1 to 40 µg/ml. Results analysed in triplicate. Mean values are shown with range shown as error bars.

**Figure 3.29** *MP added to whole blood culture with LPS at doses 0, 0.5, 2, 5, 10, 20, 40 mg/l*

MP added at lower doses (0.5mg/l to 40 mg/l) to whole blood culture with LPS (1µg/ml). Results are compared to unstimulated samples and stimulated samples without MP. Results are in triplicate with mean values given with range shown as error bars.
In vitro MP added in lower doses 0.5mg/l to 40mg/l to whole blood culture 3 hours after LPS added. Results analysed in triplicate with mean given and with range shown in error bars.

3.4.2.3 Methylprednisolone ex vivo

5 mls of blood was taken from patients before each of three pulsed MP doses. The only exclusion to the study were patients who had received a previous MP bolus in the last three months. All other patients were included. Data was collected on clinical features such as number of joints involved, medication taken on admission and markers of inflammation such as ESR and CRP. The ESR, CRP and FBC were measured on the day of admission and on the day of discharge. IL-10 production was corrected for the lymphocyte and monocyte count measured on the day of admission.

To date, 11 patients have been entered into the study. The study is on going. Complete data has been collected and analysed on 8 patients. The clinical and laboratory characteristics of the patients are given in table 3.19 below. There was a wide range of variability in constitutive IL-10 production which probably reflects the different treatment regimes and variations in the degree of inflammation. Results were therefore analysed as a ratio of stimulated/constitutive IL-10 production (see figure 3.31). IL-10 production was reduced in all patients at the end of the full course of treatment i.e. at 52 hours after the first sample was taken. In 3 patients, IL-10 production increased at 24 hours, in one patient it was unchanged and in 4 patients it decreased. There was no significant difference between the dose of methylprednisolone given in each group. In the patients in whom stimulated IL-10 production decreased after the methylprednisolone, admission CRP and ESR was generally higher and pre-admission oral prednisolone dose was lower however these results were not statistically significant. However, there was a strong correlation between the change in stimulated
IL-10 production and the change in ESR after the methylprednisolone bolus (see figure 3.32) (correlation coefficient 0.922, p<0.009). This is statistically significant even after a correction for multiple testing.

**Figure 3.31  Effect of methylprednisolone on IL-10 production ex vivo**

IL-10 production for each patient is plotted at three time points: Pre MP, 24 hours post MP which is 24 hours after the first bolus dose of methylprednisolone and 52 hours post MP. The last time point is taken after the methylprednisolone course is finished. All results are expressed as a ratio of stimulated/constitutive IL-10 production corrected for monocyte and lymphocyte count. This is because there was large variability in constitutive IL-10 production.

**Figure 3.32  Change in stimulated IL-10 production correlates with change in ESR after treatment with Methylprednisolone**

The change in stimulated IL-10 production from pre to 24 hours post MP treatment is plotted with the change in ESR for each of 8 patients. There is a high degree of correlation (correlation coefficient 0.922, p<0.009)
Although the daily dose for MP is 30 mg/kg for children over 15 kg, a standard dose of 500 mg is given. In our study, all but one child had a MP dose of less than 30 mg/kg.

### Table 3.19: Clinical and Laboratory Characteristics of Patients in Methylprednisolone Study

<table>
<thead>
<tr>
<th>CRP</th>
<th>ESR</th>
<th>Age</th>
<th>Disease MODIFYING Drugs (mg/kg/day)</th>
<th>Methylprednisolone (mg/kg/day)</th>
<th>Methylprednisolone (mg/kg/day)</th>
<th>Days of Admission</th>
<th>Discharge Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4</td>
<td>&gt;0.3</td>
<td>67</td>
<td></td>
<td></td>
<td></td>
<td>52</td>
<td>0.47</td>
</tr>
<tr>
<td>8.2</td>
<td>&gt;0.3</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td>127</td>
<td>0.22</td>
</tr>
<tr>
<td>7.9</td>
<td>&gt;0.3</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td>131</td>
<td>0.01</td>
</tr>
<tr>
<td>5.6</td>
<td>&gt;0.3</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td>5.7</td>
<td>0.04</td>
</tr>
<tr>
<td>4.3</td>
<td>&gt;0.3</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td>88</td>
<td>0.4</td>
</tr>
<tr>
<td>0.7</td>
<td>&gt;0.3</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td>6.1</td>
<td>0.45</td>
</tr>
<tr>
<td>0.3</td>
<td>&gt;0.3</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td>1.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Note: Methylprednisolone dose given in mg/kg/day. Days of Admission refer to the day Methylprednisolone was started.
3.5 FACS analysis of cells in whole blood culture for IL-10 intracellular localisation

3.5.1 Rationale
The aim of FACS analysis of cells from whole blood culture was to identify which cells in the peripheral blood produced IL-10 after LPS stimulation in both controls and children with JIA. This would then have allowed us to correct total IL-10 production measured by ELISA for the number of IL-10 producing cells. FACS analysis has been successfully used to detect intracellular cytokines in PBMC culture and in cell lines (Prussin and Metcalfe 1995; de Caestecker, 1992; Jung, 1993; North, 1996; Inoges, 1999). One of the intracellular cytokines it has been used to detect is IL-10 and this has been successful in PBMC (Inoges, 1999).

3.5.2 Principles of method
Flow cytometry measures certain physical and chemical characteristics of cells as they pass a laser. The scattered and emitted fluorescent light is collected by two lenses at right angles to each other and a series of optics such that the beam is split and filtered and bands of fluorescence measured. Specific reagents, usually monoclonal antibodies conjugated to fluorochrome moieties, are used to label individual cells. The fluorescence measured depends on the fluorochrome used as well as properties of the cells studied allowing characteristics such as cell size, shape and intracellular components to be determined.

To determine both cell type and the presence of intracellular IL-10, a three colour staining technique was used. The fluorochromes used were cytochrome (Cy), phycoerythrin (PE) and fluorescein isothiocyanate (FITC) directly conjugated to specific monoclonal antibodies.

As the production and secretion of cytokines is an active process, measurement of intracellular cytokines is improved if cytokine secretion is inhibited. This can be achieved using monensin, a carboxylic ionophore, which disrupts intracellular protein transport causing cytokines to accumulate in the Golgi apparatus increasing the cytokine signal detected without altering cell viability (Jung, 1993; North, 1996). Another protein transport inhibitor is Brefeldin-A, which appears to inhibit protein secretion early in a pre-Golgi compartment (Fujiwara, 1988). The relative effectiveness of these protein transport inhibitors appears to be time and cytokine dependant (Jung, 1993; Fujiwara, 1988).
If FACS analysis is to be done on whole blood culture (as opposed to PBMC), it is necessary to lyse the red blood cells because otherwise they overlap the lymphocyte-monocyte area of the leukocyte population in the FACS analysis (Tiirikainen 1995). The FACS lyse method has been shown not to alter intracellular antigenic sites of leukocytes (Tiirikainen 1995).

3.5.3 Monoclonal antibodies used
T cells were identified using an anti-CD3 antibody conjugated to cyochrome. Anti-CD64 was initially used to identify monocytes. CD64 is a 72 kDa glycoprotein (cell surface antigen FcR1) that is constitutively expressed on monocytes and macrophages and can be induced on neutrophils. Anti CD68 was also used to identify monocytes. CD68 is a transmembrane protein and at the time experiments were performed, CD68 expression had not been described on neutrophils. Monocytes were not identified using the CD14 antibody because LPS was used to stimulate the whole blood culture and CD14 is an LPS receptor. Anti-IL-10 conjugated with PE was used for intracellular IL-10 detection.

3.5.4 Optimising whole blood collection
3.5.4.1 Increase in “monocyte” staining with time
Initial experiments were conducted to compare collection of blood into heparin or EDTA in order to ensure that there was no significant loss of monocytes due to adherence to the universal tubes (made of polystyrene) used for blood collection. Blood was therefore collected into universal tubes containing either preservative free heparin or EDTA. The monocyte and lymphocyte count was analysed by staining for CD64 and CD3 positive cells at 0, 3 and 5 hours.

Analyses revealed a surprising result as the CD64 + count increased rather than decreased over time as shown in table 3.20 and figure 3.33. The CD3 count however did not increase with time. It was also noticed that between 1 and 5% of cells were double positive for both CD64 and CD3.
Table 3.20  **CD64+ time course in whole blood drawn into heparin or EDTA double stained for CD64 and CD3**

<table>
<thead>
<tr>
<th>Time</th>
<th>Heparin</th>
<th>EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hours</td>
<td>CD64 = 10.7%</td>
<td>CD64 = 12.9%</td>
</tr>
<tr>
<td></td>
<td>CD3 = 26.7%</td>
<td>CD3 = 22.8%</td>
</tr>
<tr>
<td>3 hours</td>
<td>CD64 = 23.2%</td>
<td>CD64 = 8.0%</td>
</tr>
<tr>
<td></td>
<td>CD3 = 26.9%</td>
<td>CD3 = 45.3%</td>
</tr>
<tr>
<td>5 hours</td>
<td>CD64 = 27.6%</td>
<td>CD64 = 8.0%</td>
</tr>
<tr>
<td></td>
<td>CD3 = 25.7%</td>
<td>CD3 = 45.3%</td>
</tr>
</tbody>
</table>

**Figure 3.33 Change in CD64 positive cells with time**

Results from one typical experiment showing increase in CD64 positive cells over a 3 hour period. This is contrast to the CD3 positive cells which remain constant over 3 hours.

The possible reasons for the double staining are that:

1. As CD64 is an Fc receptor, it is possible that the CD3 monoclonal antibody could be binding non-specifically to it.
2. The CD64 antibody is non-specific and binds to cells other than monocytes.

Non-specific binding by anti-CD64 could also explain the increase in CD64 positive cells as CD64 is expressed on activated neutrophils and neutrophils are activated by the heparin as. This is consistent with the fact that the increase in CD64 positive cells was not seen in samples collected into EDTA.

These possibilities were then tested by using mouse serum as a first blocking stage to prevent non-specific binding of the CD3 antibody to the CD64 and then by co-staining with CD66b which is expressed on neutrophils.
3.5.4.2 Mouse serum layer

Venous blood was collected into a universal tube with preservative free heparin and left to stand for five hours. 3 μl of mouse serum was added to 100μl of blood and left to incubate for 10 minutes prior to cell surface staining. There was no difference in the increase in the double staining observed when this was compared to cells which had not been incubated with mouse serum as shown in table 3.21 below. It is therefore unlikely that the double staining was due to non-specific binding of the CD3 antibody to CD64.

Table 3.21 Results of prior incubation of sample with mouse serum on double staining of CD3 and CD64

<table>
<thead>
<tr>
<th></th>
<th>CD3</th>
<th>CD64</th>
<th>CD3+CD64</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 &amp; CD64</td>
<td>35</td>
<td>18.4</td>
<td>1.36</td>
</tr>
<tr>
<td>CD3 &amp; CD64 with mouse serum</td>
<td>32.04</td>
<td>23.82</td>
<td>0.96</td>
</tr>
</tbody>
</table>

3.5.4.3 CD66b (FITC) co-staining

If the increase in CD64 positive cells was due to an increase in activated neutrophils over time, an equal increase in time with double positive CD66b and CD64 cells would be expected. The experiment was therefore repeated with monoclonal antibodies to both CD66b (FITC) and CD64(PE) in the same tube. There was an increase in cells which were double positive for both CD66b and CD64 over time (figure 3.34). The experiment was repeated twice and the results were reproducible.

Figure 3.34 Increase in CD64 and CD66b positive cells over a three hour period

The increase in cells staining with both CD64 and CD66b correlates with the increase in CD64 suggesting that the increase in CD64 positive cells is due to an increase in activated neutrophils. Results are typical and are from one (of three) typical experiments.
Using anti-CD68 co-staining to identify monocytes

Anti-CD68 was then used to try and improve identification of monocytes (see section 2.4.3 for method). However the percentage of CD68 positive cells was higher than predicted at all time points as shown in table 39 below:

Table 3.22 CD68 staining over 5 hours

<table>
<thead>
<tr>
<th>Time</th>
<th>% of positive CD68 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hrs</td>
<td>77.1</td>
</tr>
<tr>
<td>3 hrs</td>
<td>70.8</td>
</tr>
<tr>
<td>5 hrs</td>
<td>60.1</td>
</tr>
</tbody>
</table>

These levels are much higher than the monocyte concentration in the blood and therefore the CD68 antibody must be binding non-specifically. Granulocytes have low levels of intracellular CD68. As it was possible that the CD68 antibody was entering granulocytes during the permeabilisation stage non-specifically and not being washed out, a variety of methods were tried to improve the anti-CD68 antibody specificity. These are listed in table 3.23 below. Increasing the number of washes did not improve the non-specific binding of the CD68 antibody in that total numbers of CD64 cells remained the same. However, if the method is altered so that the CD68 is added with the permeabilising buffer and incubated in the dark, a very different picture emerges with a shift down in terms of brightness for most of the positive cells ($10^3$ to $10^2$) leaving a small percentage of cells brightly positive. It appears that this method therefore can differentiate between the granulocytes and monocytes in terms of the number of CD68 positive bound antibody per cell i.e. the relative brightness of the cell.

Table 3.23 Positive CD68 staining with different methods

<table>
<thead>
<tr>
<th>Method</th>
<th>% of positive CD68 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal method</td>
<td>72.2</td>
</tr>
<tr>
<td>Add anti CD68 to permeabilising buffer</td>
<td>5.87</td>
</tr>
<tr>
<td>Wash with permeabilising buffer</td>
<td>71.3</td>
</tr>
<tr>
<td>Add to permeabilising buffer and wash with permeabilising buffer</td>
<td>8.84</td>
</tr>
</tbody>
</table>

The method as described in section 2.4.3 was altered to try and reduce the non-specific binding of the anti-CD68 antibody. Adding the antibody during the permeabilisation stage and incubated in the dark for 10 minutes at room temperature decreased the CD68 positive cells from over 70% to less than 10%. The cells which were positive however were brighter.

Back gating confirmed that the CD68 positive cells as defined above were indeed monocytes as defined by forward scatter (FSC) and side scatter (SSC).
Increasing the incubation of the cells with antibody in permeabilising buffer from 10 minutes to 20 minutes did not improve CD68 staining (see table 3.24). After 30 minutes there was a decrease in the number of CD3 and CD68 cells which was thought to be due to the toxicity of the permeabilising buffer.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>CD3 positive cells</th>
<th>CD68 positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB incubation 10 minutes</td>
<td>14.37</td>
<td>9.15</td>
</tr>
<tr>
<td>PB incubation 20 minutes</td>
<td>15.38</td>
<td>7.7</td>
</tr>
<tr>
<td>PB incubation 30 minutes</td>
<td>2.42</td>
<td>1.98</td>
</tr>
</tbody>
</table>

Table 3.24 Change in CD68 staining with increased incubation in permeabilising buffer

Altering incubation time with permeabilising buffer (PB) does not alter the number of CD68 positive cells except an incubation of 30 minutes. At this time point there is a decrease in CD3 positive cells as well, suggesting that the PB buffer is toxic when cells are exposed to it for 30 minutes.

3.5.5 Intracellular IL-10 staining

Cells were then stained for intracellular IL-10 as described in section 2.4.3. Monensin was used to disrupt intracellular protein transport. No intracellular IL-10 was detected either constitutively or after stimulation with LPS or with PMA and Ionomycin at 0, 4 or 8 hours (results not shown). The experiment was repeated 3 times in three different individuals (who were known to make IL-10 by using the whole blood culture method see section 3.2.5 for results) with no successful intracellular staining for IL-10 (see section 2.4.4 for stimulation methodology). The experiment was repeated using brefeldin A (which is also a golgi stopper). Brefeldin A was added at the start of simulation with either LPS or PMA and ionomycin at a final concentration of 5ng/ml. There was no successful staining of intracellular IL-10 (results not shown). The same anti-IL-10 mAb was used by others within the group to successfully stain PBMC during the course of this thesis.

Other groups have had similar problems detecting IL-10 using FACS analysis on whole blood culture samples (Huizanga, personal communication). The reasons for this are discussed in section 4.1.3. The method was therefore abandoned.
3.6 Transient Transfections

3.6.1 Rationale

Our collaborator Richard Kay showed, during the course of this thesis, that the ATA haplotype was associated with lower reporter gene assay expression than the GCC haplotype in U937 cells stimulated with dibutyladenosine 3':5' - cyclic monophosphate (dbcAMP, see ref (Hurme, 1990)). This result is now published in our joint paper (Crawley, 1999c). Transient transfections were therefore designed to see if this was reproducible using LPS and in a different cell lines and to investigate how the polymorphisms affect gene expression.

3.6.2 Choice of Cell type

HeLa cells were initially used but as discussed below, it was not possible to increase reporter gene expression using LPS in HeLa cells. It was therefore decided to use another cell line. THP-1 cells were chosen as they are a human monocytic cell line derived from a child with acute monocytic leukaemia (Tsuchiya, 1980). Although mouse monocytic cell lines are easier to transfect than THP-1 cells, they were not used because the IL-10 5' flanking region (and therefore possibly the transcriptional mechanisms used) is not homologous with the human IL-10 5' flanking region.

3.6.3 Transfection of HeLa cells using Calcium Phosphate method

Initial transfection experiments were performed using the calcium phosphate method to transfect HeLa cells. The GCC construct was used and luciferase expression was measured after stimulation with LPS at 1μg/ml and 10μg/ml for 8, 12, 16 and 24 hours. LPS was chosen because it is known to induce IL-10 production in vitro and in vivo (van der Poll, 1994). Many of the intracellular pathways involved in LPS stimulation of monocytes have been identified (see section 1.10.1.4). LPS was used in the whole blood culture method where differences in IL-10 production were observed to be associated with different genotypes (see section 3.2.5). The LPS dose used was 1μg/ml which is within the range expected during septic shock or close to an infectious focus (Focà, 1998).

The results are shown in figures 3.35, 3.36 and 3.37. Reporter gene expression, (shown in the figure as relative light units) decreased in all cases with time. Overall expression was reduced in stimulated samples when compared to unstimulated samples. There was no reproducible difference between the 1μg/ml and the 10μg/ml doses of LPS.
Figure 3.35 Time course of reporter gene expression in HeLa cells stimulated with LPS

The HeLa cells were transfected with the GCC construct. Two doses of LPS were used, 1μg and 10μg. Experiments are performed in duplicate with range shown in error bars.

Figure 3.36 Reporter gene expression in HeLa cells stimulated with LPS

Hela cells transfected with the GCC construct and stimulated with either 1μg or 10μg of LPS over 30.5 hours. Experiments were performed in duplicate. Mean results are shown with range as error bars.
Figure 3.37  Reporter gene expression in HeLa cells transfected with GCC construct

Comparison of HeLa cells stimulated with LPS (10μg/ml) or unstimulated over 24 hours. Notice that overall, the RLU are all very low with a wide degree of variability. Experiments are performed in triplicate with range shown by error bars.

When the IL-10 5' flanking region haplotype constructs (GCC, ACC and ATA) were compared with each other in both stimulated (LPS 1μg/ml) and unstimulated HeLa cells, there was no significant reproducible difference between the constructs (figure 3.38).

Figure 3.38  Reporter gene expression in HeLa cells using GCC, ACC or ATA IL-10 constructs

HeLa cells have been transfected with either the GCC, ACC or ATA constructs. The stimulated cells are stimulated with 1μg/ml of LPS. Results are expressed as relative light units. Overall transfection results are low and there is no significant difference in reporter gene expression between the constructs.
It is possible that this inability to increase reporter gene expression in HeLa cells was because the cells did not have the transcriptional machinery to increase IL-10 transcription in response to LPS. The transfections experiments were therefore repeated in a monocytic cell line, as monocytes are known to produce IL-10 in response to LPS. The THP-1 cells were transfected using both liposomal and DEAE-dextran methods.
3.6.4 Transfection of THP-1 cells with liposomes

3.6.4.1 Choice of method

Liposomes are formed from a combination of a cationic amphiphile and a neutral lipid. They mediate gene delivery by interacting electrostatically with negatively charged DNA sequences, forming complexes which enter cells by either endocytosis or phagocytosis (Cooper, 1998; Feigner, 1994).

THP-1 cells are notoriously difficult to transfect. Other investigators were successfully using liposomal methods to transfect mouse monocytic cell lines and advances in liposomal technology had reportedly been successful in transfecting human monocytic cell lines. Liposomes were therefore used for transfection.

For each method, 1,25-dihydroxycholecalciferol was used to induce cell differentiation (Miyaura, 1981). Differentiation is associated with CD14 expression and cell adhesion. 1,25-dihydroxycholecalciferol is the most biologically active metabolite of vitamin D and has many biological effects in addition to its role in calcium homeostasis (Clavreul, 1998; Rigby, 1984; Rigby, 1987).

3.6.4.2 Choice of liposome

There is little experience of transfection of THP-1 cells with liposomes and therefore all commercially available liposomes were tested. For each different preparation, cells were transfected with a wide range of DNA to liposome ratio using the pGL3 luciferase positive control vector (Promega). If transfection was successful then time courses and LPS dose response experiments were also carried out. The In-house Liposomes and Transfext (Promega) were unsuccessful at transfecting THP-1 cells despite testing a wide range of variables such as DNA concentration and liposomal volume (results not shown). Successful transfection was achieved with Fugene 6 (Beohringer Mannheim), Superfect (Qiagen) and Effectene (Qiagen) and these results are presented.

3.6.4.3 Optimisation of transfection with Fugene

Incubation time of the DNA/Fugene complex with the cells was investigated using 2μg of DNA (pGL3 luciferase control vector) and 2μl of Fugene reagent over 4 to 31 hours. See figure 3.39. Maximum reporter gene expression was seen at 8 hours. Transfection efficiency was low at all time points (β galactosidase readings average 0.29). To try and improve results the ratio of DNA to Fugene was tested as shown in table 3.25 below, however this did not improve transfection efficiency (β galactosidase readings average 0.36).
Figure 3.39  *Time course after transfection using Fugene Liposomes*

Reporter gene expression over 4 to 31 hours in THP-1 cells transfected with the pGL3 control construct and Fugene liposomes. Experiments performed in duplicate with range shown in error bars.

Table 3.25  *Results of altering conditions in transfection of THP-1 cells using Fugene*

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Relative light units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1μg DNA/2ul Fugene</td>
<td>1.7 (1.1-2.3)</td>
</tr>
<tr>
<td>1μg DNA/3ul Fugene</td>
<td>2.7 (2.7-2.7)</td>
</tr>
<tr>
<td>1μg DNA/6ul Fugene</td>
<td>3.0 (2.8-3.2)</td>
</tr>
<tr>
<td>2μg DNA/2ul Fugene</td>
<td>0.6 (0.5-0.7)</td>
</tr>
<tr>
<td>2μg DNA/3ul Fugene</td>
<td>2.07 (1.09-2.53)</td>
</tr>
<tr>
<td>2μg DNA/6ul Fugene</td>
<td>1.7 (1.7-1.8)</td>
</tr>
<tr>
<td>0.75 μg/DNA/6ul Fugene</td>
<td>2.6</td>
</tr>
</tbody>
</table>

The results show average relative light units using Fugene 6 at a range of DNA (0.75 to 2μg) and Fugene (2 to 6μl) doses. Two experiments performed in duplicate. Range given in ( ).

3.6.4.4  *Optimisation of transfection with Superfect (Qiagen)*

The initial transfections with Superfect used a total of 2μg of DNA (1.5μg construct) and 10 μl of Superfect. To establish whether this method was suitable for THP-1 cells, changing the medium at 3 hours and harvesting at 24 hours (as recommended by the manufacturer) was compared with changing the medium at 18 hours and harvesting at 24 hours and not changing the medium. Transfection efficiency improved if the medium was not changed (figure 3.40). When the wells were inspected at 18 hours 95% of the THP-1 cells were no longer adherent. This could have been due to several reasons: a toxic effect of either the liposomes or DNA or a vitamin D deficiency as vitamin D was only used until the time of transfection and not subsequently.
Figure 3.40  Reporter gene expression after using different transfection methods to try and improve transfection efficiency with Superfect

THP-1 cells transfected using Superfect liposomes and either the positive (pGL3 control) or negative (pGL3 basic) constructs. Experiments performed in duplicate with range shown in error bars. Transfection is improved if the medium is not changed. Manufacteres method suggests changing the medium at 3 hours.

Vitamin D was then added to the media when the cells were incubated after transfection and a range of DNA concentrations (1µg, 2 µg and 4µg) and a range of Superfect volumes of 2, 5 10 and 20µl was tested (see figure 3.41). The best conditions for transfection were 2 µg of DNA per well in a ratio of 1:5 with the Superfect and an incubation time of 24 hours. In case the liposomes were toxic to the cells a shorter incubation time was tried (2 hours Vs 24 hours) but this did not improve reporter gene assay expression at 24 hours (results not shown).
Figure 3.41 Optimization of transfection using Superfect liposomes

The following variables were optimized: change in DNA concentration from 1µg DNA per well to 4µg/DNA per well, change in DNA/Superfect ratio and incubation time 24 or 48 hours. Each experiment performed in duplicate with range shown as error bars.

3.6.4.5 Optimisation of transfection with Effectene

3.6.4.5.1 Dose response of DNA and Effectene/DNA ratio

Initial optimisation experiments used the pGL3 positive control vector. 0.4µg of DNA per well was compared with 0.2µg and 0.8µg of DNA and the following DNA (µg)/Effectene(µl) ratios: 1:10, 1:25 and 1:50. The results are shown in figure 3.42. 0.4µg of DNA was the most effective quantity of DNA to use with either 1:10 or 1:25 ratio DNA/Effectene. 0.4 µg of DNA with 4µl (1:10) of Effectene was then used for further optimisation experiments.
Figure 3.42  Range of DNA dose and ratio of DNA Effectene of transfection efficiency
THP-1 cells are transfected with pGL3 positive control vector using the Effectene method. The experiments are performed in duplicate. Mean given with range in error bars.

3.6.4.5.2  Dose response and Time course of LPS

Initial experiments using the pGL3 luciferase positive control vector demonstrated a significant increase in reporter gene expression when the cells were stimulated. In this experiment there was an 11 fold increase when cells were stimulated for 20 hours but no increase at 8 hours and only a marginal increase at 28 hours. See table 3.26.

Table 3.26  Reporter gene expression (RLU) in stimulated and unstimulated THP-1 cells transfected using Effectene and the pGL3 control construct at different time points

<table>
<thead>
<tr>
<th>Length of Time</th>
<th>Mean reading</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstimulated</td>
</tr>
<tr>
<td>8 hours</td>
<td>0.66 (0.62-0.72)</td>
</tr>
<tr>
<td>20 hours</td>
<td>1.94 (1.08-2.07)</td>
</tr>
<tr>
<td>28 hours</td>
<td>7.89 (6.82-8.95)</td>
</tr>
</tbody>
</table>

In this experiment there is an 11 fold increase in reporter gene expression after stimulation with LPS at 1µg/ml.

The manufacturers protocol suggests that whilst complex formation takes place, the growth medium should be gently aspirated from the plate and the cells should be washed once with PBS and fresh growth medium replaced just prior to adding the transfection mixture. In fact when this method was compared to just changing the medium, or not changing the medium the results suggested that the best method was to change the medium and not to wash the THP-1 cells (table 3.27). This method was therefore adopted for all experiments.
Table 3.27 Different methods used in transfecting THP-1 cells with Effectene

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean reading</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstimulated</td>
<td>Stimulated</td>
</tr>
<tr>
<td>Cells washed (Manufacturers method)</td>
<td>6.71 (5.52-7.9)</td>
<td>11.0 (7.31-14.7)</td>
</tr>
<tr>
<td>Media changed but cells not washed</td>
<td>1.94 (1.08-2.07)</td>
<td>22.7 (20.38-25.02)*</td>
</tr>
<tr>
<td>No media change</td>
<td>4.3 (4.1-4.5)</td>
<td>2.45 (2.68-3.12)</td>
</tr>
</tbody>
</table>

*In this experiment it can be seen that is a 20 fold increase in reporter gene expression using the pGL3 positive control vector with a method were the media is changed but the cells are not washed. This is different to the manufacturers recommended method.

In two separate experiments therefore reporter gene expression increase with LPS stimulation using the pGL3 control vector, however this increase in reporter gene expression could not be replicated when the IL-10 5'flanking region constructs were used. In fact multiple LPS dose response experiments suggested that LPS may be toxic as increasing doses of LPS resulted in decreasing expression of reporter gene assay (figure 3.43) and decreasing transfection with the β galactosidase vector (used as a marker of transfection efficiency) see figures 3.43 and 3.44. Examination under microscopy, showed that there were fewer adherent cells when LPS had been added.

Figure 3.43 Decrease in reporter gene expression with increasing LPS doses

THP-1 cells are transfected with the ACC construct using Effectene and stimulated with LPS at doses shown. Reporter gene expression decreases with increasing LPS dose. Experiments are performed in duplicate with range shown as error bars.
Figure 3.44 Decreasing $\beta$ galactosidase expression with increasing LPS doses

Each experiment represented as one point. Overall $\beta$ galactosidase expression decreases with increased LPS suggesting either decreased transfection or increased cell death.

Detailed time courses using the 5' flanking region constructs did not show any change in transfection efficiency (table 3.28).

Table 3.28 Time course with LPS comparing unstimulated and stimulated results

<table>
<thead>
<tr>
<th>Time</th>
<th>Unstim.</th>
<th>Stimulated (4$\mu$L Effect)</th>
<th>Stimulated (10$\mu$L Effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 hr</td>
<td>2.15(1.39-2.9)</td>
<td>1.93(1.56-2.99)</td>
<td>1.21(1.15-1.27)</td>
</tr>
<tr>
<td>18 hr</td>
<td>1.26(1.09-1.43)</td>
<td>1.5(1.27-1.78)</td>
<td>0.87(0.78-0.95)</td>
</tr>
<tr>
<td>21 hr</td>
<td>1.83(1.91-1.75)</td>
<td>2.0(1.9-2.09)</td>
<td>1.04(1-1.07)</td>
</tr>
<tr>
<td>25 hr</td>
<td>1.25(1.38-1.11)</td>
<td>1.42(1.42-1.41)</td>
<td>0.91(0.85-0.96)</td>
</tr>
<tr>
<td>49 hr</td>
<td>0.69(0.65-0.73)</td>
<td>0.83(0.79-0.86)</td>
<td>0.86(0.76-0.96)</td>
</tr>
</tbody>
</table>

Effect = volume of Effectene used per well

In this study overall reporter gene assay expression (as relative light units) was lower and there was no significant increase in reporter gene expression with the addition of LPS. There was no apparent difference between using 4 and 10$\mu$L of Effectene (i.e. a 1:10 or 1:25 ratio).

The LPS (dose range 1 to 1000ng/ml) was also added at either 4 or 20 hours after transfection in case early stimulation after transfection was the problem. There is no
dose response to LPS but LPS added at 20 hours appears to be better than LPS added at 4 hours (figure 3.45). With the LPS added at 20 hours, shorter time of stimulation (2 to 6 hours rather than 8 hours) made no difference to transfection efficiency, 4 experiments per construct, results not shown. Dose response experiments were repeated 6 times but there was no increase in reporter gene expression when the cells were stimulated with LPS. This is in contrast to experiments when the pGL3 positive control construct was used. The experiments were repeated with fresh (low passage numbers) THP-1 cells but the results remained the same.

![Bar chart showing reporter gene expression with LPS added at either 4 or 20 hours after transfection.](chart)

**Figure 3.45** *Reporter gene expression with LPS added at either 4 or 20 hours after transfection*

LPS added at doses shown at either 4 or 20 hours after transfection with the pGL3 ACC construct. Although there is no significant differences between the different LPS doses, reporter gene expression is higher when the LPS is added at 20 hours. Experiments performed in duplicate with mean given and range shown in error bars.

3.6.4.6 *Comparison of constructs*

When the results were reviewed it was noted that although there was no increase in reporter gene expression with LPS, there was overall an increase in reporter gene expression with the GCC construct when compared to the ATA construct. The results have been compiled together to obtain an estimate on GCC/ATA transfection efficiency. Analysis was performed on 28 experiments where all three constructs were used to optimise conditions. Despite the wide range of conditions used, the GCC construct was associated with higher reporter gene expression than the ATA construct in each
experiment (ratio GCC/ATA has a median 1.71, range 1.12 – 5.49). This was true for both stimulated and unstimulated experiments.

The following experiments were performed to analyse the apparent lack of response in terms of reporter gene expression to LPS.

3.6.4.7 Measurement of CD14 ligand on THP-1 cells

In order to ensure that the THP-1 cells had the potential to respond to LPS, cell surface CD14 levels were measured in both transfected and non-transfected cells using FACS analysis.

THP-1 cells were made adherent as described using vitamin D. Transfected and non-transfected cells were analysed separately. CD14 antibody was titrated as described (section 2.4.2). The THP-1 cells were then stained with anti CD14 antibody and CD14 surface expression measured by FACS analysis (section 2.4.3). 50% of the cells that were adherent expressed CD14 and there was no difference between transfected and non-transfected cells (results not shown).

3.6.4.8 Comparison of different LPS: rough and smooth

Wild type bacteria which synthesise antigenic O-polysaccharides grow as smooth colonies and produce what is called smooth LPS. This is the type of LPS that had been used in experiments to date (S.marcescens, Sigma). Rough LPS is produced by mutant strains which do not have the antigenic O-polysaccharides and have rough colonies. Although smooth strains are considered to be more virulent than rough ones, both types of LPS play a role in infection, trauma and shock (Focà, 1998). Both types of LPS stimulate protein tyrosine phophorylation and JNK activation in macrophages and THP-1 cells (Weinstein, 1993; Hambleton, 1996). A recent report suggested that rough LPS stimulated higher levels of IL-10 production in whole blood culture than smooth LPS (2606 ± 959 Vs 1111. ± 196, results ± SEM) (Focà, 1998). Rough LPS was therefore compared to smooth LPS during transient transfection.

Rough LPS (S. minnesota R595, Sigma) was diluted in RPMI to a final concentration of 1mg/ml and stimulation of THP-1 cells with rough LPS was compared to smooth LPS. There was no difference as measured by RLU between cells stimulated by smooth LPS and those stimulated by rough LPS at either 2 ng/ml or 20ng/ml. There was no increase in reporter gene expression when compared to cells which had not received any stimulation using either rough or smooth LPS (results not shown).
3.6.4.9 Measurement of IL-6 cDNA in THP-1 preparations

To find out whether the transfection process itself stimulated THP-1 cells, (which could explain why no increase in reporter gene expression was seen in the presence of LPS), IL-6 cDNA was measured in transfected and non transfected THP-1 cells. Neither experiment was stimulated with LPS. There was no IL-6 identified in transfected or the non-transfected THP-1 cells. There was however IL-6 cDNA in the positive control (see fig 52). IL-6 mRNA is produced when THP-1 cells are stimulated. Therefore THP-1 cells are not stimulated by the transfection process itself.

![PCR of IL-6 in transfected THP-1 cells](image)

Lane A and B, mRNA extracted from THP-1 cells which have not been transfected. Lanes C and D, mRNA extracted from THP-1 cells which have been transfected. Lane E, negative control. Lane F positive control.

**Figure 3.46. PCR of IL-6 in transfected THP-1 cells**

THP-1 cells do not produce IL-6 mRNA constitutively. There is no IL-6 mRNA production after transfection suggesting that transfection does not stimulate THP-1 cells significantly.
3.6.4.10  Stimulation of THP-1 cells with IL-1β, TNFα and IL-6

THP-1 cells were stimulated with IL-1β, TNFα and IL-6 for either two or four hours to determine responsiveness of the IL-10 5' flanking region reporter gene constructs to cytokines. There was no increase in reporter gene assay when compared to unstimulated cells. There was however lower reporter gene assay expression after 4 hours of stimulation compared to 2 hours for all three cytokines (figure 3.47, 3.48 and 3.49).

**Figure 3.47** Stimulation of THP-1 cells with IL-1β.

The THP-1 cells are transfected with the ACC construct. Different doses of IL-1β are compared after incubation of either 2 or 4 hours. Each experiment was performed in duplicate with ranges shown as error bars.
Figure 3.48 Stimulation of THP-1 cells with IL-6
The THP-1 cells are transfected with the ACC construct. Different doses of IL-6 are compared after incubation of either 2 or 4 hours. Each experiment was performed in duplicate with the ranges shown as error bars.

Figure 3.49 Stimulation of THP-1 cells with TNF-α.
The THP-1 cells are transfected with the ACC construct. Different doses of TNF-α are compared after incubation of either 2 or 4 hours. Each experiment was performed in duplicate with ranges shown as error bars.

In summary therefore, although transfection was successful using Effectene liposomes, LPS did not increase reporter gene expression. Moreover, results were not comparable...
between experiments. The DEAE dextran method was therefore optimised and used for further transfection.
Transfection of THP-1 cells has been reported to be more efficient with the DEAE-dextran method than with either calcium-phosphate or liposomal methods (Rupprecht and Coleman 1991; Mack, 1998). For this thesis, the THP-1 cells were initially transfected using published methods (Ma, 1996). Initial experiments were designed to see if there was a dose response to LPS in terms of reporter gene expression after transfection. Although transfection was reliably efficient, initial dose response experiments did not show any increase in reporter gene expression with increased LPS dose or time of exposure to LPS (figure 3.50).

![Figure 3.50](image)

**Figure 3.50** *Reporter gene expression with different LPS doses over 8 hours*

THP-1 cells transfected using the DEAE transfection method and the ACC construct. Dose response to LPS at 2, 4, 6 and 8 hours. There is no significant difference between stimulated and unstimulated cells. There is no difference between different doses of LPS. There is no significant difference if the cells are stimulated for either 2, 4, 6 or 8 hours.

It was noted that adherence increased in the flasks where LPS was added. There was a dose response to LPS with increased protein measurements from protein assays in the adherent cells and decreased protein in the supernatant (figures 3.51 and 3.52).

---

1 Because the quantities used in this method were so large, experiments were not duplicated unless stated
Figure 3.51 *Protein levels in supernatant after LPS stimulation*

Protein levels measured in the supernatant of THP-1 cells transfected with the ACC construct after stimulation. This graph shows a decrease in protein levels with increasing doses of LPS. Each protein level was measured in duplicate. Mean results are shown here with the range shown in error bars.

Figure 3.52 *Protein levels in adherent THP-1 cells after stimulation with LPS*

Protein levels measured in adherent THP-1 cells transfected with the ACC construct and stimulated with LPS. This graph shows an increase in protein levels with increasing doses of LPS. Each protein level was measured in duplicate. Mean results are shown here with the range shown in error bars.
When adherent cells only were analysed, there was a small increase in reporter gene assay expression after stimulation with LPS. This was not seen when non-adherent cells were analysed but this difference was not significant or reproducible (results not shown).

DMSO has been reported to increase transfection efficiency in some studies but not in others (O'Connell, 1998; Rupprecht and Coleman 1991; Mack, 1998). Transfection efficiency was therefore compared with and without DMSO and the results were analysed separately for adherent and non-adherent cells. The LPS dose used was 1μg/ml. DMSO consistently improved transfection efficiency over Vitamin D alone in cells in both adherent and cells in supernatant (figures 3.53 and 3.54). Experiments repeated in duplicate twice, results from a typical experiment shown.

![Graph showing transfection efficiency]

**Figure 3.53** *Comparison of transfection: DMSO Vs vitamin D in THP-1 cells in the supernatant*

THP-1 cells transfected with the ACC construct in the presence or absence of either LPS or DMSO or vitamin D in cells in the supernatant.
Figure 3.54 *Comparison of transfection: DMSO Vs vitamin D in adherent THP-1 cells*

THP-1 cells transfected with ACC construct in the presence or absence of DMSO, vitamin D or LPS in adherent cells.

Comparison of different constructs showed no difference between constructs but as there was no difference upon stimulation with LPS it may not have been possible to detect a difference with this system (figure 3.55).

Figure 3.55. *Comparison of different constructs in THP-1 cells*

Comparison of each IL-10 5' flanking region construct. Experiments are performed once. There is no significant difference between different constructs.
3.6.5.2 Measurement of IL-10 mRNA in THP-1 cells

It was possible that the reason there was no increase in reporter gene expression with LPS was either because THP-1 cells used did not produce endogenous or stimulated IL-10 or because they were unable to respond to LPS. To investigate the former, endogenous IL-10 mRNA was measured in THP-1 cells before and after stimulation with LPS. To investigate the latter, IL-6 mRNA was measured as a positive control.

THP-1 cells were made adherent using 1,25-dihydroxycholecalciferol (Calbiochem) as described and stimulated with LPS (1 μg/ml). One flask did not have vitamin D added (in case this made a difference to IL-10 mRNA production). In this flask, mRNA was extracted from cells in suspension after 24 hours of LPS stimulation. In all other flasks the cells were stimulated for either 0, 4, 8 or 24 hours. The experiment was performed in duplicate at 8 and 24 hours. Constitutive IL-10 mRNA was measured at 0 hours and after 24 hours in unstimulated cells. mRNA was extracted as described and cDNA isolated. PCR was performed for Actin mRNA, IL-10 mRNA and IL-6 mRNA.

The results are shown in Figure 3.56. Actin mRNA was present in all samples. IL-6 mRNA was present in samples stimulated with LPS for 4, 8 and 24 hours although levels are low at 24 hours. This indicates that the cells were stimulated (i.e. could respond to LPS) and mRNA extraction was successful.

IL-10 mRNA was present in the unstimulated samples indicating that THP-1 cells produce low levels of IL-10 mRNA constitutively. The presence or absence of 1,25-dihydroxycholecalciferol does not appear to affect IL-10 mRNA production in stimulated samples. IL-10 mRNA appears to be upregulated after 4, 8 and 24 hours of LPS stimulated (see figure 3.56).
Lane A= Time 0. Lane B = 4 hours, C & D = 8 hours, E & F = 24 hours stimulation with LPS 1µg/ml. G = no vitamin D (time 24 hours 1µg/ml). H = Time 24 but no LPS. I = control

**Figure 3.56 Actin, IL-6 and IL-10 PCR from THP-1 cDNA**

mRNA is extracted after stimulation with LPS (1µg/ml) for 0, 4, 8 or 24 hours or from unstimulated samples. PCR of Actin cDNA is used to standardise results. PCR of IL-6 cDNA is measured as a positive control. IL-10 mRNA is present constitutively in THP-1 cells and IL-10 mRNA production appears to increase after 4, 8 and 24 hours of LPS stimulation. The presence or absence of vitamin D dose not appear to affect IL-10 mRNA.
3.6.6 Summary of transfection results

3.6.6.1 Transfection of HeLa cells using the Calcium Phosphate method

Although transfection was successful using this method, reporter gene expression was decreased when LPS was used to stimulate cells.

3.6.6.2 Transfection of THP-1 cells using liposomal preparations

The In-house Liposomes and Transfext (Promega) preparations were unsuccessful at transfecting THP-1 cells. Successful transfection was achieved with Fugene 6 (Beohringer Mannheim), Superfect (Qiagen) and Effectene. The best results were achieved with Effectene. LPS increased reporter gene expression when the pGL3 control vector was used but not when the IL-10 5' flanking region constructs were used. Overall however, reporter gene expression was higher with the GCC than the ATA construct (median fold difference 1.71, range 1.12 - 5.49). This was for both stimulated and unstimulated experiments (see section 4.1.1 for discussion).

The lack of response of THP-1 cells to LPS was not due to lack of CD14 receptors as 50% of adherent cells expressed CD14. The transfection process itself did not stimulate THP-1 cells as there was no increase in IL-6 mRNA after transfection. There was no improvement in reporter gene expression using other stimulants such as rough LPS or the cytokines IL-1β, TNFα and IL-6.

3.6.6.3 Transfection of THP-1 cells using DEAE-dextran

Transfection of THP-1 cells with DEAE-dextran was efficient but once again there was no increase in reporter gene expression with LPS even though the THP-1 cells were shown to produce endogenous IL-10 mRNA and respond to LPS in terms of IL-6 mRNA production.
CHAPTER 4: GENERAL DISCUSSION
4.1 Methodology

4.1.1 Genotype data

4.1.1.1 Patient source

Our patients were derived from tertiary level referral units from within the UK. In terms of the JIA samples, there was a large bias to patients from Great Ormond Street and the Middlesex hospitals. The samples from patients with SLE were obtained from London teaching hospitals. Samples from patients with asthma were obtained from patients attending the Outpatient department at the Royal Brompton Hospital in London. Several studies have shown that genetic associations are different if the patients are derived after hospital referral or from the general population or recruited through the media (Symmons, 1994; de Jongh, 1984; Thomson, 1999; MacGregor, 1995). Recruiting patients from the general population, genotyping them and then prospectively analysing those who developed disease would be possible for patients with asthma, but not for patients with JIA. This is because JIA is a relatively rare disease with a prevalence of only approximately 1:1000 in children. This means that if 90 patients with the disease are required for sufficient power for a case cohort or association study, this would require genomic sample collection from the childhood population of 16,000 (for oligoarticular JIA) and 24,000 (for extended oligoarticular JIA). We felt this was not a practical approach.

4.1.1.2 Methods used

PCR and SSOP is rapid and allows a larger throughput of patients. Since starting this thesis it has been validated in genotyping the IL-10 5’ flanking region SNPs in a large number of patients in a variety of disease groups. In the work presented in this thesis, the results were always read blind and analysed by two independent investigators. The results generated are reproducible and this appears to be an efficient method to carry out the analyses. The methodological problems with this part of the work do not relate to the genotyping but to the patient classification and the statistical analysis. The problems with classification have already been discussed (section 1.3.1). In designing this study we felt it was important to have genetic data that related to clinical subgroups and therefore used the ILAR classification of JIA in our analyses. There are three main aspects to this work however which have posed particular challenges and should be dealt with in future studies. These are the temporal element of the classification, the quantification of severity and the analysis of individual traits.
4.1.1.3 The temporal element

The ILAR classification differentiates between extended oligoarticular and polyarticular onset disease on the basis of whether more than 4 joints were involved before or after 6 months after the onset of disease. If patients are first seen more than 6 months after the onset of disease this can be impossible to determine. Until patients are seen earlier during the course of their disease however, this may be irresolvable.

Another problem is that children can extend any time after 6 months of disease. As blood is frequently taken early in the onset of disease, it is likely that many of the children who are currently classified as having oligoarticular disease may extend. In addition, uveitis may develop many years after the onset of disease. It is therefore possible that some of the children who have been genotyped as children without eye disease may ultimately develop it.

However, another issue with temporal data is that it may be the temporal nature that is itself genetically determined. For example, children who develop eye disease early on in the course of the disease may be different to those who develop it later. Likewise, it may not be a simple less or more than 4 joints but how long did it take for more than four joints to become involved. Although we have age of onset of disease, we are not prospectively gathering data in terms of progression of disease or on length of time to develop complications.

4.1.1.4 Quantification of severity

In this thesis, the ILAR classification has been used to determine severity in that we have compared oligoarticular JIA with extended oligoarticular JIA. An alternative method would have been to enrich for severity. Enriching can mean either including patients who have severe disease or examining the group that rarely has the disease but has developed it. Selecting patients for severity increases power in most studies including the extended TDT (Morris and Whittaker 1999). In the case of JIA, investigating genetic associations by including only those with severe disease could include, for example, children with more than 8 joints and comparing them with those with a monoarthritis. This would have greater power than comparing those with more or less than 4 joints. An alternative would be to compare children with systemic manifestations of disease such as growth retardation or high inflammatory markers with those with just local symptoms.

The alternative would be to examine genetic factors in the group that rarely has the disease because in these individuals, a higher threshold requirement for disease may be
necessary. This could explain the differences in HLA haplotypes between children with RA and adults. It may also explain the difference in HLA and TNF microsatellite distribution between males and females with RA (MacGregor, 1995; Hajeer, 1997). In the latter case, males may have a greater genetic component because they do not have the environmental (e.g. oestrogen) risk factors for developing disease. In the case of JIA, it is possible that greater genetic factors will play a role in, for example, young boys developing oligoarticular JIA than in young girls. But at the moment, the increase in power obtained is off-set by the decreased numbers that are obtainable.

4.1.1.5 The analysis of individual traits

In this thesis, the association of IL-10 5’ flanking region SNPs was examined in relation to uveitis. Uveitis was examined because the literature suggests that IL-10 may play a role in the development of uveitis in animal models. The role of IL-10 however is complicated by the fact that uveitis occurs across all disease groups and although IL-10 may be beneficial in joint disease it may be harmful in uveitis (section 1.8.7.2). The statistical analysis is therefore extremely complicated.

An alternative method of analysis is to define patients in terms of genotype and then to retrospectively analyse in terms of disease characteristics or disease severity. This has produced interesting results in analysis of genotypes in adult onset RA (de Jongh, 1984). This may be more relevant form of analysis in terms of our long-term objective which is to find genotypes that predict disease.

4.1.1.6 Statistical methods

The genotype data presented in this thesis was analysed as a case cohort or association study with all the inherent problems of stratification. This is a particular problem with our patient population which is from a wide geographical area (the whole of the UK) whilst the control population is drawn mainly from the Greater London region. There are several possible solutions to this problem. The study could be repeated as a cohort study in a different population or the TDT could be performed (in the same or a different population) to confirm or refute the association. The former is impossible in the UK as there are insufficient patients. We have therefore chosen to use the TDT and preliminary results have been presented.

4.1.2 Whole blood culture

Since this thesis was started, several groups have used the whole blood culture method to measure cytokine production. There are several advantages to the whole blood culture method, it is simple, fast, reliable and reproducible. Comparisons with PBMC
culture have shown that individual variation is less and the capacity for cytokine production is higher (De Groote, 1997). The whole blood culture method only requires small samples of blood which is an advantage for work in children where total blood volume is smaller. Because it does not involve a separation procedure and therefore stimulation of cells and because all cell components are present, it is also felt to be more physiological. We optimised the system in terms of IL-10 production which involved diluting the blood 1:1 with RPMI and stimulating with LPS (1μg/ml) for 24 hours.

Although several investigators have used similar conditions (Westendorp, 1997; Focà, 1998), some have used lower doses of LPS to measure stimulated cytokine production (van der Poll, 1996; van der Pouw Kraan, 1995; Swaak, 1996; Entzian, 1996; Swaak, 1997) or have diluted samples 1:5 (Benbernou, 1995) or 1:10 (Jacobs, 1998; Hober, 1998; van der Pouw Kraan, 1995; Swaak, 1996; Swaak, 1997) with RPMI. In each case, the reason these conditions were chosen is not clear and there do not appear to be any additional benefits over our method.

In the studies presented in this thesis, the IL-10 produced was corrected for the monocyte and lymphocyte count on the day culture was started. Some studies have not corrected for monocyte and lymphocyte count (Westendorp, 1997; Eskdale, 1998) but this means that variations in IL-10 production could be entirely due to variations in cell count which in itself could be a component of disease. We chose to correct for monocyte and lymphocyte count because there is a considerable body of evidence indicating that these are the main cells involved in IL-10 production (Coze 1994; Yssel, 1992; Cohen, 1995; Mosmann 1994; Stordeur, 1995; Frankenberger, 1996; Llorente, 1997; Bourrie, 1995; Llorente, 1994). It is arguable that we should have corrected for just the monocyte count as SCID mice challenged with LPS produce the same amounts of IL-10 as control mice (Bourrie, 1995). This suggests that in mice at least, the monocyte is the primary source of IL-10 when LPS is used to stimulated IL-10 production. This is consistent with studies in humans where FACS analysis of LPS stimulated PBMC detect IL-10 only in monocytes (Frankenberger, 1996; Visser, 1998). However, not all studies agree with this and depending on the method of analysis, stimulation and other factors such as the age of the patient, IL-10 has been detected in monocytes and lymphocytes (Stordeur, 1995; Llorente, 1997; Llorente, 1994). There is considerable evidence that IL-10 is produced by T cells but this is under different conditions than those used in our analysis (Yssel, 1992).
This question would have been resolved if we had been able to determine which cells produced IL-10 using FACS analysis.

The cell count was only checked on the day the blood was taken. This is analogous to setting up PBMC culture to a specific concentration prior to starting the study. It is however possible that changes in cell concentration during the 24 hour incubation could be different between different individuals.

4.1.2.1 Ex vivo methylprednisolone study

In this study, the FBC was only measured at the start of treatment which extended over 3 days. Although it is possible that the MP altered the monocyte and lymphocyte count during the course of the study, the analysis is performed on paired samples and therefore these differences are unlikely to affect the analysis. In addition, it was felt to be unethical to take further samples of blood from children during the course of the study.

4.1.3 FACS analysis

Although the problems with non-specific cell surface staining were overcome and clear identification of both monocytes and T cells was achieved in the whole blood, we were unable to identify intracellular IL-10. This is despite the fact that the individuals who were used were known to produce IL-10 after stimulation with LPS as measured by ELISA. The anti-IL-10 monoclonal antibody was used to detect intracellular IL-10 in PBMCs by other members of the laboratory. The monencin and brefeldin A were also used by other members of the laboratory.

Intracellular IL-10 has been detected by other groups by using FACS analysis in PBMC (Inoges, 1999) but at the time the study was undertaken, FACS analysis had not been used successfully to identify intracellular IL-10 in whole blood culture.

PBMC culture is almost entirely monocytes and lymphocytes whilst whole blood culture also contains granulocytes and platelets. This means that in whole blood culture, only a very small percentage of cells produce IL-10. IL-10 is produced mainly by monocytes (Visser, 1998; Bourrie, 1995), but after LPS stimulation it is only produced in easily detectable amounts by CD14+/CD14+ monocytes which account for 82% of monocytes (Frankenberger, 1996). However, it may be that fewer monocytes produce IL-10 as in LPS stimulated PBMC culture, IL-10 was only produced by a maximum of 0.9% monocytes (detected at the single cell level by UV microscopy of permeabilised cells stained with anti IL-10 mAb) (Andersson, 1992). As blood monocytes will only be between 5 and 15% of the white cells in whole blood culture
(cf 10 -35 % in PBMC (Meisel, 1996)), it may be possible to detect IL-10 in PBMC culture but not in whole blood culture.

Recently however successful intracellular staining of IL-10 in whole blood culture has been reported (Hodge, 1999). In this experiment, whole blood culture was stimulated for 4 or 24 hours with 100ng/ml LPS (rather than 1 μg/ml LPS) in the presence of Brefeldin A (as opposed to Monencin). The investigators did not dilute the whole blood with RPMI as in this thesis and do not describe a lysis stage, otherwise the methods used for staining were almost identical. The investigators however used CD 14 mAb to identify monocytes. This was not used in this thesis because CD 14 is the receptor for LPS and can be blocked by excess LPS or down regulated in the presence of LPS as described in this paper (Hodge, 1999). Intracellular staining of IL-10 in whole blood culture is therefore possible and this could be due either to improvements in technology and antibodies or the slight differences in the methodology.

4.1.4 Transfections

Two different cell lines (HeLa and THP-1) and 4 different methods were used in transfection studies with the IL-10 5' flanking region construct. Transfection was successful in each case with adequate expression of the reporter gene construct. LPS increased reporter gene expression more than 10 fold when the pGL3 positive control vector was used. We were unable to increase reporter gene expression for either of the 3 IL-10 5' flanking region constructs using either LPS (rough or smooth), IL-1β, TNFα or IL-6. This was not due to deficient LPS receptors on the THP-1 cells as 50% of the cells expressed CD14 and the THP-1 cells responded to LPS in that they differentiated and became adherent. The results were not improved using the DEAE Dextran method although overall transfection efficiency improved. Using this method, the THP-1 cells produced IL-10 mRNA constitutively and after stimulation with LPS and respond to LPS in terms of IL-6 mRNA production. It is therefore probable that they have the appropriate transcriptional machinery for IL-10.

As reporter gene expression was increased 10 fold by LPS when the pGL3 positive control vector was transfected and not when the IL-10 5' flanking region constructs were used, this suggests that the latter cannot respond to LPS. This may be because the constructs are too short and do not contain the necessary sequence for LPS to exert its effect.

The intracellular pathway by which LPS stimulates IL-10 transcription in monocytes and by implication THP-1 cells is not totally clear but it is likely that LPS binds to
CD14 and activates AP-1. A potential AP-1 binding site exists between –2423 and –2429 which is upstream from the IL-10 5’ flanking region constructs used. In addition, induction by LPS can involve sequences both up and downstream from the coding region and it is therefore possible that there are several sites required for LPS induction outside the constructs used (Han, 1991). Our collaborator, Richard Kay, showed significant upregulation of reporter gene expression using these constructs after stimulation with dibutryl cyclic AMP (dbcAMP) (Crawley, 1999c). dbcAMP is a cell permeable structural analogue of cAMP (Hurme, 1990) and may act by up-regulating AP-2 and AP-1 for which there is a putative binding site within the construct (Kube, 1995). This may explain why up-regulation using dbcAMP was possible but not using LPS. Interestingly Richard Kay has also shown that LPS synergises with dbcAMP to up-regulate reporter gene expression (Richard Kay, personal communication).
4.2 Summary and implications of research findings

4.2.1 Genotype results

4.2.1.1 JIA

We have shown that children with extended oligoarthritis are more likely to have an ATA containing genotype than patients with oligoarticular JIA. We have shown a similar distribution of genotypes in patients with asthma. The ATA haplotype appears to be the low IL-10 producing haplotype confirmed by our collaborator using transient transfection studies (Crawley, 1999c) and the ATA/ATA genotype is associated with low IL-10 production in whole blood culture (section 3.2.5) (Crawley, 1999c). These association studies therefore suggest that in two different inflammatory diseases, severity is associated with a low IL-10 producing genotype. As IL-10 acts mainly as an anti-inflammatory cytokine, this association is biologically plausible.

The Odds Ratio (OR) for this association is 1.9. This means that a child who presents with oligoarthritis and a genotype containing an ATA haplotype has nearly twice the chance of extending as a child who does not possess an ATA containing genotype. Although this OR is biologically important, it is also consistent with polygenic gene effects.

As this analysis was performed blind, we have genotype data on all children who presented to the out-patient department during the study period. As the initial aim of the study was to look at IL-10 5’ flanking region genotypes in patients with oligoarticular and extended oligoarticular disease we have not performed a Bonferroni correction. This is a valid decision as we had one hypothesis and one test. However, in the context in which this study was performed, the significance of other associations described is contentious. In association studies there is considerable controversy about stringency and power. This is because associations are frequently tested between many potential genes and many different disease phenotypes and whilst controlling the rate of false positives is essential, it should not be done at the cost of missing real genes i.e. false negatives. There are two possible approaches to this problem. The results can be interpreted with liberal significance levels and then the sample is replicated before claims are made or a single larger study is performed with stricter significant levels. There are advantages and disadvantages of both methods (Todorov and Rao 1997). In JIA however there are insufficient numbers for the latter method. In this study we have
not reported the associations observed between patients with polyarticular RF negative arthritis with the GCC/GCC genotype (p=0.05) as we believe this must be repeated before any conclusions can be drawn.

4.2.1.2 Asthma

The same pattern of genotype distribution for patients with asthma was seen as for those with oligoarticular JIA in that those with severe asthma were more likely to have a genotype containing one or two ATA haplotypes. There is also an increase in genotypes with no GCC haplotype in patients with severe asthma. The GCC haplotype has been shown by our collaborator to be associated with higher IL-10 production in transient transfection studies (Crawley, 1999c). This means that patients with severe asthma are more likely to possess a genotype which predisposes the patients to low IL-10 production. Once again this is biologically plausible given the role IL-10 is known to play in controlling inflammation in asthma (see section 1.9.5.2).

The OR for those with severe asthma is 1.6 for an increase in ATA containing genotypes and 1.6 for no GCC containing genotypes. These suggest that there is a protective effect for the GCC containing genotypes and a deleterious effect for ATA containing genotypes. Once again this level of increased risk is biologically important although small enough to suggest that there is a polygenic effect.

This is the first time a genotype has been shown to be associated with severity in asthma. Since this report was published, Borish et al have published an association between patients whose genotype possess the A allele at −571 (which is the same as the ATA haplotype) and increased total serum IgE (Hobbs, 1998). This is consistent with our results.

4.2.1.3 SLE

We did not show an association in genotype distribution between patients with SLE and controls. There was no difference in genotype distribution between any of the disease phenotypes studied which included renal disease, anti ds-DNA and anti Ro antibodies. This is in disagreement with previous reported associations (Mok, 1998; Lazarus, 1997). It is possible in the study by Mok et al (Mok, 1998) that this difference is due to ethnicity as their population was Chinese (see letter (Crawley, 1999b)). The other associations described appear to retrospective associations that have not been repeated in our larger analysis. As these reported associations are not reproducible, it is possible that they have either occurred by chance or are due to other factors, for example stratification (see section 1.5.2.1).
4.2.2 Implication of genotype associations in terms of disease management

There are two important implications in terms of disease management. The first is that being able to predict disease outcome early will allow earlier treatment of patients who are likely to develop severe disease. This is especially important in JIA where there is anecdotal evidence that early treatment with disease modifying drugs may alter prognosis. The second implication is that replacement treatment may be a logical therapeutic option in patients genetically predisposed to low IL-10 production. Whether this should be given systemically or locally still needs to be determined.

4.2.3 Transmission disequilibrium test

4.2.3.1 Oligoarticular and extended oligoarticular JIA

The TDT did not confirm the association of the ATA haplotype with extended oligoarticular disease as there was no increased transmission of the ATA haplotype. However there was increased transmission of the ATA haplotype to all patients in both disease groups although this did not hold true after a Bonferonni correction.

There are several possible explanations.

1. The association described is a type 1 error (false positive).

2. There are insufficient numbers in each disease group for us to have sufficient power to use the TDT (type II error).

3. The ATA haplotype is a susceptibility haplotype but a positive association was seen in patients with extended oligoarticular JIA because we essentially enriched for severity.

In the current analysis there are 48 and 47 patients in the two disease groups (oligoarticular and extended oligoarticular respectively). We cannot therefore exclude either a type II error in terms of the TDT or by implication a type 1 error for the association study. It will only be possible to draw any sort of conclusions when the numbers are increased.

4.2.3.2 Uveitis

Transmission of both the ACC and the ATA haplotype were significantly increased in patients with uveitis. This is consistent with the trend seen in the association studies but again no conclusions can be drawn until the numbers are increased.

The possible reason for the discrepancy is:
1. The association study negative result is a type II error. This is possible as the TDT is a powerful test and with these haplotype frequencies in a non-biallelic system, may be more powerful than an association study.

2. The TDT is a type I error. Numbers are still small however the significance level is high and makes this unlikely.

The results from the TDT suggest that it is possession of the A allele at -1082 that is associated with disease as increased transmission was described for both ACC and ATA.

4.2.4 Linkage analysis with IL-10 5' flanking region microsatellites

We have shown strong linkage disequilibrium between the microsatellite at -1000 kb and the SNP haplotypes. This is not surprising as the microsatellite is less than 50 bp away from the SNP at -1082. Confirming linkage disequilibrium is important because associations have been described between the microsatellites and patients with SLE and RA (Eskdale, 1998; Eskdale, 1997). It is possible that these associations are due to linkage disequilibrium with functional polymorphisms such as the SNPs described.

The association between the microsatellite and the SNP haplotypes is consistent with our data because the strongest association is between the G8 microsatellite allele and the GCC haplotype. The G8 microsatellite allele is decreased in patients with either oligoarticular, extended oligoarticular or polyarticular JIA suggesting a global importance in developing either of these diseases with this allele.

When children with JIA were examined different associations were seen. The possible reasons for this are discussed in section 3.1.7.2. Although it is possible that this association is due to chance or stratification it warrants further study as it is also possible that there is a synergistic effect between the microsatellite and the SNP alleles or that the two co-segregate. Either of these latter possibilities are important in terms of the pathophysiology of the disease.

4.2.5 IL-10 production and relationship to genotype

Stimulated IL-10 production is lower in healthy controls with the ATA/ATA genotype than in those with other genotypes and this is statistically significant. This is consistent with transient transfection results from our collaborator Richard Kay who showed that the ATA haplotype was associated with lower reporter gene expression (Crawley, 1999c). It is also consistent with a recent report investigating IL-10 production by
PBMC where high, medium and low IL-10 production were associated with GCC, ACC and ATA haplotypes (Edwards-Smith, 1999).

This disagrees with results produced by Keijsers et al (Keijsers, 1998) who describe an association between the GCC haplotype and low IL-10 production using the whole blood culture method. In this study however they did not correct for cell count which could explain the differences in results. It is also conceivable that the different results are because of genetic differences between the two populations studied. Keijsers work has not been published formally in terms of the SNP haplotypes although they have published with regard to the microsatellite haplotypes (Eskdale, 1998). It now appears to be generally accepted that the GCC haplotype is associated with high IL-10 production and the ATA haplotype with low IL-10 production (Helminen, 1999).

4.2.6 Parental IL-10 production

Stimulated IL-10 production was higher in the parents of children with oligoarticular JIA when compared to those with extended oligoarticular JIA. As an estimated 84% of the variability of IL-10 is genetically regulated, this result suggests that stimulated IL-10 production is higher in the children with oligoarticular JIA than in those with extended oligoarticular JIA. It is important to note that this difference is in stimulated IL-10 production. In effect we are measuring the ability to respond to inflammation. The ability to respond to inflammation in terms of IL-10 production appears to be lower in children with more severe disease than in those with mild disease.

This is an important result in the context of this thesis, because a genetic association is meaningless unless there is a biological correlate. In other words, there is little point seeking an association with genes that alter cytokine production if that cytokine is not important in terms of pathogenicity.

We did not show that the lower IL-10 production observed was due to a statistically significant increase in ATA containing genotypes. This could be due to a type 2 error in that we did not have sufficient samples or it could be that other factors influence IL-10 production in these parents. These could be other polymorphisms regulating IL-10 production or altered production of for example, other cytokines that influence IL-10 production e.g. TNFα.

An additional important observation during the course of this study was the high incidence of familial autoimmunity in the parents and first degree relatives studied. This was despite the fact that no other family member in the parents who took part had JIA. This increase in familial autoimmunity has been noted with other autoimmune
diseases (Ginn, 1998) but has not been described with JIA. It is possible that autoimmune disorders share genes that act together as polygenic risk factors for autoimmunity. This is particularly interesting as we also found that all parents of children with JIA had lower IL-10 production when compared to healthy laboratory controls.

4.2.7 Cytokine time course in whole blood culture
Although there are no published reports on cytokine production using the whole blood culture method, the results we present are consistent with cytokine production using other methods such as PBMC or monocyte culture. These time courses were measured in order to investigate the balance of cytokines using the whole blood culture method and as such, they must be regarded as preliminary results. However it is interesting to note that pro-inflammatory cytokine production (IL-1α, IL-1β, IL-6 and TNFα) was increased in the sample with the ATA/ATA (low IL-10) genotype compared to the GCC/GCC (high IL-10) genotype. This is consistent with the fact the IL-10 decreases pro-inflammatory cytokine production. If this is reproducible then this suggests that there is an association between IL-10 genotype and pro-inflammatory cytokine production. The result is especially interesting as pro-inflammatory cytokine production occurs before peak levels in IL-10 suggesting that if IL-10 genotype is important in determining pro-inflammatory cytokine production, it must be endogenous levels of IL-10 which are important.

4.2.8 Environmental effects on IL-10 production: Ethanol and Methylprednisolone
We were able to show in vitro that both ethanol and methylprednisolone synergised with LPS to increase IL-10 production. This is consistent with data published during the course of this thesis (see section 1.10.4). We were unable to show that alcohol increased stimulated IL-10 production ex vivo. This could be due to a variety of reasons. Some of these include the time delay between drinking the alcohol and when the sample was taken (usually approximately 10 hours), the small amounts of alcohol drunk (blood ethanol levels were always undetectable) and the small numbers tested. We were also unable to determine a consistent effect of methylprednisolone on ex vivo IL-10 production although we showed a consistent effect on in vitro IL-10 production. One reason for this could be that in some children there is altered excretion of methylprednisolone so that the ex vivo blood levels were not those estimated using the in vitro work. In addition the children admitted for intravenous methylprednisolone
were sick with an active inflammatory response and a host of factors both stimulating and inhibiting IL-10 production. It is therefore perhaps more surprising that there was a strong correlation between change in IL-10 production and change in ESR. The change in IL-10 production occurred over 24 hours whilst the change in ESR over 4 days. It is becoming increasingly clear that MP interacts at several levels in determining cytokine balance and one of its many actions is to increase IL-10. It is possible that measuring the change in cytokine balance will allow us to determine which children will respond clinically to MP.

4.2.9 Transfection results

When all the results were analysed for transfections using Effectene, overall reporter gene expression was higher for the GCC construct than the ATA construct. This analysis was performed on 28 experiments where all three constructs were used to optimise conditions. Although this included both stimulated and unstimulated experiments, there was no increase in reporter gene expression with stimulation so in effect this can be regarded as constitutive expression of the reporter gene construct.

In vitro therefore, there appears to be a difference in constitutive expression of the GCC construct compared to the ATA construct. The difference is small however (median 1.7, range 1.12 — 5.49) and there is a wide confidence interval. Clinically the relevant question is “is there a difference in the transcription of IL-10 from each of the three haplotypes during inflammation”. We were unable to answer this question during the course of this study.
CHAPTER 5: FUTURE WORK
6.1 Future work arising from this thesis

6.1.1 Genotype associations

For some analysis reported in this thesis, there has been insufficient power to be sure that the negative result is not a type 2 error. This includes association studies between patients with uveitis and controls. Data continues to be collected and will be reanalysed when sufficient samples are available.

The associations described (both those reported e.g. ATA and extended oligoarticular JIA and those unreported e.g. GCC homozygote and polyarticular RF negative arthritis) need to be confirmed. This will be done using the TDT and work has already started on this, preliminary results have been described. For future studies of other polymorphisms, association studies and the TDT will be undertaken concurrently. The concept of performing an association study and then confirming results with a TDT study has already been used in the investigation of the association of HLA alleles with JIA (Moroldo, 1998).

6.1.1.1 Control population

The problems of adequate controls have been discussed (see section 1.5.2.1). An improved control population is being collected from first time blood donors from the blood transfusion service in London. To date we have ~ 200 new samples of genomic DNA. Data on ethnic origin and date of birth has been collected. It is important to collect first time blood donors because the blood transfusion service recalls donors at a different rate depending on their blood group. Ultimately these control groups will allow us to do association studies on patients of different ethnic groups which we have been unable to do to date. In addition, they will be a well matched control group for patients from the Greater London region.

6.1.1.2 The database

Because of the changing nature of JIA, follow up of the clinical status of children at extended intervals (e.g. 5 and 10 years) after the onset of disease is necessary to avoid the problems of misclassification discussed in section 1.3.1. This is currently underway. Changes in the ILAR subgroup or in the development of disease characteristics such as uveitis need to be notified to those managing the database so that the data can periodically be reanalysed.
6.1.1.3 Association of SNPs and microsatellites

The different association described between the IL-10 flanking region SNPs and microsatellite needs to be investigated. This will require repeating the association in a control group that is matched geographically with our patients and enlarging the patient samples. If the association holds then the function of haplotypes containing the microsatellite and SNPs could be investigated in vitro to see if there is a synergistic effect of both alleles being present that predisposes to disease.

6.1.2 Parental IL-10 production

Overall, parents of children with JIA produce lower levels of stimulated IL-10 production than laboratory volunteers. This could be due to a number of reasons but it is important to know whether the predilection to low IL-10 production predisposes to the development of JIA. Parents of children with JIA therefore need to be compared to age matched controls (parents of children without JIA) to see if IL-10 production is different.

6.1.3 The effect of Methylprednisolone on cytokine production ex vivo

This work is continuing as a larger sample is needed for adequate analysis.

The demonstration of low or high IL-10 production must be put into context as it is the balance of cytokines that will determine outcome. This was demonstrated elegantly by Tillie-Leblond et al in their investigation of cytokine production in patients with status asthmaticus. In this study they showed that patients with status asthmaticus had increased levels of IL-1, IL-6, TNFα in bronchial alveolar fluid whilst that of IL-1Ra and IL-10 were not significantly different compared to ventilated patients but were higher than controls. They then measured the net inflammatory activity by measuring the capacity of bronchial alveolar fluid to modulate intracellular adhesion molecule-1 (ICAM-1) expression. A strong pro-inflammatory activity was demonstrated and this was shown to be mainly due to bioactive IL-1β and TNFα (Tillie-Leblond, 1999). This demonstrates that high levels of anti-inflammatory cytokines can still exist when net activity is pro-inflammatory and it is the balance of cytokines that is important.

As part of our ex vivo investigation on the effect of methylprednisolone on cytokine production, we will also measure constitutive and LPS stimulated pro-inflammatory cytokine levels on the stored serum. This will allow us to determine the effect that MP has on altering CK balance in children who receive pulsed MP.
6.1.4 Transfection studies

Future studies should therefore repeat the above experiments using longer constructs that include the potential AP-1 binding sites (-2423 and -2429).

In human monocytes, the addition of IL-1 or TNFα did not up-regulate IL-10 production unless the cells were primed with LPS (Foey, 1998). This may explain why the addition of IL-1 and TNF in the absence of LPS did not up-regulate reporter gene expression in the THP-1 cells. The experiment could be repeated using IL-1 and TNFα in the presence of LPS.

The experiments could also be repeated by transfecting human monocytes rather than cell lines. Transfection of human monocytes using adenoviral methods has already been described (Bondeson, 1999). It is important because transfection of human monocytes may give different results to transfection of cell lines (Bondeson, 1999). Transfection of THP-1 cells will be repeated using a larger constructs with some containing the AP-1 binding site. If reporter gene expression was shown to be different between the three different haplotypes then further experiments to define which transcription factors were involved in binding to the polymorphic sites would be performed.
6.2 The future of genetic studies in JIA

The ultimate aim of all genetic studies is to be able to create a model that will allow us to predict outcome in patients that we see early in the course of disease. This is especially important in JIA where it seems likely that early treatment may prevent long term damage. We already have the technological ability to screen hundreds of genes in thousands of patients and to perform association studies and family based studies such as the TDT. At the moment however we are unable to make sense of the data generated. We do not currently have the mathematical models that will allow us to analyse many genes together in terms of a variety of disease traits or severity markers.

The future of genetic studies in JIA is therefore likely to take the following stepwise approach.

1. Improve the clinical database. Many of the questions raised in Chapter 5 cannot be answered because there is insufficient clinical information.

2. Enlarge the database. As JIA is a polygenic disease, the OR and attributable risk for each gene is small and therefore patient numbers need to be increased to provide sufficient power.

3. Update the database. The database needs to be updated when a patients diagnosis changes. This is especially important for temporal aspects of the disease that define subgroups or disease traits such as uveitis that may only appear after a period of time.

4. Develop methods that will allow us to analyse multiple genes in relation to gene severity. This will include enriching for severity and the analysis of quantitative traits. Both of these methods require mathematical models that are not yet available and more clinical data as well as an increased sample size.

5. Develop methods that will allow us to analyse multiple genes in terms of disease traits. These traits may cross different ILAR subgroups. Analyses will again require the use of different mathematical models to avoid the problems inherent with multiple testing.
Appendices

Appendix A

*Making competent cells – method used by collaborator Richard Kay*

E coli JM109 was cultured overnight without ampicillin at 37°C in a shaking incubator before 4 mls was diluted into 200 mls of L broth so that the E coli could grow to an optimum density. The culture was then placed into four 50 ml Falcon tubes and placed on ice for 15 minutes before being centrifuged at 4000g for 15 minutes at 4°C. The supernatant was discarded and each pellet was resuspended into 16 mls of transformation buffer 1 (RbCl₂ 3.8g, MnCl₂.4H₂O 2.48g, CaCl₂.2H₂O 0.38g, Fac (pH 7.5), 7.5 ml of 1M stock, glycerol 37.5 ml made up to 250 mls of H₂O). The suspension was incubated on ice for 15 minutes, centrifuged at 4000g for 15 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 16 mls of transformation buffer 2 (MOPS (pH 6), 5 mls of 0.5 M stock, RbCl₂ 0.3g, CaCl₂.2H₂O 2.75g, glycerol 37.5 ml made up to 250 mls with H₂O). Cells were aliquoted and frozen at -70°C.

*Transformation of competent cells: method used by collaborator Richard Kay*

Aliquots of cells were thawed slowly on ice and 50µl was added to pre-chilled 15 mls polypropylene centrifuge tube. 0.7 µl of beta-mercaptoethanol was added and the suspensions gently swirled every 2 minutes for 10 minutes. 10 to 50ng of plasmid was added and the solutions were incubated on ice for 30 minutes with occasional swirling. The suspension was then incubated at 42°C for 45 seconds and then on ice for 2 minutes before 450µl of SOC (heated to 42°C) was added ad the cells shaken at 225 rpm at 37°C for 1 hour. The cells were then centrifuged at 4000rpm for 5 minutes on a microfuge. 300µl of supernatant was removed, the cells were resuspended and then plated out onto agar plates containing ampicillin (80 mg/ml) and methicillin (20mg/ml) and incubated overnight at 37°C.

SOB medium: Tryptone 20g, yeast extract 5g, NaCl 0.5g, made up to 1L with distilled water and autoclaved before 10 mls of MgCl₂ (1M) and MgSO₄ (1M) was added.

SOC Add 2mls 20% glucose to 100 mls SOB

LB agar: NaCl 10g, Tryptone 10g, yeast extract 5g, Agar 20g, made up to 1L with distilled H₂O. Antibiotics added when cooled.
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Appendix B

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Revision of the proposed classification criteria for Juvenile Idiopathic Arthritis: Durban, 1997 Taken from (Petty, 1998)

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Criteria definitions for proposed classification criteria for Juvenile Idiopathic Arthritis: Durban, 1997

Arthritis: Swelling within a joint, or limitation in range of joint movement with joint pain or tenderness, which persists for at least 6 weeks, is observed by a physician, and which is not due to primary mechanical disorders (also see exclusions list in ARA criteria for juvenile arthritis)

Number of affected joints: Joints that are able to be individually evaluated clinically will be counted as separate joints

Sacroiliac joints arthritis: Presence of tenderness on direct compression over the sacroiliac joint

Quotidian fever: Daily recurrent fever that rises to 39°C or above once a day and returns to or below 37°C between fever spikes.

Serositis: Pericarditis, pleuritis, and/or peritonitis

Enthesitis: Tenderness at the insertion of tendon, ligament, joint capsule or fascia to bone

Psoriasis; family history of psoriasis: Diagnosed by a dermatologist

HLA-B27 associated disease: Ankylosing spondylitis; sacroiliitis with inflammatory bowel disease; acute (symptomatic) anterior uveitis

Dactylitis: Swelling of one or more digits, usually in an asymmetric distribution, that extends beyond the joint margin

Positive rheumatoid factor: At least 2 positive results (as routinely defined in a laboratory using the WHO standard) 3 months apart during the first 6 months of observation

Nail pitting: A minimum total of 2 pits on one or more nails at any time

Inflammatory spinal pain: Pain in the spine at rest with morning stiffness in the spine that improves on movement

Uveitis: As diagnosed by an ophthalmologist

Spondlyoarthropathy: Inflammation of entheses and joints of the lumbrosacral spine

Terms used in descriptors

Features of systemic disease: Fever, rash, serositis, hepatomegaly, splenomegaly

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HLA Class I or II predisposing or protective alleles: Description of alleles positively or negatively associated with the category

Limb predominance: Description of any definite predominance of arthritis in upper or lower limbs

Positive test for antinuclear antibody: At least 2 positive results (as routinely defined in the laboratory you use) 3 months apart, during the first 6 months of observation. Indicate technique and titre

Oligoarthritis: Arthritis in a cumulative total of 1 to 4 joints

Polyarthritis: Arthritis in cumulative total of more than 4 joints

Large joints: Hip, knee, ankle, wrist, elbow, glenohumeral

Small joints: All others

Symmetry: A predominantly symmetrical or predominantly asymmetrical pattern of joint involvement

Appendix C:

Method of DNA extraction used prior to this thesis for DNA obtained from Northwick Park

One volume of whole blood was diluted with 9 volumes of cold buffer containing 0.32M sucrose, 5mM MgCl₂, 1% Triton and 0.01M Tris-HCL pH 7.6 and centrifuged to produce a nuclear pellet. This was resuspended in 25 ml of cold buffer containing 75mMNaCl and 24mM NaEDTA, pH 8, 1.25ml of 20% sodium dodecylsulphate and Protease. The DNA was extracted from the solution by phenol:choloroform extraction and ethanol precipitation at -20°C. It was washed in 70% ethanol, dried, resuspended in TE buffer and stored at 4°C.
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PUBLICATIONS
POLYMORPHIC HAPLOTYPES OF THE INTERLEUKIN-10 5' FLANKING REGION DETERMINE VARIABLE INTERLEUKIN-10 TRANSCRIPTION AND ARE ASSOCIATED WITH PARTICULAR PHENOTYPES OF JUVENILE RHEUMATOID ARTHRITIS

ESTHER CRAWLEY, RICHARD KAY, JAMES SILLIBOURNE, PRITASH PATEL, IAN HUTCHINSON, and PATRICIA WOO

Objective. To determine the distribution of the interleukin-10 (IL-10) 5' flanking region haplotypes in children with arthritis and in controls, and to investigate the functional significance of each haplotype.

Methods. Sequence-specific oligonucleotide probing was used to determine haplotype frequency. Transient transfection studies were used to investigate the transcription of reporter genes driven by each haplotype. Whole blood cultures were performed to assess IL-10 production by each genotype.

Results. Patients with arthritis involving >4 joints were more likely to have a genotype with an ATA haplotype than those whose arthritis remained restricted to <4 joints. This ATA haplotype was associated with lower transcriptional activity than the GCC haplotype (P = 0.02), and the ATA/ATA genotype was associated with lower IL-10 production under lipopolysaccharide stimulation than other genotypes (P < 0.02).

Conclusion. The results of this study demonstrate the functional significance of the ATA haplotype and reveal a significant association of genotypes containing this haplotype with extended oligoarthritis.

Dr. Crawley's work was supported by Arthritis Research Campaign (ARC) grant no. W0546. Dr. Kay's work was supported by ARC grant no. K0539. Mr. Patel's work was supported by an ARC intercalated BSc studentship. Dr. Woo's work was supported by an MRC program grant.

Esther Crawley, BA(Hons), BM BCh, MRCP; Pritash Patel, BSc(Hons), Patricia Woo, PhD, FRCP, FRCPCH: University College London Medical School, London, UK; Richard Kay, MB ChB, PhD, James Sillibourne, BSc(Hons): Ninewells Medical School, Dundee, UK; Ian Hutchinson, PhD: University of Manchester, Manchester, UK.

Drs. Crawley and Kay contributed equally to this work.

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Submitted for publication July 31, 1998; accepted in revised form January 21, 1999.

Juvenile rheumatoid arthritis (JRA) is defined as the development, in children under the age of 16 years, of arthritis in 1 or more joints lasting for more than 6 weeks (1). The reported prevalence of JRA varies between 1 per thousand and 10 per thousand (2), with the disease progressing to cause severe functional limitation (classes III and IV, Steinbrook classification [3]) in up to 49% of children (4). JRA is not a homogeneous disease, and these idiopathic arthritides have been reclassified by the International League of Associations for Rheumatology (ILAR) Task Force on Paediatric Rheumatology (5). Both proinflammatory and anti-inflammatory cytokines can be found in synovial tissue and synovial fluid in the joints of patients with both adult- and juvenile-onset arthritis (6-8). Proinflammatory cytokines such as interleukin-1β (IL-1β), IL-1α, tumor necrosis factor α (TNFα), IL-6, IL-12, and interferon-γ (IFNγ) promote inflammatory changes leading to bone and cartilage degradation (9-13). This destruction can be inhibited or controlled by receptor antagonists, soluble receptors, and antiinflammatory cytokines such as IL-10 and IL-4 (12,14-20). Inflammation persists when there is an imbalance between proinflammatory cytokines and their inhibitors. This has been shown to occur in juvenile chronic arthritis, Lyme disease, RA, and reactive arthritis (8,12,19,21).

IL-10 is a potent regulator of proinflammatory cytokines and is produced by monocytes and lymphocytes (22-25). It exerts its antiinflammatory effect by inhibiting the synthesis of proinflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, IL-12, and TNFα in activated macrophages (14,26-30), IFNγ production by T cells (28,31), and TNF, IL-1β, and IL-8 release from human polymorphonuclear leukocytes (32). IL-10 also blocks the action of the proinflammatory cytokines.
IL-1α and IL-1β by increasing the release of soluble IL-1 receptor antagonist (14,33,34). IL-10 suppresses intercellular adhesion molecule 1 expression by synovial cells, thereby inhibiting mononuclear cell traffic into synovial tissue (20,35).

Several studies have suggested that in vitro IL-10 is more effective than IL-4 in decreasing proinflammatory cytokine production by mononuclear cells from synovial fluid and synovial cells and peripheral blood of patients with RA (14,15). Furthermore, IL-10 appears to be more effective than IL-4 in ameliorating inflammation in the collagen-induced arthritis animal model (16). IL-10 can also reverse cartilage degradation by mononuclear cells from RA patients in tissue culture and appears to be more effective than IL-4 in this respect (15). Addition of exogenous IL-10 to synovial fluid monocytes or synovial membrane cultures from patients with RA decreases the production of proinflammatory cytokines, suggesting that IL-10 may play an antiinflammatory role in arthritis and that the endogenous IL-10 is insufficient to control the proinflammatory milieu (29,36). Preliminary work on children with systemic JRA suggests that production of IL-10 from whole blood culture is reduced (37).

Twin studies and family studies have suggested that ~75% of the variation in IL-10 production is genetically determined (38). IL-10 production appears to be controlled at the transcriptional level (39). The IL-10 5’ flanking region, which controls transcription, is polymorphic, with 2 microsatellites between −4000 and −1100 (40) and 3 single-basepair substitutions that were previously assigned to positions −1082, −819, and −592 (41,42). The G to A nucleotide polymorphism occurs within a putative Ets transcription factor–binding site. The C to T nucleotide polymorphism lies within a putative positive regulatory region (43), and the C to A polymorphism lies within a putative STAT 3 binding site and a negative regulatory region (43). There is linkage disequilibrium between the alleles with the 2 downstream polymorphisms occurring in tandem, and only 3 of 4 possible haplotypes have been found in Caucasian populations: GCC, ACC, and ATA. Initial in vitro work on peripheral blood mononuclear cell (PBMC) cultures has suggested that the GCC/GCC genotype is associated with higher IL-10 production than other genotypes (41), although the results of preliminary studies using the whole blood culture method appear to contradict this (44).

Associations between different IL-10 5’ flanking region haplotypes and systemic lupus erythematosus (SLE) and RA have been reported. In patients with SLE there appears to be an association between anti-Ro antibodies and the GCC or ACC haplotype (42), although the numbers studied were small and these results need confirmation from larger population association studies. An association between lupus nephritis and the ATA haplotype in a southern Chinese population has also been described. However, the distribution of haplotypes in the Chinese population is so different from those described in Caucasian populations that it is not clear whether this is due to ethnic differences or the different methodology used (45). There is a report of increased frequency of the ACC and ATA haplotypes in patients with RA who are positive for IgA rheumatoid factor but negative for IgG rheumatoid factor (46), although once again the numbers for this patient subgroup were small and these results need to be verified with larger patient and control groups. We have recently shown an association of the ATA haplotype in patients with severe asthma, when compared with patients with mild asthma or controls (47).

In this study we investigated whether there was an association between JRA and the various IL-10 haplotypes. Our hypothesis was that genetically determined lower production of IL-10 might influence disease susceptibility and/or severity. We also examined the functional significance of the haplotypes using transient transfection studies, and that of the genotypes using whole blood culture in healthy controls.

**PATIENTS AND METHODS**

**Patients and controls.** All patients and controls were Anglo Saxon Caucasians from the UK. The controls were recruited from 2 sources. One group of controls consisted of laboratory personnel from central and northern London (n = 127). The other consisted of male patients from 2 general medical practices in northern London (n = 147). There was no difference in haplotype distribution between female and male controls (χ² = 3.6, P = 0.98). All patient samples were obtained from pediatric rheumatology clinics held at Great Ormond Street Hospital for Sick Children (London, UK) or the Middlesex Hospital (London, UK), or from the United Kingdom Paediatric Rheumatology HLA Database (Arthritis Research Campaign epidemiology unit, Manchester, UK). Informed consent was obtained.

Genomic DNA was either extracted as previously described (48,49) or prepared using the Nucleon BACC I kit (Scotlab, Coatbridge, UK). This method enabled us to produce genomic DNA from 300 μl of EDTA-anticoagulated blood (final concentration ~60 μg/ml). We used the remainder of samples that had been used to measure complete blood counts or erythrocyte sedimentation rates and then stored in the hematology department at either the Great Ormond Street Hospital for Children or the Middlesex Hospital. Using the proposed ILAR criteria (5), JRA was classified as oligoarticular (<5 joints involved at onset), extended oligoarticular (>5
joints after the first 3 months), polyarticular rheumatoid factor negative, polyarticular rheumatoid factor positive, or systemic, enthesitis-associated, or psoriatic arthritis.

**Sequence-specific oligonucleotide probing (SSOP).** The SSOP method to define the IL-10 5' flanking region genome has been described previously (41). Briefly, the IL-10 5' flanking region was amplified between -1122 bp and -533 bp by polymerase chain reaction (PCR). PCR was performed with a total volume of 30 μl and 2 μl of sample genomic DNA (~0.1 μg). Final concentrations were 10 mM Tris HCl, pH 8.8, 50 mM KCl, 1% Triton, dNTPs at 0.2 mM each, 2.5 mM MgCl₂, and 1M Betaine with 1 unit Taq (Promega, Madison, WI) per reaction. The following cycles were used: 95°C for 4 minutes, and 30 cycles of 95°C, 55.5°C, and 72°C for 1 minute each followed by 72°C for 4 minutes. For each PCR product, 6 oligonucleotides (20 ng/ml) were used to probe for the 6 possible alleles, using a dot-blot technique. Detection using a chemiluminescent detection kit (Kirkegaard & Perry, Gaithersburg, MD) was carried out according to the manufacturer's recommendations. All samples were numbered before analysis to prevent bias in the interpretation of SSOP results.

**PBMC culture.** Blood was collected into a 20-ml universal tube (Sterilin; Bibby Sterilin, Staffordshire, UK) with preservative-free heparin (30 μl, 30 units; CP Pharmaceuticals, Wrexham, UK) and then diluted with an equal volume of plain RPMI 1640 (with glutamine, penicillin, and streptomycin but no fetal calf serum [FCS]). The blood/RPMI was then cushioned above 5 ml of Histopaque (Sigma, St. Louis, MO) and centrifuged at room temperature (20°C, 400g for 30 minutes). The cells at the interface were recovered, washed twice in plain RPMI/FCS at 1.5 X 10⁶ cells/ml. The cells were centrifuged at room temperature (20°C, 400g for 30 minutes). The cells were then incubated at 37°C in 4% CO₂. The suspensions were washed, counted, and resuspended to a concentration of 6.25 X 10⁶/ml in cRPMI. After 24 hours in culture, the cells were centrifuged, counted, and resuspended to a concentration of 6.25 X 10⁶/ml in cRPMI. Eight hundred microliters of the cell suspension was electrophoresed with 35 μg of pGL3 basic and 2 μg of pRL.TK (Promega) as a transcription efficiency control at 280V, 900 microfarads, and infinite resistance using the Easyjet Plus electroporator (EquiBio, Milton Keynes, UK). After electroporation, the cells were immediately diluted to a concentration of 5 X 10⁶/ml in 10 ml of warm cRPMI. These cells were placed in a single well of a 6-well plate for 16 hours in the presence of 1 mM dbcAMP (lot no. 01103EV; Aldrich, Milwaukee, WI). Cells were harvested, lysed, frozen overnight at ~80°C, and analyzed for both firefly and renilla luciferase activities using the dual luciferase assay kit according to the manufacturer's instructions (Promega). Luciferase activities, expressed in relative light units, were obtained using an L9501 luminometer (Berthold, Milton Keynes, UK).

**Statistical analysis.** To test our hypothesis that disease severity is associated with an IL-10 haplotype, the genotypes defined by SSOP were expressed as 0, 1, and 2 ATA haplotypes and compared between different groups using the chi-square test. Based on power size calculations, it was estimated that 90 patients were needed in each patient group for the test to have an 80% chance of demonstrating a 10% difference between groups. The results from the whole blood culture and PBMC culture were not normally distributed, and therefore the Mann-Whitney U test was used to compare PBMC and whole blood culture results as well as to compare results obtained using whole blood culture between subjects with different genotypes. For the transient transfection experiments, variance between groups was analyzed nonparametrically using the Kruskal-Wallis test. If groups were significantly different using
Table 1. Interleukin-10 genotypes in controls and in children with juvenile rheumatoid arthritis (JRA)*

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>GCC/ ACC</th>
<th>GCC/ ATA</th>
<th>GCC/ ACC</th>
<th>ACC/ ATA</th>
<th>ACC/ ATA</th>
<th>ATA/ ATA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (274)</td>
<td>70 (25.5)</td>
<td>25 (9.2)</td>
<td>54 (19.7)</td>
<td>24 (8.8)</td>
<td>38 (13.9)</td>
<td>18 (6.6)</td>
</tr>
<tr>
<td>Oligoarthritis (86)</td>
<td>22 (25.6)</td>
<td>23 (26.7)</td>
<td>20 (23.3)</td>
<td>9 (10.5)</td>
<td>9 (10.5)</td>
<td>3 (3.5)</td>
</tr>
<tr>
<td>Extended oligoarthritis (78)</td>
<td>16 (20.5)</td>
<td>16 (20.5)</td>
<td>24 (30.8)</td>
<td>5 (6.4)</td>
<td>11 (14.1)</td>
<td>6 (7.7)</td>
</tr>
<tr>
<td>Polyarticular, RF−(64)</td>
<td>24 (37.5)</td>
<td>13 (20.3)</td>
<td>12 (18.8)</td>
<td>3 (4.7)</td>
<td>7 (10.9)</td>
<td>5 (7.8)</td>
</tr>
<tr>
<td>Polyarticular, RF+(17)</td>
<td>5 (29.4)</td>
<td>3 (17.6)</td>
<td>4 (23.5)</td>
<td>2 (11.8)</td>
<td>2 (11.8)</td>
<td>1 (5.9)</td>
</tr>
<tr>
<td>Systemic arthritis (104)</td>
<td>22 (21.2)</td>
<td>28 (26.9)</td>
<td>24 (23.1)</td>
<td>9 (8.7)</td>
<td>17 (16.3)</td>
<td>4 (3.8)</td>
</tr>
<tr>
<td>Enthesitis associated (56)</td>
<td>17 (30.4)</td>
<td>11 (19.6)</td>
<td>8 (14.3)</td>
<td>4 (7.1)</td>
<td>12 (21.4)</td>
<td>4 (7.1)</td>
</tr>
<tr>
<td>Psoriatic arthritis (30)</td>
<td>12 (40)</td>
<td>8 (26.7)</td>
<td>3 (10)</td>
<td>2 (6.7)</td>
<td>3 (10)</td>
<td>2 (6.7)</td>
</tr>
</tbody>
</table>

* Values are the number (%). There were no statistically significant differences between groups. JRA was categorized as follows, according to the International League of Associations for Rheumatology proposed classification (5): oligoarthritis (<5 joints involved at onset); extended oligoarthritis (oligoarthritis extending to >5 joints after the first 3 months); polyarticular arthritis (>4 joints at onset; can be rheumatoid factor [RF] negative or RF positive); systemic arthritis (arthritis with quotidian fever and rash); psoriatic arthritis; or enthesitis-related arthritis.

The gene dosage of ATA haplotypes in controls and in patients with oligoarthritis and extended oligoarthritis is shown in Table 2. Children with oligoarthritis were more likely to have 0 ATA-containing genotypes (i.e., GCC/GCC or GCC/ACC or ACC/ACC genotypes) than patients with extended oligoarticular JRA ($P < 0.05$). Although a similar picture was seen in patients with eye disease, the difference was not statistically significant (Table 3).

Comparison of whole blood culture and PBMC culture for IL-10 production. PBMC culture was performed on 15 healthy Caucasians and whole blood culture on 45 healthy Caucasians living in northern London. Subjects were excluded if there was a history of arthritis or if they had a symptomatic viral infection. IL-10 production in the unstimulated samples was significantly higher in the PBMC cultures than in the whole blood cultures at 24 hours ($P < 0.001$). This probably reflects stimulation of the cells during cell separation for

Table 2. Interleukin-10 haplotypes in controls and in children with juvenile rheumatoid arthritis*

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>0 ATA haplotypes</th>
<th>1 ATA haplotypes</th>
<th>2 ATA haplotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (274)</td>
<td>164 (59.9)</td>
<td>92 (33.6)</td>
<td>18 (6.6)</td>
</tr>
<tr>
<td>Oligoarthritis (86)</td>
<td>54 (62.8)</td>
<td>29 (33.7)</td>
<td>3 (3.5)</td>
</tr>
<tr>
<td>Extended oligoarthritis (78)</td>
<td>37 (47.4)</td>
<td>35 (44.9)</td>
<td>6 (7.7)</td>
</tr>
</tbody>
</table>

* "0 ATA haplotypes" includes the following genotypes: GCC/GCC, GCC/ACC, and ACC/ACC; "1 ATA haplotype" includes GCC/ATA and ACC/ATA; "2 ATA haplotypes" is ATA/ATA. Values are the number (%).

† Genotype distribution in patients with extended oligoarthritis was significantly different from that in those with oligoarthritis ($P < 0.05$), with a decrease in the proportion with 0 ATA haplotypes and increases in the proportions with 1 or 2 ATA haplotypes.

Table 3. Interleukin-10 haplotypes in juvenile rheumatoid arthritis patients with eye disease

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>0 ATA haplotypes</th>
<th>1 ATA haplotypes</th>
<th>2 ATA haplotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligoarthritis (18)</td>
<td>10 (55.6)</td>
<td>7 (38.9)</td>
<td>1 (5.6)</td>
</tr>
<tr>
<td>Extended oligoarthritis (23)</td>
<td>9 (39.1)</td>
<td>10 (43.5)</td>
<td>4 (17.4)</td>
</tr>
<tr>
<td>Total (41)</td>
<td>19 (46.3)</td>
<td>17 (41.5)</td>
<td>5 (12.2)</td>
</tr>
<tr>
<td>No eye disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligoarthritis (41)</td>
<td>28 (68.3)</td>
<td>12 (29.3)</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td>Extended oligoarthritis (45)</td>
<td>22 (48.9)</td>
<td>21 (46.7)</td>
<td>2 (4.4)</td>
</tr>
<tr>
<td>Total (86)</td>
<td>50 (58.1)</td>
<td>33 (38.4)</td>
<td>3 (3.5)</td>
</tr>
</tbody>
</table>

* Values are the number (%). Patients with eye disease were less likely to have 0 ATA haplotypes and more likely to have 2 ATA haplotypes than those without eye disease, but the difference was not statistically significant.
IL-10 levels measured at 24 hours were corrected for the lymphocyte and monocyte count as measured on the day the culture was started. The results are shown in Figure 1. There were at least 5 subjects with each genotype. The production of IL-10 was lowest in those with the ATA/ATA genotype, and this difference was statistically significant ($P < 0.02$).

**Transient transfection.** The relative transcriptional activities of the different IL-10 5′ flanking region haplotypes were assessed by cloning the first 1047 bp of the IL-10 5′ flanking region from GCC, ACC, and ATA homozygous individuals into the pGL3 luciferase vector and transiently transfecting them into the U937 monocytic cell line. This region was specifically chosen because it avoided the highly polymorphic CA microsatellite region IL-10.G (51) and enabled the effect of these biallelic polymorphisms to be assessed in isolation.

Using this system, we were able to confirm that stimulated U937 cells are capable of transcribing genes using 1047 bp of the IL-10 5′ flanking region. IL-10 5′ flanking region reporter constructs were transcribed 20 times more efficiently than the vector alone in all cases ($P < 0.01$). When the relative transcriptional activities of the different constructs were compared, the haplotype ATA conferred significantly weaker transcriptional activity than GCC ($P = 0.02$) (Figure 2). The transcriptional activity of the ACC haplotype, whose relative activity lies between that of GCC and ATA, did not significantly differ from the activity of either of the other 2.

**DISCUSSION**

In this study we found that 59.9% of the control population had the genotype 0 ATA, 33.6% had 1 ATA, and 6.6% had 2 ATA. The frequencies of haplotypes were similar to those noted in previously published reports (41,50).

Our hypothesis was that cytokine genotype/haplotype dosage is associated with disease severity in JRA. A significant association between an ATA-containing genotype and extended oligoarticular JRA, compared with persistent oligoarticular JRA, was demonstrated. Since this was only a case-controlled or cohort study, we are currently using the transmission disequilibrium test (TDT) to test this association. It appears that the ATA haplotype is associated with severity and not susceptibility, since the presence of the ATA haplotype was not increased in patients with oligoarticular JRA when compared with controls. This is not the first single nucleotide polymorphism (SNP) to be shown to alter gene expression. Investigators at our laboratory have found an IL-6 5′ flanking region SNP to be associated with systemic JRA (52).

Several 5′ flanking region polymorphisms have been shown to be associated with other disease pheno-
To demonstrate transcriptional variations between the 3 haplotypes, we used U937 cells in our transfection studies because the activated monocyte is a major producer of IL-10 in arthritis. DbcAMP was used to stimulate U937 cells because it is an analog of cAMP, which is thought to play an important role in IL-10 stimulation in vivo via cAMP-responsive elements in the IL-10 5' flanking region (54,55). Using this model, we demonstrated that the ATA haplotype was associated with lower transcription than either the GCC or the ACC haplotype. This is the first time that the IL-10 promoter haplotypes have been shown to possess different transcriptional response to a given stimulus.

To investigate whether genotype affects IL-10 production in response to a known stimulus such as LPS, the whole blood culture method was used instead of PBMC culture. This is because we found lower constitutive levels of IL-10, but the same order of magnitude of stimulated IL-10, using the whole blood culture method, probably because mononuclear cells are stimulated during the separation of cells for peripheral blood culture. There also appears to be less coefficient of variation in the whole blood culture results (56), and it could be argued that the whole blood culture method mimics the in vivo state more closely. We showed that under the culture conditions chosen, maximal IL-10 production occurred at 24 hours. Interestingly, other groups have independently also chosen these conditions to assess IL-10 production ex vivo (35).

When whole blood cultures from different genotypes were compared for IL-10 production upon stimulation with LPS, IL-10 production was significantly lower in those with the ATA/ATA genotype. This is in contrast to results reported by Keijsers et al (44) that suggested that the A allele at -1082 was associated with higher IL-10 production than the G allele. In their study, however, IL-10 levels were much lower across all genotypes than in our study. Those authors also analyzed allele distribution and not genotype.

Although the association studies predict lower IL-10 production for those with the ATA/X genotype, we were unable to demonstrate this. This is probably because the whole blood culture method is not sensitive enough to detect small genetically determined differences in IL-10 production.

In the mouse and rat models of collagen-induced arthritis, recombinant IL-10 decreases the incidence, delays the onset, and reduces the severity (measured by paw swelling) of arthritis (17,18,57). The histologic findings are consistent with the notion of a chondroprotective effect of IL-10, associated with suppressed TNFα and IL-1 (16). In the mouse, anti-mouse IL-10 antibodies have been shown to accelerate the onset and increase the severity of the arthritis (16,58). Although IL-4 appears to have a synergistic effect with IL-10 in reducing paw swelling, IL-10 has the dominant role (16). Our findings are therefore consistent with the hypothesis that lower endogenous production of IL-10 would lead to a more severe form of disease such as extended oligoarthritis. Clearly these results have implications for arthritis in humans since a relative deficiency of endogenous IL-10 could be corrected using exogenous IL-10, with an expectation that there should be a reduction in inflammation.

The association with IL-10 haplotypes or a cytokine "genotype" could provide a prognostic indicator that is biologically plausible and may ultimately enable
us to define subgroups of patients who are at risk of developing severe disease as well as those to whom specific treatments should be targeted. The association does, however, need to be confirmed in other patient populations. Further family studies using the TDT methodology are already under way to confirm the association of the ATA haplotype in patients with either extended oligoarticular arthritis or eye disease.

In summary, we have established the haplotype frequency for IL-10 in a UK Caucasian control population. We have shown that there is transcriptional variation between the different haplotypes, and variation in IL-10 production under LPS stimulation among the different genotypes. Our findings reveal an association between the "low" IL-10-producing haplotype and disease severity in children with oligoarticular-onset JRA that extends to polyarthritis.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Steve Humphries, University College London, for supplying control genomic DNA, Prof. Alan Ebringer for supplying samples from patients with arthritis-associated arthritis, and Drs. Wendy Thompson and Rachelle Donn for supplying genomic DNA from the UK Paediatric Rheumatology HLA Database.

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association of the IL-10 polymorphic haplotypes in the proximal 5' promoter with SLE or a particular disease phenotype of SLE. The high IL-10 levels observed in patients with SLE could be due to other regulatory elements, another pathologic mechanism, or other candidate genes found in association with SLE (13).

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Single nucleotide polymorphic haplotypes of the interleukin-10 5' flanking region are not associated with renal disease or serology in Caucasian patients with systemic lupus erythematosus

Serum titers of interleukin-10 (IL-10) are higher in patients with systemic lupus erythematosus (SLE) compared with controls, positively correlated with anti-double-stranded DNA (dsDNA) antibody titers and the SLE Disease Activity Index, and negatively correlated with C3 levels (1). In multiplex families, IL-10 production by peripheral blood mononuclear cells (PBMC) is increased in healthy relatives, which suggests that the increase in IL-10 production seen in patients may be genetically controlled (2).

IL-10 production is mainly controlled at the transcriptional level (3). Within the IL-10 5' flanking region (which controls transcription), there are 2 microsatellite regions and 3 single nucleotide polymorphisms (SNP). The 2 microsatellite loci are at 1.1 kb and 4.0 kb 5' of the transcription initiation site, while the 3 SNP are found at the −1082, −819, and −592 positions, respectively (4,5).

Whole blood culture analysis (uncorrected for cell count) has suggested that variation in IL-10 production is associated with alleles at both the 1.1- and 4.0-kb microsatellite loci (6). A large study of Mexican Americans has shown an association of SLE with the 127-basepair allele at the 1-kb microsatellite locus (7). An initial study of Caucasian patients in the UK also suggested an association of this microsatellite with SLE (8). Since the 1.1-kb microsatellite is close to the −1082 SNP, and only 3 SNP haplotypes have been described in Caucasian populations (GCC, ACC, ATA), it is likely that IL-10 G alleles and these SNP haplotypes are in linkage disequilibrium. Findings in PBMC cultures and whole blood culture suggest that the GCC/GCC genotype is associated with high IL-10 production (4), and that the ATA/ATA genotype is associated with low IL-10 production (9). Transient transfection studies suggest that the GCC haplotype is associated with high IL-10 production and the ATA haplotype with low IL-10 production (9). There have been 2 reports of associations between different IL-10 haplotypes and disease phenotypes of patients with SLE (5,10). Our hypothesis is that there is an association between the putative high IL-10-producing haplotype GCC and SLE in patients with either dsDNA antibodies or renal disease.

All patients (n = 120) and controls (from 2 north London populations [n = 127, n = 147]) were Anglo-Saxon Caucasians from the same geographic area in the UK. All of the patients with SLE met ≥4 of the American College of Rheumatology revised criteria for the disease (11). They were all examined by 1 of us (DAI), and full historical details of their disease were recorded. Renal disease (n = 50) was assessed histologically. A patient's serum was considered to be anti-dsDNA positive if ≥3 measurements by enzyme-linked immunosorbent assay (ELISA) yielded twice the recommended upper limit for normal anti-dsDNA antibody titer (50 IU/liter). In addition, virtually all of these patients' sera were positive by *Crithidia luciliae* testing. The IL-10 genotype was identified for patients and controls using sequence-specific oligonucleotide probing as described previously (4). The results are shown in Table 1. There was no difference in genotype distribution between patients with SLE and controls for any of the disease phenotypes shown. There was no association between any of the 3 haplotypes and renal disease or any laboratory marker including anti-Ro antibodies, which were also measured by ELISA (Shield Diagnostics, Dundee, Scotland).

Our results differ from those of the 2 previously published reports of IL-10 haplotypes in patients with SLE (5,10). In their study of 76 patients, Lazarus et al describe an association between anti-Ro positivity and the CC alleles, i.e., the ACC and GCC haplotypes (5). Those authors also report that “the production of Ro autoantibodies was confined to those patients who had inherited the GCC haplotype either in homozygous fashion or heterozygotes.” However, their report does describe a patient with the ACC/ATA genotype whose serum was anti-Ro positive. We have not found an association between anti-Ro positivity and either the GCC or the ACC haplotype. Mok et al describe an association between the ATA haplotype and renal disease in southern Chinese patients with SLE (10). We have found no such association for Anglo-Saxon Caucasians. This could be due to a methodologic or population difference (12). Our patient sample size was greater and therefore likely to have less ascertainment bias.

In summary, this is the largest study of IL-10 genotypes in Caucasian patients with SLE. We have not shown an
was maintained, and there was no fibrosis. Liver iron concentration was 11335 µg/g dry weight (206 µmol/g N<1700 µg/g, 31 µmol/g). Serum ferritin was raised (table), but the liver copper concentration was unchanged.

At the age of 40, this patient shows no clinical evidence of haemochromatosis, the diagnosis being based on molecular and biochemical findings. Over a period of 14 years on treatment with trientine, his liver histology has improved, his transferrin saturation has fallen, and his liver function tests have remained normal, but the ferritin concentration has increased from 239 to 806 µg/L. The liver iron is markedly raised, but there is no earlier figure for comparison. The copper concentration has remained at a relatively low level for Wilson's disease. Treatment of patients with Wilson's disease with trientine has been shown to result in iron-deficiency anaemia: in 19 patients studied, the serum iron fell from 16-9 to 10-7 µmol/L, the difference being statistically significant. The possibility exists, therefore, that treatment with trientine has slowed down—if not arrested—the progress of this patient's haemochromatosis. Trientine may be the drug of choice for treating patients with Wilson's disease and iron overload, whatever the pathogenesis. Long-term treatment with penicillamine does not depress serum iron, but zinc therapy has been shown to induce iron-deficiency anaemia.

There are no corresponding studies for tetrathiomolybdate.


**Haplotype associated with low interleukin-10 production in patients with severe asthma**

Sam Lim, Esther Crawley, Patricia Woo, Peter J Barnes

Asthma is characterised by the expression of multiple inflammatory genes. Interleukin-10 (IL10), an anti-inflammatory cytokine, inhibits the synthesis of proinflammatory cytokines, chemokines, and inflammatory enzymes in activated macrophages, T cells, and human polymorphonuclear leucocytes. Production of IL10 is decreased in alveolar macrophages and peripheral blood mononuclear cells of patients with asthma, which could explain the proinflammatory profile in these patients and may lead to the development of more severe disease. IL10 production seems to be controlled at a transcriptional level and low concentrations of IL10 mRNA have been found in patients with asthma compared with controls. The IL10 5' flanking region, which controls transcription, is polymorphic. In addition to two microsatellites between −4000 and −1200, there are three single base-pair substitutions between −1117 and −627 that produce three different haplotypes, GCC, ACC, and AAT. These polymorphisms are in close proximity to several transcription factors that may interfere with gene transcription. Previously, the GCC/GCC genotype has been associated with higher and the AAT haplotype with lower production of IL10 in peripheral blood mononuclear cells than other genotypes. We investigated whether the difference in IL10 production between asthmatic and normal patients was due to a difference in the distribution of these haplotypes.

We used sequence-specific oligonucleotide probing to screen for the three different haplotypes in 240 white controls, 82 patients with mild asthma, and 113 patients with severe asthma. The control population were matched for geography, ethnic origin, and had no symptoms or diagnoses of asthma. Patients with mild asthma (mean age 27-6 [SD 0-85] years, 35 women, 47 men) had normal lung function (mean forced expiratory volume in 1 s [FEV1] 90-1 [0-095]% predicted), intermittent symptoms, and required no maintenance corticosteroids. Those with severe asthma (mean age 44-9 [1-39] years, 58 women, 55 men) had daily symptoms requiring regular β2-agonist therapy and high-dose inhaled (>800 µg/day beclomethasone dipropionate, or equivalent) or oral corticosteroids and impaired lung function (mean FEV1 64-4 [2-06]% predicted). Sequence-specific oligonucleotide probing by a dot blot technique with specific oligonucleotides for each of the six possible alleles in the IL10 5' regulatory region. We compared haplotype frequency in the groups with the χ2 test with Yates' correction.

The distribution of haplotypes in those with mild asthma and controls did not differ (table). However, fewer patients with severe asthma than controls had the putative high IL10-producing haplotype GCC (p=0-01, odds ratio 1-5) and more had the putative low IL10-producing haplotype AAT (p=0-01, odds ratio 1-6).

Associations between promoter polymorphisms and disease are common. Differential expression of IL10 has been implicated in several inflammatory diseases, including rheumatoid arthritis. An association has been shown between the IL10 promoter in patients with systemic lupus erythematosus and the GCC haplotype, which is consistent with the high IL10 concentrations seen in these patients, and an association between the low-IL10-producing haplotype (ATA) and a more severe form of arthritis. Therefore, the IL10 haplotype has a role in determining disease severity but does not seem to be important in susceptibility. In asthma, the low IL10-producing haplotype is more likely to be associated with severe disease.

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Juvenile Dermatomyositis: Epidemiology and Diagnostic Laboratory Data

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Clinical Description and Epidemiology Data

Juvenile dermatomyositis (JDM), with its hallmark rash, is the most common of the pediatric inflammatory myopathies, affecting three to four per million children/year and is at least 20 times more common than juvenile polymyositis (JPM), in which the rash is absent. The cutaneous manifestations of JDM include edema and telangiectasia surrounding the eyes, capillary drop out and dilation (best seen in the eyelids and eyelid margins, hard palate, edge of the finger nails, and redened “alligator skin” over the metacarpal, interphalangeal joints (Gottron’s papules), elbows, knees, and medial aspect of the ankles. The primary clinical feature if both JDM and JPM is chronic and progressive weakness of proximal muscles, resulting in difficulty in getting up from a chair or the floor, climbing stairs, and in activities of daily living such as dressing or feeding. In severe disease, the muscles affecting breathing and swallowing are impaired. Four diagnostic criteria (elevated serum levels of muscle derived enzymes, electromyographic evidence of inflammatory myopathy, positive muscle biopsy, proximal muscle weakness) are commonly used to make a diagnosis: definite JDM if, in addition to the typical rash, three of the four criteria are identified; definite JPM (no rash) but three of the four criteria are present. The affected area of inflammatory myopathy is often quite focal in distribution, which may impede the process of obtaining sufficient evidence to fulfill the criteria for diagnosis if an uninvolved area is inadvertently sampled. As a result, these criteria for diagnosis, while moderately useful, are imperfect, and depend on selection of an area of inflammation for two (electromyogram, muscle biopsy) of the four criteria. In early or mild disease, diagnosis and institution of therapy may be delayed with less than fortunate consequences. Because myositis is often a component of other autoimmune rheumatic diseases, it is essential to exclude such conditions as pediatric Systemic Lupus Erythematosus (SLE) (see article by Dr. Klein-Gitelman), mixed connective tissue disease, chronic arthritis in... Continued on page 106

Cytokines in Children with Arthritis

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Juvenile Idiopathic Arthritis

The prevalence of Juvenile Idiopathic Arthritis (JIA) is between 1:1000 and 1:10,000. Whilst many children have a good prognosis and ultimately lead a normal active life, a ten year review by Wallace and Levinson showed that 49% of these children have a poor prognosis and progress to severe functional limitation (class III and IV, Steinbrocker classification). In fact, chronic arthritis is one of the commonest causes of physical disability in childhood. JIA is not a homogenous disease and research into the aetiology and pathogenesis of JIA has been hampered by different classification systems using different criteria for defining JIA and its subgroups. The International League Against Rheumatism (ILAR) Taskforce on Pediatric Rheumatology has recently proposed a new unifying classification which will be used in this... Continued on page 112


cA2 was used to treat a teenager with systemic onset JIA, there was rapid and complete control of fever for five days after treatment and improvement in anorexia, chest, and abdominal pain suggesting that TNF-α is also important pathologically in systemic onset JIA.\textsuperscript{13} However, the fever recurred and the arthritis was unaffected throughout; therefore, it is unlikely to be the only cytokine that is relevant in this disease. Pro-inflammatory cytokines such as interleukin IL-1β, IL-1α, TNF-α, IL-6, IL-12, and IFN-γ promote immunological and inflammatory changes leading ultimately to bone and cartilage degradation.\textsuperscript{1,16-18} Many of these cytokines have been found in serum and synovial fluid of patients with JIA, and serum levels of IL-2R, IL-6, and the p55 soluble TNF-R correlate with inflammatory markers in children with JIA where the soluble TNF-R and IL-2R correlated very closely with disease severity and were more sensitive parameters than CRP. A further consequence of pro-inflammatory cytokines in vivo was the up-regulation of the acute phase protein, serum amyloid A.\textsuperscript{20,21} This protein polymerizes to produce amyloid fibers resulting in systemic amyloidosis in susceptible individuals.

### Anti-inflammatory Cytokines in Arthritis

The destruction caused by pro-inflammatory cytokines can be inhibited or controlled by naturally occurring antibodies, receptor antagonists, soluble receptors, and anti-inflammatory cytokines such as IL-10, IL-4, and TGF-β.\textsuperscript{11,12,22,29} IL-10 is a major anti-inflammatory cytokine since it inhibits the synthesis of pro-inflammatory cytokines from activated macrophages, T-cells, and human polymorphonuclear leukocytes.\textsuperscript{13,30-32} IL-10 also blocks the action of the pro-inflammatory cytokines IL-1α and IL-1β by increasing the release of soluble IL-1 receptor antagonist (IL-1RA).\textsuperscript{23,33,34} IL-10 suppresses ICAM-1 expression by synovial cells thereby inhibiting mononuclear cell traffic into synovial tissue.\textsuperscript{20,35} IL-4 is similar to IL-10 in that it inhibits the production of pro-inflammatory cytokines such as IL-1 and TNF-α.\textsuperscript{11,23,24,36} and appears to stimulate the production of cytokine inhibitors such as IL-1 receptor antagonist (IL-1RA) and soluble IL-1R and soluble TNF receptor.\textsuperscript{11} Several in vitro studies have suggested that IL-10 is more effective than IL-4 in decreasing pro-inflammatory cytokine production by mononuclear cells from synovial fluid and peripheral blood of patients with rheumatoid arthritis.\textsuperscript{23,24} Furthermore, IL-10 appears to be more effective than IL-4 in ameliorating inflammation in the collagen induced arthritis animal model.\textsuperscript{25} Both IL-4 and IL-10 can reverse the cartilage degradation by mononuclear cells from patients with rheumatoid arthritis in tissue culture although IL-10 appears to be more effective than IL-4.\textsuperscript{24,29,36}

### TH1 or Th2 Response?

Little is known about the cytokine production from T-cells from patients with JIA although one study has reported a prevalent TH0/TH1 pattern of cytokine secretion from T-cell clones from the synovial fluid of five patients with oligoarticular JIA.\textsuperscript{37} Work from our lab has shown a prevalent TH1 pattern of intracellular cytokines in synovial T-cells from patients with oligoarticular JIA (unpublished observations). It is possible that the type of response varies in patients with different JIA phenotypes as described below.

### Cytokines in Different Types of JIA

#### Oligoarticular and Extended Oligoarticular JIA

Oligoarticular JIA is defined as arthritis which affects four or less joints. Not only are fewer joints involved than in other forms of JIA, but also the arthritis appears to follow a more benign course with less destruction of the joint and a better long term prognosis.\textsuperscript{29} Murray et al. have recently demonstrated that children with oligoarticular JIA appear to have more IL-4 mRNA in the synovial tissue biopsies than children with polyarticular onset JIA. In their study, they also found the combination of IL-4 and IL-10 mRNA more frequently in non-erosive disease when compared to erosive disease.\textsuperscript{29} These observations may indicate that IL-4 is protective in oligoarticular JIA limiting the destructive effects of pro-inflammatory cytokines. Genetic differences could determine the prognosis of these two subgroups exhibiting mild or severe disease with similar onset. Our work has shown an association between children who develop extended oligoarticular JIA and the ATA haplotype in the 5' flanking region of the IL-10 gene (Table 2). This region has been shown to control IL-10 gene transcription, and the ATA haplotype is associated with low IL-10 production in transient transfection studies. Healthy individuals who are homozygous for this haplotype have lower IL-10 production in whole blood cultures stimulated with LPS.\textsuperscript{29} Another genetic association has been described between early onset oligoarticular JIA in Norwegian children and an allele in the IL-1α 5' flanking region. This association was particularly strong for children with chronic iridocyclitis.\textsuperscript{40} The functional relevance of this allele is not known and the finding was not confirmed in a UK population subsequently.\textsuperscript{41} Several studies have shown associations between children with oligoarticular JIA uveitis and HLA antigens.\textsuperscript{42-44}

#### Polyarticular, Rheumatoid Factor Negative JIA

In contrast to oligoarticular JIA, polyarticular JIA is frequently associated with joint damage and erosive change. This laboratory has shown that serum TNF-α

### Table 1. Examples of Pro- and Anti-Inflammatory Cytokines

<table>
<thead>
<tr>
<th>Pro-inflammatory cytokines</th>
<th>Anti-inflammatory cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon gamma (IFN-γ)</td>
<td>Interleukin 1 receptor antagonist (IL-1RA)</td>
</tr>
<tr>
<td>Interleukin 1 (IL-1)</td>
<td>Interleukin 4 (IL-4)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Interleukin 10 (IL-10)</td>
</tr>
<tr>
<td>Interleukin 6 (IL-6)</td>
<td>Interleukin 13 (IL-13)</td>
</tr>
</tbody>
</table>

Transforming growth factor β (TGFB)
Soluble TNF receptors (sTNF-R)
levels are higher in patients with poly-
articular JIA than in those with oligo-
articular disease or spondyloarthopathies. Furthermore, the balance of TNF-α with
sTNF receptors I and II in synovial fluid is
skewed towards high TNF-α in chil-
dren with polyarticular onset disease.9
Other cytokines such as IL-6 and IL-2R
appear to be elevated in active disease in
subtypes with different age of onset.43

**Polyarticular, Rheumatoid Factor
Positive JIA**

The clinical picture of early onset
polyarticular, rheumatoid factor positive JIA is identical to that seen in adults and
it is likely that the pathology is similar.
Cells isolated from the synovium of adult
patients with rheumatoid arthritis express
higher levels of IL-6 and IL-6 like cyto-
kine mRNAs, and spontaneously secrete
greater quantities of these proteins into
culture than cells isolated from the syn-
ovium of patients with osteoarthritis.46
Leistad et al.47 have compared mRNA
expression in the synovium of patients
with rheumatoid arthritis and osteoartri-
tis. Patients with rheumatoid arthritis
expressed IL-6, IL-6R IL-7 IL-8, IL-10,
and IL-12 whilst patients with osteoarthritis
only expressed IL-8 and IL-10. This
suggests a pro-inflammatory response within the synovium of patients with
rheumatoid arthritis. In patients with
rheumatoid arthritis, exogenous IL-10
decreases the production of pro-inflam-
matory cytokines (TNF, IL-1, and IFN-γ)
and neutralizing anti-IL-10 monoclonal
antibodies increases the production of
these cytokines from stimulated synovial
and peripheral blood mononuclear
cells.23,24,31,48 These results suggest that
the IL-10 response to stimulus is genetically deter-
mined and the reduced frequency of the
CC genotype (low IL-6 secretors) in
young systemic onset JIA patients may
suggest that IL-6 contributes to its patho-
gegenesis, in addition to other genetic fac-
tors such as HLA type. In a study of 13
systemic JIA patients and ten age-matched
controls such as HLA type. In a study of 13
systemic JIA patients and ten age-matched
controls showed that the CC genotype was
associated with significantly lower levels
of plasma IL-6. The genotype association
in systemic JIA is shown in Table 3.

**Systemic JIA**

IL-1β, IL-6, and TNF-α levels are increased
in peripheral blood and synovial fluid of
children with systemic JIA.19,49,50 IL-6
levels correlated strongly with ESR and
when IL-6 levels were measured during
febrile episodes, IL-6 closely mirrored
body temperature whilst the TNF-α levels
rise and fall out of phase with the temper-
ature with the peak being five hours after
the peak of fever.49 From this lab, Fishman
et al.51 investigated the possibility that
this cytokine profile resulted from a var-
iation in the control of IL-6 expression
leading to relative over-production of IL-
6 in children with systemic JIA. A G/C
polymorphism was detected at position
−174 in the 5′ flanking region of the
IL-6 gene. Transient transfection studies
showed that the C allele was associated
with lower expression than the G alleles
both constitutively and when stimulated
by LPS or IL-1. Plasma levels in 102 con-
trols showed that the CC genotype was
associated with significantly lower levels
of plasma IL-6. The genotype association
in systemic JIA was shown in Table 3.

These results suggest that the IL-6
response to stimulus is genetically deter-
mined and the reduced frequency of the
CC genotype (low IL-6 secretors) in
young systemic onset JIA patients may
suggest that IL-6 contributes to its patho-
genesis, in addition to other genetic fac-
tors such as HLA type. In a study of 13
systemic JIA patients and ten age-matched
controls, whole blood cultures from the
patients produced lower levels of IL-10
(anti-inflammatory) than samples from
the controls when stimulated with LPS.53
The IL-6 agonist soluble IL-6 receptor
(sIL-6R), however, has been shown to be
elevated54 and furthermore de Benedetti
et al. showed increased IL-6-sIL-6R com-
plexes at the height of fever.55 Thus there
may be an imbalance in systemic patients
with increased production of IL-6 and its
soluble receptor and reduced production
of IL-10 resulting in the persistence of
inflammation.

**Psoriatic Arthritis**

The synovitis of psoriatic arthritis is sim-
ilar but not identical to other forms of
arthritis with less synovial lining cell
hyperplasia and increased vascularity.56
Measurement of cytokine production in
psoriatic synovial membranes has shown
increased production of IL-10, IL-18, IL-
2, IFN-γ, and TNF-α.57 The production of
IL-18 and IFN-γ was strongly correlated
with IL-10. The cytokine levels were
higher in patients with psoriatic arthritis
despite higher histopathological scores in
patients with rheumatoid arthritis.57 The
balance of pro- versus anti-inflammatory
cytokines has not been defined.
Spondyloarthropathies

Little is known about cytokines in children with spondyloarthropathies. Rooney et al. showed that patients with spondyloarthropathies had higher synovial TNF receptors I and II/TNF-α than patients with polyarticular disease. This higher level of anti-inflammatory cytokines is after the disease is established. In a recent double-blind cross over placebo controlled trial, inhaled corticosteroids taken for four weeks were shown to increase IL-10 release from macrophages. Methylprednisolone has also been shown to increase IL-10 in murine endotoxaemia if given at high dose (50 mg/kg) but not at low dose.

Lyme Arthritis

When synovial fluid mononuclear cells from patients with Lyme arthritis are
genes have been found to be associated with disease subgroups and may well be important in the pathogenesis or disease severity of these JIA subgroups. Further studies of other cytokine genes within the cytokine network are needed. Drugs used in the treatment of JIA may act partly by altering cytokine production and restoring cytokine homeostasis. In the future, genetic studies may allow one to give a patient a reliable prognosis based on accumulated odds ratios for different cytokine genotypes as well as other associated genes. The elucidation of cytokine imbalances will also provide molecular targets for the development of more specific and safer means of therapy.

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Pediatric Lupus: Similarities and Differences from Adult Onset Lupus

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Systemic lupus erythematosus (SLE) is a disease of immune dysregulation which affects approximately one in 2,000 individuals. It is a prototypic autoimmune disease characterized by the production of an array of autoantibodies in association with clinical disease manifestations. The disease strikes young women most frequently, although the disease has been diagnosed in patients of all ages, all ethnic groups, and both sexes. In fact, 20% of lupus is diagnosed during the first two decades of life. Given the wide range of age at disease onset, one wonders whether there are differences in the clinical immunology of this disease depending on age at onset. I will review this complex issue in regards to both clinical disease and laboratory testing. The cause of SLE has yet to be elucidated; however, current research supports the idea that it is multifactorial and includes genetic predisposition, environmental signals, and hormonal influences. Hormonal changes in the female patient are correlated with disease onset which peaks during the child bearing years (15 to 40). It is also interesting to note that the female to male disease distribution changes from approximately 2:1 in the first decade of life to 9:1 during adolescence only to return to a ratio of 2:1 after the menopausal years.

A variety of studies have evaluated the role of female hormones in lupus disease onset and activity. Clearly, as seen above, there is a female disease predominance. One pediatric study revealed higher FSH, LH, and lower free androgens in postpubertal boys and girls with SLE.\(^1\) A recent study compared survival statistics of lupus patients belonging to a high or low female sex hormone at onset grouped according to age at diagnosis.\(^2\) Thus, children with lupus at onset under the age of 14 were grouped with women whose lupus occurred after the age of 51 in comparison to women whose lupus onset was between ages 17 to 47. Patients who had disease onset between 15 to 16 years and 48 to 50 years were excluded. Life table analysis was performed on these two groups compared to healthy controls and rheumatoid arthritis patients treated with methotrexate or gold salts: preliminary report. Inflamm Res 44:23-24, 1995.
Concentration of chromium in the quenching solution per unit surface area of coated specimen at the end of each thermal cycle

Figure 3.4.1.1 Amount of Chromium leaching out of samples after each cycle (Cl = Quenched in 3.5%wt NaCl solution).

Weight changes after each cycle

Figure 3.4.1.2 Weight changes after each cycle (Cl = Quenched in 3.5%wt NaCl solution).