Immune-phenotyping of B cells using LEGENDScreen™ to investigate the role of B cells in immunogenicity, and immune regulation by Bregs, in patients with rheumatoid arthritis

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Declaration

‘I, Laura Magill, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

Abstract

Rheumatoid arthritis (RA) is a multifactorial disease associated with failure of immune tolerance. B cells play a prominent role in the pathogenesis of this disease, via the production of auto-antibodies, pro-inflammatory cytokines, and an impaired regulatory B cell (Breg) response.

Biologic drugs have significantly advanced the treatment of RA but are not always effective and indeed the development of anti-drug antibodies (ADA) has been associated with a poorer clinical outcome. Here I set out, firstly, to identify a predictive biomarker that discriminates RA patients who are more likely to develop ADA in response to adalimumab, a human monoclonal antibody against tumor necrosis factor (TNF)α. By taking advantage of an immune-phenotyping platform, LEGENDScreen^{TM}, I measured the expression of 332 cell surface markers on B and T cells in a cross-sectional adalimumab-treated RA patient cohort with a defined ADA response. The analysis revealed seven differentially expressed markers between the ADA^{+} and ADA^{-} patients. Validation of the differentially expressed markers in an independent prospective European cohort of adalimumab treated RA patients, revealed a significant and consistent reduced frequency of signal regulatory protein (SIRP)α/β-expressing memory B cells in ADA^{+} versus ADA^{-} RA patients. I also assessed the predictive value of SIRPα/β expression in a longitudinal RA cohort prior to the initiation of adalimumab treatment. I showed that a frequency of less than 9.4% of SIRPα/β-expressing memory B cells predicts patients that will develop ADA, and consequentially fail to respond to treatment, with a receiver operating characteristic (ROC) area under the curve (AUC) score of 0.92. Thus, measuring the frequency of SIRPα/β-expressing memory B cells in patients prior to adalimumab treatment may be clinically useful to identify a subgroup of active RA patients who are going to develop an ADA response and not gain substantial clinical benefit from this treatment.

Secondly, further mining of the results from the LEGENDScreen^{TM} has allowed me to identify a signature of RA. Comparison of HCs to RA patients identified 16 differentially expressed markers associated with RA, and not with disease activity or treatment. Validation in an independent cohort determined that the combined

expression of CD97, CD170 and CD11c on B cells may identify individuals that are at risk of developing RA.

Thirdly, I have identified a novel marker of Bregs. Here I show that $CD19^{hi}CD170^{hi}$ B cells capture the majority of IL-10 producing B cells. This subset and not its negative counterpart ($CD19^{+}CD170^{int/low}$) suppress IFNγ and IL-17 production by $CD4^{+}$ T cells, in an IL-10 and CD170 dependent manner. $CD19^{hi}CD170^{hi}$ B cells are numerically reduced and functionally defective in RA patients. Preliminary data suggests that the aberrant production of IL-10 by Bregs in RA patients could be attributed to a defect in CD170 recycling. I propose CD170 may aid future immunological studies of Bregs, and that targeting CD170 therapeutically could improve disease in RA.

Impact Statement

The work presented in this thesis constitutes three main findings which have implications both for research in the lab, and in the clinic. Firstly, I have identified a novel predictor of anti-drug antibody (ADA) development in response to adalimumab treatment in RA. The development of ADA has been found to be associated with poorer clinical outcomes and failure of treatment. Therefore, the ability to predict ADA development within the clinic could inform treatment decisions, for example by not administering a drug where there is a high risk of developing ADA for a given individual. This would contribute to a more personalised approached to medicine; an important goal for conditions that require long-term management with drugs. Furthermore, immunogenicity i.e. the development of ADA, is often a neglected field for both basic immunologists as well as clinical scientists. The work presented in this thesis looking at ADA forms part of a wider project called ABIRISK (Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK). ABIRISK is a consortium of scientists and clinicians working collaboratively to address the issue of immunogenicity. What's more, the platform (LEGENDScreen^{TM}) and the strategy for identification of the predictive markers is novel and could inform future investigations into protein cell-surface biomarkers, and compliment other "-omics" techniques such as genomics and metabolomics.

Our second finding is a "signature of RA". This signature has three potential uses; 1. enhancing our understanding of the role of B cells in the fields of immunology and rheumatology; 2. providing novel candidate therapeutic drug targets; 3. predicting onset of RA in at-risk individuals. While these points have not been investigated to any great extent within the scope of this thesis, future investigations would aim to better address these points. Of note, the extensive amount of data obtained as part of this thesis provides the option for further investigations, with data on T cells, B cells and their subsets, in healthy individuals, and RA patients treated with numerous drugs.

Finally, I propose a novel marker of Bregs. There is currently no universal marker of Bregs and the classification of Bregs by the production of the cytokine IL-10 increases the complexity of their investigation in the lab. A novel marker of Bregs could help facilitate the progression of the field of Breg biology and could provide a novel drug target in patients with diseases in which a dysregulation of Bregs has been demonstrated.

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List of abbreviations

Ab	Antibody
ABIRISK	Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK
ABT	Antigen-biding test
ACPA	Anti–citrullinated protein antibodies
ACR	American College of Rheumatology
ADA	Anti-drug antibodies
AIA	Adjuvant-induced arthritis
AID	Activation-induced cytidine deaminase
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APRIL	A proliferation inducting ligand
ATP	Adenosine triphosphate
AUC	Area under the curve
B-1	Natural antibody secreting cells
BAFF	B cell activating factor
BCMA	B cell maturation antigen
BCR	B cell Receptor
BLIMP-1	B lymphocyte-induced maturation protein-1
BM	Bone marrow
BMI	Body Mass Index
Breg	Regulatory B cell
Ca^{2+}	Calcium ions
CCP	Cyclic citrullinated peptides
CD	Cluster of differentiation
cDMARD	Conventional disease-modifying anti-rheumatic drugs
CIA	Collagen-induced arthritis
CRP	C-reactive protein
CXCL	Chemokine ligand
D	Diversity (ref. gene rearrangement)
DAS28	Disease activity score 28
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
EAU	experimental autoimmune uveitis
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDC	Follicular dendritic cell
FLS	Fibroblast-like synoviocytes
FMO	Fluorescence minus one
FO	Follicular B cells
FoxP3	Forkhead box P3
FSC	Forward scatter
GBS	Group B Streptococcus
GC	Germinal centre
GWAS	Genome-wide association study
GZMB	Granzyme B
HC	Healthy control
HEL	Human Erythroleukemia Cell Line
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMI	Innovative Medicines Initiative
IRAS	Integrated Research Approval System
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
IU	International Unit
IV	Intravenous
J	Joining (ref. gene rearrangement)
JAK	Janus kinase
KO	Knock Out
LPS	Lipopolysaccharide
LRS	Leukocytes Reduction System
mAb	Monoclonal antibody
MFI	Median fluoresce intensity

MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
MSD	Meso Scale Discovery
MTX	Methotrexate
MZ	Marginal zone B cells
NICE	National Institute for Health and Care Excellence
NK cell	Natural Killer cell
OD	Optical Density
P/S	Penicillin/Streptomycin
PALS	Periarterial lymphatic sheaths
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PC	Phosphorylcholine
PCA	Principle component analysis
PCR	Polymerase chain reaction
PIA	pH-shift anti-idiotype antigen-binding test
PtC	Phosphatidylcholine
PTEN	Phosphatase and tensin homolog
RA	Rheumatoid arthritis
RA-D	Rheumatoid arthritis - cDMARDs treated
RAG	Recombination-activating genes
RANKL	Receptor activator of nuclear factor kappa-B ligand
RBL	Rat basophil leukemia
REC	Research Ethics Committee
RF	Rheumatoid factor
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
ROUT	Robust regression and Outlier removal
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SCF	Stem-cell factor
SEM	Standard error mean
SFA	Systematic framework analysis
SHM	Somatic hypermutation
SHP	Src homology region 2 domain-containing phosphatase
SIGLEC	Sialic acid binding Ig-like lectins

SIRP	Signal regulatory protein
SLE	Systemic lupus erythematosus
SNP	Single-nucleotide polymorphism
SSC	Side scatter
t-SNE	T-distributed Stochastic Neighbor Embedding
T1	Transitional-1 B cell
TACI	Transmembrane activator and CAML interactor
TCR	T cell receptor
TDM	Therapeutic drug level measuring
TGF	Transforming growth factor
Th	T helper
TIM	T-cell immunoglobulin and mucin domain
TLR	Toll-like receptors
TNF	Tumor necrosis factors
TNFi	TNF inhibitor
Tr1	T-regulatory 1 cell
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UCLH	University College London Hospital
ULN	Upper limit of normal
UV	Ultraviolet
V	Variable (ref. gene rearrangement)
WT	Wild Type

1. Introduction

1.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease that leads to destruction of cartilage and bone erosion, and manifests as pain, swelling and stiffness of the joints (1). This occurs as a result of inflammation of the synovial membrane called synovitis (Figure 1.1). According to most epidemiological studies, RA affects 0.5-1% of the adult population, with women at greater risk than males, and an average age of onset typically between 30-50 years (2-4). Disability associated with joint damage in RA reduces quality of life and leads to a significant socio-economic burden (3). In addition to joint inflammation, patients with RA may also develop systemic complications including cardiovascular, pulmonary, psychological and skeletal disorders (5). Individuals with RA have a 1.5 fold greater risk of cardiovascular diseases (6). If left untreated or poorly managed, RA is associated with increased mortality as a result of both the disease itself but also from the associated co-morbidities (7).

Classification of RA is defined based on the revised American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR) classification (revised 2010, 1992 original) (Table 1.1) (8). The diagnosis of RA combines several factors including: presence of clinical synovitis; involvement of two or more joints; confirmed presence of serological and inflammatory markers; and duration of symptoms. Auto-antibodies against citrullinated proteins (anti-CCP) and the Fc portion of IgG known as rheumatoid factor (RF), are found in patients with RA and are used as a diagnostic to confirm disease. However, it is noteworthy that RF and anti-CCP are not present in all RA cases, with around 31% of RA patients reported to be sero-negative for RF (9). In addition to RF and anti-CCP, autoantibodies against citrullinated fibrinogen (10) and carbamylated proteins (anti-CarP) (11) are also associated with RA. C-reactive protein (CRP) levels and erythrocyte sedimentation rate (ESR) are used both in diagnosis and to monitor disease activity in RA. Both CRP and ESR are indicators of inflammation.

Disease activity in RA is measured by the disease activity score (DAS)28. This score is calculated based on: the number of tender and swollen joints out of a possible 28 joints, a global health assessment (gVAS), and either raised levels of ESR or CRP.

Table 1.1. American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR) classification of RA (revised 2010, 1992 original)

	Score
Target population (who should be tested?): patients who	
1) have at least one joint with definite clinical synovitis (swelling)*	
2) with the synovitis not better explained by another disease†	
Classification criteria for RA (score-based algorithm: add score of categories A–D a score of ≥6/10 is needed for classification of a patient as having definite RA)‡	
A. Joint involvement§	
1 large joint¶	0
2−10 large joints	1
1−3 small joints (with or without involvement of large joints)**	2
4−10 small joints (with or without involvement of large joints)	3
>10 joints (at least one small joint)††	5
B. Serology (at least 1 test result is needed for classification)‡‡	
Negative RF *and* negative ACPA	0
Low-positive RF *or* low-positive ACPA	2
High-positive RF *or* high-positive ACPA	3
C. Acute-phase reactants (at least one test result is needed for classification)§§	
Normal CRP *and* normal ESR 0	0
Abnormal CRP *or* normal ESR 1	1
D. Duration of symptoms¶¶	
<6 weeks	0
≥6 weeks	1

* The criteria are aimed at classification of newly presenting patients. In addition, patients with erosive disease typical of rheumatoid arthritis (RA) with a history compatible with prior fulfilment of the 2010 criteria should be classified as having RA. Patients with long-standing disease, including those whose disease is inactive (with or without treatment) who, based on retrospectively available data, have previously fulfilled the 2010 criteria should be classified as having RA.

† Differential diagnoses differ in patients with different presentations, but may include conditions such as systemic lupus erythematosus, psoriatic arthritis and gout. If it is unclear about the relevant differential diagnoses to consider, an expert rheumatologist should be consulted.

‡ Although patients with a score of less than 6/10 are not classifiable as having RA, their status can be reassessed and the criteria might be fulfilled cumulatively over time.

§ Joint involvement refers to any swollen or tender joint on examination, which may be confirmed by imaging evidence of synovitis. Distal interphalangeal joints, first carpometacarpal joints and first metatarsophalangeal joints are excluded from assessment. Categories of joint distribution are classified according to the location and number of involved joints, with placement into the highest category possible based on the pattern of joint involvement.

¶ 'Large joints' refers to shoulders, elbows, hips, knees and ankles.

** 'Small joints' refers to the metacarpophalangeal joints, proximal interphalangeal joints, second to fifth metatarsophalangeal joints, thumb interphalangeal joints and wrists.

†† In this category, at least one of the involved joints must be a small joint; the other joints can include any combination of large and additional small joints, as well as other joints not specifically listed elsewhere (eg, temporomandibular, acromioclavicular, sternoclavicular, etc.).

‡‡ Negative refers to international unit (IU) values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; low-positive refers to IU values that are higher than the ULN but three of less times the ULN for the laboratory and assay; high-positive refers to IU values that are more than three times the ULN for the laboratory and assay. When rheumatoid factor (RF) information is only available as positive or negative, a positive result should be scored as low-positive for RF.

§§ Normal/abnormal is determined by local laboratory standards.

¶¶ Duration of symptoms refers to patient self-report of the duration of signs or symptoms of synovitis (eg, pain, swelling, tenderness) of joints that are clinically involved at the time of assessment, regardless of treatment status.

ACPA, anti-citrullinated protein antibody; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

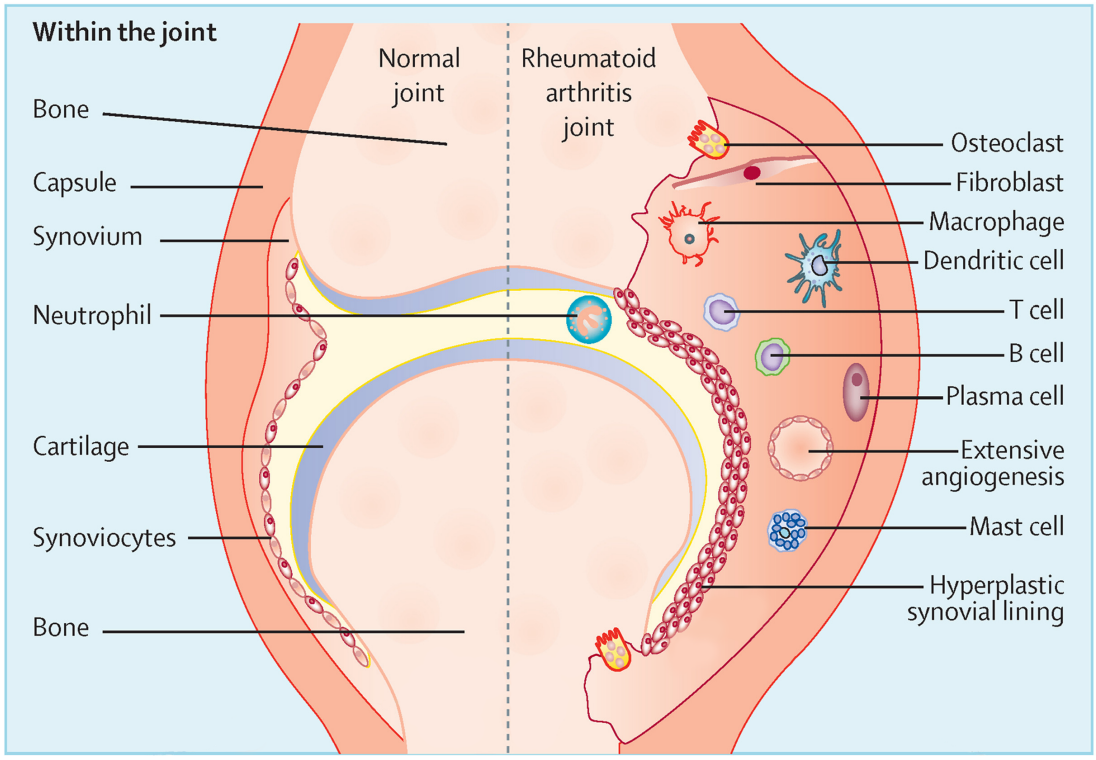

Figure 1.1 Joint damage in rheumatoid arthritis.
Normal healthy joint, and joint in rheumatoid arthritis, showing immune cell infiltrate and degradation of bone and cartilage (12).

1.1.1. Risk factors for RA development

Several genetic and environmental factors have been associated with an increased risk of rheumatoid arthritis (RA). Amongst these the strongest associations have been identified with gender, genetic susceptibility, exposure to external environmental factors and composition of mucosa-microbiota.

1.1.1.1. Genetic risk factors

The genetics of RA have been extensively investigated by both conventional and genome-wide approaches, with considerable evidence in support of a genetic association, and heritability estimated to be around 60% (13). Hundreds of single nucleotide polymorphisms (SNPs) have been identified to be associated with RA, many of which have been ascribed with immune effector or regulatory functions (14). Prominent amongst these genetic associations is the Human Leukocyte Antigen (HLA)-DR gene, in particular *HLA-DRB1* (15). This association has suggested a role for antigen presenting cells (APCs) governing T cell repertoire selection as well as their hyper-activation in RA pathogenesis, for example by increasing presentation of altered peptides including citrullinated proteins (16). Protein tyrosine phosphatase non- receptor 22 (PTPN22), which acts to regulate TCR signalling and is thought to lead to a decreased threshold for immune activation of T cells (17), has been shown to be highly associated with RA and is associated particularly with RF+ patients (18). Additional associations have been identified with genes that are involved in inflammatory pathways, or in the dysregulation of these pathways, in RA. These include CTLA-4, the inhibitory receptor expressed on T cells (19), REL, which encodes c-Rel a NF-κB family transcription factor, and is involved in cell signalling (20), STAT4, a transcription factor involved in the differentiation and proliferation of T cells (21), and IL6ST, which encodes the IL-6 transducer protein (22). Despite our identification of many genetic factors associated with RA, their functional role, and impact on the development and their contribution in the pathology of RA remains unclear.

1.1.1.2. Environmental risk factors

Several environmental factors, including smoking, dietary and other lifestyle factors have been associated to RA (23). One of the most well documented environmental risk factors for RA is exposure to tobacco. Several studies have identified a consistent association with RA and reported that smoking may lead to a 2-fold increase risk of developing disease (24, 25). Smoking is thought to induce tissue stress at the lung mucosa leading to an increase in post-translation modifications of peptides, including citrullination, and consequently the generation of anti-CCP antibodies (26-28). Interestingly, a link between smoking, RA, and an increased prevalence of periodontitis has been also been reported (29, 30). Smoking however, is not the only "environmental-culprit", with occupational exposure to silica dust also having been associated with an increased incidence of RA (31).

There has been a long-standing interest with respect to the role that infections may play in the development to RA. Molecular mimicry, whereby peptides share similar amino acid sequence or structure with auto-antigens, leads to loss of tolerance, and production of autoantibodies (32). Thus, molecular mimicry between pathogen-proteins and self-antigens provides a possible model for disease induction. Amongst many pathogens, RA patients have increased titres of Epstein-Barr virus antibodies in the serum as well as an increase in EBV-infected B cells compared to healthy controls (33, 34). In addition to a possible role that viral infections play in the induction of disease, in the last ten years there has been an increased interest in the role that microbes play in the pathogenesis of RA, in particular those colonising the mucosa. In individuals at risk of developing RA, those that go on to develop RA had higher levels of mucosal-associated immunoglobulin A (IgA)- antibodies in their blood prior to onset of RA (35). This supports the current working hypothesis that exposure to microbiota or microbial components in the mucosa, potentially together with other environmental factors in genetically susceptible individuals, leads to mucosal inflammation and the break of tolerance necessary for the initiation of RA (35). The majority of the interactions between a host and its commensal microbiota are symbiotic. However, several studies have found that the composition of intestinal microbials is altered in patients with RA (36). A pioneering study shows that *Prevotella copri*, a commensal bacteria resident in human gut, was found to be

strongly correlate with new-onset untreated RA, with an over-expansion of *P. Copri* present in the stool of new-onset compared to in chronic RA patients, psoriatic arthritis patients, and HCs (37). Two further studies provide support for this finding identifying increased *P. Copri* in a third stool samples from recent onset RA patients, and elevated levels of *P. Copri* in stool samples from patients during the first year of disease (38). Changes in oral microbiota composition, in addition to the involvement of gut-dysbiosis, have also been associated with changes in immunological responses at the mucosa, with a high prevalence of periodontitis observed in new-onset RA patients (39).

There are no definitive studies yet in humans confirming the role of microbial dysbiosis in RA, and indeed the majority of conclusive studies are from experimental mouse models of arthritis, where more evidence in support of a role for microbiota in the pathogenesis of arthritis have been found. For example, it has been shown that germ-free mice reconstituted with microbiota from collagen induced arthritis (CIA)-susceptible mice resulted in an increased susceptibility to arthritis, compared to germ-free mice reconstituted with microbiome from CIA-resistant mice (40). In addition colonisation of SKG mice (an animal model that spontaneously develops arthritis), with fecal samples from RA patients where *Prevotella copri* is dominant, develop exacerbated arthritis compared to fecal microbiota from HCs, and exhibited an increase in Th17 cells (41). Furthermore, *Prevotella histicola* has also been proposed to play a protective role in arthritis, with treatment of arthritis-susceptible HLA-DQ8 mice with *Prevotella histicola* isolated from the human gut, resulting in a decreased incidence and severity of arthritis compared to controls (42).

Many of the environmental risk factors for RA are events that occur at the interface between the external and the immune system, such as in the lungs, the oral mucosa and the gastrointestinal tract. However, the full extent of the role environmental factors play in RA is yet to be fully elucidated, with a combination of multiple genetic and environmental risk factors at play.

1.1.2. The immune-pathogenesis of rheumatoid arthritis

1.1.2.1. The breaking of immune tolerance and auto-antibodies in rheumatoid arthritis

RA arises as a result of failure of immune tolerance. Tolerance is the mechanisms whereby the immune system distinguishes self from non-self, leading to the removal or control of potentially harmful, auto-reactive immune cells. This occurs firstly during development of either B cells in the bone marrow or T cells in the thymus through a process called central tolerance. In the thymus the gene *AIRE* regulates the expression of tissue-specific proteins by thymic medullary cells, resulting in the deletion of tissue-reactive T cells (43). However, not all self-reactive lymphocytes are removed at this stage with a proportion escaping into the periphery. Here there is a second checkpoint of immune tolerance called peripheral tolerance, which keeps in check self-reactive cells via mechanisms including anergy, control by regulatory cells, and clonal deletion (described in more detail in Chapter 1.3). Immune tolerance strikes a balance between preventing autoimmunity whilst not impairing the immune defence. A breakdown in immune tolerance can occur as a result of the environmental and genetic risk factors described above, ultimately leading to altered post-transcriptional regulation of self-proteins. These altered self-proteins are subsequently recognised by autoreactive T and B cells that have escaped tolerance mechanisms, and thus a breakdown in tolerance occurs and leads to the production of auto-antibodies by plasma cells (44). Findings have shown that auto-antibodies, produced by self-reactive B cells, are present prior to onset joint inflammation, suggesting that the breaking of tolerance and activation of immune responses that lead to the development of RA occur well before the onset of clinical symptoms (57).

One post-translational modification that is thought to play a major role in the onset of RA is citrullination. This is the process whereby an arginine residue is converted to a citrulline, and leads to novel peptide-MHC interactions that may drive auto-antibody production (45). Citrullination is catalysed by the calcium-dependent enzyme protein arginine deiminase (PAD), and its expression by neutrophils, monocytes and macrophages is thought to play a role in RA (46, 47). Notably

citrullinated proteins are not inherently immunogenic and the process of citrullination has several roles including regulation of gene expression, terminal differentiation of epithelial cells, protein demethylation, and apoptosis (48). None-the-less, citrillunation is thought to increase the affinity of a peptide to particular MHC II molecules (e.g. HLA-DRB1) and hence increases the chance of self-peptide recognition and presentation by an MHC, and therefore activation of auto-reactive T cells (49). Auto-antibodies against citrullinated proteins (anti-CCP), as mentioned are one of the hallmarks of disease, however not all individuals with RA are found to have anti-CCP antibodies, with higher serum levels thought to predict greater severity of disease (50).

In addition to anti-CCP antibodies, the autoantibody RF, is present in an estimated 80% of RA patients. However, it needs to be noted that RF antibodies are also present in around 1-30% of healthy individuals dependent upon ethnic background (51). RF, typically a pentameric IgM antibody, is able to bind the Fc portion of an IgG, and is produced in response to polyclonal activation of B cells and exposure to antigen-antibody complexes such as occurs in response to citrullinated proteins and anti-CCP development (52, 53). RF also contributes to the formation of large immune complexes, which can activate complement as well as trigger neutrophils to produce degradative enzymes which can lead to tissue damage (54).

Alongside RF and anti-CCP there is a growing list of other autoantibodies found to be associated with RA, including anti-carbamylated proteins (anti-CarP) (11), anti-type II collagen antibodies (55) and anti-alpha-enolase antibodies (56). It is interesting to note that these antibodies target proteins that are distributed throughout the whole body and are not joint specific, and it remains unclear exactly how a systemic loss of tolerance leads to arthritis within the joints.

1.1.2.2. From systemic loss of tolerance to joint specific disease

RA is characterised by the development of synovitis, a term which refers to the thickening of the synovial lining, infiltration of immune cells including monocytes, macrophages, dendritic cells (DCs), natural killer cells (NKs), innate like lymphoid

cells (ILCs), B cells, T cells and plasma cells (the roles of these various cell types are explored in more detail below), neovascularization and lympho-angiogenesis. It is thought that anti-CCP antibodies may directly contribute to joint damaged via the activation of osteoclasts, and consequential induction of pain, inflammation, and bone loss (58, 59), and therefore may in part explain the involvement of the joints in RA. Notably, studies of synovial fluid from RA patients show a greater abundance of citrullinated antigens in the joints compared to in the serum (60, 61). A more recent study has identified two auto-antigens, N-acetylglucosamine-6-sulfatase (GNS) and filamin A (FLNA), which are highly expressed in the synovium of RA patients and have high sequence homology with gut commensal bacteria (62). This may indicate a link between events at the gut mucosa and joint inflammation in RA. It is also possible that trauma events within the joints could contribute to the onset of RA by initiating joint localised inflammation, post-translational modifications, and antigen recognition by circulating auto-antibodies (63).

While no specific link has been identified, DCs are good candidate cells for drivers of progression to RA (64). Abundant frequencies of DCs are present in the synovium of RA patients, and they are able to present auto-antigen and drive inflammatory T cell progression (65). Furthermore, intra-articular injection of inflammatory, collagen-pulsed DCs was sufficient to initiate arthritis in DBA/1 mice, a collagen-induced model of arthritis (66).

The development of auto-antigens and the breaking of immune tolerance leads to the activation of resident cells within the joint and subsequently a cascade of inflammatory immune events and the development of synovitis, cartilage damage and bone destruction; the hallmarks of RA. Fibroblast-like synoviocytes (FLSs) within the joint are thought to be important contributors to early initial inflammation within the joints (67), although the complete mechanism remains unclear. By taking advantage of imaging techniques such as ultrasound and MRI, abnormalities in the joints are reported prior to clinically apparent arthritis. (68, 69). More work needs to be done to better understand the onset of chronic joint inflammation associated with RA, which may lead to novel therapeutic strategies for the treatment of this disease.

1.1.2.3. Inflammation in the joint

The activation of resident cells within the joint by inflammatory mediators, leads to the recruitment of both innate and adaptive cells to the joint, fueling the inflammation, and thus creating a positive feedback loop (12). Cytokines and chemokines within the synovial compartment regulate inflammation, attracting immune cells to the joints as well as activating tissue resident cells. In addition to their articular effects, pro-inflammatory cytokines promote the development of systemic effects, including production of acute-phase proteins (such as CRP) (70), anemia (71), cardiovascular disease (72, 73), and osteoporosis (74), and may affect the hypothalamic–pituitary–adrenal axis, resulting in fatigue and depression (75).

Recruitment of innate cells, in particular neutrophils, characterises the early stage of RA. Neutrophils resident within the joints secrete proteases, prostaglandins and reactive oxygen intermediates, which contributes to tissue damage within this organ (1, 54). Neutrophils also secrete chemokines CCL2 and CXCL8, responsible for further recruitment of monocytes and neutrophils respectively (54). Both joint resident and monocyte-derived macrophages recruited by neutrophils in the synovium produce several cytokines including TNFα, IL-1 and IL-6 (76). TNFα and IL-1 drive the production of pro-inflammatory cytokines and chemokines, and the up-regulation of adhesion molecules on vascular endothelial cells and fibroblast-like synoviocytes (FLSs) within the synovium that leads to the recruitment and retention of circulating leukocytes (77). Whereas, IL-6 is a growth factor and promotes B cell differentiation and production of auto-antibodies (78). Furthermore, joint resident DCs as well as contributing to local inflammation and the recruitment of leukocytes via cytokine production, also present autoantigens to autoreactive T cells (79).

In response to inflammatory stimuli, joint endothelial cells upregulate adhesion molecules, integrins and selectins, thus facilitating the recruitment of circulating adaptive immune cells from the blood to the joint (1, 77). Selectins mediate the initial adhesion of leukocytes to the endothelium and integrins facilitate leukocyte arrest that leads to the transmigration of cells into the joint (77).

FLS, are specialised cells within the joint tissue that have been shown to play a role in RA via the production of cytokines and chemokines, that lead to the

recruitment, retention and activation of leukocytes (67, 80). At least in the early stage of disease the main role of FLS is the production matrix metalloproteinases (MMPs), enzymes that degrade the matrix, driving bone erosion and cartilage damage (81). FLS also promote the activation of chondrocytes, cells responsible for the maintenance of cartilage matrix, but in RA lead to joint damage (82).

Differentiation of autoreactive T cells plays a major role in driving RA, forming part of the adaptive immune response that leads to disease (83). Following their migration to the joint, directed by cytokines from tissue resident and infiltrated cells, T cells in response to auto-antigen presented by dendritic cells (DCs), macrophages and other antigen presenting cells, will differentiate into T helper 1 (Th1) and T helper 17 (Th17) cells, which produce IFNγ and IL-17 respectively and contribute to inflammation and tissue damage (84). In particular, IL-17 produced by Th17 cells has been shown to activate synovial fibroblasts, chondrocytes, and osteoclasts, resulting in cartilage degradation and bone erosion (85, 86). In turn, Th17 cells drive activation and differentiation of B cells into plasma cells via the production of IL-21 (87). In addition to the recruitment of pro-inflammatory cells, a failure of suppression by regulatory T cells (Tregs) has been reported in RA. Tregs are important in the maintenance of tolerance, and in RA it has been demonstrated that they are unable to suppress TNFα and IFNγ production from T cells (88). The function of Tregs has been shown to be restored after infliximab treatment, suggesting that the latter may work via restoration of the tolerogenic activity of these cells (89).

B cells contribute to the pathogenesis of RA via antigen presentation (90), autoantibody production (91) and secretion of pro-inflammatory cytokines (91). The accumulation of activated T cells and B cells within the lymph nodes leads to the formation of germinal centres. Here, mature B cells go through somatic hyper-mutation and produce autoantibodies (92). Autoantibodies drive bone erosion and osteoclastogenesis; anti-CCP positive patients have more pronounced bone erosions than anti-CCP negative RA patients (93, 94). The formation immune complexes by autoantibodies drives synovitis via the activation of macrophages, and enhances osteoclastogenesis by activating osteoclasts via Fc binding (93). In the joint, B cell survival and proliferation is maintained by the abundant quantities of

BAFF, APRIL and IL-6 produced by joint resident FLS, macrophages and stromal cells, in addition to infiltrating lymphocytes, monocytes and dendritic cells (67, 95, 96). More recently a role for B cells that exert regulatory function via the secretion of the anti-inflammatory cytokine IL-10 (regulatory B cells, Bregs) has been described (97). Bregs are fewer in number in RA, and produce overall less IL-10 than B cells from healthy individuals (98); the role of Bregs in RA is described in more detail in Chapter 1.4.

1.1.3. Treatment of RA, cDMARDs and biological therapies

RA is typically treated with conventional disease modifying anti-rheumatic drugs (cDMARDs) and/or biologics. cDMARDs act directly on the immune cells leading to a reduction in swelling and stiffness of joints over periods of weeks or months. The most commonly used cDMARDs in RA include methotrexate (MTX), prednisolone, sulfasalazine and hydroxychloroquine, with MTX usually being the first therapeutic choice. MTX is a folate analogue known to inhibit the metabolism of folic acid (99). The rational for the use of MTX for the treatment of RA was based on it's ability to reduce cell proliferation including immune cells involved in the autoimmune inflammatory response. However, other mechanisms are likely responsible for MTXs capacity to specifically reduce inflammation (100).

Often a combination of cDMARDs and MTX is required. In some cases, sufficient control is not achieved with these drugs. In patients with severe, active and progressive disease and where treatment with cDMARDS has failed, biologics are used. In the UK, choice of biologic is defined by the National Institute for Health and Care Excellence (NICE) guidelines (Rheumatoid arthritis in adults: management, NICE guidelines [CG79] February 2009).

Table 1.2. Biological therapies commonly used to treat RA.

Name	Target	Antibody construct	Action	Administration
Etanercept (trade name: Enbrel)	TNFα	Receptor-construct fusion protein	TNFα inhibition by acting as a decoy receptor	Subcutaneous injection weekly
Adalimumab (trade name: Humira)	TNFα	Human mAb	Binds soluble, transmembrane and receptor bound. Neutralising Ab	Every 2 weeks, given by subcutaneous injection
Infliximab (trade names: Remicade, Inflectra or Remsima)	TNFα	Chimeric mAb	Binds soluble, transmembrane and receptor bound. Neutralising Ab	Intravenous infusion about every 8 weeks
Rituximab (trade name: Rituxan, MabThera)	CD20	Chimeric mAb	B cell depletion	IV Infusion - two infusions are given 2 weeks apart, which is repeated when the improvement is wearing off (generally 6 months to 3 years later)
Tocilizumab (trade name: RoActemra)	IL-6R	Humanised mAb	Binds soluble and membrane bound IL-6 receptors reducing IL-6 inflammatory responses	Once every 4 weeks by intravenous infusion, or weekly by subcutaneous injection
Abatacept (trade name: Orencia)	CD80 and CD86	CTLA-4 based fusion protein	Blocks interactions between B and T cells	Once every 4 weeks by intravenous infusion, or weekly by subcutaneous injection

The first biological drug to be licensed for treatment in RA was the TNF inhibitor (TNFi) infliximab (101). TNFα, an inflammatory cytokine, has a major role in RA in driving inflammation and promoting disease, thus providing an attractive target for targeted monoclonal antibody therapy. Since the discovery of infliximab, further biologics have been developed also targeting TNFα or it's receptor, and consequently TNF inhibitors are the most commonly used first line biologic treatments for RA (101). Infliximab is a chimeric antibody, generated by combining mouse heavy and light chain variable regions with the constant region of a human antibody (101). Subsequently, advances in technology lead to the development of the fully human anti-TNF mAb treatment, adalimumab, which is a completely human derived recombinant antibody, and thus reducing the inherent immunogenicity associated with a chimeric antibody (102). Both infliximab and adalimumab bind soluble, trans-membrane and receptor bound TNFα acting to neutralise its effects, but each drug has it's own specific TNFα epitope target (103). Another TNFi used to treat RA is etanercept, a receptor construct fusion protein that acts as a decoy receptor for TNFα (101). Thus, all three drugs reduce excessive inflammation in RA by blocking the inflammatory effects of TNFα.

An alternative to the anti-TNFα-based therapeutics, that has also shown success in the treatment of RA, is rituximab, a chimeric antibody (104). Rituximab targets CD20, which is expressed on B cells from early stages of development, on pre-B cells to mature B cells, but it is not expressed on plasma cells. Rituximab treatment results in the depletion of the majority of B cells in circulation, but not plasma cells, with levels of autoantibodies often remaining unchanged (104, 105). The success of rituximab in RA supports the notion that B cells play a pathogenic role in RA beyond antibody production. Rituximab is often used in patients following failure of TNF inhibitors or as first line therapy when TNF inhibitors are not suitable.

As previously described IL-6 is an important driver of inflammation in RA, tocilizumab is a humanized anti-IL-6 receptor antibody (IL-6R) (106). IL-6 promotes the activation of leukocytes and osteoclasts, therefore targeting IL-6 not only acts to reduce bone erosion but also prevents inflammatory effects due to B cell activation, including auto-antibody production (78). Due to the success in controlling inflammation newer guidance from NICE now allows the use of tocilizumab as a

first biologic (2016 NICE technology appraisal guidance 375). A more recent study has shown that tocilizumab is more successful than anti-TNFs when used as a first line therapy after cDMARD failure (107).

CD80 and CD86 are constitutively expressed on all antigen presenting cells (APCs) including activated B cells and monocytes, and provide a co-stimulatory signal required for the activation and differentiation of autoreactive T cells (108). Abatacept is a CTLA-4 based fusion protein that binds CD80 and CD86, and thus inhibits the activation of autoreactive T helper cells, and has shown to improve symptoms in RA (101, 109).

Janus kinases (JAK) are part of the JAK-STAT signaling pathway which is involved in development and homeostasis, and can lead to the transcription of a wide array of cytokines and growth factors (110). This pathway is continuously activated in RA and for example promotes the production of inflammatory cytokines and an overexpression of MMPs (111). Small molecule inhibitors targeting JAK, including tofacitinib (112) and baricitinib (113), have shown efficacy in RA.

IL-17 is enriched in the synovium of RA patients and activates synovial fibroblasts, chondrocytes, and osteoclasts (114, 115). Drugs targeting IL-17 have been approved for the treatment of active psoriatic arthritis and ankylosing spondylitis (secukinumab), and psoriasis (ixekizumab) (116). While there has been some limited success in targeting IL-17 in RA patients, these treatments have not been licensed for the treatment of RA patients (117, 118). Similarly, levels of the inflammatory cytokine IL-1β are elevated in RA (119, 120). The drug anakinra targets IL-1β and is licensed for the treatment of RA, however, it shows lower efficacy and a higher withdrawal rate than other RA biologics (121, 122).

1.2. Immunogenicity and anti-drug antibodies (ADA)

Despite biologics having improved patient care for RA patients on the whole (123), not every patient responds to treatment. Immunogenicity is an immune response against a biological drug that results in infusion reactions (124), hypersensitivity reactions and loss of efficacy of the drug (125). ADA can form immune complexes that block the drug from interacting with its target and increase its clearance. This results in lower blood drug levels and therefore reduces treatment efficacy (126, 127). Multiple observational clinical studies have shown the development of ADA against adalimumab occurs in up to around a third of adalimumab treated patients (128, 129); ADA have been reported to occur in as high as 63% of infliximab treated RA patients (124, 130, 131); ADA against rituximab in up to 11% of patients (132, 133); and ADA against tocilizumab up to 8% of patients (134, 135). Some studies have identified ADA against etanercept (136, 137), however, the results are inconclusive with other studies detecting no ADA at all (137). RA patients with ADA typically have a worse clinical outcome and are more likely to fail the drug (124, 137, 138). Indeed, lower drug levels and ADA presence at 3 months was shown to predict lack of response to the drug at 12 months (139).

1.2.1. Risk factors contributing to ADA development

Despite the association of ADA with loss of response to treatment, there have been very few investigations into the immunological mechanisms that drive ADA development and/or what may predispose a patient to develop ADA. Not all individuals develop ADA, making it likely there are specific predisposing risk factors. Therefore, there is a need to identify predictive biomarkers to define individuals at risk of developing ADA, and to improve the clinical management of RA to prevent unnecessary treatment of patients that may not respond to a particular therapy.

The development of ADA has been broadly attributed to factors relating to the patient, the drug, and the course of treatment (140). RA patients with ADA, have been observed to have higher baseline disease activity (141). Furthermore, patients who have developed ADA against one anti-TNFα are more likely to develop ADA

against a second anti-TNFα drug (142, 143). In addition, patients that were homozygous for the same IgG allotype as adalimumab (G1m17) were more likely to develop ADA (41%), and those homozygous for G1m3 had the lowest frequency of ADA^+ individuals (10%) (144). In MS (multiple sclerosis), which is commonly treated with and antibody therapy against interferon-β, ADA development against the drug was associated with HLA-DR4 (145).

Anti-drug antibodies, like all antibodies can be neutralising or non-neutralizing (140). Neutralizing antibodies will directly interfere with the activity of the drug by blocking binding of the drug-antibody to its target e.g. TNFα. Non-neutralizing antibodies cause the formation of immune complexes (127) that mediate the clearance of the drug from the system, thus reducing its half-life. Immune complexes facilitate faster clearance of the drug, and hence explain why lower serum drug levels are observed in the presence of ADA (128).

The construct of a drug, e.g. full antibody versus fusion protein, is a major contributing factor to immunogenicity, and explains in part the variance in immunogenicity between drugs as described above (140). Biologics are intrinsically immunogenic although some constructs are less immunogenic than others. Both, infliximab and rituximab are chimeric antibodies, and are considered to be the most immunogenic (101). Tocizilumab, a humanized mAb, and adalimumab a fully human mAb are less immunogenic (101), with etanercept, a receptor construct fusion protein (136), least likely to of elicit an immune response (140).

Finally, treatment related factors can include a variety of aspects including regime, route of administration, duration of treatment and co-treatment (140). High doses of infliximab have been shown to maintain clinical response in the presence of ADA (146) and induce immunological tolerance via the induction of unresponsive T cells (anergy) (147). Careful monitoring of dose in line with individual responses will also help minimise unwanted immunogenicity effects. Co-treatment of anti-TNFs with the cDMARD MTX has been associated with reduced ADA levels (148), however, absolute numbers of patients developing ADA remains the same (124).

1.2.2. Measuring ADA

The true extent of the impact of ADA on clinical outcome is still open for debate (149). Currently there are many methods of measuring ADA with varying accuracy and sensitivity (149). Bridging enzyme-linked immunosorbent assays (ELISAs) are most commonly used to measure ADA; both free and bound ADA are detected using a labeled version of the therapeutic antibody (140). However, detection of bound ADA is compromised by 'drug inference'. Therefore, if ADA testing is done by competitive binding assay directly after treatment administration, the high levels of drug in the body could mask presence of ADA. Alternative assays include radio-immunoassays such as the antigen-binding test (ABT), which uses a radioactively labeled version of the therapeutic (140). Similar to ELISA based techniques, radioimmunoassays predominately detect free ADA. A better alternative is the pH-shift anti-idiotype antigen-binding test (PIA), which is able to detect free and bound ADA, however, few studies have used this technique (140). Furthermore, most of these assays do not distinguish between neutralising or non-neutralising antibodies, and therefore if the ADA will directly interfere with the drugs activity or simply reduce the half-life of the drug (140). The ability to accurately detect ADA could potentially deliver precision medicine to heterogeneous diseases such as RA and increase the efficiency of clinical decision-making.

1.3. The biology of B cells in health and in disease

B cells are a population of immune cells fundamental in the host response against pathogens. So called due to their origin in the *bone* marrow (BM), B cells contribute to the clearance of pathogens via the production of antibodies against foreigner antigens, antigen presentation, and secretion of a vast array of cytokines (90, 91, 150). Broadly defined as immunoglobulin (Ig)-expressing cells, their origin can be traced back to jawed vertebrates (151). Their initial discovery came following the identification of Ig/antibodies in the serum (152), with plasma cells (terminally differentiated B cells) later defined as the producers of antibodies (153). Different B cell stages of differentiation and maturation can be distinguished based on the expression of different surface molecules, as outlined in Table 1.3. These phenotypes allow us to identify and study the different B cell types.

Table 1.3. B cell subtypes.
Expression (+) or no expression (-), at high, intermediate (Int) or low levels, of cell-surface proteins on human B cells.

	CD19	CD20	CD38	CD24	CD27	IgM	IgD
Immature	+	+	High	High	-	High	High
Mature	+	+	Int	Int	-	Int	+
Memory	+	+	-	High	+	+/-	-
Plasmablast	Low	Low	High	-	High	+/-	-
Plasma Cell	Low	-	High	-	+	-	-

1.3.1. Early B cell development

B cells derive from hematopoietic stem cells in the BM where they undergo initial maturation, developing in direct contact with the stromal cells that provide many of the signals required for their development (154). Within the BM, growth factors and cytokines, including CXCL12, Fms-related tyrosine kinase 3 (FLT3), thymic stromal lymphopoietin (TSLP), IL-7, stem-cell factor (SCF), and receptor activator of nuclear factor-κB ligand (RANKL), are required for successful differentiation and maturation of B cells in the early stages of development (155-159). Transcription factors including E2A, EBF and PAX5 also promote B cell maturation, stabilising B cell lineage commitment and differentiation (160, 161). In particular, PAX5 deletion in mice leads to the generation of uncommitted cells, which frequently results in development of lymphomas in these mice (162). Furthermore, PAX5 mutations have been identified in childhood B-lineage acute lymphoblastic leukaemia (163).

As part of their development, B cells acquire the expression of an Ig molecule, the B cell receptor (BCR). The BCR is membrane bound and comprises two heavy and two light chains, with a unique variable domain and hyper-variable regions that specifically bind to antigen. The gene encoding the heavy chain consists of multiple variable (V), diversity (D), and joining (J) gene segments whereas the gene encoding the light chain has multiple V and J segments. The random rearrangement of these segments during B cell early development contributes to the diversity of the BCR (164). Heavy chain rearrangement occurs first, with D-J, and then V-DJ rearrangements, at the pro-B cell stage of development. These VDJ rearrangements are driven by the recombinases RAG-1 and RAG-2 (165). After VDJ rearrangement of the heavy chain, a surrogate light chain is expressed alongside the heavy chain generating the pre-BCR (pre-B cells). Successful expression of the pre-BCR triggers VJ arrangement of the light chain and the expression of the BCR, a functional IgM or IgD, in immature B cells, which can now leave the BM and migrate to the lymph nodes and spleen (166). Once in the periphery, immature B cells continue their maturation programme in response to environmental stimuli. An overview of early B cell development is shown in Figure 1.2.

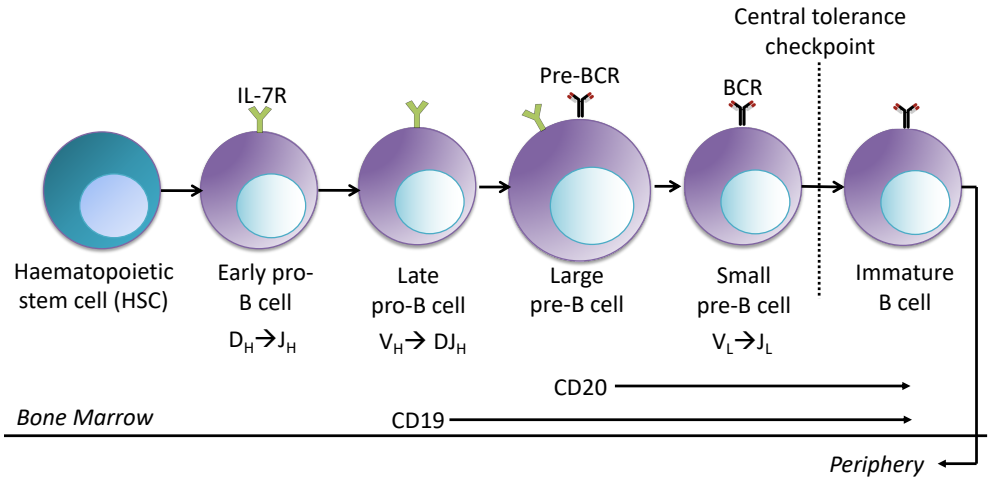

Figure 1.2. Early B cell development.
Overview of the different B cell stages of development in the bone marrow. B cells develop from haematopoietic stem cells, undergoing D-J (early pro-B cell) then V-DJ (late pro-B cells) recombination of the heavy chains (H) of the BCR, and leading to the expression of a pre-BCR (large pre-B cell). IL-7 drives the differentiation and development of B cells. Following VJ recombination of the light chain (L) B cells express a mature BCR (small pre-B cell). Immature B cells are subject to central tolerance and may undergo clonal deletion, receptor editing or anergy; those that pass the central tolerance checkpoint enter the periphery as immature B cells.

1.3.2. Central tolerance

Immunological tolerance refers to the mechanisms by which the immune system prevents the effects of auto-reactive immune cells, including the release of auto-reactive lymphocytes into the periphery and the production of autoantibodies. Nearly 50-70% of newly formed immature B cells in the BM are either polyreactive or autoreactive (167). Central tolerance occurs in the BM and prevents the escape of auto-reactive B cells into the periphery by three mechanisms: receptor editing, clonal deletion and anergy. Receptor editing is a process involving the internalization of an autoreactive BCR and rearrangement of a subsequent light chain gene, which leads to a BCR with a new specificity. Receptor editing removes about 30-35% of autoreactive B cells (167). If receptor editing fails to remove an autoreactive B cell, B cells are deleted via a process called clonal deletion (167). Autoreactive B cells can also enter a state of unresponsiveness known as anergy, whereby B cells fail to respond to antigen stimulation (168). These anergic B cells are relatively short-lived**.**

1.3.3. Transitional B cells

Immature cells that have overcome central tolerance are classified as immature Transitional-1 cells (T1) and leave the BM and migrate to the spleen where they further differentiate into T2 B cells (166, 169). The architecture of the spleen is divided into two main defined regions; the red pulp containing red blood cells and the white pulp in which the lymphoid cells reside. T cells reside in the T cell areas, which centre around arterioles and make up the periarterial lymphatic sheaths (PALS). The B cell follicles surround the PALS, and B cells can also be found in the marginal zone of the white pulp of the spleen. T1 B cells migrate to the B cell follicles surrounding the PALS within the spleen (170). Transitional B cells are functionally immature and short-lived, with T2 cells having an increased survival compared to T1 cells as a result of interactions with T cells via IL-4 and CD40L, illustrating the importance of T cell help in B cell development (171). BCR signalling is required for T1s to develop into T2s (172), with BAFF also playing a role as demonstrated by a lack of T2 B cells in mice with BAFF and BAFFR deficiency (173, 174). While signalling via the BCR remains critical for survival of T1 and T2 B cells, both respond differentially to antigen encounter, whereby T2 B

cells proliferate but T1 B cells undergo apoptosis (175). Neurogenic locus notch homolog protein 2 (Notch2) is important in controlling cell fate decisions; based on the strength of the signal via the BCR engagement and Notch2 signalling, T2 B cells will develop further into mature follicular (FO) or marginal zone (MZ) B cells (176). Notch2 is thought to be critical for MZ B cell development with studies in Notch2 KO mice demonstrating a loss of MZ B cells (177). T2s can also develop into non-antibody producing B cell phenotypes, including Bregs, in response to environmental stimuli such as ligation of CD40, TLR and the BCR, and in response to cytokines including IL-21, IL-1β, IL-6, IFNγ and IL-35 (see Chapter 1.4 for more details) (178, 179).

1.3.4. B cell activation and maturation

Following a successful BCR development, B cells exit the BM and enter the periphery where they are exposed to stimuli that ensure their survival. B cells require constitutive signalling by the BCR (in the absence of bound antigen) for their survival. The BCR interacts with the co-receptor CD45 (180) which contains an immunoreceptor tyrosine-based activation motif (ITAM). This interaction actively regulates down-stream BCR signalling, and provides a positive BCR signal for B cell survival (181). Conversely, interaction with CD22 or CD72, both which possess an immunoreceptor tyrosine-based inhibition motif (ITIM), act to negatively regulate signalling via the BCR (182, 183). In addition to the IgM-BCR, mature B cells in the periphery also express an IgD-BCR, however, a distinct role for the IgD is not clearly understood (184). More recent evidence has suggested that the chemokine receptor CXCR4 interacts specifically with IgD and not the IgM, via modulation of the actin skeleton, and that signaling via CXCR4 requires the IgD (185).

B cell activation factor (BAFF), a cytokine produced by BM stromal cells, monocytes, DCs and B cells, has been demonstrated to have an important role in promoting B cell survival (186, 187). BAFF-deficient mice, for example, lack mature B cells (174, 188). APRIL (a proliferation-inducing ligand, TNFSF 13a), which is related to BAFF, also plays a role in B cell development (186). Both BAFF and APRIL bind the receptors BCMA (B cell maturation antigen, TNFRSF 17) and

TACI (transmembrane activator and CAML interactor, TNFRSF 13b), with BAFF also binding BAFF-R. The expression of the three receptors varies across different B cell subsets; BCMA is expressed on mature B cells, plasmablasts and plasma cells; TACI is on memory B cells, plasmablasts and plasma cells; and BAFF-R on immature and mature B cells but not plasmablasts and plasma cells (189). Interaction of these growth factors with their receptors leads to signalling via NF-kB pathways, with BAFF thought to signal via both the RelB and cRel NF-kB pathways (190). Whilst, ligation of BAFF-R promotes immature B cell survival and maturation, ligation of BCMA is required for plasma cell survival. Ligation of TACI is instead involved in T-cell independent antibody responses, B cell regulation, and class-switch recombination (191).

1.3.4.1. T cell independent activation

B cell activation does not always require T cell help. B cells can undergo a more rapid activation independent of T cells and the formation of GCs. T cell independent activation still requires two signals following antigen encounter. The second signal is provided either by activation of toll-like receptors (TLRs) e.g. by LPS expressed on the surface of bacteria, or via cross-linking of several BCRs, which can be achieved with repetitive polysaccharides or polymeric antigens such as flagellin (192).

1.3.5. Peripheral tolerance

A proportion of immature B cells leaving the BM are auto-reactive despite central tolerance. Peripheral tolerance keeps in check auto-reactive B cells that made it past the tolerance checkpoints within the BM and have entered the periphery. There are several mechanisms of peripheral tolerance including anergy, suppression by regulatory cells and clonal deletion. Anergy, the state of unresponsiveness, is characterised by the down regulation of the BCR, which makes the B cell unresponsive to auto-antigen encounter (168). Poorer binding of the antigen to the BCR can induce anergy, and whilst strong antigen binding results in exclusion of the B cell from the B cell follicles, preventing their proliferation and expansion (168). Transgenic mice with BCRs specific for hen egg lysozyme (HEL), demonstrated that low avidity binding by monovalent antigens typically resulted in anergy, whereas

high avidity binding by multivalent antigens leads to clonal deletion (193). Studies in a BCR signalling reporter mouse model suggest that antigen-encounter in the spleen during B cell maturation, influences the responsiveness of the BCR via the down-modulation of IgM expression on the surface of the cell and by having an effect on modifying basal calcium levels (194). Anergy can also occur as a result of prolonged exposure to an antigen, which results in an increase in intracellular free calcium and tyrosine phosphorylation (195).

BCR signalling is also regulated by interactions with receptors containing ITIMs, including CD22, via the recruitment of SH2 domain-containing phosphatases (SHP) such as SHP-1 (196). These proteins act to dephosphorylate protein tyrosines that have been phosphorylated after BCR signalling. Interaction of the BCR with these ITIM bearing molecules is thought to be important in the maintenance of anergy following antigen encounter (168). In addition, the recruitment of SH2 domain-containing inositol 5'-phosphatase 1 (SHIP1) and phosphatase and tensin homolog (PTEN) are believed to be key players in maintaining anergy and restraining auto-immunity development (197, 198). Regulatory T cells are able to directly suppress auto-reactive B cells thus preventing immune reactions against self (199). Finally, auto-reactive B cells can undergo programmed cell death (apoptosis) via the process of clonal deletion as a result of the lack of appropriate co-stimulation signals or survival signals such as BAFF (186).

1.3.6. Germinal centre formation and antibody production

MZ B cells are mature B cells that reside within the marginal zones of the spleen. While mouse MZ B cells are non-circulating, human MZ B cells are able to recirculate through the blood (176). MZ B cells respond rapidly, quickly differentiating into plasmablasts and secreting antibody. They are therefore a useful first line of defence against pathogens circulating in the blood (200). MZ B cells express high levels of the transcription factor Blimp-1, which is critical for differentiation of plasma cells. This allows them to rapidly differentiate into plasma cells. MZ B cells are typically $IgM^{hi}IgD^{lo}CD21^{hi}CD23^{-}$ (201). They also express high levels of CD1d, an MHC class I-like molecule involved in lipid antigen presentation and the activation of invariant natural killer T cells (iNKT) (202, 203).

FO B cells are mature B cells that constitutively circulate between the follicles in the lymph nodes, and in the absence of antigen will live only for a few days (204). Within the follicles are follicular dendritic cells (FDCs) which play an important role in the activation of B cells (205). FO B cells express CXCR5, which binds the chemokine CXCL1 and is secreted by FDCs as well as marginal reticular cells, and importantly drives migration of B cells to the follicles (205, 206). The FDCs express Fc receptors CD23 and CD32 which allow them to capture soluble antigen and present it to B cells on their surface in its native form (207). Alternatively, larger antigens are brought into the follicles by conventional DCs (205). FO B cells that have encountered antigen are retained in the follicle; antigen encounter causes B cells to upregulate the expression of integrins, which facilitate their adhesion to the extracellular matrix, and allow the B cells to migrate to the interface between the T and B cell zones (208). They start to express CCR7 which binds CCL19 and CCL21, driving migration towards the T-cell zone and thus the interaction with T cells at the B-T cell border within the follicle (205). Helper follicular T cells within the follicles (Tfh) that have recognised the same antigen will, upon encounter with the B cell, promote proliferation and further maturation of the FO B cell into short-lived antibody secreting plasmablasts or go on to form germinal centres (GC) (205). Short-lived plasmablasts provide an immediate IgM antibody response. Those B cells that do not encounter their cognate antigen continue to recirculate (205).

Antigen-activated FO B cells that have not differentiated into plasma cells, migrate to the lymphoid follicles and form GCs. GCs are formed by proliferating B cells, as well as FDCs and antigen-specific T cells that provide help to B cells, and are composed of a dark zone and a light zone (92, 209). It is in the GC that B cells become memory B cells expressing high affinity BCR and long-lived plasma cells producing switched antibodies, which provide long-term protection against pathogens (92, 209). Within the dark zone of the GC, B cells become activated proliferating B cells called centroblasts. Here they undergo somatic hypermutation (SHM), affinity maturation and isotype switching. Affinity maturation is the process whereby B cells express BCR of increasing affinity to the target antigen; this is achieved as a result of SHM and selection for antigen binding (92). The process of SHM involves point mutations in the hypervariable regions of the BCR which can

generate Ig with differing affinity for the antigen and is driven by the enzyme activation-induced cytidine deaminase (AID) (166). The somatic hypermutated B cells stop proliferating and become centrocytes upon migration into the light zone. The light zone contains FDCs and Tfh cells. Here, centrocytes that bind antigen presented by FDCs with low affinity are outcompeted by B cells with higher affinity for their antigen and undergo apoptosis (210). Tfh cells promote survival of the B cells via ligation of CD40 expressed on B cells and by producing the cytokine IL-21, which drives proliferation of the B cells and promotes the retention of the B cells within the GC (211). Only the B cells with highest affinity go onto proliferate (clonal expansion) and differentiate into long-lived plasma cells and memory cells (166).

Also, only centrocytes with highest-affinity BCRs undergo class switching. Isotype class switching alters the antibody type relevant to the particular antigen, and was first demonstrated in chickens (212). B cells constitutively express IgM unless they receive the necessary signals for class switching, which results in the expression of another Ig. Therefore IgM is considered the primary response antibody (213). There are 5 different immunoglobulins: IgM, IgA, IgG, IgE, IgD, with differing and specialised functions (Table 3.3). Mature B cells express both IgM- and IgD- BCRs and while both are also secreted the role of secreted IgD is less clear. It has been proposed that IgD may have a possible role in response to microbiota, with a deficiency of the DNA damage-response protein 53BP1 leading to an upregulation of IgD on B cells that is dependent upon an intact microbiome (214). IgG antibodies are the most abundant, and able to neutralize toxins and viruses and fix complement, and can be sub-divided into 4 sub classes: IgG1, IgG2, IgG3 and IgG4 (215). IgA antibodies are involved in the mucosal immune response and provide protection against pathogens at mucosal surfaces (216). Finally, IgE antibodies protect against helminths and are also involved in the allergic responses (217).

Numerous transcription factors tightly regulate plasma cell development including most notably Blimp-1, and Bcl-6. Bcl-6 is a transcriptional repressor and mice lacking Bcl-6 fail to form GCs (218). Amongst other roles, Bcl-6 acts to silence Blimp-1 the master regulator of plasma cells, and therefore inhibits B cells leaving the GC as plasma cells (219). The architecture of the GC has been demonstrated to be in part determined by the transcription factor FoxO-1 (220, 221). Mice lacking

FoxO-1 on GC B cells are unable to develop a dark zone and B cells fail to undergo affinity maturation and class-switching (221). FoxO-1 was also found to promote CXCR4 transcription, which has previously been reported to be necessary for dark and light zone formation (222). Chip-seq analysis of human GC B cells identified shared binding regions for FoxO-1 and Bcl-6 further demonstrating that FoxO-1 works alongside Bcl-6 (221).

Table 1.4 Antibody types and their role in the immune response.

Antibody	Structure	Role
IgD	Monomer	Microbiota recognition
IgM	Pentamer	Neutralising
IgG	Monomer	Neutralises toxins and viruses, able to cross the placenta, fixes complement, opsonising.
IgA	Dimer	Mucosal response
IgE	Monomer	Allergy, protects against helminths

1.3.7. Long-lived plasma cells and memory B cells

The high-affinity B cells produced from a GC reaction develop into either plasma cells or memory cells (166). Following their proliferation and expansion within the GC, B cells migrate to peripheral tissues or return to the BM. In the BM they become long-lived plasma cells, which are terminally differentiated and non-proliferative. Those in the periphery develop into memory B cells. Immunological memory allows the rapid response of the immune system upon reencounter with the same antigen. Following antigen encounter, the memory B cells differentiate into high-affinity plasma cells and produce antibody against the re-offending pathogen (223). In addition to GC-derived memory B cells that are considered class-switched memory B cells, there also exists IgM memory B cells, derived from T-independent antigen encounter and activation (224, 225).

1.3.8. Natural antibody secreting cells (B-1 cells)

In contrast to typical FO-derived antibody-secreting cells, there is a sub-group of B cells that are able to spontaneously secret antibodies (226). These B cells, known as B-1 B cells (as opposed to "B-2" which encompasses all other 'conventional' B cells), secret natural antibodies (IgM antibodies present naturally in the blood without prior immunization). They were first identified in mice, defined by their expression of CD5 and existing predominantly in the peritoneal cavity (227). Human B-1 cells were more elusive than their mice counterparts, later defined as $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ (228). In mice only B-1 cells express the marker CD5, whereas CD5 expression in humans is not restricted to B-1 cells.

Given their ability to spontaneously secrete antibodies, B-1 cells do not therefore require T cell help, nor do they undergo an adaptive response and hence cannot form memory-like B cells (226). Thus, they are considered 'innate' B cells. B-1 cells are deemed to have two major functions: 1. The rapid or immediate defence against microbial pathogens, and 2. Housekeeping, in the form of facilitating removal of dead cells and debris. This is due to the polyreactive nature of natural antibodies and the ability to bind broadly expressed components of microbial pathogens such as phosphorylcholine (PC), and for example phosphatidylcholine (PtC), which is a key

component of the membrane of red blood cells (229, 230). B-1 antibodies do not undergo SHM are more limited in their V(D)J combinations. They predominately express IgM antibodies but have been demonstrated to have some ability to class-switch; it is thought that a high proportion of IgM and IgA in the serum is B-1 cell derived (231, 232). It is considered that B-1 cells develop early in life and are maintained through self-renewal, with declining numbers found with age (228, 233, 234). However, while B-1 cells have a useful role in the first line defence against microbial pathogens, they are thought to play a pathogenic role in autoimmune diseases, for example via the production of auto-antibodies (235, 236).

An overview of B cell maturation is show in Figure 1.3.

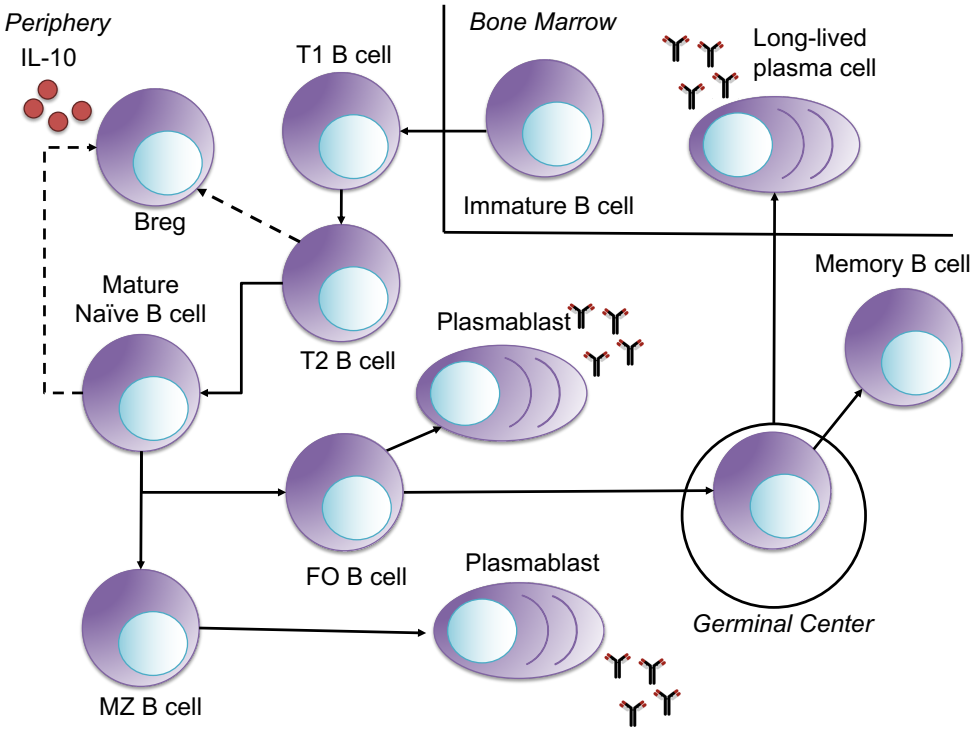

Figure 1.3 B cell maturation.
Overview of the stages of B cell development and maturation once immature cells have left the bone marrow and following antigen encounter. Having undergone development in the bone marrow immature B cells enter the periphery and become T1 B cells and migrate to the B cell follicles within the spleen where they further differentiate into T2 B cells. Upon antigen activation of mature B cells, they either differentiate into mature follicular (FO) or marginal zone (MZ) B cells. MZ B cells rapidly differentiate into short lived antigen producing plasmablasts. FO B cells will either develop into plasmablasts as part of the primary immune response, or form germinal centres (GCs) within the lymphoid follicles. Within the GCs B cells undergo somatic hypermutation and antigen selection, resulting in high affinity BCRs and will develop either into long-lived plasma cells or high affinity memory B cells. Alternatively, T2s can also develop into Bregs, and more recently a plasmablast-like IL-10 producing B cell subtype has been described.

1.3.9. Role of B cells beyond antibody production

1.3.9.1. Antigen presentation by B cells

Immunological studies of patients treated with rituximab (a B-cell depletion anti-CD20 antibody) have highlighted other aspects of B cell biology beyond the production of antibodies that are important in auto-immune conditions. In rituximab treated patients, although there is sometimes a reduction in auto-antibody levels, these levels are not dramatically reduced as rituximab does not deplete antibody producing plasma cells (104). B cells express major histocompatibility complex (MHC) class II molecules, allowing them to process and present antigen, and are considered professional antigen presenting cells (237). B cells become activated following encounter with antigen. Binding of antigen to the BCR triggers a signal-transduction cascade that results in the transcriptional activation of genes that lead to B cell activation. The BCR-antigen complex is internalised and trafficked towards newly synthesised MHC class II molecules within the intracellular compartments. Here the antigen is processed into peptides, and the peptides form complexes with the MHC. This MHC-peptide complex is transported to the surface of the cell where it can encounter T cells. Recognition of the peptide by the T-cell receptor (TCR) on the surface of T-helper cells (Th cells), leads to their activation. The depletion of B cells from mice, has demonstrated that B cells are required for optimal activation and proliferation of $CD4^+$ T cells (238). In turn the activated antigen specific T cell provides "help" to the B cell via ligation of co-receptors on B cells such as CD40 and via the production of cytokines such as IL-2, IL-4 and IL-5 (239). These cytokines promote both the proliferation and survival of B cells, driving their differentiation into plasma cells (171, 240, 241). Bregs are also involved in lipid presentation and the activation of natural killer T cells, with anti-inflammatory capacity (242).

1.3.9.2. Cytokine producing B cells

B cells have been demonstrated to produce a variety of cytokines (243). Those that produce inflammatory cytokines are broadly termed effector B cells (243). Effector B cells can be subdivided into B effector (Be)1 and Be2 cells. Be1 cells develop

following Th1 help, and produce IFNγ and IL-12, which in turn continue to promote Th1 cells (244). Th2 help leads to the development of Be2 cells that produce IL-2, IL-4 and IL-13, and which also help promote Th2 cells. Both Be1 and Be2 cells are able to produce TNFα and IL-6. In addition to the release of pro-inflammatory cytokines, B cells mediate immune regulation via the production of the anti-inflammatory cytokine IL-10, these cells named as Bregs are described in more detail below (245). The multiple pathogenic and regulatory functions of B cells are summarized in Figure 3.3.

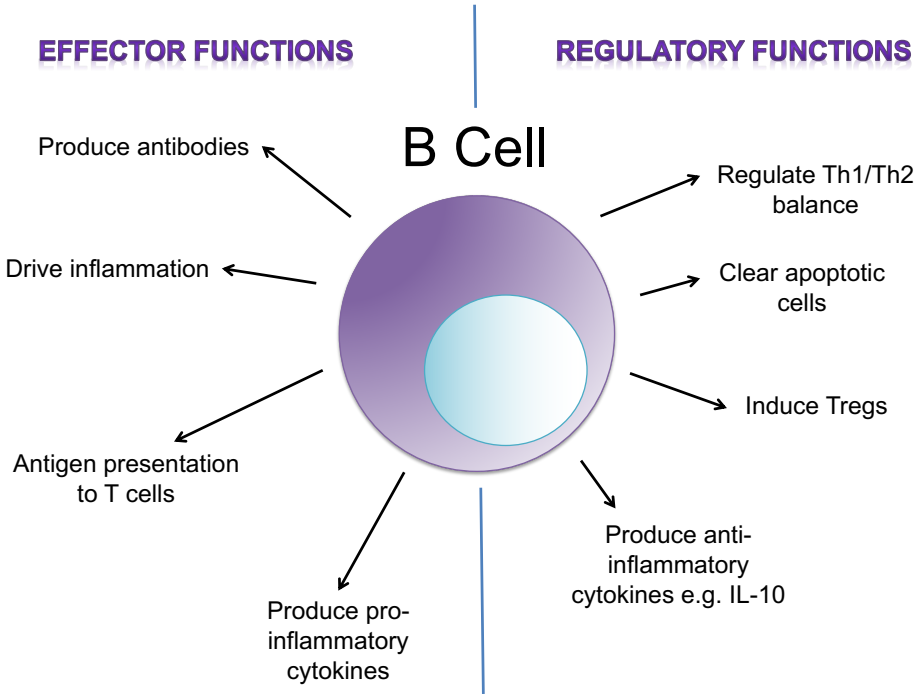

Figure 1.4. Pathogenic and regulatory functions of B cells.
Overview of effector functions of B cells that drive the elimination of pathogens, and of regulatory functions that control excessive inflammation. These varying functions are often implicated in auto-immune conditions leading to excessive inflammation through over activation of effector functions and failure of regulatory functions.

1.4. Regulatory B cells

The very first observation that suggested that B cells may have regulatory capacity dates back to 1974 (246). Adoptive transfer of lymphoid cells from sensitised mice was shown to suppress delayed type hypersensitivity (DTH), and suppression was greater when the transferred cell population was enriched with B cells. However, it was much later that they were identified as Bregs, and shown that they restrain excessive activation of the immune system, via the provision of IL-10 (247). This was demonstrated in a mouse model of colitis, where a subset of IL-10 producing B cells that suppress intestinal inflammation was reported (247). Shortly following, Fillatreau and colleagues showed that mice with experimental autoimmune encephalomyelitis (EAE) with IL-10-deficient B cells were unable to recover from disease (248). Bregs are thought to represent less than 1% of the peripheral blood B cell population (249).

1.4.1. Breg activation

Subsequently, it was demonstrated that Bregs require activation in order to produce IL-10. In collagen-induced arthritis (CIA), stimulation of B cells with an agonistic anti-CD40 antibody gave rise to a population of B cells with potent suppressive capacity. These B cells were able to produce IL-10 and both inhibited Th1 differentiation and prevented arthritis development (250). Furthermore, mice lacking CD40 exclusively on B cells develop an exacerbated autoimmune disease compared to wild type (248). In addition to CD40 engagement, stimulation of TLRs or the BCR results in the differentiation of Bregs (251, 252). Defects in BCR signalling were associated with a reduction in IL-10-producing B cells and exacerbated disease in EAE mice (178).

Bregs have also been shown to differentiate in response to multiple cytokines, including IL-21 in the EAE model (253); Breg expansion in response to IL-1β and IL-6 induced by gut microbiota (179); immature B cell expansion and increased IL-10 production in response to IFNγ in humans and in EAE mice (254); and the expansion of IL-10^{+} B cells by IL-35 in the experimental autoimmune uveitis (EAU) mouse model (255). Production of IL-10 by B cells therefore can be achieved

by a variety of stimuli suggesting there are multiple mechanisms by which Bregs can be induced *in vivo*.

1.4.2. Bregs in human health and disease

In humans, IL-10$^+$ B cells were first described in multiple sclerosis (MS) patients. B cells from patients with MS were shown to produce less IL-10 than their healthy counterparts (256). Further work looking at MS patients showed better clinical outcomes in patients with helminth infections. This is likely due to an accumulation of IL-10$^+$ B cells in response to the infection (257).

Our group has demonstrated that in healthy individuals, CD19$^+$CD24^{hi}CD38^{hi} immature B cells were able to suppress IFNγ, TNFα and IL-17 production by CD4$^+$ T cells, but that these B cells were functionally impaired in systemic lupus erythematous (SLE) (258). Immature B cells isolated from SLE patients produced less IL-10 than healthy B cells upon CD40L stimulation, despite normal CD40 expression levels. This was found to be due to an impaired phosphorylation of STAT3, which is involved in signalling following CD40 ligation. The deficiency was found to be in the B cells themselves rather than in the T cells, as healthy CD19$^+$CD24^{hi}CD38^{hi} B cells were able to suppress IFNγ produced by CD4$^+$ T cells from SLE.

Unlike in SLE patients where the number of immature B cells is normal, rheumatoid arthritis (RA) patients with active disease have fewer CD24^{hi}CD38^{hi} B cells and CD19$^+$CD24^{hi}CD38^{hi}IL-10$^+$ B cells (98). The number of CD24^{hi}CD38^{hi} B cells was shown to correlate negatively with CRP, but no correlation was observed with ESR, RF, or anti-cyclic citrullinated peptide (CCP) antibodies (98). Interestingly, in patients with active RA, CD19$^+$CD24^{hi}CD38^{hi} B cells were able to suppress IFNγ and TNFα production by CD4$^+$ T cells, however, they fail to inhibit the polarisation of naïve CD4$^+$ T cells into Th17 cells or convert them into suppressive Tregs (98).

Several more recent papers have also studied the role of Bregs in RA. Three independent studies reported fewer Bregs in RA patients (259-261) whereas one study reported an increased percentage of Bregs in RA patients (262). However, in the latter paper, only three out of ten RA samples showed proportionally more IL-10

production than in healthy individuals (262). IL-10 also correlated inversely with disease activity score (DAS) in all four studies, suggesting that IL-10-producing B cells are more effective in regulating disease in patients with better clinical scores. Furthermore, in RA patients receiving anti-TNF therapy, there was an increase in Bregs at 6 months following commencement of anti-TNF treatment compared to baseline (263).

The ability of $CD24^{hi}CD38^{hi}IL\text{-}10^{+}$ Bregs to suppress Th1 responses via IL-10 is partially mediated by the engagement of CD80 and CD86. Inhibition of TNFα and IFNγ production by $CD4^{+}$ T cells in humans is reduced in the presence of antibodies against CD80 or CD86 (98, 258). $IL\text{-}10^{+}$ Bregs have also been shown to inhibit $CD8^{+}$ T cells, suppressing IFNγ production in response to hepatitis B virus infection (264), and TNFα production following stimulation with LPS and CpG (249). Both in mouse and in humans, it has been shown that IL-10 produced by Bregs converts naïve and activated T cells into Tr1 and FoxP3 Treg cell subsets, which are important in the maintenance of tolerance (98, 265). In patients with active RA, Bregs fail to convert effector T cells into Tregs, thus further suggesting a defect in the tolerogenic status in these patients (98).

Bregs are also thought to induce tolerance and promote survival of transplants. Two major initial findings were indicative of an important role for B cells in transplantation. Firstly, in patients treated with rituximab there was a very high rate of acute rejection of renal transplants (266). Secondly, patients tolerant to renal transplants showed higher numbers of B cells and exhibited an increased expression of genes related to B cell differentiation (267, 268). Thus, the increase in $CD24^{hi}CD38^{hi}$ B cells present in tolerant patients supports a role for Bregs in transplantation (267, 269). Furthermore, several studies have also specifically linked an increase in IL-10 production by B cells to tolerance in transplantation. In renal transplant patients, tolerant individuals exhibit a frequency of $CD24^{hi}CD38^{hi}$ B cells that is comparable to that in healthy individuals, but those with stable grafts but under immunosuppression and those with chronic rejection exhibit a reduced frequency of these B cells (269). Furthermore, in a separate study of renal transplants, $CD24^{hi}CD38^{hi}$ B cells in tolerant individuals produced more IL-10 following 4 days stimulation to induce Bregs, than either HCs or those with stable

transplants (270). Evidence of the protective role of Bregs in transplantation is also supported by experiments in mice. For example, anti-CD20 treatment in ovalbumin (OVA) sensitised mice with skin grafts lead to accelerated rejection of skin grafts from mice expressing the OVA gene (271). It was also shown that tolerance could be transferred via transfer of splenic B cells from cardiac allograft tolerant mice to recipient mice, which lead to an increased graft survival (272). Transfer of B cells expressing Tim-1, a marker of Bregs, also increased survival of islet allografts in WT mice (273). Finally, an increased frequency of T2 B cells was observed in a mouse model of transplantation tolerance, with the survival of skin grafts by B cells shown to be antigen-specific, with only B cells from tolerised and not naïve mice able to prolong skin graft survival in naïve mice. (274). However, T2 B cells from IL-10 KO mice could also induce tolerance, suggesting that IL-10 may not be the only mechanism by which Bregs induce tolerance (274).

1.4.3. Phenotype of Bregs

There is currently no consensus on what cell surface markers define Bregs, and while Bregs typically produce IL-10, not all IL-10^{+} cells are suppressive and not all regulatory B cells achieve suppression via IL-10 (258, 275). Thus, the gold standard to identify Bregs remains the measurement of IL-10 production and the assessment of their suppressive capacity. There are several proposed phenotypes for Bregs (described below), however, none of the phenotypic markers currently available capture the entirety of IL-10 producing B cell population. Therefore, it is important to continue to investigate whether there are surface markers or a combination, that can allow a more accurate identification of Bregs. This is important if we are to better understand their function and role in human health and disease. A further limitation to the study of Bregs using IL-10 as their marker, is the technical restraint of isolating cells using IL-10 due the intracellular location of IL-10. Furthermore, IL-10 production requires stimulation; the ability to identify a cell type that is 'primed' to produce IL-10, without stimulation, would allow us to identify Bregs *ex vivo*.

1.4.3.1. CD24^{hi}CD38^{hi}IL-10^{+} Bregs

Our group has previously shown that amongst the various subsets of B cells in circulation, immature CD24^{hi}CD38^{hi} B cells produce the majority of IL-10 following *in vitro* stimulation with an agonistic anti-CD40 antibody (258). This population was originally described in PBMCs from healthy individuals and later found to be decreased in patients with SLE (258). As described above CD19^{+}CD24^{hi}CD38^{hi} B cells are able to suppress IFNγ, TNFα and IL-17 production by CD4^{+} T cells (258). Furthermore, they are able to inhibit the polarisation of naïve T cells into Th1 or Th17 cells (98).

1.4.3.2. B10 Bregs

IL-10 expressing CD19^{+}CD24^{hi}CD27^{+} memory B cells, termed B10 cells, have been shown to down-regulate the production of cytokines by monocytes in humans (249). Of note, these cells were first described in the spleens of normal and auto-immune mice as CD1d^{hi}CD5^{+} B cells (276), with parallel functions exhibited by CD19^{+}CD24^{hi}CD27^{+} human B10 cells, following stimulation of PBMCs with LPS, CD40L and CpG (249). However, these Bregs do not suppress T cell cytokine production to any greater extent than their negative B cell counterparts. However, monocyte-derived TNFα was reduced when monocytes were cultured with B10 cells, and this effect could be reversed by blocking IL-10, although IL-10 alone did not suppress TNFα production (249).

1.4.3.3. B regulatory 1 Bregs

In humans, inducible IL-10^{+}B cells were designated as B regulatory 1 (BR1) cells, defined as CD25^{hi}CD71^{hi}CD73^{lo}IL-10^{+} (277). Br1 cells are able to suppress antigen-specific CD4^{+} T cell proliferation via IL-10 production, and were originally identified in non-allergic beekeepers, tolerant to the bee venom allergen phospholipase A2 (PLA), and in allergic individuals before and after immunotherapy. Br1 cells produce allergen-specific IgG4 antibodies, implicating a role in allergen tolerance, as well as high levels of IL-10. Tolerant individuals exhibit an increased frequency of Br1 cells compared to controls, and interestingly

an increased expression of IL-10 and IgG4 by B cells, and an increase in number of IL-10^+ PLA-specific B cells, was observed in allergic individuals following allergen-specific immunotherapy (278).

1.4.3.4. IL-10^+ plasmablasts

Matsumoto *et al.* first described a subset of regulatory plasmablasts in IL-10 reporter mice, whereby $CD138^+$ plasmablasts in the draining lymph nodes expressed IL-10 in response to inflammation via induction of experimental autoimmune encephalomyelitis (EAE) (279). Furthermore, they demonstrated that in the absence of plasmablasts, as a result of genetic ablation of BLIMP-1 and IRF4 in B cells, that disease was more severe compared to control mice. In humans, stimulation of isolated B cells with CpGC plus IL-2, IL-6 and IFNγ lead to an expansion of $CD27^{int}CD38^+$ plasmablasts that secreted IgM (279). In this study the majority of IL-10^+ B cells were found to be captured within this $CD27^{int}CD38^+$ cell population. More recently a natural regulatory plasma cell population has been described in *Salmonella* infected mice, with expansion of IL-10^+CD138^{hi} B cells in response to the infection (280). These cells were also found to express the inhibitory receptor LAG-3, and a population of LAG-3^+CD138^{hi} B cells was further described in naïve mice, which up-regulated IL-10 in response to infection. Finally, an accumulation of LAG-3^+CD138^{hi} B cells in CD72 KO mice was observed, with those mice demonstrating a reduced control of infection, which was restored following treatment with anti-IL-10 and anti-IL-10R antibodies (280).

1.4.3.5. TIM-1^+ Bregs

Tim-1^+ IL-10^+ B cells exhibiting regulatory capacity were first described in the spleens of naïve mice, and were expanded in response to immunisation with an islet allograft or OVA (273). Subsequently, B cells in C57BL/6 mice with a mutation in the Tim-1 gene conferring a loss-of-function were shown to produce less IL-10 (281). More recently Aravena *et al.* (282) have translated these findings into humans, and characterised this Tim-1^+ population in patients with systemic sclerosis. $Tim1^+$ B cells were able to suppress IL-17, IFNγ and TNFα production by $CD4^+$ T cells. In

line with $CD24^{hi}CD38^{hi}IL10^{+}$ Bregs, immature ($CD24^{hi}CD38^{hi}$) B cells express greatest levels of Tim-1, which was upregulated upon activation via engagement of the BCR and TLR9. Furthermore, systemic sclerosis patients show reduced numbers of $Tim\text{-}1^{+}IL\text{-}10^{+}$ B cells (282).

1.4.3.6. iBregs, GZMB^{+} B cells, and CD39^{+}CD73^{+} Bregs

While the majority of Bregs, although phenotypically quite varied, mediate suppression primarily via the production of IL-10, other regulatory B cells suppress by alternative IL-10-independent mechanisms.

iBregs (induced Bregs) were identified in co-cultures of B cells isolated from PBMCs from healthy individuals stimulated with CpGC and co-cultured with autologous T cells. They require CTLA-4 stimulation by T cells, and were demonstrated to regulate T cell proliferation via the cytokine transforming growth factor (TGF)-β and the enzyme indoleamine 2,3-dioxygenase (IDO), leading to the expansion of regulatory T cells (283). Another subtype of B cells able to suppress T cell proliferation in an IL-10 independent manner are characterised by the expression of the protease Granzyme B (GZMB) and were identified in solid tumours. In this context, Linder *et al.* showed that GrB production was expanded by IL-21 and characterised these GrB^{+} B cells as $CD38^{+}CD1d^{+}IgM^{+}CD147^{+}$(284).

A final Breg subset that also acts to regulate inflammation via the expression of enzymes is characterised by $CD19^{+}CD39^{+}CD73^{+}$ expression. This population was first described by Saze and colleagues in B cells from PBMCs isolated from healthy donors (285). CD39 and CD73 are nucleotide-metabolizing ecto-enzymes and are both part of a pathway that converts ATP to AMP and AMP to adenosine (286). The balance of ATP to adenosine conversion favours either a pro- or anti-inflammatory environment, thus acting as a regulatory pathway.

1.5. Siglecs and CD170/Siglec-5

CD170/Siglec-5 (Sialic acid-binding Ig-like lectin-5) is a member of the Siglec family, which are sialic acid-binding immunoglobulin-like lectins. Siglecs are found in all mammals and can be broadly classified as those that are conserved across all mammals, and the more variable CD33-related Siglecs (287). Most immune cells express Siglecs including monocytes, NK cells, T cells and B cells. Siglecs are cell-surface trans-membrane receptors, they have from two up to 17 extracellular Ig domains, and a cytoplasmic domain with an ITIM or an ITAM motif. The terminal Ig domain is an amino-terminal V-set domain that has the sialic acid binding site. The majority of sialic acids have an ITIM and are negative regulators of signalling, with a few Siglecs having an ITAM domain and act as activating receptors. Many Siglecs have been described to be endocytic receptors either constitutively recycling via the endosomes or endocytosed following ligation. For example, CD22 following ligation has been shown to co-localise with the transferrin receptor suggesting trafficking through the endosomal system, and has been shown to subsequently recycle back to the surface (288, 289).

Siglecs bind sialic acid residues or sialyated proteins, which are expressed on all cells, with each Siglec having preferential binding to particular sialosides; CD170 preferentially binds alpha 2-8 sialic acid (Neu5Ac2-8-Neu5Ac) and Sialyl-Tn (Neu5Alpha2-6-GalNAc) (290, 291). Regulation of signalling via Siglecs can occur as a result of both cis and trans interactions with sialic acids (290).

B cells uniquely express Siglec-2 (CD22), CD170 (292), and Siglec-10 (290). CD22 is the most well characterised Siglec on B cells and has a prominent functional role due to its ability to negatively regulate BCR signalling, by both binding to sialic acids on the receptor thus inhibiting signalling, or by sequestering sialic acids away from the receptor to allow signalling (183, 293, 294). CD22 is able to form homo-oligomers with other CD22 molecules by binding sialic acids on their surface, upon antigen binding to the BCR these homo-ologomers are able to bind the BCR via the sialic acids expressed on the surface of the BCR. This leads to the phosphorylation of the ITIM of CD22, leading to the inhibition of calcium signalling from the BCR (295).

CD170, in addition to being expressed by B cells, has been described on neutrophils and monocytes (290, 296). It possesses four extracellular Ig domains, one ITIM and one ITIM-like motif, which allows CD170 to negatively regulate signalling (296). Following ligation, CD170 undergoes phosphorylation of it's ITIM leading to the recruitment of the protein tyrosine phosphatases (PTPs), Src homology-2-containing tyrosine phosphatases 1 (SHP-1) and SHP-2 (297). This ability of CD170 to act as an inhibitory signalling receptor was first demonstrated in CD170-transfected rat basophil leukemia (RBL) cells, which showed reduced calcium signaling and serotonin release, suggesting a less activated cell state (298).

CD170 shares a very similar extracellular structure with Siglec-14, exhibiting 99% sequence homology of the first two Ig domains (299). However, unlike CD170 which has an ITIM, Siglec-14 has an intracellular domain that associates with the ITAM containing the adapter protein DAP12 (287). Ligation of Siglec-14 leads to the phosphorylation of the ITAM allowing the binding of proteins involved in signalling; thus Siglec-14 acts to positively regulate signalling (299). Siglec-14 however, is not expressed on B cells, whereas Siglec-14 and CD170 are co-expressed on granulocytes (292). It is likely that these two receptors work together to regulate signalling providing the respective positive and negative signals (300).

While some studies have investigated the role of CD170, overall there is scarce evidence to demonstrate a role for CD170 on B cells (298-300).

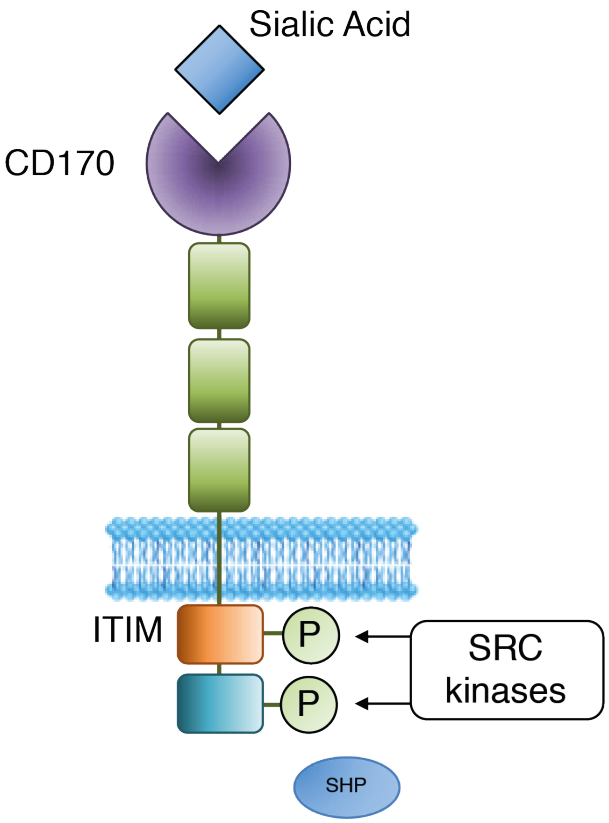

Figure 1.5 Structure of CD170.
CD170 is a transmembrane receptor; it has four extracellular Ig domains, including one V-set sialic acid binding Ig domain (purple ¾ circle) and three C2-set Ig domains (green rectangle). Intracellularly CD170 has one immune-receptor tyrosine-based inhibitory motif (ITIM) (orange rectangle) and one ITIM-like motif (blue rectangle); following ligation of the Siglec the ITIM and ITIM-like domains become phosphorylated (P) by SRC kinases, which leads to the recruitment of SHP proteins, including SHP-1 and SHP-2, and the negative regulation of signalling.

1.5.1. The evolution of CD170 and Siglec-14, and the SIGLEC-14-null mutation

Siglec-14 is thought to have evolved from CD170, in response to the exploitation of Siglecs by pathogens (301). However, in some individuals Siglec-14 is absent. The *SIGLEC5* gene, which is adjacent to the *SIGLEC14* gene, is undergoing partial gene conversion with *SIGLEC14* (299). The resulting "*SIGLEC5/14*" gene has the same coding sequence as *SIGLEC5*, however, it is under the control of the *SIGLEC14* promoter (292). An individual may express both *SIGLEC5* and *SIGLEC14* or lack one or both of the alleles for *SIGLEC14*, replaced by the *SIGLEC5/14*. The immunological consequences of this Siglec-14-null mutation are not clearly understood, however, the Siglec-14 null phenotype has been hypothesized to play a role in bacterial infections. Individuals lacking *SIGLEC14* show an impaired response to group B Streptococcus (GBS) (300), and the null allele was associated with varied response to vaccination and susceptibility to *Mycobacterium tuberculosis* (302).

1.5.2. The role of Siglecs in health and disease

Sialic acids, the ligands for Siglecs, are expressed across vertebrates, but are not conventionally found on microorganisms (303). Sialic acids can therefore be considered to denote self, thus the ligation of Siglecs by sialic acids has implicated Siglecs in immune regulation and tolerance, such as the regulation of BCR activation by self antigen (304). If an autoreactive BCR binds an auto-antigen presented by another cell, sialic acids expressed on the opposing cell can recruit CD22, thus inhibiting auto-reactive signaling via the BCR (295). This role of CD22 in tolerance is supported by studies in CD22 deficient autoimmune mouse strains, which developed exacerbated disease compared to control mice (305). In KO mice lacking CD22 and/or Siglec-G (orthologue of human Siglec-10), there was an increase in calcium signalling and higher IgM levels (303). Thus, both CD22 and Siglec-G can act as negative regulators suppressing antibody production in B cells. Of interest, bacteria including *C. jejuni,* and *N. meningitide,* and some viruses have evolved to exploit Siglecs. Either by *de novo* biosynthesis or by the scavenging of sialic acids

from the environment, the expression of sialic acids on the surface of these pathogens can lead to evasion of immune responses (306).

Siglecs may also be involved in immune tolerance via regulation of the anti-inflammatory cytokine IL-10. The monocyte RAW246 cell line overexpressing CD170 or Siglec-9 showed increased IL-10 production following stimulation with LPS (307). Furthermore, the flagellum from C. *jejuni* was found to induce IL-10 production by DCs via binding to Siglec-10 (308).

CD170 was originally described as a myeloid specific marker (309, 310), with its expression in B cells having only more recently been described (311). There are limited functional studies of CD170 on immune cells, beyond its ability to bind sialic acids and signal via the ITIM motif. This is in part due to the lack of a direct orthologous gene in mice. One study has described CD170 function on T cells, demonstrating a better survival of CD170-expressing T cells following HIV-1 infection. In addition HIV-1 infected patients had more CD170 expressing T cells (312). Another study showed that stimulation with the N-formyl peptide fMLP or TNFα, enhanced neutrophil CD170 expression, and that anti-CD170 monoclonal antibodies reduced oxidative burst activity by neutrophils (309).

Although no association between aberrant expression of CD170 and RA pathogenesis has been made, several reports have described CD170 expression as being associated with various pathologies, which could give us an insight into its function in RA. Aberrant expression of CD170 was observed in acute myeloid leukaemia and a complete lack of expression in acute lymphoblastic leukaemia (313). CD170 has been identified as a marker of critical limb ischemia in patients with diabetes (314). Overexpression of CD170 was associated with increased replication of Mycobacterium tuberculosis (302). A GWAS study looking at periodontitis identified CD170 as a risk loci for this disease (315). Interestingly the occurrence of periodontitis has also been demonstrated to be higher in patients with RA, suggesting a role for oral microbiota in disease, and may be a linked to the ability of Siglecs to bind pathogens expressing sialic acids (290, 316).

1.5.3. Regulation of TLR signaling by Siglecs

Regulation of the adaptive immune response by Siglecs has been demonstrated for CD22 and its role in modulating BCR signaling (294). Further to this, Siglecs have also been shown to play a role in innate immune responses by regulating signaling via Toll-like receptors (TLRs). TLRs are able to recognize, for example, LPS expressed by bacteria, and CpG-DNA/ ssRNA released intracellularly by bacteria and viruses, thus allowing a cell to respond independently of BCR ligation/activation. CD22 KO B cells from mice show increased proliferation in response to TLR stimulation (Poly(I:C)/LPS/CpGC), greater activation (measured by CD86 expression) and more MHC class II expression (317). Confirming this, Paulson and colleagues showed that by expressing CD22 on a $CD22^{-/-}$ murine B cell line, there was a decrease of MHC class II expression, whilst subsequently blocking CD22 with an anti-CD22 antibody resulted in an increase in proliferation (317). Furthermore, in the CD22 KO B cells there was a reduced production of suppressors of cytokine signaling (SOCs) compared to WT mice, which was more pronounced following CpG stimulation (317). Moreover, B cells from Siglec-G x CD22 double deficient mice showed increased proliferation compared B cells from to control mice in response to TLR stimulation (303). Thus further supporting a role for negative regulation of signaling in response to TLR engagement by siglecs. In bone marrow derived macrophages (BMDMs) an up-regulation of Siglec-E (orthologue of human Siglec-9) was observed in response to LPS stimulation, and was associated with reduced NF-κB signaling, and dependent on the signal transduction adapter MyD88 (318). Furthermore, crosslinking of Siglec-E reduced TNFα and IL-6 cytokine production by the BMDMs. Overall siglecs appear to have a role in regulating signaling in response to TLR stimulation, although a direct interaction between TLRs and siglecs has not yet been described.

1.5.4. Siglecs as targets for therapeutics

CD22 is expressed across all B cells subsets, thus making it an attractive target for B cell depletion therapy. Epratuzumab is a monoclonal antibody based drug against CD22, which has shown efficacy in cancer (B-cell non-Hodgkin lymphoma (319))

and in SLE (320). However, it has been shown to regulate BCR signaling rather than leading to a depletion of B cells (321). In a mouse model expressing humanized CD22, B cells showed no change in proliferation following epratuzumab treatment but instead show a decrease in calcium signaling in response to anti-IgM stimulation (322). CD33 (Siglec-3) is also targeted for treatment of acute myeloid leukemia by the mAb gemtuzumab, an antibody-drug conjugate against CD33. In this regard and in light of their association with numerous diseases, and their ability to regulate both innate and adaptive immune responses, other Siglecs may prove attractive targets for therapeutics. Finally, Siglec-15, which is highly expressed in differentiating osteoclasts, can be targeted to inhibit bone loss associated with some cancer and other pathologies (323).

1.6. ABIRISK

The ABIRISK project (Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK), was funded by The Innovative Medicines Initiative (IMI) in March 2012, part of the EU Seventh Framework Programme for Research and Technological Development (FP7). It aims to understand the immune response to biologics, and to understand how an individual patient responds to a specific treatment. The work in this report forms part of work package 2 that aims to evaluate biomarkers as potential predictors of immunogenicity, and evaluate functionally and numerically, the role that B cells and in particular Bregs, play in the pathogenesis of RA and formation of ADA. The project as a whole combines, scientists, statisticians and clinicians from both academia and industry, and looks at both cross-sectional as well as longitudinal data collected through clinical studies.

1.7. Aims and objectives

In order to address the questions posed by ABIRISK, I employed the use of a high-throughput flow cytometry platform, LEGENDScreen^{TM}. This tool takes advantage of flow cytometry technology to provide a unique opportunity to investigate the expression of 332 proteins on the surface of immune cell subsets from PBMCs from both healthy individuals and RA patients. From this I generated a wealth of data that I mined to investigate several aims as outlined below.

1.7.1. Aims:

1. To validate and optimize the LEGENDScreen^{TM}, a novel high-throughput flow cytometry platform that had not previously been published, as a tool to extensively immune-phenotype T and B cells from healthy individuals and patients with RA.
2. To use LEGENDScreen^{TM} to analyse the immune cell profiles in ADA^{+} and ADA^{-} adalimumab treated RA patients and identify markers associated with ADA formation.
3. To validate identified ADA markers in a prospective cohort of adalimumab treated patients.
4. To use LEGENDScreen^{TM} to compare the immune cell profiles of HC versus RA patients and to identify markers uniquely associated with RA, generating a 'signature of RA'.
5. To investigate the potential of the RA signature to predict development of RA in a pilot cohort of at-risk individuals.
6. To mine the LEGENDScreen^{TM} results to identify a novel marker for Bregs.

2. Materials & Methods

2.1. Ethical Approval

Ethical approval was obtained from the ethics committee of University College London Hospitals Health Service Trust under REC reference no. 14/LO/0506 and IRAS project number 10126303 for Prospective Study, and REC reference no. 14/SC/1200 IRAS project number 142793 for Cross-Sectional study. Ethical approval was also obtained from the ethics committee of; CPP, Ile de France VII (13-048), Academic Medical Centre, Amsterdam (METC 2013_304), and Azienda Ospedaliero Univeritaria Careggi, Italy (2012/0035P82). Patients and controls were recruited after providing informed consent (Appendix A.1 and A.2). Storage of samples collected complied with the requirements of the Data Protection Act 1998.

2.2. Patients and healthy volunteers

A UK cross-sectional cohort of 124 RA patients (treated with cDMARDs, adalimumab and tocilizumab) and a European prospective cohort of 37 RA patients switching to adalimumab treatment, were recruited for this study as part of the ABIRISK consortium (Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK; www.abirisk.eu/). Further RA patients were recruited to the prospective cohort that were treated with rituximab, etanercept, infliximab or tocilizumab, for use by other members of the consortium. Peripheral blood was obtained from RA patients attending the rheumatology clinic at University College London Hospital, London or at other ABIRISK centers in France, Netherlands and Italy. All RA patients matched the definition of RA, as outlined in the ACR/EULAR classification (revised 2010, 1992 ACR original). Sample collection was done in accordance with the study protocol for the clinical study of rheumatoid arthritis with respect to response to biological treatment, as part of the ABIRISK consortium. Samples were collected as part of either the cross-sectional or prospective study ABIRISK studies (see below). Detailed clinical and laboratory information corresponding to day of sampling, was collected from clinical records where available including: DAS28 score, CRP, RF, ESR, and anti-CCP, smoking status, gender, age, and BMI. DAS28 is used as the measure of disease activity – it

combines the number of swollen joints and the number of tender joints out of a total of 28 defined joints, as well as ESR/CRP levels and a self-assessment of health made by the patient out of 10. A score of 5.1 or over is considered active disease, less than 3.2 is considered low disease activity and below 2.6 remission. Full clinical and demographic details can be found in the results section. Age and sex matched healthy controls were recruited and studied in parallel for the cross-sectional study (n=49).

2.2.1. Cross-sectional

Patients recruited as part of the cross-sectional study had blood samples taken at a single time point. Samples were collected from RA patients treated with biologics (adalimumab, or tocizilumab) or cDMARDs, and from treatment naïve patients. 50ml of blood was collected in Heparin/Sodium Vacutainer blood collection tubes for isolation of peripheral blood mononuclear cells (PBMCs) (BD) and a further 5ml serum sample was obtained in serum Vacutainer Serum Separator Tubes (BD).

2.2.2. Prospective

Patients with RA that were switching treatment onto a biologic, either from previous treatment by a different biologic or from previous treatment with cDMARDs only, were recruited for this arm of the study. These patients were followed longitudinally with collection of serum and PBMC samples at baseline prior to starting the new treatment, and subsequently at one, three, six and 12 months post commencement of treatment. RNA was also collected at each time point using PAXgene collection tubes (PreAnalytiX), and an additional 2ml of blood taken in EDTA vacutainer tubes (BD) for DNA analysis at month six only. To minimise batch effect, PMBCs and serum were frozen at each visit to allow simultaneous assessments to be performed for a given individual.

2.3. Leukocytes reduction system (LRS) cones

For experiments that required large quantities of B cells it is not possible to obtain enough cells from 50ml of blood from healthy donors. For these experiments

therefore, PBMCs were extracted from leukocytes reduction system (LRS) cones using density gradient centrifugation with Ficoll®-Paque Plus (GEHealthcare), as described below. The leukocyte cones were obtained from single donors from NHS Blood and Transplant (NHSBT). These leukocyte cones have previously been shown to yield viable human PBMCs and generate similar results to those observed from standard PBMC extraction form healthy human donor blood (324).

2.4. Sample preparation, processing and storage

2.4.1. PBMC isolation and freezing

PBMCs were isolated from peripheral blood via density gradient centrifugation with Ficoll®-Paque Plus. The Standard Operating Procedure was used as defined by the ABIRISK consortium to ensure all samples were processed in the same way across the consortium. Samples were processed as soon as possible after collection, within the same day. Whole blood was centrifuged at 400 x g, at room temperature (RT) (18-21^{o}C) for 10 minutes with slow acceleration and minimal brake. The plasma layer was transferred into a sterile 50ml falcon and placed in a 56^{o}C water bath to heat inactivate, for a minimum of 35 minutes. After the heat inactivation the plasma was refrigerated to cool it to 4^{o}C. The remaining blood sample was diluted 1:1 with Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine and $NaHCO_3$ medium (Sigma-Aldrich) and layered over 50ml SepMate™ (STEMCELL Technologies, Canada) tubes filled with 15ml of Ficoll. For cones, no plasma was extracted and blood was diluted 1:5 before layering on Ficoll due to the increased concentration of cells compared to standard blood samples. SepMate tubes were centrifuged at 1200 x g (RT) for 10 minutes then the tubes were inverted emptying the upper fraction, containing PBMCs, into empty sterile 50ml falcon tubes. Cells were washed with RPMI by centrifuging at 400 x g for 10 minutes at 4^{o}C, then the cell pellets combined and suspended in 20ml RPMI ready for counting. Alternatively, diluted blood was layered directly on to 15ml of Ficoll in 50ml falcon tubes and centrifuged at 800 x g, room temperature (RT) for 25 minutes with minimum acceleration and brake. The lymphocyte layer was extracted using sterile pasteur pipettes into a 50ml falcon tube. Extracted cells were then washed and resuspended in RPMI for counting. The cooled plasma was spun at 2400 x g, 4^{o}C,

for 10 minutes, to separate out complement. Live cells were counted (see later) and frozen down at a concentration of $1x10^7$ cells/ml in complement-free supernatant from inactivated autologous plasma (upper layer) and 10% DMSO (Sigma-Aldrich), in 1ml aliquots. PBMCs from cones were frozen in fetal calf serum (FCS) (Biosera, France) with 10% DMSO. Cells were initially frozen down at -80°C for at least 24 hours, before being transferred into liquid nitrogen (-196°C) for long-term storage. Freezing down at -80°C was done in a Nalgene® Mr. Frosty® (Thermo Scientific), containing isopropyl alcohol, which creates a controlled freezing rate of 1°C/minute.

2.4.2. Thawing of PBMCs

When required, frozen cells (stored in liquid nitrogen) were rapidly thawed by diluting in warmed (37°C) RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 100 U/μg/mL penicillin/streptomycin (p/s) (Sigma-Aldrich). Cells were centrifuged at 500 x g, 4°C for 8 minutes and washed with the supplemented RPMI and counted before proceeding with experiments. Cells were counted using a Neubauer Haemocytometer and a light microscope. Cells were diluted 1 in 10 in Trypan blue (Sigma-Aldrich) which stains the nuclei of dead cells. The total number of live cells was calculated by multiplying the number of cells in one corner of the grid by $x10^4$ (to account for the volume in the Haemocytometer), by the dilution factor, and the total volume that the cells were suspended in.

2.4.3. Serum processing and freezing

Serum blood collection tubes were centrifuged at 1500 x g for 10 minutes at 4ºC. Serum was aliquoted into cryovials, and frozen down at -20°C for 24 hours and transferred to -80°C for long-term storage.

2.4.4. Processing of EDTA and PAXgene™ tube samples

Blood samples collected in EDTA blood collection tubes for DNA were aliquoted directly into cryovials. Both DNA aliquots and RNA tubes were frozen down at -20°C for 24 hours and transferred to -80°C for long-term storage.

2.5. Shipment of samples to ABIRISK partners

EDTA DNA samples and PAXgene™ RNA samples were shipped on dry ice to ABIRISK partners for other analyses. Selected PBMCs were also sent using a liquid nitrogen dry shipper. Serum samples were sent for anti-drug antibody testing by ABIRISK partners; the results informed samples selection of patients for experiments, and data analysis, according to the presence or absence of ADA.

2.6. Anti-drug antibodies measurements

Adalimumab ADA were measured using MSD GOLD 96-well Streptavidin SECTOR Plates (L15SA) and a Meso Scale Discovery (MSD) MESO® QuickPlex SQ 120 Instrument (Meso Scale Diagnostics) by collaborators in the ABIRISK consortium.

2.7. B cell isolation using magnetic cell sorting

B Cells were isolated from PBMC samples, which were thawed, and isolated by negative selection using an EasySep™ Human B Cell Enrichment Kit (Stemcell Technologies). Isolation was done with cells in MACS buffer; 1X PBS (Sigma-Aldrich), 0.5% FCS and 2mM ethylenediamine tetra-acetic acid (EDTA) (Gibco, Invitrogen). PBMCs were thawed as described, washed in MACS buffer, counted and resuspended at $5x10^7$ cells/ml in MACS buffer in polystyrene round bottom FACS tubes. 50µl/ml of enrichment cocktail was added to each sample and cells incubated at RT for 10 minutes. After incubation 75µl/ml of magnetic particles which have been vortexed to achieve uniform suspension, are added and samples incubated for a further 5 minutes. Tubes were topped up with MACS buffer to a total volume of 2.5ml and placed into the EasySep™ magnet without lid for 5 minutes. Unwanted cells bound to the magnetic beads are attracted to the tube wall and bound there by the magnet. Samples and magnets were inverted and the solution containing unbound B cells was poured into a fresh tube. Cells were counted and resuspended at the required volume for further experiments in supplemented RPMI.

2.8. Stimulation of cells for IL-10 production

To induce production of IL-10 by B cells, PBMCs and isolated B cells need to be stimulated. PBMCs are thawed and plated in 96 well plates, alternatively B cells can be isolated prior to plating. Stimulations were added as described below and cells were incubated at 37°C 5% CO_2 for 72h.

2.8.1. CpG and mCD40L stimulation

Cells were cultured with final concentration of 1μM CpGC (ODN 2395) (InvivoGen), or 10μg/ml MEGACD40L® (soluble, human, recombinant) (Enzo Life Sciences). CpGC stimulates both pDCs which produce IFNα that promotes IL-10 production by B cells, and the B cells themselves. Cells were incubated for 72 hours at 37°C 5% CO_2.

2.8.2. CD3 stimulation

For PBMCs αCD3 can be used to activate T cells, which in turn stimulate B cells via CD40. 96 well plates were pre-incubated with 0.5μg/ml anti-CD3 (Hit-3a) (BD Biosciences), for 2 hours at 37°C 5% CO_2 to allow to adherence to the plate. Cells are subsequently added and incubated for 72h.

2.9. Stimulation with PMA, Ionomycin, and Brefeldin for detection of cytokines by flow cytometry

For analysis of cytokines for flow cytometry cells were incubated with 0.05μg/ml PMA, 0.25μg/ml Ionomycin and 5μg/ml Brefeldin (all Sigma-Aldrich), for the final 5 hours of culture. At the 67 hour timepoint, the cell culture supernatant was carefully removed from the wells and replaced with supplemented RPMI, and PMA, Ionomycin and Brefeldin, and incubated for a final 5h at 37°C, 5% CO_2. The supernatant was transferred into a new 96 well plate and can be frozen and -80°C for cytokine analysis by ELISA.

2.10. Flow Cytometry

Samples were stained with antibodies for flow cytometry as per the table below. All samples were run on the BD LSR II (BD) and acquired using BD FACS Diva except the LEGENDScreen™, which was run on the Verse (BD) and acquired using BD FACSSuite™ v1.0 (BD). Full details of all antibodies used can be found in Table 4.1. For panel setup and compensation single stains were made using compensation beads (Anti-Mouse Ig, κ/Negative Control Compensation Particles Set, BD), by adding one drop of each of the beads (positive and negative control) and 1µl of antibody in 500µl of FACS buffer. Before acquisition of samples, voltages for the lasers were adjusted to minimise compensation between samples, then a compensation matrix was generated using the single stain controls. All flow cytometry data was analysed using FlowJo 8.7. or 10.5 (Treestar), fluorescence minus one (FMO) controls were used to set gating for analysis to define positive staining. T-distributed Stochastic Neighbor Embedding analysis and generation of vSNE plots was performed in FlowJo 10.5.

Table 2.1 Antibodies for flow cytometry.
Target, fluorochrome colour, isotype, clone and supplier for antibodies used for cell surface markers and cytokines, for analysis by flow cytometry.

Cell Surface Marker	Colour	Isotype	Clone	Company	Dilution
CD11c	BV510	Mouse IgG1, κ	3.9	BioLegend	1 in 50
CD11c	BV421	Mouse IgG1, κ	3.9	BioLegend	1 in100
CD127	BV510	Mouse IgG1, κ	A019D5	BioLegend	1 in 50
CD127	BV421	Mouse IgG1, κ	A019D5	BioLegend	1 in 100
CD138	APC	Mouse IgG1, κ	DL-101	BioLegend	1 in 50
CD150	BV421	Mouse IgG1, κ	A12	BD	1 in 100
CD150	PE	Mouse IgG1, κ	A12 (7D4)	BioLegend	1 in 100
CD158d	APC	Mouse IgG1, κ	mAb 33 (33)	BioLegend	1 in 50
CD167a	PE	Mouse IgM, κ	51D6	BioLegend	1 in 100
CD170	PE	Mouse IgG1, κ	1A5	BioLegend	1 in 100
CD172α/β (SIRPα/β)	APC	Mouse IgG1, κ	SE5A5	BioLegend	1 in 50
CD19	BV785	Mouse IgG1, κ	HIB19	BioLegend	1 in 50
CD19	APC-Cy7	Mouse IgG1, κ	SJ25C1	BD	1 in 100
CD1a	PE/Dazzle 594	Mouse IgG1, κ	HI149	BioLegend	1 in 100
CD1a	BV421	Mouse IgG1, κ	HI149	BioLegend	1 in 100
CD1c	BV421	Mouse IgG1, κ	L161	BioLegend	1 in 100
CD226	PerCP/Cy5.5	Mouse IgG1, κ	11A8	BioLegend	1 in 50
CD226	APC	Mouse IgG1, κ	11A8	BioLegend	1 in 50
CD24	PE-Cy7	Mouse IgG2a, κ	ML5	BD	1 in 25
CD307e	APC	Mouse IgG2a, κ	509f6	BioLegend	1 in 50
CD324	APC/Fire 750	Mouse IgG1, κ	67A4	BioLegend	1 in 50
CD324	APC	Mouse IgG1, κ	67A4	BioLegend	1 in 50
CD335	PerCP/Cy5.5	Mouse IgG1, κ	9E2	BioLegend	1 in 50
CD335	BV421	Mouse IgG1, κ	9E2	BioLegend	1 in 100
CD338	FITC	Mouse IgG2b, κ	5D3	BioLegend	1 in 50
CD338	PE	Mouse IgG2b, κ	5D3	BioLegend	1 in 100
CD38	BV605	Mouse IgG1, κ	HIT2	BioLegend	1 in 25
CD38	FITC	Mouse IgG1, κ	HIT2	BD	1 in 100
CD4	V500	Mouse IgG1, κ	RPA-T4	BD	1 in 100
CD62L	BV421	Mouse IgG1, κ	DREG-56	BioLegend	1 in 100
CD97	FITC	Mouse IgG1, κ	VIM3b	BioLegend	1 in 50
CD97	PE	Mouse IgG1, κ	VIM3b	BioLegend	1 in 100
DR3	PE	Mouse IgG1, κ	JD3	BioLegend	1 in 100

Ig light chain κ	BV421	Mouse IgG1, κ	MHK-49	BioLegend	1 in 100
NOTCH2	APC	Mouse IgG2a, κ	MHN2-25	BioLegend	1 in 50

Cytokine	Colour	Isotype	Clone	Company	Dilution
IFNγ	BV510	Mouse IgG1, κ	4S.B3	BioLegend	1 in 25
IL-10	APC	Rat IgG2a	JES3-19F1	BD	1 in 25
IL-17a	BV711	Mouse IgG1, κ	BL168	BioLegend	1 in 25
IL-6	FITC	Mouse IgG1, κ	MQ2-13A5	eBiosciences	1 in 50
TNFα	eFlour450	Mouse IgG1, κ	MAb11	eBiosciences	1 in 25

2.10.1. Ex-vivo staining

Cells for staining were plated in a 96-well plate at a $5x10^5$ cells per well. Cells were washed twice with 200µl/well 1X PBS (1in 10 dilution of 10X PBS in double distilled (dd) H_2O) by centrifugation at 500 x g, for 5 minutes. Samples were stained with LIVE/DEAD™ Fixable Blue Dead Cell Stain (Invitrogen) (1:500 dilution in PBS) and the plate incubated for 20 minutes at room temperature (RT). Cells were subsequently washed twice in FACS buffer (1X PBS plus 1% FCS and 0.01% sodium azide) and stained with cell surface antibodies at the required concentration (see Table 2.1) in a volume of 50µl and incubated at 4^oC for 30 minutes. Appropriate FMO controls were also generated. After incubation with antibodies, cells were washed twice in FACS buffer and fixed with 100µl/well fixation buffer (BioLegend) for 10 minutes at 4^oC. Finally cells were washed and resuspended in FACS buffer and data was acquired on the LSR II flow cytometer (BD).

2.10.2. Intracellular staining after culture

Cells were cultured with stimulations as required (e.g. CpGC for 72h), followed by 0.05µg/ml PMA, 0.25µg/ml Ionomycin and 5µg/ml Brefeldin for final 5 hours of culture for detection of cytokines, as described above. Cells were stained with LIVE/DEAD™ Fixable Blue Dead Cell Stain and cell surface markers as described above. After staining of cell surface markers, cells were washed twice with FACS buffer, and then incubated in 100µl of intracellular fixation buffer (eBiosciences) at 4^oC for 20 minutes. After incubation, cells were washed twice with 1X permeabilisation buffer (diluted 1 in 10 from 10X stock) (eBioscience) and incubated for 5 minutes with 25µl of permeabilisation buffer at 4^oC. 25µl of permeabilisation buffer containing the cytokine antibodies diluted at double concentration was added to the cells and they were incubated for a further 40 minutes at 4^oC. Finally, cells were washed and resuspended in FACS buffer, and data acquired on the LSR II.

2.11. LEGENDScreen™

LEGENDScreen™ (BioLegend) kits were used to preform high throughput flow cytometry using the BD FACSVerse (BD). Peripheral blood mononuclear cells (PBMCs) from HCs and RA patients were analysed using the LEGENDScreen™ as per the manufacturer's protocol with several modifications as described below. The kits were validated before use as a tool for evaluating the expression of cell surface markers in PBMCs and on specific cells subtypes within PBMCs (T cells and B cells). This work was done in in collaboration with William Sanderson (previous PhD student in the laboratory), Dr Marsilio Adriani (previous Post-Doc at UCL), and in connection with BioLegend; when this project began the LEGENDScreen™ had not been published. LEGENDScreen™ staining was done on total approximate 40 million PBMCs; this allowed for staining of 120,000 cells per LEGENDScreen™ PE-antibody and provided sufficient resolution for analysis of the smallest B cell population of interest (immature B cells), which represent around 5-10% of the total B cell population. Inter-assay variability was validated by comparing data from the same sample ran repeatedly at three different timepoints, and intra-assay variation was analysed by comparing average expression across the four plates (see Chapter 3 Results I for more details). This confirmed the reproducibility of LEGENDScreen™ for the analysis of PBMCs and is outlined in more detail in Chapter 3 Results I.

The kit contains lyophilized PE-antibodies, with one PE-conjugated antibody per well across 4 x 96-well plates (Figure 2.1). The kit contains antibodies against 332 different cell surface proteins and appropriate isotype controls. The lyophilized antibodies were reconstituted with sterile-filtered water (Sigma) prior to the addition of sample. Plates were spun at 600 x g for 5 minutes, then reconstituted with 25µl of deionised water. Reconstituted LEGENDScreen™ plates were split into two new sets of 4 plates suitable for the Verse FACS machine, to allow the simultaneous staining of two samples. Since I was using the minimum recommended number of cells for staining, the PE-conjugated antibodies would be in excess, and therefore the split plates still contain sufficient antibody (in excess) for accurate staining. Reconstituted plates were kept at 4^{o}C in the dark. An additional step was included at this stage whereby PBMCs were stained in a volume of 10ml using LIVE/DEAD™ Fixable Blue Dead Cell Stain (1 in 500 in PBS) in the dark at RT for 20 minutes to

allow exclusion of dead cells during analysis (Dead Cell Stain not included within the kit). The cells were washed, resuspended in the Cell Staining Buffer provided within the kit and seeded at a density of 120,000 cells/well ($2.4x10^6$/ml) into 96 well plates containing the PE antibodies for the 332 markers and isotype controls. Plates containing the PBMCs were incubated at 4^oC in the dark for 30 minutes. A second additional step was included to allow identification of markers on specific immune cells; cells were washed by centrifuging plates at 500 x g for 6 minutes and 50 µl of the following antibodies diluted in FACS buffer were added per well; CD4 V500 (1 in 100), CD19 APC-Cy7 (1 in 100), CD24 PE-CY7 (1 in 200), CD38 FITC (1 in100) (not included in the kit). Cells were incubated at 4^oC for 20 minutes in the dark to allow for staining. Hence, cells were stained with a total of 5 different conjugated antibodies, plus a Dead Cell Stain, for flow cytometry. This allowed analysis of each of the 332 different markers on PBMCs total, $CD4^+$ T cells, $CD19^+$ B cells and $CD19^+CD24^{hi}CD38^{hi}$ immature B cells, $CD19^+CD24^{int}CD38^{int}$ mature B cells and $CD19^+CD24^{hi}CD38^{lo}$ memory B cells (see Chapter 3 Results I Figure 3.1 for further details of gating strategy). Cells were washed and fixed with Fixation Buffer contained within the LEGENDScreen™ kit, for 15 minutes at 4^oC in the dark before being washed again and resuspended in 120µl of Cell Staining Buffer. Appropriate single stain controls were also generated for calibrating the flow cytometer (V500, APC-Cy7, PE-Cy7, FITC, Blue UV and PE). Plates were run on the Verse FACS machine (BD), using an automated plate reader. Labels for each well with the antibody from the LEGENDScreen™ kit were inputted into the Verse software and saved as a template that could be used for each sample run. Each plate took approximately 2 hours to run, with 4 plates per sample and two samples stained simultaneously, totaling approximately 8 hours run time. See Figure 2.1.

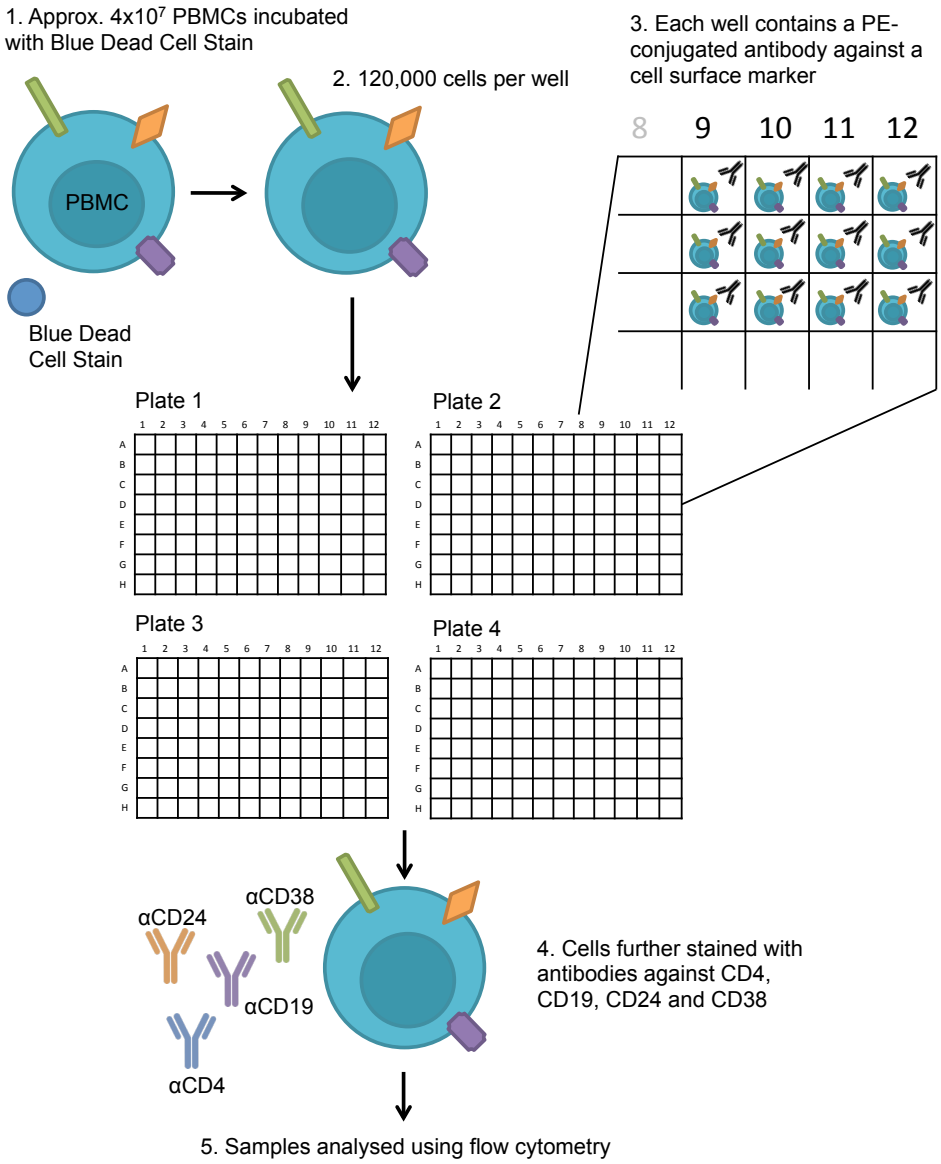

Figure 2.1. Overview of the LEGENDScreen™ platform.
The LEGENDScreen™ kit consists of four 96-well plates with lyophilized PE-conjugated antibodies against 332 cell surface marker proteins and appropriate isotype controls, with one antibody per well. PBMCs from HC and RA patient samples are first stained with a Blue Dead Cell Stain (1), before being seeded at a density of ~120,000 cells per well into the LEGENDScreen™ plates (2). Each well contains one PE-conjugated antibody (3), and cells are further stained with antibodies against CD4, CD19, CD24 and CD38 to delineate $CD4^+$ T cells, $CD19^+$ B cells and $CD19^+CD24^{hi}CD38^{hi}$ immature B cells, $CD19^+CD24^{int}CD38^{int}$ mature B cells and $CD19^+CD24^{hi}CD38^{lo}$ memory B cells (4). Samples are analysed using flow cytometry (5).

2.12. Isolation of cell subsets using fluorescence activated cell sorting

Cells were sorted using the FACS Aria (BD). PBMCs or isolated B cells as required were stained with fluorescence conjugated antibodies against specific cell surface proteins that delineate required cells types, and thus allow the cells to be sorted. PBMCs were thawed and counted, as described; if required B cells were isolated using the EasySep™ Human B Cell Enrichment Kit, as described, and/or cultured. Cells for sorting are washed twice (500 x g, 8 minutes) and resuspended in MACS buffer (PBS + 1% FCS, 0.4% EDTA) for staining at $1x10^8$ cells/ml. Conjugated antibodies are added, at a concentration of 10µl of antibody per 20 million cells, and incubated at 4^oC for 20 minutes. Cells are washed twice in MACS buffer (500 x g, 8 minutes) and resuspended in a volume of $1x10^8$cells/ml. Cells were filtered into blue filter capped FACS tubes and flushed through with MACS buffer so cells were at a final concentration of $5x10^7$/ml for sorting. Single stains were made up in 500µl of MACS buffer. Directly prior to the sort DAPI was added to the cells (1 in 10,000 dilution) to detect dead cells. For collection of sorted cells, polypropylene FACS tubes were prepared with media for collection (RMPI + 50% FCS); one tube per population to be sorted. Cells were sorted using the FACS sorter, drawing gates on the software to define required populations to be sorted. After sorting, cells were counted, washed twice (500 x g, 8 minutes) and resuspended at the volume required for the experiment, in the required media.

2.13. T cell suppression assay with FACS sorted B cells

T cells and sorted B cell population's were cultured at a ratio of 1:1 for 72h. Cells were stimulated with 0.5µg/ml anti-CD3 (to activate T cells) with or without CpGC (1µM) (to activate B cells) and 0.05µg/ml PMA, 0.25µg/ml Ionomycin and 5µg/ml Brefeldin added for the final 5h of incubation (all as described). Cells were also cultured with/without 10µg /ml LEAF™ Purified anti-human IL-10 and IL-10R (CD210) (both BioLegend), as required. Furthermore, B cells were incubated with 5µg/ml human Siglec-5/Siglec-14 antibody or 5µg/ml monoclonal mouse IgG_1 isotype control (both R&D systems) for 30 minutes at 37^oC and 5%CO_2 prior to their addition to T cells, as required. $CD4^+$ $CD25^-$ T cells were sorted from freshly defrosted PBMC samples. $CD19^{hi}CD170^{hi}$ and $CD19^+CD170^{int/low}$ B cells were

sorted from B cells that were purified using the EasySep™ Human B Cell Enrichment Kit as described and cultured at 1 million cells per well in 200µl supplemented RPMI, with CpGC for 72h at 37^{o}C, to induce expansion of the CD170^{hi} population. Cytokine production was assessed by intracellular cytokine staining for flow cytometry as described and supernatants were collected and analysed for IL-10 by ELISA.

2.14. ImageStream®

ImageStream is a flow cytometry based imaging technique. PBMCs were defrosted and counted as described and plated at 1x10^{6} per well in a 96 well plate. Cells were either rested overnight at 37^{o}C or cultured for 72h with 1µM CpGC, and 0.05µg/ml PMA, 0.25µg/ml Ionomycin and 5µg/ml Brefeldin for the last 5 hours of culture for cytokine production. Cells were stained with antibodies against cell surface and intracellular proteins as described above. Stained samples are suspended in 30µl FACS buffer in 1.5ml eppendorfs and analysed using the ImageStream^{X} Mark II (Amnis). Analysis was done using IDEAS software (Merck). Co-localisation of proteins was determined by calculating Bright Detailed Similarity score; Pearson's correlation coefficient was calculated for localized bright spots with a radius of 3 pixels or less, and log transformed to give the Bright Detailed Similarity score. A score of around greater than two can be thought of as co-localised. Internalisation of a protein was determined by calculating the internalised protein as a ratio of the total protein. The Internalization score is a log of this ratio, with a higher number suggesting greater internalisation.

All samples were stained with LIVE/DEAD Fixable Violet Dead Cell Stain (Thermo Fisher) and the antibodies outlined in Table 2.2

Table 2.2. Fluorochrome conjugated antibodies used for ImageStream experiments.

Recycling, and co-localisation with endosomes/lysosomes

Marker	Colour	Isotype	Clone
CD19	Alexa Fluor® 488	Mouse IgG1, κ	HIB19
CD170	PE	Mouse IgG1, κ	1A5
TfR	PE/Cy7	Mouse IgG2a, κ	CY1G4
LAMP-1	Brilliant Violet 605™	Mouse IgG1, κ	H4A3

Co-localisation with IgM

Marker	Colour	Isotype	Clone
CD19	PECy7	Mouse IgG1, κ	HIB19
CD170	PE	Mouse IgG1, κ	1A5
CD22	APC	Mouse IgG1, κ	HIB22
IgM	FITC	Mouse IgG1, κ	MHM-88

IL-10

Marker	Colour	Isotype	Clone
CD19	Alexa Fluor® 488	Mouse IgG1, κ	HIB19
CD170	PE	Mouse IgG1, κ	1A5
CD24	PE/Cy7	Mouse IgG2a, κ	ML5
CD38	Brilliant Violet 605™	Mouse IgG1, κ	HIT2
IL-10	APC	Rat IgG2a	JES3-19F1

2.15. Cytokine detection by ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to measure and quantify IL-10 in the supernatants of cultured cells (Human IL-10 DuoSet ELISA R&D systems). Supernatants were collected from cultured cells, stimulated to produce IL-10, prior to replacement of media with media containing 0.05µg/ml PMA, 0.25µg/ml Ionomycin and 5µg/ml Brefeldin for final 5h of stimulation. Supernatants were carefully transferred into a new 96-well plate and frozen at minus 80^{o}C until required. Supernatants were left to defrost at RT before using in the ELISA. The ELISA was performed as per the manufacturers instructions on undiluted culture supernatants. Flat-bottomed 96-well plates were coated overnight with 50µl/well of the 2µg/ml capture antibody. The plate was then washed four times with 200µl/well PBS-Tween and blocked with 1% Bovine Serum Albumin (BSA) (Santa Cruz Biotechnology) in PBS (=Reagent Diluent) for one hour. The plate was washed (x4) again and undiluted supernatant samples added for 2 hours at RT or 4^{o}C overnight. In addition, an 8-fold serially diluted recombinant human IL-10 standard (31.2-200pg/ml) was also added as a control from which to generate the standard curve. After incubation the plate was washed (x4), incubated with 100µl of 75ng/ml detection antibody for 2h at RT, washed, and incubated with 100µl working dilution (1 in 40) Steptavidin-HRP for 20 minutes at RT in the dark, before washing (x4) a final time. 100µl substrate solution (TMB Stabilized Chromogen, Life Technologies) was added and cells incubated for 10-15 minutes in the dark until the colour had developed sufficiently, as indicated by a gradual colour gradient across the serially diluted samples. At this point, 50µl of Stop Solution 2N Sulfuric Acid (R&D) was added to quench the reaction. The optical density (OD) was read immediately using a microplate reader at 450nm.

2.16. Recycling and internalisation assays

PBMCs were thawed and counted as described. B cells were isolated using the EasySep™ Human B Cell Enrichment Kit as described. B cells were plated at 200,000 cells per well in a total 100µl volume in a 96 well plate in supplemented RPMI. Cell surface CD170 was blocked by adding saturating amounts of anti-CD170 antibody on ice for 30 minutes (10µg/ml). Cells were washed twice in

supplemented RPMI, and resuspended in supplemented RPMI. Cells were incubated for 60 minutes (37^{o}C) to allow CD170 to be recycled to the surface. Control cells were left on ice. Internalised (un-blocked CD170) recycled to the surface can be stained using CD170-PE and analysed by flow cytometry (as described). Recycling was measured as the increase in CD170 MFI (median fluoresce intensity) compared to cells left on ice.

Alternatively, PBMCs were plated at 1x10^{6} per well in 100µl of supplemented RPMI and left to rest overnight at 37^{o}C. After resting cells were stained with the PE conjugated antibody against CD170 in supplemented RPMI, on ice for 30 minutes. Excess CD170-PE was washed off and cells were either left on ice or incubated for 60 minutes at 37^{o}C. Cells were further stained for flow cytometry and internalisation of CD170 analysed by ImageStream® (as described).

2.17. Calcium Flux

PBMCs were thawed and counted as described. B cells were isolated using the EasySep^{TM} Human B Cell Enrichment Kit as described. After isolation cells were counted, washed and resuspended in supplemented RPMI, and plated in a 96 well plate at 1 million cells per well. As required B cells were blocked prior with anti-CD170 (5µg/ml), for 30 minutes at 37^{o}C, 5% CO_2. After blocking cells were washed in RPMI + Probenecid (5µl in 5ml) (an organic anion transporter inhibitor) (Invitrogen). Cells were loaded with Fluo-4 – AM dye for Ca^{2+} flux (Invitrogen), by incubating cells with 1µM Fluo-4 – AM diluted in RPMI +Probenecid for 30 minutes at 37^{o}C, 5% CO_2. Cells were washed twice in RPMI +Probenecid and left to rest for minimum 30 minutes at 37^{o}C, 5% CO_2. Baseline Flou-4 MFI was recorded for 30 seconds before adding AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgA +IgG +IgM (H+L) (Jackson ImmunoResearch) (final concentration 20µg/ml, diluted in PBS, warmed to RT before adding), and then the sample was recorded for a further 3 minutes. Samples were analysed using the kinetics function in FlowJo v8.7.

2.18. SIGLEC5/14 genotyping

2.18.1. DNA extraction

DNA was extracted from thawed frozen PBMC samples using the QIAamp DNA Mini Kit (Qiagen), as per the manufacturers instructions. $5x10^6$ cells were resuspended in 200µl of PBS in 1.5ml eppendorfs. To lyse cells, 20µl of QIAGEN Protease was added and mixed by pipetting, followed by 200µl of Buffer AL. Samples were then vortexed for 15 seconds to mix, and incubated at 56°C for 10 minutes. To extract the DNA 200µl of ethanol as added to each sample, and samples were vortexed again to mix. Next the samples were pipetted onto QIAamp Mini spin columns with a 2ml collection tube. Columns were centrifuged at 6000 x g for 1 min; the collection tube containing filtrate was discarded and replaced with a clean one. Next, 500µl of Buffer AW1 was added, and samples centrifuged at 6000 x g for 1 minute and the collection tube discarded. Then 500µl of Buffer AW2 was added, samples centrifuged at 17,000 x g for 3 min, and the collection tube discarded again. To elute QIAamp Mini spin columns were placed in a clean 1.5ml eppendorf and 200µl Buffer AE added. Samples were incubated at room temperature (15–25°C) for 5 minutes. Finally, columns were centrifuged at 6000 x g (8000 rpm) for 1 minute and filtrate collected.

To assess quality and quantity of yield, samples were analysed using the NanoDrop™ Spectrophotometer ND-1000 (Labtech) and ND-1000 v3.7.1 software. DNA samples were stored at minus 20^oC.

2.18.2. PCR for genotyping of SIGLEC5 *and* SIGLEC14

The following 15µl reaction mix was used for each sample; 1µl DNA (50-70ng/µl) (or water; no DNA control), 11.01µl nuclease free water (Ambion), 3µl 5x GC Buffer (OneTaq Polymerse kit), 0.3µl dNTPs (NEB), 0.09 µl OneTaq Polymerase (NEB), plus either 0.3µl hSig14 fwd (10pM) and 0.3µl hSig14 rev (10pM), or 0.3µl hSig5 fwd (10pM) and 0.3µl hSig5 rev (10pM), or 0.3µl hSig14 fwd (10pM) and 0.3µl hSig5 rev (10pM).

The following primers were used (5'→ 3');
hSig14 fwd AAA GTG CTG CAG CTA TGG GAC,
hSig14 rev TCC TCT CCC AAT GCT GAA CC,
hSig5 fwd ACT GCC GTC CCA CAA GAC C,
hSig5 rev ACA GAA ACC CAC CAA GCG GG.

PCR was performed using the OPTICON PCR instrument (BioRad). Samples were run on the following PCR cycle; 94°C 1 minute 30 seconds, [94°C 20s, 57.5°C 30s, 68°C 2m30s]x35 cycles, 68°C 3m00s.

2.18.3. Agarose gel electrophoresis

A 1% agarose gel was made by heating 1g of agarose in 100ml of Tris-Acetate EDTA buffer (10x Tris Acetate-EDTA (Sigma) diluted to 1X in PBS) until it dissolved. 10µl of SYBR® Safe DNA gel stain (Invitrogen) was added and the mixture poured into the gel casing with a comb for up to 20 wells, and left to cool for minimum 20 minutes until set. The set gel was placed in the gel electrophoresis apparatus and the apparatus filled with Tris-Acetate Buffer until the gel was completely covered. 5µl of 1Kb Hyper Ladder (BioLine), or 15µl of sample plus 1.5µl of loading dye (5X sample loading buffer, BioLine), were added to the wells. Samples were run at 100 volts for approximately 40 minutes, until the loading dye reached the far end of the gel. The gel was read on a UV transilluminator (BioRad) using Quantify One software. The expected bands are as follows;

Wild type	Sig5 2,097 bp + Sig14 1,671 bp
Heterozygous	Sig5 2,097 bp + Sig14 1,671 bp + Sig5/14 1,491 bp
Homozygous	Sig5/14 1,491 bp

2.19. Statistical Analysis

Statistics were performed using Prism (GraphPad) unless otherwise mentioned. P values were calculated using two-tailed *t*-test, ANOVA (Analysis of variance) (with

multiple comparisons) or multiple *t*-tests, or equivalent non-parametric tests, as required and as described below. Values are presented as dot plots with mean ± standard error mean (SEM), or as box and whisker plots with whiskers showing minimum and maximum values, and box showing upper and lower quartiles and median value. P values less than 0.05 were considered significant with $p<0.05$*, $p<0.01$** and $p<0.001$***.

FACS files were analysed using FlowJo version 8.7. and version 10.5 (TreeStar). tSNE analysis was performed using the tSNE plugin for FlowJo 10.

Volcano plots for LEGENDScreen™ data were generated by plotting p value (unpaired multiple *t*-test) versus fold-change. Fold change was determined by calculating the difference between groups as a ratio.

Outliers were removed in the validation analysis using the ROUT method (Robust regression and Outlier removal) in Prism (Q=1%), where stated.

Generation of heatmaps was performed using Multiple Experiment Viewer_4_8 (MeV_4_8) (TM4). Heatmaps provide a visual overview of the differences between groups and show mean (average) expression for each marker, with each square representing one of the 332 LEGENDScreen™ markers.

Principle component analysis (PCA) was performed using JMP statistical software package Version 12.0.1 (SAS Institute). This dimensionality reduction technique allowed us to ascertain if our LEGENDScreen™ derived signatures were powerful enough to define our populations of interest (e.g. ADA^+ versus ADA^-, or HC versus RA patients).

Receiver operating characteristic (ROC) curves were generated, and area under the curve (AUC) calculated in JMP. A positive outcome was considered either an ADA^+ individual or an RA patient accordingly, with ROC curves used to determine the ability of LEGENDScreen™ derived and validated surface markers to predict these outcomes.

For two variables, paired or unpaired *t*-test analysis was performed as required, assuming normal distribution. Where samples did not exhibit normal distribution, a Mann-Whitney test was performed. Normality was tested using D'Agostino and Pearson normality test. Paired analysis was performed for paired values such as the same individual with and without treatment. For three or more variables, unpaired or paired ANOVA was performed assuming normal distribution. For non-normal data a Kruskal-Wallis test was performed.

2.19.1. Pipeline for analysis of LEGENDScreen™ data and signature generation

The pipeline for analysis of the extensive LEGENDScreen™ data set was developed as part of this project. This is also explained in the Results chapters.

Identification of differentially expressed markers was done using multiple *t*-test analysis, with markers with $p<0.05$ considered to be significant. To confirm robustness of these markers, they were validated in an independent cohort. *T*-test analysis was performed assuming normal distribution.

To exclude markers associated with inflammation I correlated the expression of each of the 332 LEGENDScreen™ markers on the total RA cohort, with the respective DAS28 score for each patient. Any markers that showed significant correlation (r), $p<0.05$, were considered to be associated with inflammation and removed.

In the ADA-signature analysis to remove treatment-associated markers, I compared expression between ADA^+, ADA^- and cDMARD treated RA patients (as a control). For normally distributed samples (tested using D'Agostino and Pearson normality test), I performed an ANOVA test to compare means; for non-normally distributed data I performed a Kruskal-Wallis test. In the RA signature analysis to exclude treatment associated markers, I selected only markers that were different between HC and adalimumab treated RA *and* between HC and cDMARD treated RA.

To confirm the selected markers were sufficient to define our populations of interest (e.g. ADA^+ versus ADA^-, or HC versus RA patients), I performed a PCA.

In the validation cohort I use an unpaired *t*-test analysis or a Mann-Whitney test to compare marker expression between the two groups. A ROC curve analysis was performed on successfully validated markers as described above to determine predictive power.

For continuous clinical parameters including DAS28, CRP, ESR, age, BMI and ADA titre, I performed a correlation analysis with SIRPα/β expression using Pearson correlation. For categorical clinical variables, MTX co-treatment, gender and smoking status I used a *t*-test analysis.

3. Results I – The LEGENDScreen™

In this chapter I establish and validate LEGENDScreen™ as a novel high-throughput platform for the analysis of PBMCs and immune cell subsets in HCs and RA patients by flow cytometry.

3.1. Establishment and validation of the LEGENDScreen™ staining

Amongst the many different cell types involved in the pathogenesis of RA, B and T cells are established key players in the initiation and chronicity of disease (1). In particular B cells are known to mediate inflammatory roles via the release of autoantibodies and pro-inflammatory cytokines, as well as inhibition of inflammation via Bregs (325). As outlined in the methods I modified the LEGENDScreen™ protocol to include the addition of antibodies to delineate $CD4^+$ T cells, $CD19^+$ B cells, and $CD19^+CD24^{hi}CD38^{hi}$ immature B cells, $CD19^+CD24^{int}CD38^{int}$ mature B cells and $CD19^+CD24^{hi}CD38^{lo}$ memory B cells. This allowed the expression of the 332 LEGENDScreen™ markers to be analysed on these different cell populations. The gating strategy used is shown in Figure 3.1. Firstly, I gated on lymphocytes using forward (FSC-A) and side (SSC-A) scatter, then I used the height and width, forward and side scatter parameters to exclude doublets, and gated out dead cells using the Dead Cell Stain. B cells were defined as $CD19^+$ and T cells as $CD4^+$. CD24 and CD38 were used to identify B cell subsets within the $CD19^+$ cell population. The frequency of expression of the LEGENDScreen™ markers was defined by gating on the appropriate isotype control contained within the kit.

Using known lineage-specific markers, the specificity of the platform for each cell subset ($CD19^+$ B cells and $CD4^+$ T cells) was corroborated (Figure 3.2). B cell and T cell populations gated based on CD19 and CD4 expression respectively showed consistent expression of their lineage specific markers, supporting the chosen gating strategy for these cell subsets.

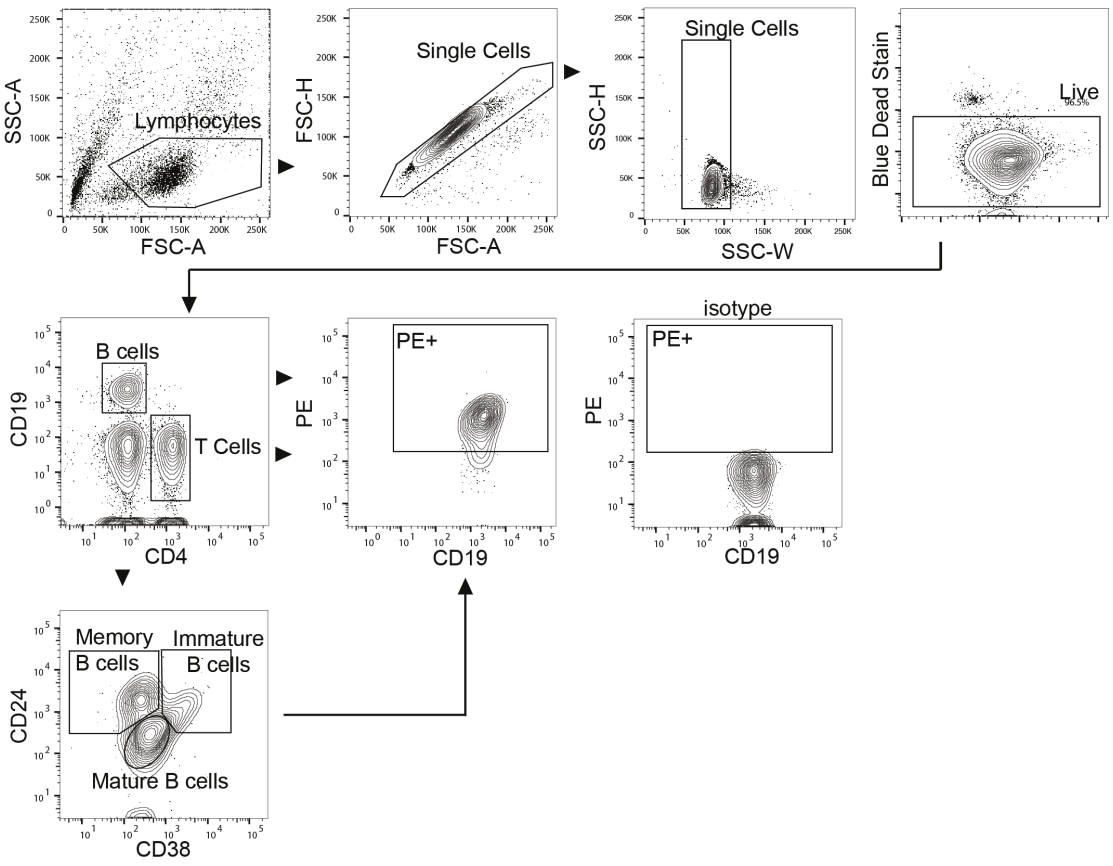

Figure 3.1. LEGENDScreen™ gating strategy.
PBMCs from frozen samples were stained with the LEGENDScreen™ kit in addition to markers against CD19, CD4, CD24 and CD38 and a Blue Live Dead stain to assess viability. Representative flow cytometry plots showing the sequential gating strategy for $CD19^+$ B cells, $CD4^+$ T cells and B cell subsets based on CD24 and CD38 expression on $CD19^+$ B cells. Cells were gated using side scatter-area (SSC-A) and forward scatter-area (FSC-A) parameters, followed by exclusion of doublets using FSC-height(H) and FSC-A, and SSC-H and SSC-width(W), and exclusion of dead cells. Immune cell subsets were gated as shown and expression of PE-conjugated LEGENDScreen™ markers gated based on specific PE isotype control contained within the kit.

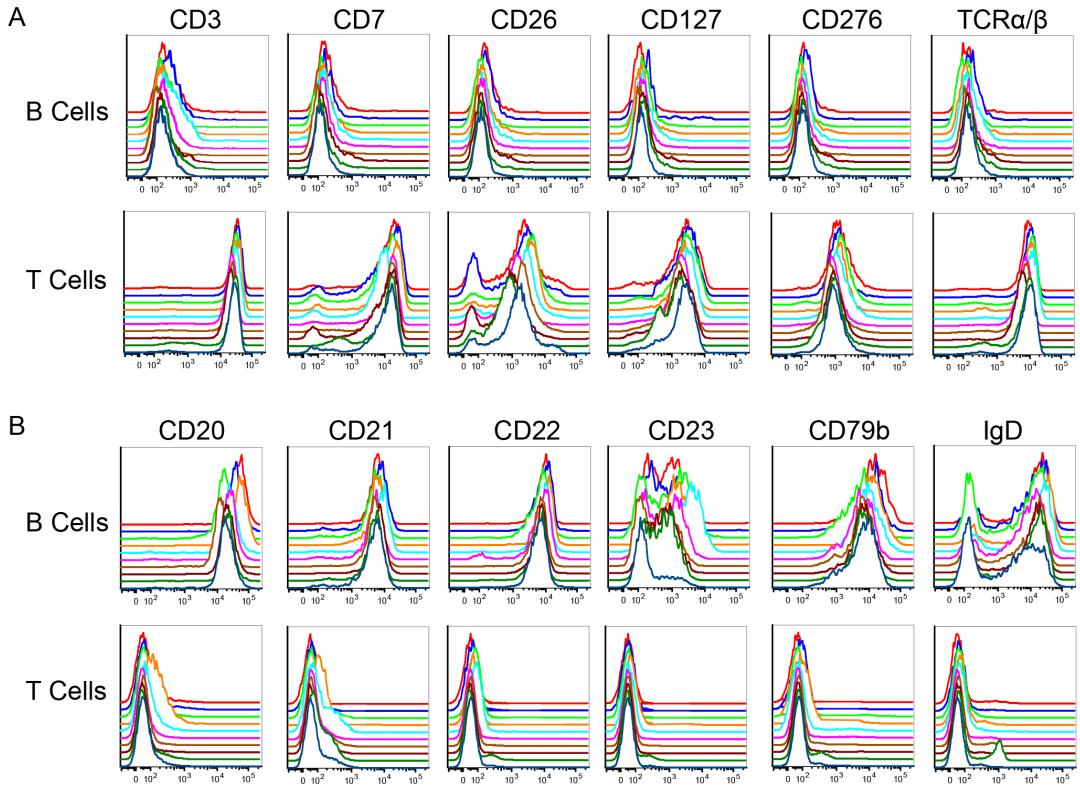

Figure 3.2. Validation of the flow cytometry gating strategy for defining CD4$^+$ T cells and CD19$^+$ B cells in the LEGENDScreen^{TM} analysis.
PBMCs from 10 HCs were stained with the LEGENDScreen^{TM} and additional antibodies to identify CD19$^+$ B cells and CD4$^+$ T cells. Overlay histograms for markers (PE MFI) reported to be specific for CD4$^+$ T cells (A), and CD19$^+$ B cells (B), showing their expression on B cells gated using CD19 and T cells gated using CD4, to validate specificity (n=10).

3.2. Validation of the LEGENDScreen™ platform in healthy individuals

As phenotyping using the LEGENDScreen™ had not previously been published, it was necessary to established the stability of this platform in terms of both intra- and inter- assay variability. This was done in collaboration with William Sanderson (previous PhD student in the laboratory), and Dr Marsilio Adriani (former Post-Doc at UCL). Validation was performed on PBMCs from 10 HCs. Inter-assay variability was assessed using PBMCs from the same HC isolated at a single time-point but analysed on three independent occasions within a month time period. MFI expression for the 332 markers on either T cells, B cells or monocytes was compared between each run and correlation calculated between the datasets (r) (Figure 3.1B). The line of regression (r) is close to one suggesting that both thawing of the cells and data collection by the FACSVerse™ remain constant over-time and did not dramatically affect the results. Very few points fall far from the line of regression, therefore I can be confident that I can compare samples run at different times and that any variation is not due to inter-assay variability. While some markers show some deviation, it is likely that these markers have particularly low MFI where a small difference is amplified in the correlation analysis but may not be biologically relevant. To assess intra-assay variability, the mean MFI for each of the plates from a single run were compared using one-way ANOVA (Figure 3.1C). This was done for three independent HCs and showed no significant difference in the MFI between the 4 plates.

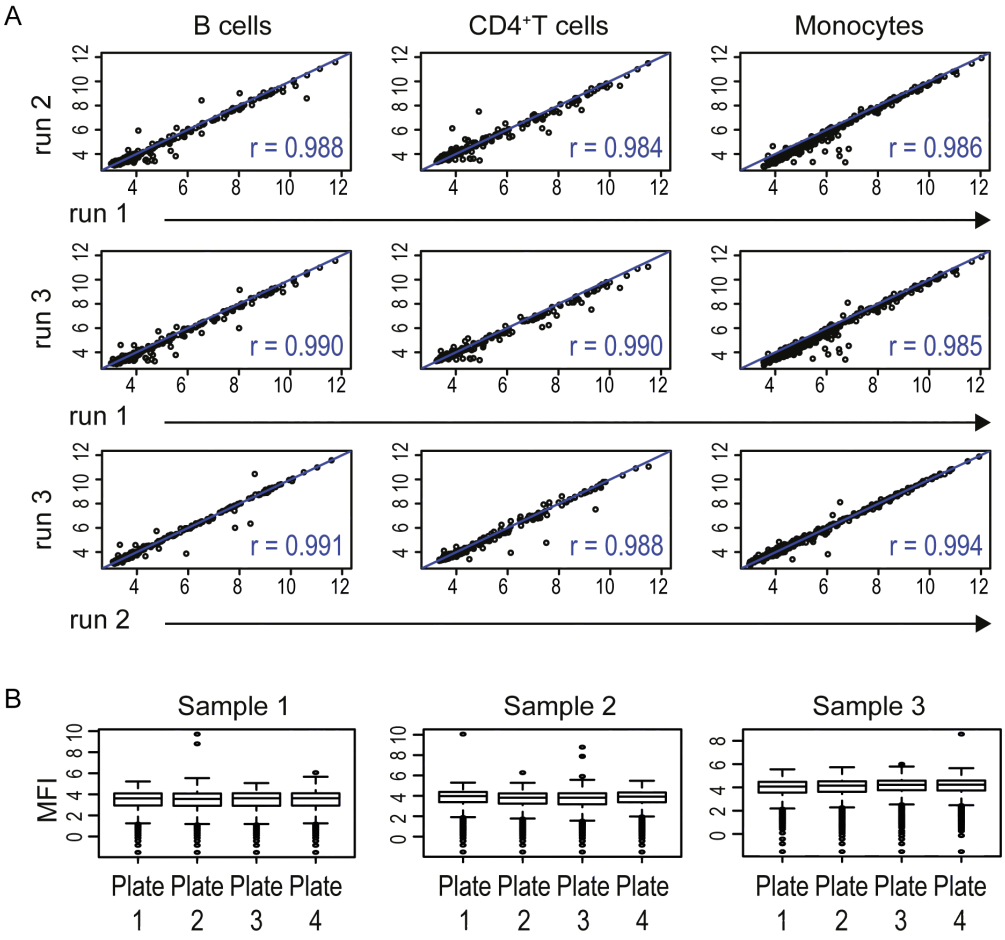

Figure 3.3. Validation of LEGENDScreen™ for inter- and intra- assay stability. PBMCs from 10 HCs were stained using the LEGENDScreen™ kit. A) PBMCs from the same HC isolated at a single time point were analysed using the LEGENDScreen™ on three independent occasions (Run 1-3), marker expression was analysed (MFI) and correlation assessed using Spearman correlation coefficients (r). (B) Mean MFI from each of the four LEGENDScreen™ plates for 3 HCs (samples 1-3). Mean ±SD, one-way ANOVA. Experiments and analysis performed by Marsilio Adriani.

3.3. Validation of the LEGENDScreen™ for analysis of patients with RA.

Our aim is to use the LEGENDScreen™ to compare differences between HCs and RA patients. To assess inter-patient variability, PBMCs isolated from 10 cDMARD treated RA patients (demographics in Table 3.1) were stained using the LEGENDScreen™ platform as described for HCs and including the addition of markers defining $CD4^+$ T cells, $CD19^+$ B cells and B cell subsets ($CD24^{hi}CD38^{hi}$ immature B cells, $CD24^{int}CD38^{int}$ mature B cells and $CD24^{hi}CD38^{lo}$ memory B cells). I compared this data to that from the 10 HCs used in the prior validation, and took the standard deviation for each marker expressed (MFI) on the B cell population for the two groups (RA and HC). Wilcoxon non-parametric test of paired data did not show statistical differences in the average variation exhibited by the LEGENDScreen™ markers between HCs and RA patients (Figure 3.4).

Table 3.1 LEGENDScreen™ RA signature discovery cohort patient demographics and disease characteristics.

	Healthy controls	DMARDs treated RA (biologic naïve)	Adalimumab treated RA
n	18	10	21*
Sex, female n (%)	15 (79)	9 (90)	17 (81)
Age (years), mean (SD)	35.2 (10.7)	45.6 (13.5)	58.5 (13.3)
DAS28 (SD)	-	3.95 (1.76)	3.26 (1.4)
Seropositive (RF+/CCP+) (%)	-	100	95
CRP mg/l (SD)	-	6.78 (4.75)	9.77 (15.6)
Current Treatment			
DMARDs (n)	-	10	4*
Adalimumab (n)	-	-	14
Etanercept (n)	-	-	1*
Tocilizumab (n)	-	-	2*

*All patients previously treated with adalimumab and tested for ADA against adalimumab. Values in the table represent mean ± standard deviation (SD), or number of patients (n) with proportion of total (%) where indicated.

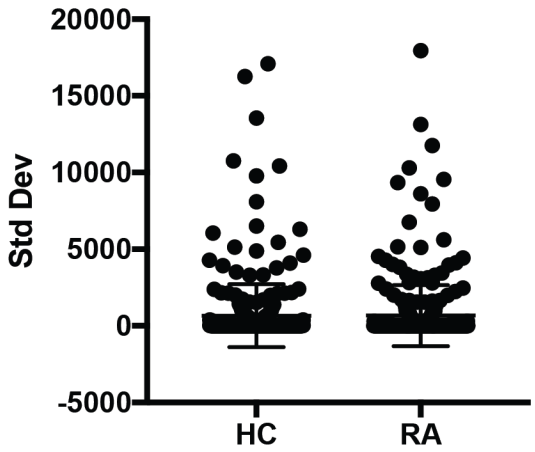

Figure 3.4. Inter-patient variation of LEGENDScreen™ marker expression (MFI) on B cells is not significantly different from variation observed within HCs.

10 HCs and 10 cDMARD treated RA patients were stained using LEGENDScreen™ with additional antibodies to identify $CD19^+$ B cells. Standard deviation was calculated for each of the 332 LEGENDScreen™ markers for either HCs or RA patients; each black dot represents one of the 332 LEGENDScreen™ markers. Wilcoxon testing showed no significant difference ($p>0.05$).

3.4. Summary

In this first chapter I have demonstrated that the LEGENDScreen™ exhibits high intra- and inter- stability, and that our gating panel is sufficient to identify T and B cells. Furthermore, I show that inter-patient variation is not significantly greater that inter-HC variation. Therefore, I believe that the LEGENDScreen™ is a stable platform that will allow the investigation of phenotypical differences between HC and RA patients, on T cells, and B cells. This tool has the potential to uncover novel markers that may have important immunological roles or provide possible novel therapeutic targets.

4. Results II – Predicting ADA in adalimumab treated RA patients

One of the major drawbacks of biological treatment is immunogenicity, the immune response against a drug, and the development of antibodies directed against the drug (anti-drug antibodies [ADA]) (140), which are often associated with a poorer clinical outcome (125, 326). Adalimumab, a fully human monoclonal antibody against tumor necrosis factor (TNF)α and a common first line biologic used in the treatment of RA, exhibits particularly high rates of immunogenicity (up to around a third of patients treated with adalimumab develop antibodies against the drug) (128, 129, 327). The identification of predictive biomarker/s that distinguish rheumatoid arthritis (RA) patients who are more likely to develop ADA in response to adalimumab, would considerably improve the clinical management of RA. To achieve this aim, I took advantage of the LEGENDScreen^{TM} and compared adalimumab treated RA patients that have developed ADA to ADA negative patients.

4.1. LEGENDScreen^{TM} analysis identifies an immune-module associated with ADA in RA patients treated with adalimumab

For the initial investigation of ADA in adalimumab treated RA patients with the LEGENDScreen^{TM} platform, I used a cross-sectional cohort of patients recruited at UCL. Patients attending the rheumatology clinics that matched our recruitment criteria (RA, adalimumab treated) were identified and approached on the day of their appointment; enrolled patients were consented and blood samples were taken for PBMCs and serum. Clinical data including DAS28 score, CRP, RF, ESR, and anti-CCP, smoking status, gender, age, and BMI were recorded on the day of the recruitment. Clinical details of patients used for LEGENDScreen^{TM} experiments can be found in Table 4.1. Serum samples were sent in batches for ADA testing by ABIRISK collaborators at GSK and the recruitment of patients continued until 10 ADA^{+} patients were identified. In our cross-sectional cohort of 55 adalimumab treated RA patients, approximately 20%, tested positive for ADA against adalimumab, this is within the expected range of ADA positivity based on reported literature of up to a third, and very close to the value found in a European

retrospective multicohort analysis, reporting an incidence of 19.2% (128, 129, 328) (Table 4.2).

Co-treatment with MTX has been shown to reduce ADA levels (148), albeit not the incidence of ADA (124). In our cohort, although our samples size is too small to obtain firm results, fewer of the patients with detectable ADA are co-treated with MTX, which is in-line with reported observations. Furthermore, while the average dose of MTX in ADA^{pos} patients that are taking the drug is higher, this is likely to reflect the worse disease exhibited in this patient group, which is in part due to a failure of adalimumab as a result of ADA.

To identify biomarker/s associated with an ADA response to adalimumab, I compared the surface immune-signature of PMBCs isolated from 10 ADA^- versus 10 ADA^+ RA patients that were treated with adalimumab for a minimum of 12 months (Table 4.1). PBMCs were stained with fluorescently-conjugated antibodies identifying $CD4^+$ T cells, $CD19^+$ B cells, and immature, mature and memory B cell subsets in addition to the 332 cell surface markers included in the LEGENDScreen™ panel (Figure 3.2, Appendix A.3, and as described in Chapter 3 Results I). The expression-pattern of surface markers on PBMCs appears to be distinct between ADA^- and ADA^+ RA patients (Figure 4.1A). The variation in marker expression is also present following analysis of $CD19^+$ B cells and $CD4^+$ T cells (Figure 4.1B), with a greater difference observed in B cells than in T cells. As many of the markers assessed by the LEGENDScreen™ platform were either not expressed or were expressed at a very low level, to increase the statistical power of future analyses, markers with less than 5% expression on all samples tested were excluded from this study. These markers were considered to not be expressed based on background staining observed for isotype controls of up to 5%. Inclusion of this criteria resulted in the removal of 74 B cell and 134 T cell-associated markers. Expression of the remaining markers was compared between ADA^+ and ADA^- RA patients using unpaired multiple *t*-test analysis to test if the differences observed were significant. B cells showed highest number of differentially expressed markers (ADA^- vs. ADA^+) (n= 22) compared to T cells (n=11) ($p<0.05$, multiple *t*-test) (Figure 4.1C). These results together with the numerical and functional imbalance in B cells associated with the pathogenesis of RA (98, 325, 329), prompted us to focus

on differentially expressed markers expressed by B cell subsets. Respectively four, seven and nineteen DEMs were identified on mature, immature, and memory B cells between ADA^+ and ADA^- RA patients (Figure 4.1D, Appendix A.4). Of note, all these differentially expressed markers are down-regulated in ADA^+ RA patients, compared to ADA^-.

Next, a systematic framework analysis (SFA) was used to distinguish between differentially expressed markers associated with the presence of ADA as opposed to disease severity and/or the effect of adalimumab therapy. The SFA is shown in Figure 4.2 and described as follows. Differentially expressed markers on memory B cells that correlated significantly with Disease Activity Score-28 (DAS28) (n=1) (Pearson correlation) were excluded from our study, as these were considered to be due to RA-related inflammation (Appendix A.5). None of the differentially expressed markers identified on mature and immature B cells correlated with DAS28. To account for treatment effect, I compared the expression of the differentially expressed markers in ADA^-, ADA^+ and cDMARD treated RA patients (RA-D). Using one-way ANOVA analysis or Kruskal-Wallis analysis for non-normally distributed markers, I excluded markers that were significantly different between ADA^- and RA-D, but not between ADA^+ and RA-D. These markers were considered to be related to treatment, based on the assumption that ADA^+ patients are typically non-responders to treatment, and therefore differences between ADA^+ and ADA^- patients may be due to the treatment not working and not ADA itself. Furthermore, patients treated with cDMARDs act as a non-adalimumab treated control to account for adalimumab specific treatment effects (Figure 4.3). I also removed markers that no longer showed significance following ANOVA analysis between ADA^- and ADA^+ samples in order to select markers that are more robustly different between ADA^+ and ADA^- and therefore more likely to be replicated in the validation cohort. Following the application of the SFA I was left with 7 differentially expressed markers; CD167a and CD1c expressed on mature; IL-7Rα, CD138 and CD324 on immature and SIRPα/β and CD1a on memory B cells (expression shown in heatmap; Figure 4.4A). These 7 markers are henceforth defined as the ADA 'module'. To ascertain if this selection of markers has sufficient statistical power to discriminate between ADA^+ and ADA^- RA patients, I performed a principal component analysis (PCA) (Figure 4.4B). This dimensionality reduction

technique demonstrated that the 7 parameters cluster ADA^+ and ADA^- patients separately with the first principle component accounting for 47% of the variation observed between the individual samples.

Table 4.1. ADA cross-sectional cohort patient demographics and disease characteristics.

	RA (adalimumab ADA^{neg})	RA (adalimumab ADA^{pos})	DMARDs treated RA (biologic naïve)
n	10	10	10
Sex, female n (%)	7 (70)	9 (90)	9 (90)
Age (years), mean (SD)	62.4 (14.8)	53.4 (10.5)	45.6 (13.5)
DAS28 (SD)	3.05 (1.06)	3.63 (1.69)	3.95 (1.76)
Seropositive (RF+/CCP+) (%)	100	90	100
CRP mg/l (SD)	14.07 (21.54)	5.64 (6.20)	6.78 (4.75)
Current treatment			
DMARDs only (n)	3*	1*	10
Adalimumab (n)	5	8	-
Etanercept (n)	1*	-	-
Tocilizumab (n)	1*	1*	-
Treatment			
MTX use, n (%)	7	5	5
Average MTX dose, mg/week, mean (SD)	15.7 (3.6)	18 (51.1)	19 (2.0)
Prednisolone use, n (%)	1	2	2
Hydroxychloroquine use, n (%)	1	2	6
Sulfasalazine use, n (%)	1	1	6

*Previously treated with adalimumab.
Values in the table represent mean ± standard deviation (SD), or number of patients (n) with proportion of total (%) where indicated.
Adalimumab dose 40mg every two weeks, tocilizumab dose 4mg/kg every 4 weeks, etanercept does 25mg twice a week.

Table 4.2. ADA results for adalimumab treated UK cross-sectional cohort of RA patients.

ABIRISK sample no.*	ADA response (positive/negative)	ADA titres (fold dilution)
08-01-0007	P	16
08-01-0008	N	n/a
08-01-0022	N	n/a
08-01-0025	P	2
08-01-0026	N	n/a
08-01-0027	N	n/a
08-01-0037	P	8
08-01-0040	N	n/a
08-01-0042	N	n/a
08-01-0046	N	n/a
08-01-0055	N	n/a
08-01-0057	N	n/a
08-01-0059	N	n/a
08-01-0063	N	n/a
08-01-0064	N	n/a
08-01-0065	N	n/a
08-01-0066	N	n/a
08-01-0068	N	n/a
08-01-0069	P	<2
08-01-0071	P	<2
08-01-0076	P	<2
08-01-0077	N	n/a
08-01-0085	N	n/a
08-01-0086	N	n/a
08-01-0087	N	n/a
08-01-0088	N	n/a
08-01-0107	P	<2
08-01-0109	P	2
08-01-0119	P	80
08-01-0123	N	n/a
08-01-0124	P	80
08-01-0125	P	500
08-01-0129	N	n/a
08-01-0134	P	<2
08-01-0135	P	320
08-01-0137	N	n/a
08-01-0138	N	n/a
08-01-0139	N	n/a
08-01-0140	N	n/a
08-01-0142	N	n/a
08-01-0143	N	n/a
08-01-0148	N	n/a

08-01-0149	P	20
08-02-0002	N	n/a
08-02-0006	P	40
08-03-0001	N	n/a
08-03-0002	N	n/a
08-03-0004	N	n/a
08-03-0006	N	n/a
08-03-0007	N	n/a
08-03-0009	N	n/a
08-03-0010	N	n/a
08-03-0012	P	20
08-03-0013	N	n/a
08-03-0014	N	n/a

Negative (N)	39	80.20%
Positive (P)	16	19.80%
TOTAL	55	

*"08" = UK sample
"01" = London, "02" = Basildon, "03" = Sheffield
"00xx" = sample no.

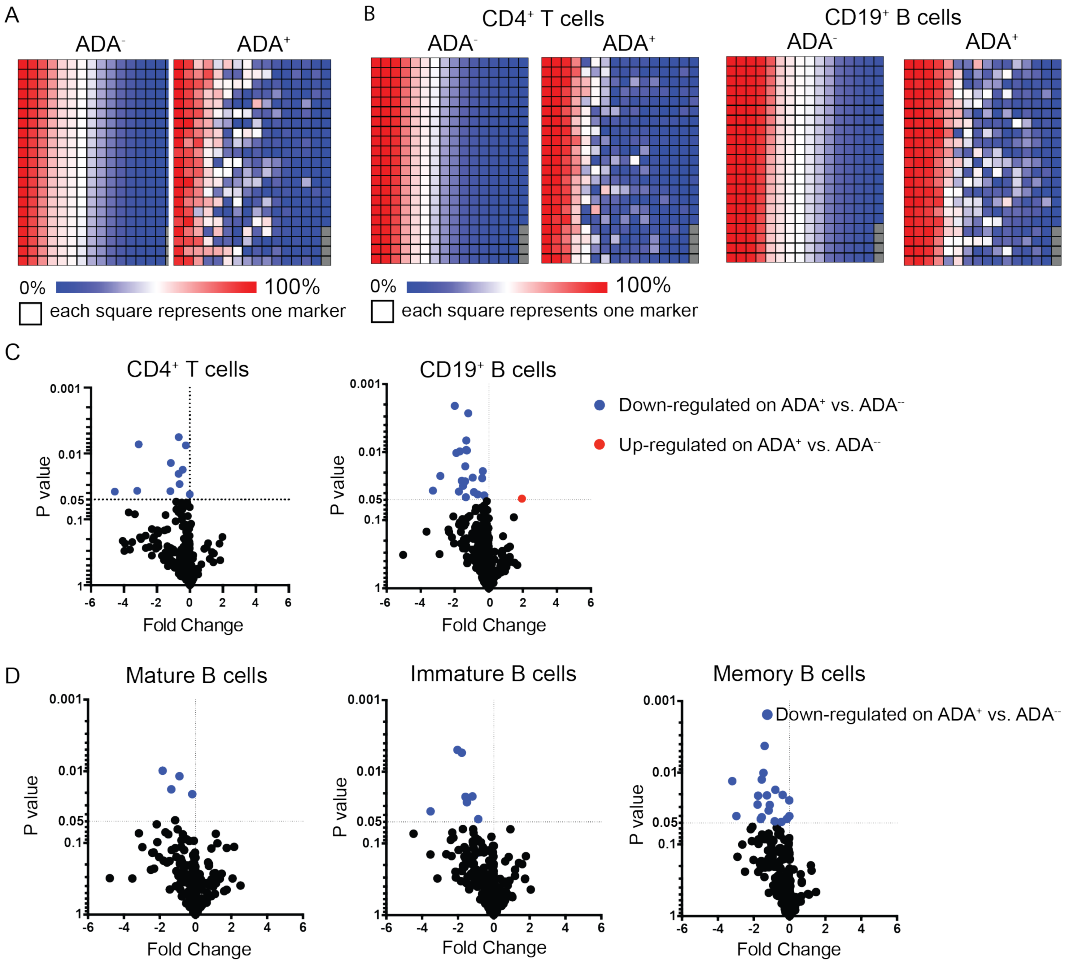

Figure 4.1. LEGENDScreen™ analysis of adalimumab treated RA patients (cross-sectional cohort) identifies cell surface markers associated with ADA.
PBMCs from patients treated with adalimumab defined as ADA^+ (n=10) or ADA^- (n=10) were stained with LEGENDScreen™ for 332 cell surface markers, in addition to antibodies against CD19, CD4 and CD24 and CD38. Heatmaps showing average frequency expression of each LEGENDScreen™ marker for each sample group (ADA^+ and ADA^-) for PBMCs (A), and $CD4^+$ T cells and $CD19^+$ B cells (B), each square represents one of the 332 markers, and are ranked according to expression in ADA^- patients. Volcano plots showing fold-change of frequency expression between patient groups (ADA^-/ADA^+) (Log_2) and p value (*t*-test) (Log_{10}), in ADA^- versus ADA^+; for $CD4^+$T cells and $CD19^+$B cells (C), and mature ($CD24^{int}CD38^{int}$), immature ($CD24^{hi}CD38^{hi}$) and memory ($CD24^{hi}CD38^{lo}$) B cells (D). No markers passed the Holm-Sidak post-hoc test. Blue circle: significantly down-regulated markers; red circle significantly up-regulated markers.

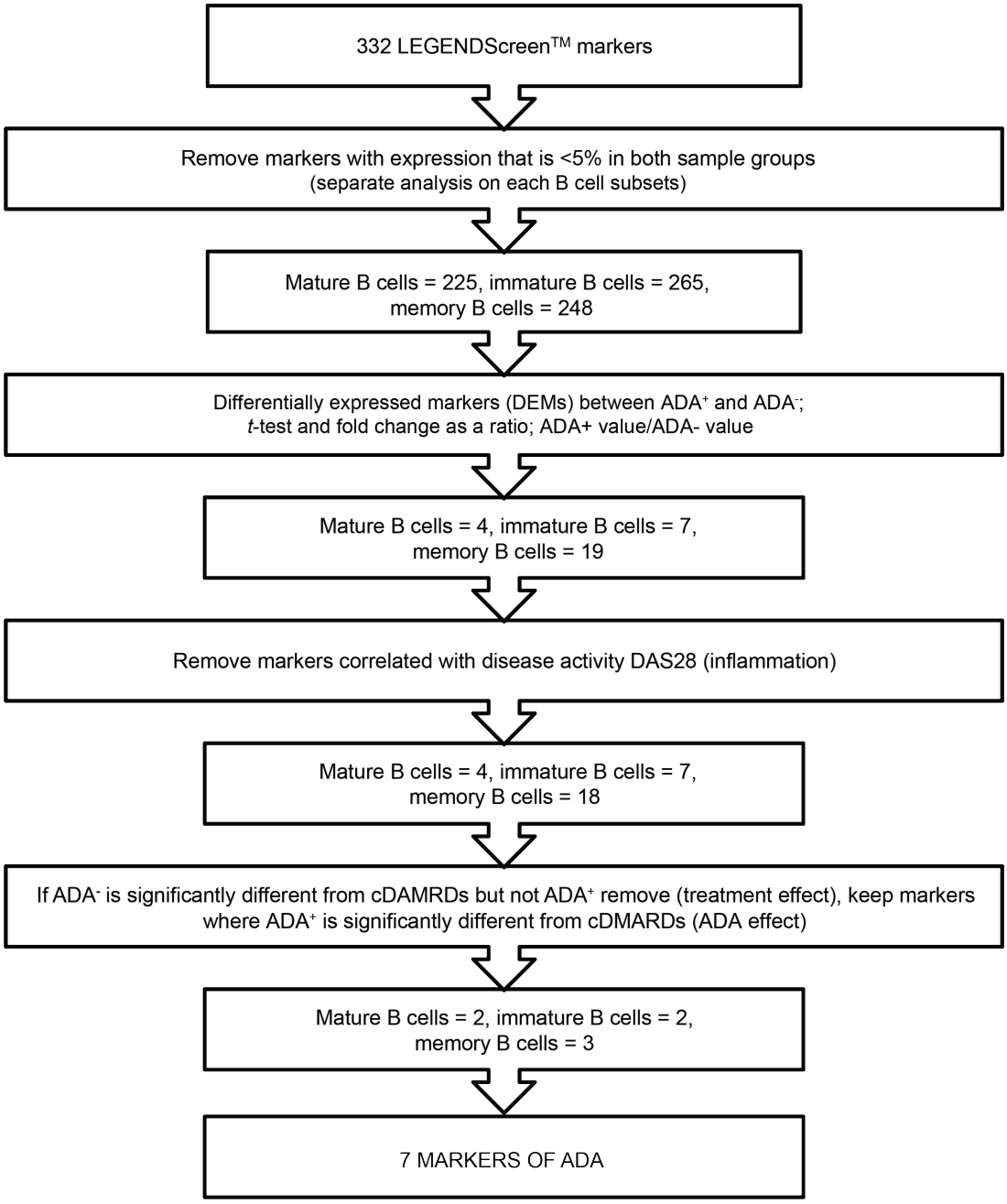

Figure 4.2. Selection of markers for ADA module from LEGENDScreen™ analysis of cross-sectional cohort.
Flow Diagram. One-way ANOVA analysis of ADA^- vs. ADA^+ vs. cDMARD treated (RA-D) RA patients (see also Figure 4.3). Fold change calculated as a ratio of the ADA^+ value divided by the ADA^- value.

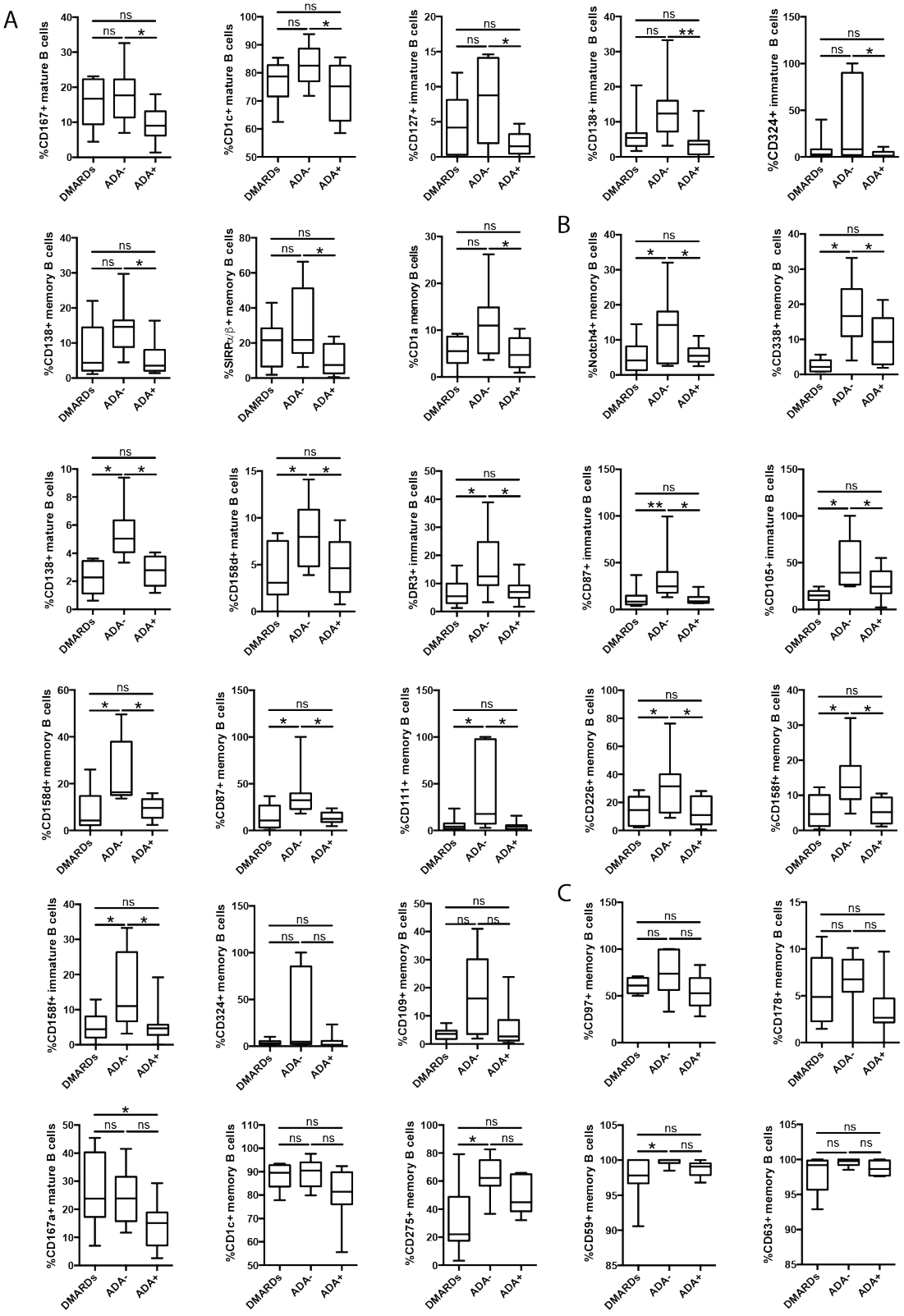

Figure 4.3. Removal of differentially expressed markers from ADA module.
One-way ANOVA analysis or Kruskal-Wallis (non-parametric data), of ADA^- vs. ADA^+ vs. cDAMRDs treated RA patients (RA-D) of LEGENDScreen^{TM} expression data for differentially expressed markers that were retained following removal of markers associated with DAS28. Normality was determined using D'Agostino and Pearson normality; CD138, CD324, CD87, CD111, CD109, CD178, CD1c, and

CD59 were not normally expressed. (A) Markers retained. (B) Markers removed due to significant difference between ADA^- and RA-D. (C) Markers removed as ADA^- vs. ADA^+ no longer significant. Box and whisker plot (min to max) *$p \leq 0.05$, ns=not significant.

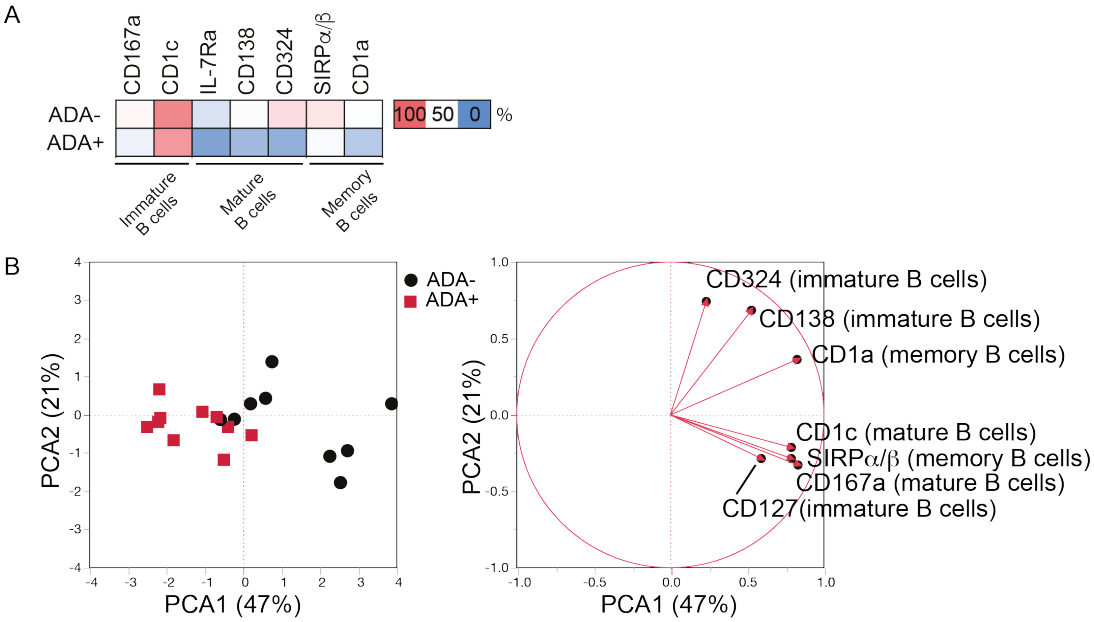

Figure 4.4. The ADA-associated "immune-module".
PBMCs from patients treated with adalimumab defined as ADA^+ (n=10) or ADA^- (n=10) were stained with LEGENDScreen™ for 332 cell surface markers, in addition to antibodies against CD19, CD4, CD24 and CD38. (A) Heatmap showing mean frequencies of differentially expressed markers (p value defined in Appendix A.4), between patient groups on B cell subsets, following exclusion of markers associated with DAS28 and 'treatment effect' (see Figure 4.2). (B) PCA of frequency of expression of the differentially expressed markers on the 20 adalimumab treated RA patients (ADA^- black circles, ADA^+ red squares) with contribution of each marker to the principal components denoted by length and direction of the corresponding red arrow.

4.2. Low frequency of SIRPα/β$^+$memory B cells constitutes a risk factor for ADA development

While previous studies have shown some molecular association with the development of ADA in RA patients (143, 144), currently there are no clinically accepted predictive biomarkers for ADA development in anti-TNFα treatment. I validated the B cell subset ADA-associated 'module' in an independent prospective European RA cohort (n=35) (map of recruitment is shown in Figure 4.5A), which was designed to assess immunogenicity development following initiation of adalimumab treatment (none of the patients included in this study had been previously treated with this biologic) (Table 4.3). These patients formed part of a wider cohort of approximately 250 RA patients recruited across ABIRISK that were switching treatment to infliximab, tocilizumab, etanercept or rituximab.

Purified PBMCs and serum were collected at three time points: baseline prior to commencement of treatment, 1 month and 12 months after treatment initiation (Figure 4.5B), and ADA was measured at each time point (Table 4.4). Out of the 37 patients recruited, 5 seronegative (RF and CCP negative) and 2 of unknown serological status were excluded from this study (Figure 4.6). A further 6 patients, which tested positive at baseline (n=5) or had transient ADA expression (n= 1) were excluded. The prospective cohort of RA patients was classified as ADA$^+$ or ADA$^-$ based on presence of ADA at the 12-month time-point.

Month 12 PBMCs from the prospective cohort were stained for CD19, CD24, CD38 and the 7 differentially expressed markers as shown in Figure 4.4A. Analysis showed that the frequency of SIRPα/β$^+$memory B cells was consistently reduced in ADA$^+$ compared to ADA$^-$ RA patients at the 1-year follow-up (*t*-test following removal of outliers (ROUT Q=1%)) (330). None of the other module markers were confirmed in this analysis (Figure 4.7A-C). To test the ability of SIRPα/β$^+$memory B cells to distinguish patients as ADA$^+$ or ADA$^-$ I generated a receiver operating characteristic (ROC) curve, plotting sensitivity against specificity for different SIRPα/β values observed in our validation cohort at month 12 (Figure 4.7D). An area under the curve (AUC) value of 0.92 was calculated, indicating that the

frequency of SIRPα/β^{+}memory B cells is highly accurate at defining ADA positivity. A cut-off value for the frequency of SIRPα/β^{+}memory B cells was determined using the calculation of sensitivity-(1-specificity), with individuals expressing less than 9.4% SIRPα/β^{+}memory B cells deemed to be ADA^{+}.

To further confirm that changes in SIRPα/β^{+}memory B cell frequencies were associated with ADA and not to any of the other clinical or demographic parameters, implicated in RA (DAS28, CRP, ESR, age, BMI, ADA titre), a correlation analysis of these variables was performed. None of the clinical parameters, DAS28, CRP, ESR, age, BMI, nor ADA titre correlated significantly with the percentage of SIRPα/β^{+}memory B cells (Figure 4.8A). Furthermore, there were no significant differences in the percentage of SIRPα/β^{+}memory B cells between patients stratified according to concomitant treatment with methotrexate (MTX), gender or smoking status (Figure 4.8B-D).

Of interest, the development of ADA in the prospective cohort was associated with non-response or partial response to adalimumab, according to the EULAR classification (67%) (Figure 4.8E). The frequency of SIRPα/β^{+}memory B cells was significantly decreased in non-responders compared to responder patients (Figure 4.8F). However, patient response to treatment was independent from the quantities of ADA in circulation, since ADA titre did not correlate with DAS28 (Figure 4.8G). I therefore hypothesise that the presence of less than 9.4% SIRPα/β^{+}memory B cells in RA patients prior to initiation of adalimumab treatment could be used as biomarker to predict ADA development in these patients.

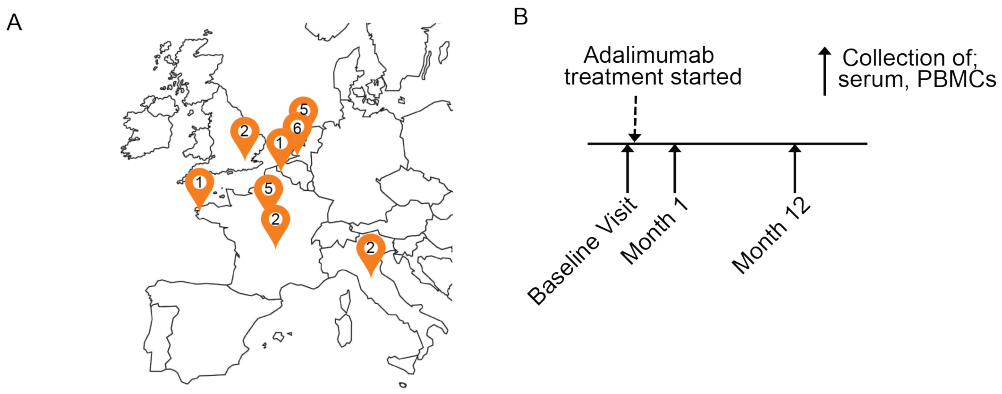

Figure 4.5. Recruitment of a European Prospective cohort of RA patients commencing adalimumab treatment.
PBMCs and serum samples were collected longitudinally (baseline, month 1 and 12 following start of treatment) from RA patients starting adalimumab treatment across Europe. For each visit ADA level was measured by Meso Scale Discovery (MSD) technology. (A) Map shows location of recruitment sites (orange marker), with the number of adalimumab treated patients recruited at each site shown within the orange marker. (B) Timeline of sample collection.

Table 4.3. Prospective European ADA cohort patient demographics and disease characteristics.
Independent prospective validation cohort (UK, France, Italy, Netherlands).

	RA (adalimumab ADA^{neg})	RA (adalimumab ADA^{pos})
n	12	12
Sex, female n (%)	8 (67)	10 (83)
Age (years), mean (SD)	59 (15.7)	45 (12.6)
DAS28 (SD)	2.45 (1.2)	3.49 (1.2)
Seropositive (RF+/CCP+) n (%)	12 (100)	12 (100)
CRP mg/l (SD)	7.5 (11.6)	9.2 (9.1)
Location		
UK	1	1
France	6	3
Italy	1	1
Netherlands	4	7
Treatment		
MTX use, n (%)	10 (83.3%)	8 (66.7%)
Average MTX dose, mg/week, mean ± SD	18.25±5.3	15.63±6.6
Prednisolone use, n (%)	1 (8.3%)	3 (25%)
Prednisone use, n (%)	3 (25%)	3 (25%)
Hydroxychloroquine use, n (%)	3 (25%)	0 (0%)
Leflunomide use, n (%)	0 (0%)	3 (25%)
Sulfasalazine use, n (%)	2 (17%)	0 (0%)

†EULAR response at month 12 visit
Values in the table represent mean ± standard deviation (SD), or number of patients (n) with proportion of total (%) where indicated.
Month 12 (M12)
All patients received 40mg adalimumab every other week.

Table 4.4. ADA results and serological status (RF/anti-CCP+) for prospective cohort of RA patients that switched to adalimumab treatment.
Neg = negative, Pos = positive, "?" = unknown. Final ADA status is negative (no ADA present at any time point), positive (ADA present at month 12 (M12) only, or M12 and month 1(M1)), transient (ADA present at M1 but not at M12) and baseline (ADA present at baseline). Sero-status "Neg" if neither RF or anti-CCP positive, "Pos" if either RF or anti-CCP pos.

ABIRISK sample no.*	Baseline	ADA status M1	M12	ADA titre (ng/ml) M12	Final ADA status	RF	anti-CCP	Sero-status
40-01-0013	Neg	Neg	Neg	n/a	Neg	n/a	n/a	?
40-01-0015	Neg	Neg	Neg	n/a	Neg	Neg	Neg	Neg
40-07-0001	Neg	Neg	Neg	n/a	Neg	Neg	Neg	Neg
40-04-0006	Neg	Pos	Neg	n/a	Transient	n/a	Neg	?
40-14-0001	Neg	Neg	Neg	n/a	Pos	Neg	Neg	Neg
40-17-0014	Pos	n/a	Pos	19.66	Baseline	Neg	Neg	Neg
40-08-0005	Neg	Neg	Pos	20.96	Pos	Neg	Neg	Neg
03-01-0005	Neg	Pos	Pos	17.78	Pos	Neg	Neg	Neg
40-05-0008	Pos	Neg	Neg	n/a	Baseline	Pos	Pos	Pos
40-01-0026	Pos	Neg	Neg	n/a	Baseline	Neg	Pos	Pos
40-08-0002	Pos	Pos	Pos	22.69	Baseline	Pos	Pos	Pos
09-01-0016	Pos	Neg	Neg	n/a	Baseline	Pos	Pos	Pos
09-01-0020	Neg	Neg	Neg	n/a	Transient	Pos	Pos	Pos
10-04-0014	Pos	Neg	Neg	n/a	Baseline	Pos	Pos	Pos
40-01-0003	Neg	Neg	Neg	n/a	Neg	Pos	Pos	Pos
40-05-0005	Neg	Pos	Pos	25.38	Pos	Pos	Pos	Pos
40-01-0017	Neg	Pos	Pos	89.34	Pos	Pos	Pos	Pos
40-11-0001	Neg	Neg	Neg	n/a	Neg	Pos	Pos	Pos
40-17-0007	Neg	Neg	Neg	n/a	Neg	Pos	Neg	Pos
40-17-0011	Neg	Neg	Neg	n/a	Neg	Pos	Pos	Pos
40-01-0022	Neg	Neg	Neg	n/a	Neg	Pos	Pos	Pos
40-01-0027	Neg	Neg	Neg	n/a	Neg	Pos	Pos	Pos
03-01-0007	Neg	Pos	Pos	242.91	Pos	Pos	Pos	Pos
03-01-0009	Neg	Neg	Neg	n/a	Neg	Pos	Pos	Pos
09-01-0012	Neg	Neg	Neg	n/a	Neg	Pos	Pos	Pos
09-01-0015	Neg	Neg	Pos	17.45	Pos	Pos	Pos	Pos
09-01-0017	Neg	Pos	Pos	1973.43	Pos	Pos	Pos	Pos
09-01-0018	Neg	Pos	Pos	31.32	Pos	Pos	Pos	Pos
09-01-0021	Neg	Pos	Pos	9.96	Pos	Pos	Pos	Pos
10-01-0011	Neg	Neg	Neg	n/a	Neg	Pos	Pos	Pos
10-01-0017	Neg	Neg	Pos	5.22	Pos	Pos	Pos	Pos
10-01-0013	Neg	Neg	Pos	8.23	Pos	Pos	Pos	Pos
10-01-0014	Neg	Pos	Pos	16.33	Pos	Pos	Pos	Pos
10-01-0020	Neg	Neg	Neg	n/a	Neg	Pos	Pos	Pos
08-01-0002	Neg	Neg	Pos	5.36	Pos	Pos	Pos	Pos
08-01-0004	Neg	Neg	Neg	n/a	Neg	Pos	Pos	Pos
40-10-0002	Neg	Pos	Pos	3.32	Pos	Pos	Pos	Pos
09-01-0023	Neg	Neg	Neg	n/a	Neg	Pos	Pos	Pos

*"03-" = Italy ,"08-" = UK , "09-"/"10-" = Netherlands, "40-" = France
"-xx-" = centre number
"-00xx" = sample no.

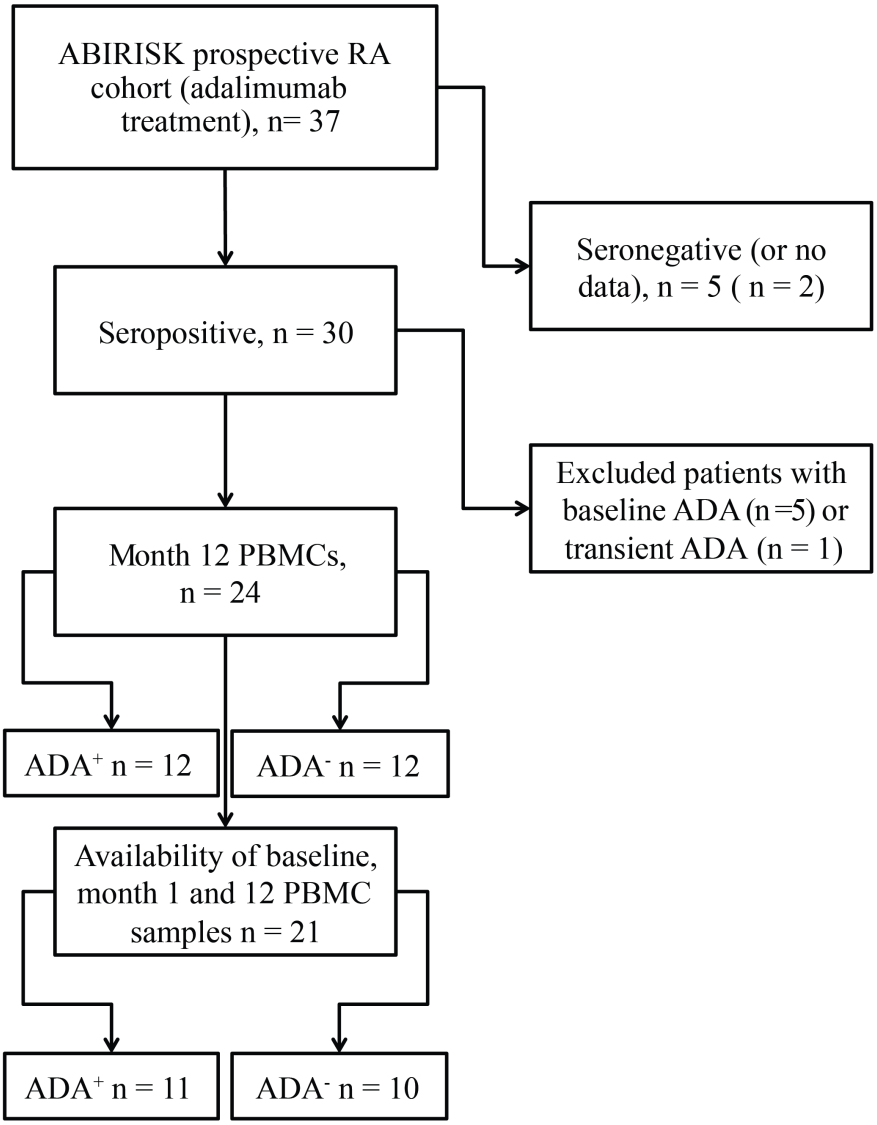

Figure 4.6. Selection of prospective patients for validation cohort.
Flow diagram detailing the selection criteria applied to the validation cohort of patients.

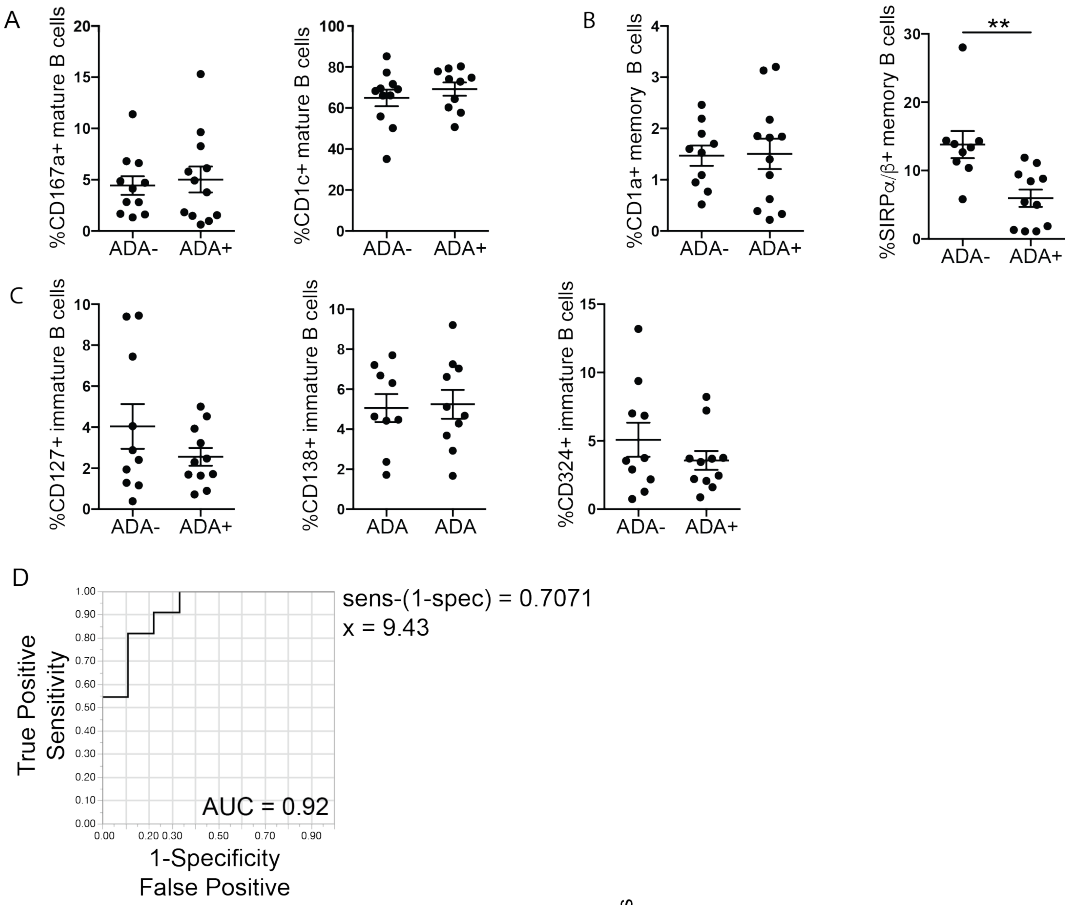

Figure 4.7. Reduced frequency of SIRPα/β$^+$ memory B cells is validated in ADA$^+$ adalimumab treated RA patients.

PBMCs and serum samples were collected longitudinally (baseline, month 1 and 12 following start of treatment), from RA patients starting adalimumab treatment across Europe. For each visit ADA level was measured by Meso Scale Discovery (MSD) technology. Month 12 PBMCs were stained for flow cytometry, for the module (7 markers), and the frequencies of cells expressing the markers analysed (n=12 ADA$^-$, n=12 ADA$^+$). Outliers removed using robust regression and outlier removal (ROUT (Q=1%)). (A-C) Frequency of expression of markers on (A) mature, (B) memory and (C) immature B cells from the prospective cohort; scatter plots showing mean ±SEM. *T*-test analysis, **p≤0.01.

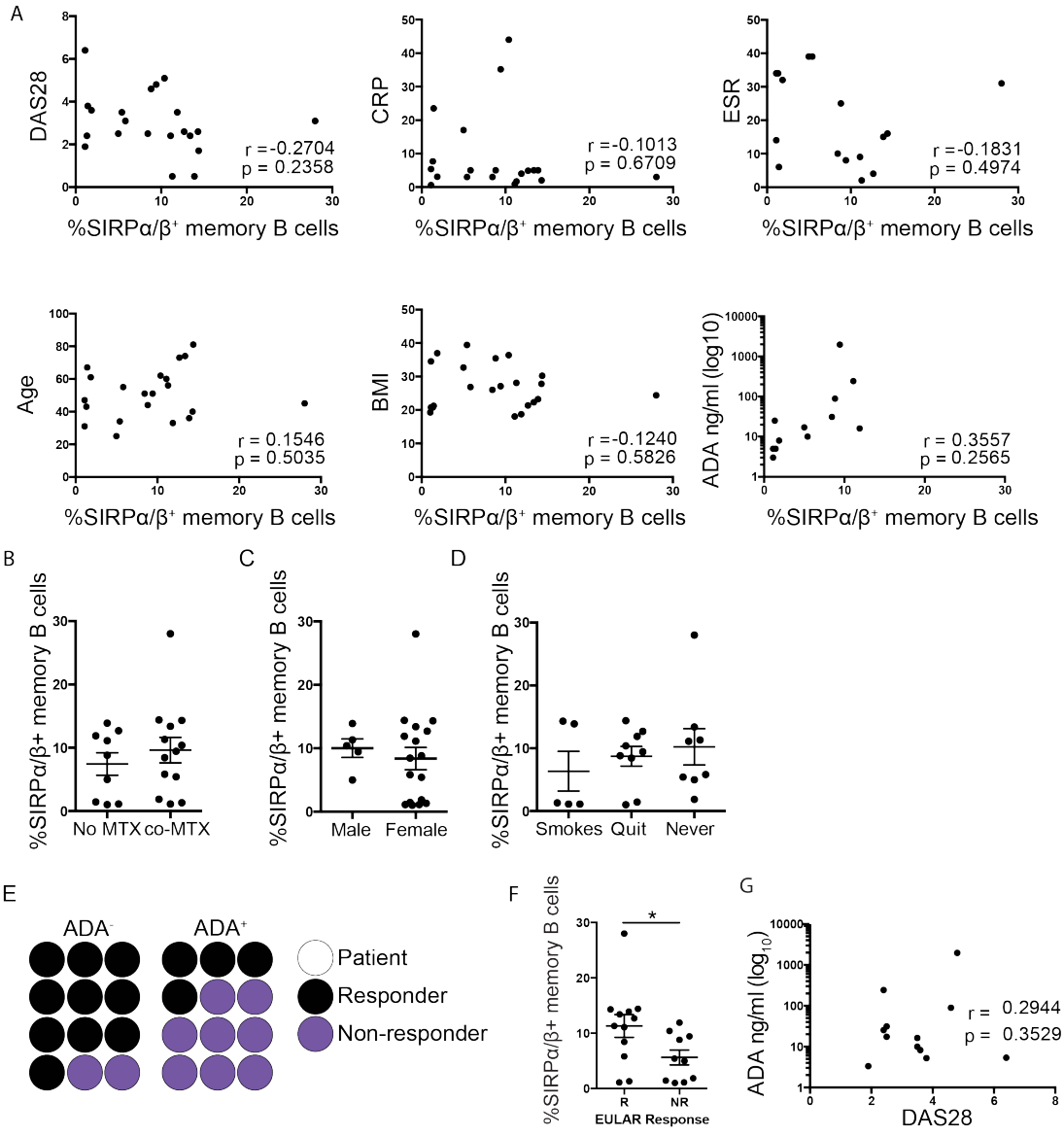

Figure 4.8. ADA$^+$ patients are poor responders to adalimumab treatment.
PBMCs and serum samples were collected from RA patients across Europe at month 12 following start of adalimumab treatment. ADA level was measured by Meso Scale Discovery (MSD) technology. PBMCs were stained for flow cytometry SIRPα/β (n=12 ADA$^-$, n=12 ADA$^+$). Clinical data was obtained from clinical records. (A) Scatter plots showing %SIRPα/β$^+$memory B cells with: DAS28 score, CRP mg/L, ESR mm/hr, age, BMI, ADA ng/ml (log_{10}), at month 12. (B-D) %SIRPα/β$^+$memory B cells in patients treated with combination therapy with methotrexate (co-MTX), or without (No MTX) (B), by gender (C) and by smoking status (D), at month 12. (E) Dot plot representing proportion of ADA$^-$ and ADA$^+$ patients that are responders (black) or non-responders (purple); each circle represents one prospective patient. (F) Scatter column plot with %SIRPα/β$^+$memory B cells for non-responder (NR) and responder (R) patients to adalimumab according to EULAR classification. (G) ADA ng/ml (log_{10}) and DAS28. All non-significant ($p>0.05$), except F where *$p<0.05$. For A and G Pearson Correlation (r) was performed, and for B, C and F *t*-test and D one-way ANOVA. Column graphs show mean and ±SEM.

4.3. Frequency of SIRPα/β$^+$memory B cells as a predictor of ADA

I envisaged two possible scenarios for the change in the frequency of SIRPα/β$^+$memory B cells: i) all patients prior to adalimumab treatment express a similar frequency of SIRPα/β$^+$memory B cells, and the frequency is down regulated concomitantly to the development of ADA; ii) patients that will go on to develop ADA have fewer SIRPα/β$^+$memory B cells at baseline compared to the ADA$^-$ patients. Using the cut-off value determined by ROC-curve analysis, described in Figure 4.7F, 9 out of 20 patients assessed at baseline showed less than 9.4% of SIRPα/β$^+$memory B cells in circulation. Strikingly, 73% of patients with SIRPα/β$^+$memory B cell frequencies below the cut-off value became ADA$^+$ after 12 months of adalimumab therapy whilst 80% of patients with SIRPα/β$^+$memory B cell frequencies above the cut-off value remained ADA$^-$ (Figure 4.9A). Representative expression of SIRPα/β on memory B cells in ADA$^-$ versus ADA$^+$ RA patients is shown in Figure 4.9B. To confirm the predictive value of reduced SIRPα/β$^+$ memory B cells frequency, baseline samples were separated according to future development of ADA by month 12. Patients that will develop ADA showed significantly lower frequencies of SIRPα/β$^+$memory B cells compared to patients that do not develop ADA ($p=0.0002$) (Figure 4.9C). Longitudinal analysis revealed no changes in the frequency of SIRPα/β$^+$ memory B cells between visits in both ADA$^-$ and ADA$^+$ patients (Figure 4.9D and 4.9E). Furthermore, analysis of the month 1 visit revealed that the majority (80%) of patients that have developed ADA by month 12 already have detectable ADA by 1 month following start of treatment (Figure 4.9E).

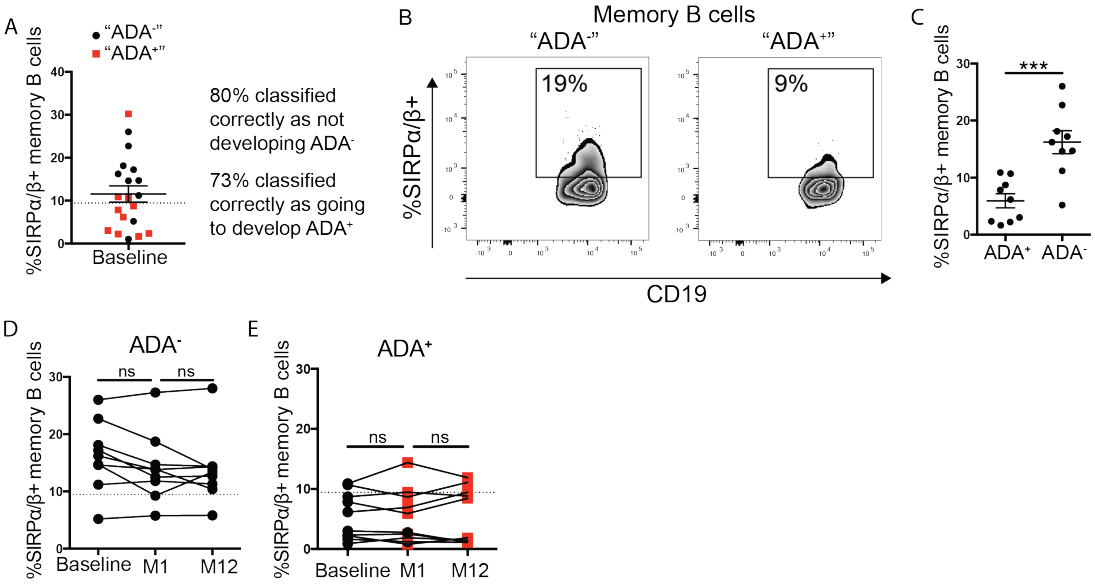

Figure 4.9. Low frequency of SIRPα/β$^+$memory B cells predicts ADA development in adalimumab treated RA patients.

PBMCs from the prospective cohort (Table 4.3) taken at baseline, month 1 (M1) and 12 (M12) visits (n = 21), were stained with SIRPα/β-APC for flow cytometry (n=11 ADA$^+$ n=10 ADA$^-$). (A) RA patients prior to commencing adalimumab (baseline, from prospective cohort) were stratified according to the frequency of SIRPα/β$^+$memory B cells. Black circle and red square correspond to individuals respectively that do not "ADA$^-$" or do "ADA$^+$" develop ADA, after unblinding. The dotted line represents the threshold value from M12 ROC curve analysis. (B) Representative flow cytometry plots showing %SIRPα/β$^+$memory B cells in "ADA$^-$" and "ADA$^+$" individuals. (C) Scatter plot showing mean±SEM of %SIRPα/β$^+$memory B cells of patients at baseline, sub-divided by ADA development by M12, *t*-test analysis, following a robust regression and outlier removal (ROUT (Q=1%)), identifying two outliers, ***p≤0.0001. (D) Graph showing longitudinally the %SIRPα/β$^+$memory B cells at baseline, M1 and M12 for ADA$^-$ RA patients, dotted line as A, one-way ANOVA. (E) As D for ADA$^+$ patients, with red squares indicating positive ADA detection and black circle shows negative ADA detection at point of sampling.

4.4. Summary

Here I identified the frequency of SIPRα/β expressing memory B cells as a predictive marker for development of ADA against adalimumab. This marker was derived using a strict analysis framework that aimed to dissect the development of ADA away from confounding factors that can arise as a consequence of ADA, such as poor response to treatment and worse disease. Hence, I removed markers associated with DAS28 score and with treatment effect. Notably while LEGENDScreen^{TM} identified several markers that showed a difference between the ADA^{+} and ADA^{-} RA patients within the cross-sectional cohort, only one marker was reproducible in the validation cohort. This could reflect the subtleties in the difference between ADA^{+} and ADA^{-} patients or perhaps that there are multiple mechanisms of development of ADA. Most significantly, the frequency of SIPRα/β^{+} memory B cells do not only define ADA in patients with detectable ADA but also predict ADA development, which could have important implications within the clinic allowing a more informed choice of biological therapeutic.

Our initial cohort was recruited in London, therefore the patients enrolled in this study are likely to reflect a mixture of backgrounds and ethnicities due to the multicultural nature of the London population and since UCLH receives referrals from across the UK. The validation of the ADA associated markers was demonstrated in a unique and independent cohort of RA patients switching to adalimumab treatment from across Europe. This cohort was part of a wider cohort of patients recruited for the study of immunogenicity by ABIRISK. Our diverse cohorts give us confidence that the frequency of SIRPα/β^{+} memory B cells is reflective of the greater population. While studies have aimed to assess incidence of ADA in diverse population cohorts (European (328), International (129)) to my knowledge no one has compared incidence between populations. Understanding if a particular population is more susceptible to ADA development may give us further clues to the mechanisms of immunogenicity.

The true clinical impact of ADA remains up for debate. While multiple studies in RA have shown a correlation between ADA and treatment failure (125, 126), due to

the variety of methods used to measure ADA across studies, and the varying associated limitation of these methods, it is difficult compare these studies, with reports of ADA prevalence varying between different studies (149). As mentioned the work presented in this thesis forms part of the project ABIRISK, a European consortium formed to address the outstanding questions regarding immunogenicity. One the major goals of ABIRISK is to accurately quantify the impact of ADA on diseases including RA, inflammatory bowel disease (IBD) and MS (149). Part of this aim includes the development of an accurate and standardised way to quantify ADA (331). Recently ABIRISK published a luciferase-bioassay for the detection of anti-interferon-beta antibodies that was found to be more sensitive than the conventional bridging ELISA (331). I am therefore confident that the detection of ADA in our cohort is an accurate reflection of the true levels and incidence of ADA within the cohort. Furthermore, our cohort also provides evidence in support of the clinical effect of ADA, with the majority (two thirds) of ADA^+ patients showing a poor response to treatment only a year after first administration of the drug, and with ADA development detectable typically as early as 1 month after starting adalimumab.

While there are many studies that quantify ADA in RA patients, far fewer studies addressed the mechanisms or aim to predict ADA development. It has recently been shown in BAFF transgenic mice that the observed reduction in ADA development when patients are co-treated with MTX could be due to high BAFF levels (332). This was also associated with increased expression of CD73 and CD39. Interestingly a regulatory subset of B cells has been described as $CD73^+CD39^+$ (285), suggesting a possible role for regulatory B cells in the protection against development of ADA. Furthermore in anti-TNF treated RA patients, higher serum BAFF levels were associated with absence of ADA (332). However, neither CD73 nor CD39 were identified in our analysis as markers associated with ADA. Furthermore, while I have not measured serum BAFF levels, the LEGENDScreen™ included the BAFF receptors BAFF-R (CD268) and TACI (CD269), however, neither of which were found to be associated with ADA in our analysis either.

Given an observed increased susceptibility to developing ADA against a second anti-TNF (142) and an increased risk of developing ADA against adalimumab in patients

homozygous for the same IgG allotype as the drug (144), it is possible that ADA development may be specific to the drug and thus require different predictors/biomarkers for different drugs. More recently within ABIRISK, Notch2 has been shown to predict ADA against IFN-beta treatment in MS (333). Notably no studies confirm these identified associations in different disease cohorts. At current, I have not investigated the expression of SIRPα/β$^+$ in different diseases or against a different treatment. If we were to find SIRPα/β$^+$ is not predictive of ADA development for example in tocilizumab treated RA patients, we can speculate that ADA development could be a treatment specific mechanism. If SIRPα/β$^+$ is predictive against other drugs in RA but not in e.g. IBD, we can hypothesize that ADA development is disease specific. Alternatively, if it is predictive against adalimumab in IBD but not for other drugs, then this would also add support towards a treatment-specific mechanism of ADA development. Nonetheless, regardless of the outcome, this information would give us a valuable insight into the mechanism of ADA development, and therefore are important to investigate.

4.5. Future Work

In light of the points discussed above regarding the mechanisms of ADA development, future work could include measuring serum BAFF levels for our cohort and correlating these results with our data. Furthermore, it would be beneficial to investigate SIRPα/β expression in RA patients treated with different biologics and in patients with other autoimmune conditions such as IBD. This would help us to establish firstly if SIRPα/β can be used more generally to predict ADA and secondly to better understand if the mechanism of ADA is generalised or treatment/disease specific.

5. Results III – The signature of RA

RA is defined by the manifestation of synovitis, however, seropositive RA onset is preceded by athe presence of specific auto-antibodies such as RF and/or anti- anti-CCP, which have been identified as many as 15 years before onset of disease (334, 335). In addition to the presence of auto-antibodies, patients with symptoms such as arthralgia and raised inflammatory cytokine levels are at risk of developing RA (336). Only 20% of at risk individuals have been reported to develop RA within 4 years (337). Prompt treatment of early arthritis leads to a better response and a longer-term positive disease outcome (338, 339). In order to implement early intervention treatment we need to be able to identify those patients that will progress to RA. Here I aimed to take advantage of the LEGENDScreen^{TM} platform to generate an immune-signature unique to RA patients.

5.1. Identification of a unique signature in RA patients

To identify biomarker/s associated with disease I compared the surface immune-signature of PMBCs isolated from 18 healthy and 31 RA patients (Table 3.1). PBMCs were stained with fluorescently-conjugated antibodies identifying $CD4^{+}$ T cells, $CD19^{+}$ B cells, and B cell subsets ($CD24^{hi}CD38^{hi}$ immature B cells, $CD24^{int}CD38^{int}$ mature B cells and $CD24^{hi}CD38^{lo}$ memory B cells) in addition to the 332 cell surface markers included in the LEGENDScreen™ panel (as described in the methods [see Figure 2.1] and in Chapter 3 Results I, [see Figure 3.1]). To assess if the LEGENDScreen^{TM} was sensitive enough to detect differences between HC and RA patients, I firstly measured the frequency of expression of the 332 LEGENDScreen^{TM} cell surface markers on total PBMCs. This data is presented as heatmaps in Figure 5.1A. The results show two unique "immune-signatures" that differentiate HCs from RA patients. I next calculated fold change to quantify the differences and identify the markers that most strongly contributed to the observed immune profile variations in PBMCs between HC and RA. To establish if these differences were significant, I performed a *t*-test analysis, thus identifying significantly differentially expressed markers that were either up or down regulated on RA compared to HC on the respective B cell subsets. This data is presented as a

volcano plot, reflecting the significance, size and direction of the change between sample groups (Figure 5.1.B).

Having established that differences between HC and RA patients at a PBMC level could be identified, I further focused my analysis to look at T and B cells separately. By assessing the frequency of the 332 markers expressed on B and T cells I was able to obtain a T cell and a B cell immune profile that distinguished HCs from RA patients (Figure 5.1C). To improve the statistical power of the future analyses performed on this dataset, markers that were expressed on less than 5% of cells were removed; this resulted in the exclusion of 115 markers on T cells and 74 on B cells. Markers that were found to be significantly differentially expressed on either T or B cells between HCs and RA patients, following multiple *t*-test analysis are quantified in Figure 5.1D (a complete list of differentially expressed markers can be found in the appendix [A.6]).

The majority of significantly differentially expressed markers were up-regulated in RA, which would match the hyper-activated state associated to B and T cells in disease (95). Notably there are more differentially expressed markers in B cells than T cells with 34 markers in B cells and only 9 markers in T cells; results that further support the pivotal role of B cells in driving RA development and progression (104). Next, I have assessed the immune profile of immature, mature and memory B cells in HCs compared to RA patients using the same gating strategy as described in Figure 3.2. The analysis of the expression of the 332 surface markers on B cell subsets revealed 40 differentially expressed markers on mature B cells, 49 on immature B cells and 72 on memory B cells (Figure 5.1C). A full list of the differentially expressed markers on the B cell subsets can be found in the appendix (A.6).

5.2. Strategy for the generation of the "immune-signature"

To identify an immune-signature unique to RA patients and to eliminate possible confounding factors, I applied a systematic framework analysis (SFA) that excludes the effect of treatment and disease activity (described in Figure 5.2). I used the frequency of expression on B cells from HCs as a baseline, generated a fold change

for each individual marker expressed on B cells or B cells subsets, and compared these values to those identified in RA patients treated with adalimumab or with cDMARDs. Markers that were differentially expressed, compared to HCs, in both cDMARDs and adalimumab treated RA were retained; this was in order to identify markers that were differentially regulated in all RA patients rather than in response to treatment with cDMARDs or adalimumab. This resulted in the identification of 5 markers on total B cells, 9 on mature, 4 on immature and 12 on memory B cells (Figure 5.2). In addition, any markers that were correlated with disease activity, a measurement that is in part calculated based on the level of inflammation (CRP/ESR), were also removed in order to exclude markers associated more generally with inflammation as opposed to inherent differences in RA patients. As none of our remaining markers correlated with DAS28, no further markers were eliminated at this stage. A full list of markers that correlated with DAS28 is reported in the appendix (Appendix A.5). Following our exclusion criteria, 16 unique markers expressed by different B cell subsets and/or total B cells were retained; Benjamini and Hochberg analysis with an FDR of 5% was applied to correct for multiple comparisons (Table 5.1). This list was further refined by including only markers that pass the Benjamini and Hochberg analysis and that meet a stringent p value ($p<0.0018$), thus resulting in the inclusion of only 10 markers, and which I refer to as the 'RA signature'. (Figure 5.3A). PCA of the frequency of expression of the 10 markers on their respective B cell subset from our LEGENDScreen^{TM} cohort dataset demonstrated that these 10 differentially expressed markers have sufficient statistical power to discriminate between HCs and RA patients (Figure 5.3B).

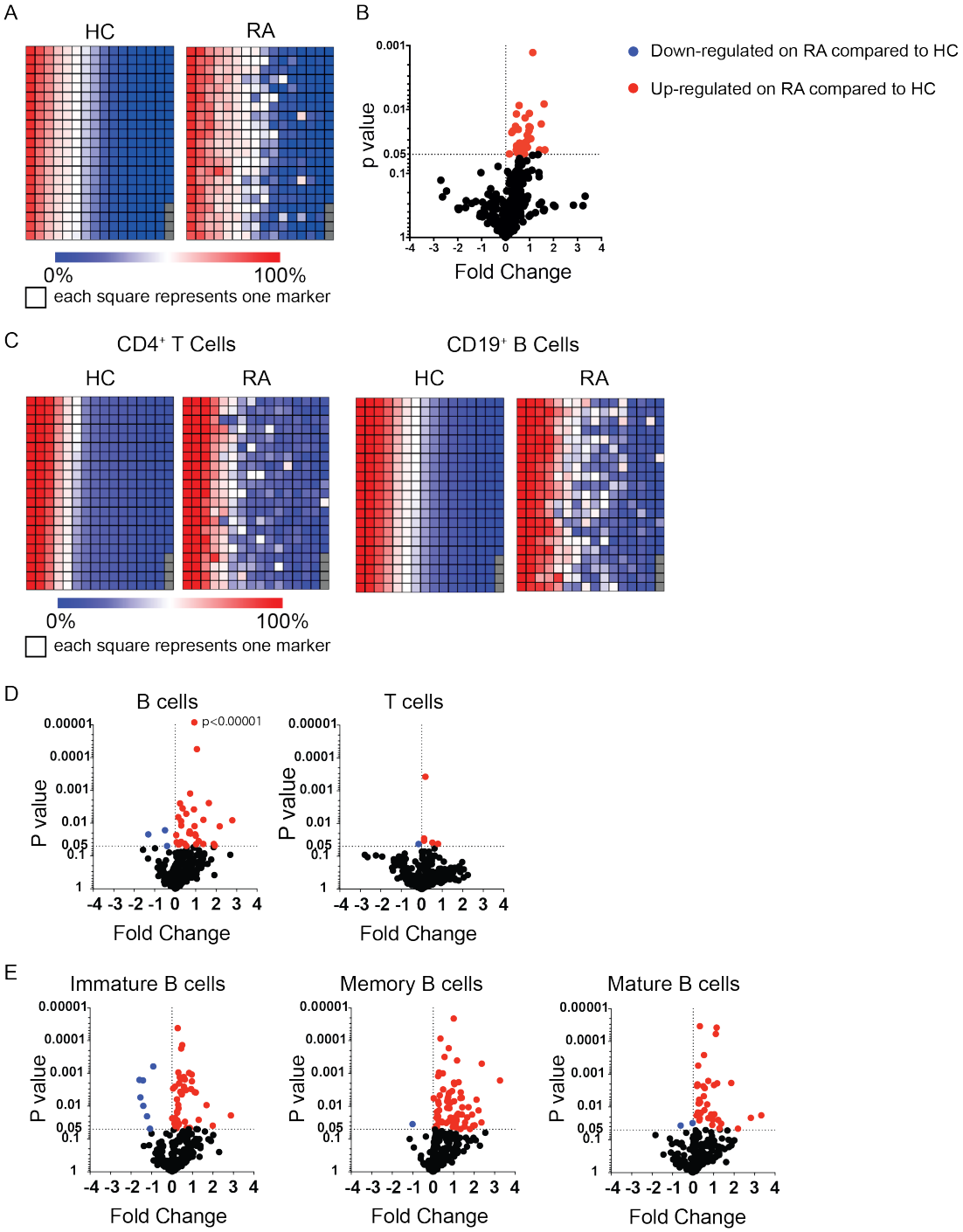

Figure 5.1. LEGENDScreen™ analysis of RA patients versus HCs identifies more differences on B cells than T cells.
PBMCs from HCs (n=10) and RA patients (n=31) were stained with LEGENDScreen™ for 332 cell surface markers, in addition to antibodies against CD19, CD4, CD24 and CD38. (A) Heatmap showing average frequency expression of each LEGENDScreen™ marker on total PBMCs for each sample group (HC and RA), each square represents one marker and are ranked according to expression in HC. B) Volcano plots showing fold-change of frequency expression between patient groups (HC/RA) (Log_2) and p value (*t*-test) (log_{10}) for the 332 LEGENDScreen™ markers on total PBMCs. Blue circle: significantly down-regulated markers; red circle significantly up-regulated markers, in RA versus HC. C) Heatmaps (as A) for

$CD4^+$ T cells and $CD19^+$ B cells. D+E) Volcano plots (as B) for $CD19^+$ B cells and $CD4^+$ T cells (D) and, and immature ($CD24^{hi}CD38^{hi}$), mature ($CD24^{int}CD38^{int}$) and memory ($CD24^{hi}CD38^{lo}$) B cells (E).

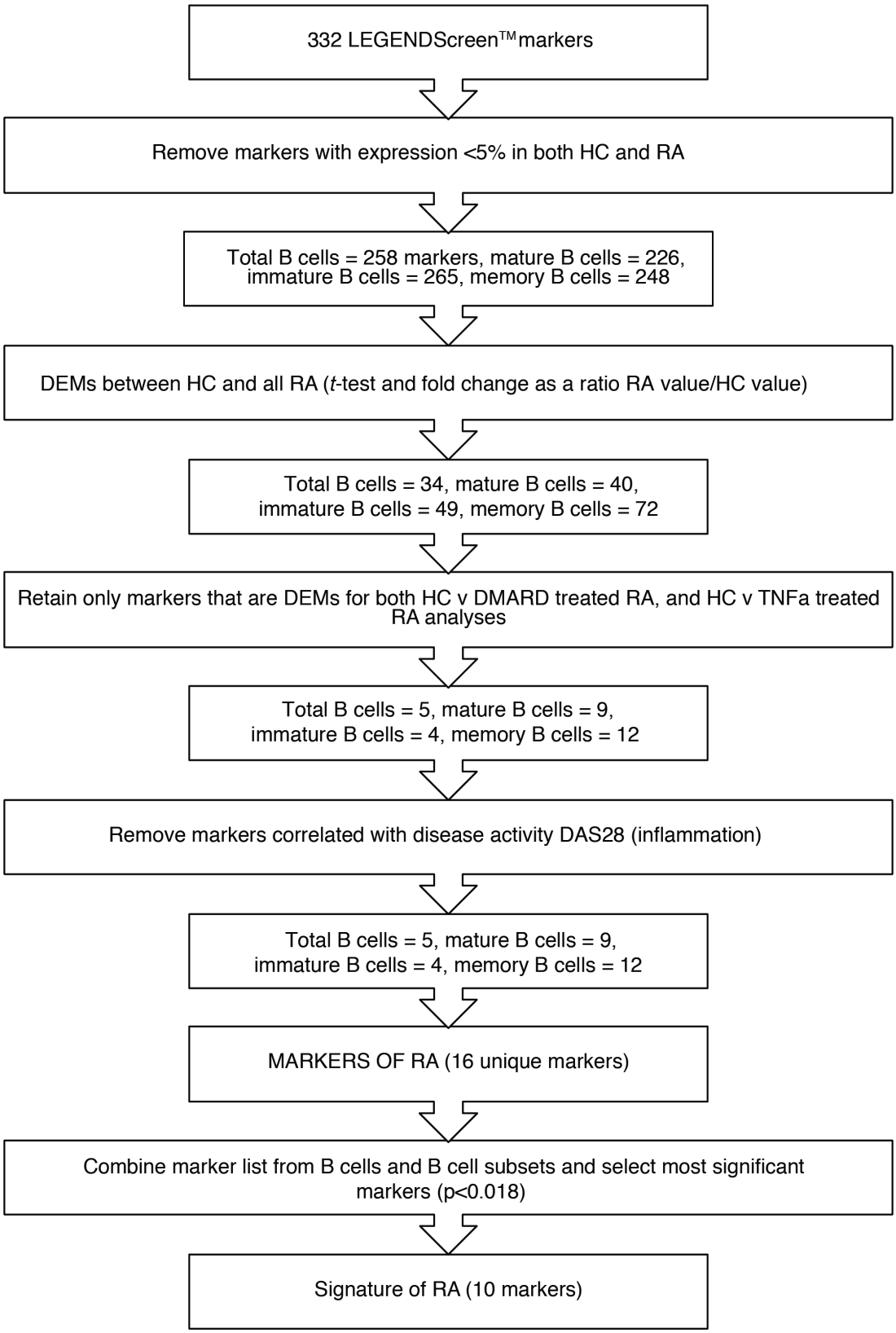

Figure 5.2. Selection of markers for RA signature from LEGENDScreen™ analysis of cross-sectional RA cohort.
Overview of systematic framework analysis applied to generate a immune-signature of markers that define RA.

Table 5.1. Complete list of RA signature markers.
Differentially expressed markers on B cells and B cell subsets (as indicated) between HCs and RA patients following exclusion of markers as per Figure 5.2. Markers listed in order of significance with most significant at the top. A false discovery rate of 5% was applied using the Benjamini Hochberg analysis' markers that passed the FDR are indicated with *.

Marker	Fold Change	P Value	Cell Type
***CD97**	1.91	0.000004	Total B cells
***CD97**	2.00	0.000021	Memory B cells
***Ig light chain κ**	1.26	0.000034	Mature B cells
***CD150 (SLAM)**	2.21	0.000038	Mature B cells
***Ig light chain κ**	1.21	0.000042	Immature B cells
***CD150 (SLAM)**	2.08	0.000055	Total B cells
***CD97**	2.15	0.000059	Mature B cells
***Ig light chain κ**	1.28	0.000086	Memory B cells
***CD11c**	1.69	0.000169	Memory B cells
***CD158d**	5.17	0.000502	Memory B cells
***CD62L**	1.19	0.000545	Mature B cells
***CD170**	1.78	0.000966	Immature B cells
***CD307**	1.95	0.001079	Immature B cells
***CD335**	9.60	0.001634	Memory B cells
***Notch 2**	1.96	0.001741	Immature B cells
***CD158d**	3.64	0.001848	Mature B cells
***CD307**	2.26	0.001885	Mature B cells
***CD307**	2.02	0.001901	Memory B cells
CD158d	3.12	0.002438	Total B cells
***CD97**	2.17	0.003736	Immature B cells
CD226	2.54	0.003746	Memory B cells
CD150 (SLAM)	2.24	0.003814	Immature B cells
CD150 (SLAM)	2.52	0.005632	Memory B cells
CD245	1.61	0.008291	Mature B cells
CD158d	3.22	0.009261	Immature B cells
CD114	4.64	0.013400	Memory B cells
CD11c	1.98	0.017330	Mature B cells
CD172a	2.25	0.018464	Memory B cells
CD172a	2.01	0.024192	Total B cells
CD62L	1.19	0.026830	Immature B cells
CD172a	2.59	0.032133	Mature B cells
CD202b	4.07	0.038068	Memory B cells
Integrin β5	2.91	0.041377	Memory B cells
CD172a	1.63	0.046593	Immature B cells

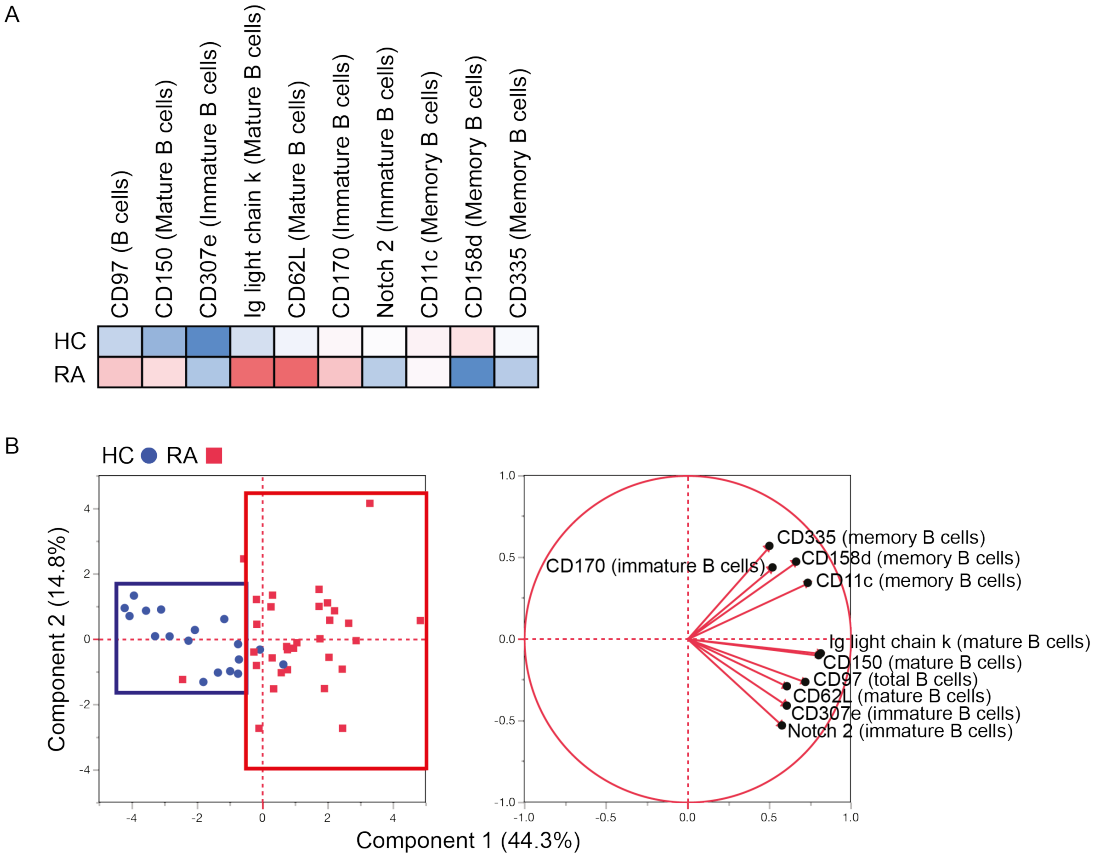

Figure 5.3. Principle component analysis of expression data for RA signature shows separate clustering of HC and RA patients.
Analysis of LEGENDScreen™ data. (A) Heatmap showing average (mean) frequency of expression for each of the 10 RA signature markers on HC and RA. (B) Principle component plot for frequency of expression of top 10 differentially expressed markers showing HC (blue dots) and RA (red squares) for the first two principle components (percentage contribution to variation in brackets). Contribution of each marker to the principle components is depicted by the length and direction of the corresponding red arrow.

5.3. Validation of the RA signature in an independent cohort of RA patients and HCs confirms five of the signature markers

Next, to ensure reproducibility of our results I validated the RA immune-signature markers in an independent European cohort. Our validation cohort (n = 88) was recruited in France, The Netherlands, Germany and in the UK (Table 5.2). The advantage of including patients from different European centres in our study is that it allows us to exclude the potential effect that ethnicity may play in the differences observed in our initial UK cohort. To allow faster acquisition of data and the analysis of a greater number of patients, I used conventional flow cytometry. PBMCs were isolated from RA patients and stained for the RA signature surface markers (as Figure 5.3). Using the cut off value of $p<0.05$ five of the ten markers identified in the initial cohort were successfully validated ($CD97^+$ B cells, $CD150^+$ mature B cells, $CD11c^+$ memory B cells, $CD170^+$ immature B cells, $CD307e^+$ B cells) (green dots), and five markers that no longer showed a significant difference were excluded (Ig light chain $kappa^+$ B cells, $CD158d^+$ memory B cells, $CD62L^+$ mature B cells, $CD335^+$ memory B cells, $Notch2^+$ immature B cells) (red dots) (*t*-test or Man-Whitney test for non-parametric data, following testing for normality using D'Agostino & Pearson test [$CD11c^+$ memory B cells, Ig light chain $kappa^+$ B cells, $CD158d^+$ memory B cells, $CD62L^+$ mature B cells, $CD335^+$ memory B cells, and $Notch2^+$ immature B cells were found to be non-normally distributed]) (Figure 5.4A+B). PCA clustering analysis demonstrates that these five validated markers are able to cluster RA patients from healthy controls (Figure 5.4C).

To test the predictive value of these markers to distinguish HC from RA, I generated a receiver operating characteristic curve (ROC curve) for each marker, plotting sensitivity against specificity, and calculated the area under the curve (AUC); the closer the AUC to 1 the better it is at distinguishing the two groups (Figure 5.5). Frequency of $CD11c^+$ memory B cells, $CD97^+$ B cells and $CD170^+$ immature B cells have high AUC of 0.74, 0.73 and 0.72 respectively and are therefore good predictors of RA. $CD150^+$ mature B cells and $CD397e^+$ immature B cells have low AUC values of 0.64 and 0.63 respectively and therefore are poor predictors of RA. Using sensitivity and specificity values for the prediction of RA for different marker

frequencies, cut off values for prediction of RA were defined, and are shown as “x” in the figure.

Table 5.2. Validation RA cohort patient demographics and disease characteristics.
Patients recruited from UK, France, Italy and the Netherlands.

	HCs	RA-D	RA-A	RA-T	At-risk	Early-RA
n	31	35	53	22	5	7
Sex, female n (%)	24 (68)	26 (81)	37 (70)	14 (64)	100 (0)	100 (0)
Age (years), mean (SD)	27 (10.7)	60 (17.9)	54 (15.1)	62 (14.7)	54 (13.9)	47 (9.0)
DAS28 (SD)	-	3.01 (1.4)	3.02 (1.7)	1.72 (1.2)	-	-
Seropositive (RF+/CCP+) n /seronegative n /sero-unknown n	-	35/0/0	45/5/3	7/5/10	5/0/0	7/0/0
CRP mg/l (SD)	-	10.3 (20.6)	8.2 (12.1)	1.3 (1.3)	2.0 (0.6)	21.0 (19.5)

RA-D = RA treated with cDMARDs
RA-A = RA treated with adalimumab
RA-T = RA treated with tocilizumab

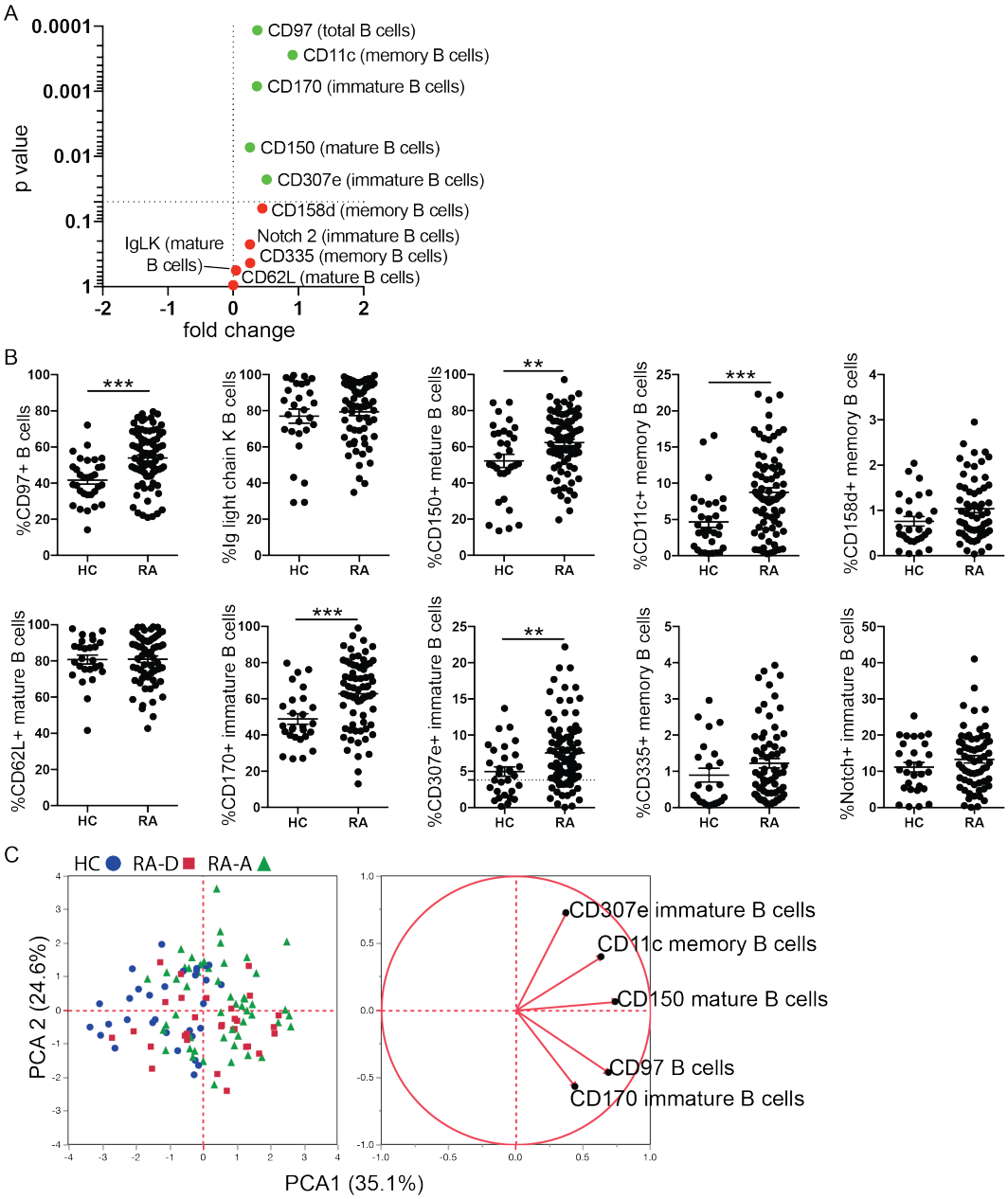

Figure 5.4. Five of the RA signature markers are successfully validated in an independent European cohort of patients.

PBMCs from HC (n=31), and cDMARD (n=35) (RA-D) and adalimumab treated (n=53) (RA-A) RA patients were stained for the RA signature markers (CD97, Ig light chainκ, CD150, CD11c, CD158d, CD62L, CD170, CD307e, CD335, and Notch 2) and CD19, CD24 and CD38 to define B cells and B cell subsets. (A) Volcano plot showing fold change versus p value (*t*-test or Mann-Whitney) for expression of each marker on the specific subset. Dotted lines denote 'zero' fold change, and p value of 0.05. Green dots are significantly different, red dots no longer significantly different in the validation cohort. (B) Individual marker frequency of expression for each RA signature marker on the relevant subset. Mean ±SEM, *t*-test analysis or Mann-Whitney, **p<0.01, ***p<0.001. (C) Principle component plot showing clustering of HC (blue dots) and RA-D (red squares) and RA-A (green triangles) for principle components 1 and 2. Contribution of each marker to the principle components is depicted by the length and direction of the corresponding red arrow, and given in brackets.

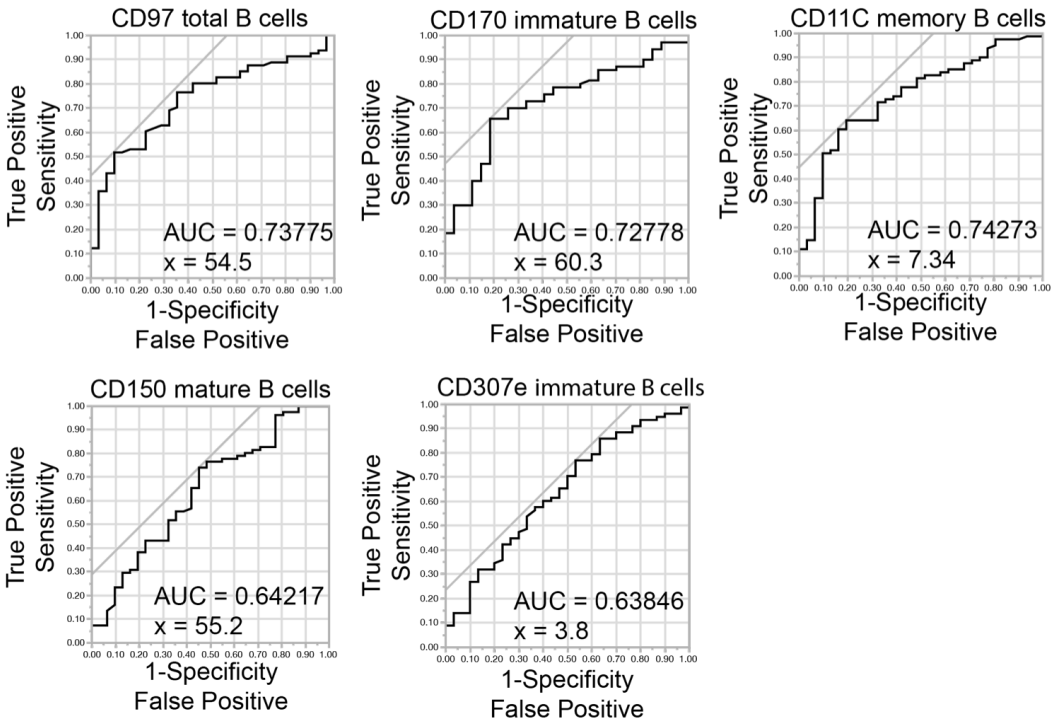

Figure 5.5. ROC curve analysis of the 5 RA signature markers, identifies CD97, CD170 and CD11c as good predictors of RA.

ROC curve analysis was performed on frequency expression data of the 5 validated RA signature markers (HC=31 and RA=88 samples). AUC is reported in the figure. A cut-off value, to predict an RA patient from a HC was determined by sensitivity-(1-specifictiy) with RA as the positive outcome, and is denoted as 'x' and reported in the figure.

5.4. Preliminary investigations into the RA signature as a tool to predict development of RA in "at-risk" individuals

Following the ROC analysis of the validated RA signature markers I hypothesise that CD97, CD170 and CD11c could be used as biomarkers for the identification of patients that are either "at-risk" of developing RA (these patients are RF or CCP positive, but lacked signs of synovitis) or at an early stage of disease (early-RA) 11 individuals fulfilling the criteria of at-risk or early RA have been recruited so far at the rheumatology clinic at UCLH and analysed (early n=5, at-risk n= 6). Although no conclusion can be made at this stage due to the low number of patients enrolled in this part of my study, the results show a significant increase of frequency of $CD97^+$ B cells in individuals that are at "at-risk" of developing RA compared to HCs. The mean expression of CD97 is above the ROC curve derived threshold value (dotted line) for both "at-risk" and early RA patients (Figure 5.7A). There is also a significant increase in frequency of $CD170^+$ immature B cells and $CD11c^+$ memory B cells in early-RA patients. Of interest, the "at-risk" patients group had a greater standard deviation than the early RA patients (for at-risk and early RA respectively for CD97 14.7 and 8.0, CD11c 4.7 and 1.9, and CD170 21.1 and 13.5), with typically half the "at-risk" patients falling above cut-off value and half below (Figure 5.7B). Therefore, I hypothesise that the "at-risk" patients with high frequencies for all three RA signature markers are most likely to develop RA.

These preliminary investigations provide encouraging results showing that the use of CD97, CD170 and CD11c expression on B cells subset could help to stratify individuals that may develop RA. In order to investigate this fully I would need to recruit a larger cohort of patients from the rheumatology clinics that are considered as "at-risk" of developing RA, and follow them longitudinally to see if they will develop RA.

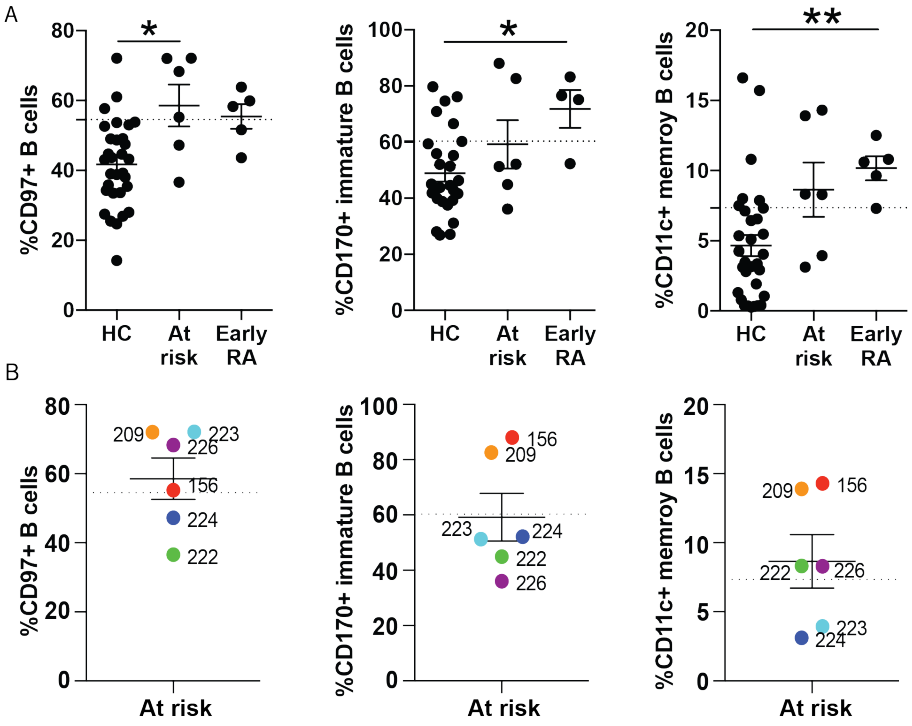

Figure 5.6. The RA signature could be predictive of RA onset.
Following ROC curve analysis, three RA signature makers (CD97, CD170 and CD11c) had an AUC value that suggested that they would be good predictors of RA. The frequency of expression of these markers was assessed in a preliminary cohort of patients who were at-risk of developing RA (n=6) or that had been recently diagnosed with RA (at time of sample collection) (early RA) (n=4) using flow cytometry and compared to HCs. (A) Frequency of $CD97^+$ B cells, $CD170^+$ immature B cells and $CD11c^+$ memory B cells. The ROC curve derived cut-off value from Figure 5.5 is indicated as a dotted line. (B) As A but showing at-risk only and labelled to indicate which dot is which sample (sample no.). Mean ±SEM, one-way ANOVA *p<0.05, **p<0.01.

5.5. Summary

In this chapter I use the LEGENDScreen^{TM} data to develop an immune-signature that uniquely identifies RA patients irrespective of their inflammation status and ability to respond to treatment. The frequency of CD97^{+} B cells, CD150^{+} mature B cells, CD11c^{+} memory B cells, CD170^{+} immature B cells and CD307e^{+} immature B cells is elevated in the UK cohort of RA patients. Further validation in an independent European cohort confirms that a higher frequency of CD97^{+} B cells, CD170^{+} immature B cells and CD11c^{+} memory B cells have sufficient predictive power to define RA patients over HCs as determined by ROC curve analysis. In order to address if these markers could be used to predict RA onset, I measured their expression in a small cohort of at-risk and early RA individuals. CD97, CD170 and CD11c all showed potential to stratify at-risk patients and warrant further investigation in a longitudinal cohort. Moreover, by using an unbiased high-throughput screening approach I have identified several markers that have not previously been associated with RA including CD150 and CD170 and provided supporting evidence for those that have been reported prior such as CD97, CD307e and CD11c (174, 190, 340-343). I propose that this unbiased approached of analysis of immune cells in disease could provide a means to identify novel therapeutic targets.

5.6. Future work

I will establish if CD97, CD170 and CD11c are able to predict development of RA. To achieve this, I will analyse their expression in a larger cohort of at-risk RA patients, which have been followed longitudinally and can therefore be stratified according to future manifestation of RA disease. Furthermore, I will investigate the functional role of CD97, CD11c, CD150 and CD307 in RA, with a long-term aim to investigate their potential as drug targets for RA. In addition to these markers, this work has identified many other markers on B cells and B cell subsets that are dysregulated in HCs compared to RA patients. Any of these molecules could warrant further investigation in an effort to reveal more about the role of B cells in RA.

6. Results IV – Identification of CD170/Siglec-5 as novel marker for Bregs

A major goal in the Bregs field is the identification of a marker that can be used as an alternative to IL-10. So far, as described in detail in the introduction, immature B cells have been ascribed with regulatory capacities. However, less that 20% of these B cells produce IL-10 (344). Our lab and others have previously reported that RA patients have a reduced number of Bregs in circulation (98, 259-261). I exploited the results from the LEGENDScreen^{TM} by selecting differentially expressed markers on immature B cells between HCs versus RA patients. Next, I excluded any of these markers that were also differentially expressed between HCs and RA patients on mature or memory B cells, resulting in the identification of 15 candidate markers for Bregs (Table 6.1). CD170, a member of the Sialic acid-binding immunoglobulin-type lectins (SIGLEC) family, was the most significantly differentially expressed marker that fitted this criteria (see Table 6.1). Siglecs are a family of sialic acid receptors that have been increasingly reported to be involved in immune tolerance, including B cell tolerance (304). As described in the introduction, human B cells have been reported to express only 3 members of this family, Siglec-2 (CD22), Siglec-10 (CD330) and Siglec-5 (CD170), all of them with inhibitory functions. For instance, Siglec-G (the murine orthologue for Siglec-10) and CD22 double-deficient mice develop a severe form of autoimmunity (303). In the case of CD170, it has been shown that monocytes overexpressing this receptor produce high amounts of IL-10 and low levels of TNFα following TLR stimulation (345). Similarly, Siglec-10 mediates an increase in IL-10 production by DCs after interaction with C. *jejuni* flagella (308). In contrast, epratuzumab, an antibody targeting CD22, inhibits the production of IL-6 and TNF, but not IL-10 by B cells (346). This evidence suggests that Siglecs can differentially modulate pro- and anti-inflammatory cytokine secretion by immune cells, including B cells. Based on this knowledge, I hypothesise that CD170 could act as a surrogate marker for the identification of Bregs.

In this chapter, I show that under Breg polarising conditions the up-regulation of CD170 parallels the induction of IL-10 production. $CD19^{hi}CD170^{hi}$ B cells prevent the differentiation of $CD4^+$ T cells into $IFN\gamma^+CD4^+$ and $IL\text{-}17^+CD4^+$ T cells in an IL-

10 and CD170 dependent manner. Furthermore, RA patients that present with a reduced frequency of IL-10-producing B cells also showed a reduced number of $CD19^{hi}CD170^{hi}$ B cells.

My initial results also show that CD170 is not only a surface marker for Bregs, but may also functionally drive the transcription of IL-10 in Bregs. Preliminary results show that in the presence of a blocking anti-CD170 antibody there is a reduction in Ca^{2+} mobilisation in B cells. Moreover, ImageStream analysis suggests that whereas CD170 does not interact directly with the BCR, unlike CD22, it is colocalised instead with the BCR adapter protein CD19 (347). Of interest, I demonstrate for the first time that CD170 is internalised and subsequently recycled to the surface via the endosomes. This recycling was found to be defective in B cells isolated from RA patients. Finally, I show that a *SIGLEC5* gene polymorphism is not associated with RA.

Table 6.1 Differentially expressed markers between HCs and RA patients unique to immature B cells.

	Fold change	P value
CD170	1.784	0.001
CD181	0.333	0.002
TRA-1-81	0.374	0.002
CD150 (SLAM)	2.239	0.004
Siglec-8	0.344	0.005
CD119 (IFNgR a chain)	1.235	0.009
Galectin-9	0.379	0.01
CD63	1.193	0.016
NPC	0.429	0.02
CD39	1.11	0.023
CD11a	1.008	0.024
CD1c	1.086	0.034
CD132	1.261	0.034
CD99	1.122	0.04
SSEA-5	0.476	0.049

6.1. $CD19^{hi}CD170^{hi}$ B cells express more IL-10 than their negative counterparts

We have previously shown, and here confirmed by ELISA (Figure 6.1A) and ImageStream (Figure 6.1B), that B cells isolated from RA patients produce less IL-10 than B cells from HCs upon stimulation with CpGC. The lack of expansion of IL-10^{+} Bregs in RA patients was further confirmed by intracellular staining (Figure 6.1C-E). Hence, I took advantage of the decreased capacity of B cells from RA patients to produce IL-10 and used isolated B cells from patients as a control for the validation of CD170 as a marker for Bregs, by hypothesising that its expression should be decreased alongside the reduced production of IL-10. Next, I stained B cells from RA and HCs for CD170 either *ex vivo* or following 72h stimulation with CpGC. The results show that under Breg polarizing conditions there is an expansion of a novel population co-expressing high levels of CD19 and CD170 (Figure 6.1F+G). In contrast to HCs, I report an impairment in the expansion of $CD19^{hi}CD170^{hi}$ B cells in RA patients (Figure 61.H).

To identify whether IL-10 producing Bregs also express high levels of CD19 and CD170, I purified B cells from HCs and RA patients and stimulated them with CpGC. This stimuli has been shown to be pivotal for the maximum production of IL-10 in humans (348). The results show that in HC B cells the $CD19^{hi}CD170^{hi}$ population captures the vast majority of IL-10 producing B cells (Figure 6.2 A+B). In addition, my results show that a decrease of CD170 expression results in a similar decrease of IL-10 production by B cells. (Figure 6.2B+C). Furthermore, despite RA patients showing an overall reduced number of Bregs, compared to healthy controls, those RA IL-10^{+}B cells are also $CD19^{hi}CD170^{hi}$ (Figure 6.2B+C).

Next, I took advantage of tSNE, a high-performance non-linear dimensionality reduction technique that in conjunction with a machine learning aided clustering algorithm objectively delineates the heterogeneity of a cellular population. tSNE analysis was applied to $CD19^{+}$ B cells at 72h post-stimulation with CpGC. This analysis identified that the IL-10^{+} B cells gate closely overlaps the $CD170^{hi}$ population (shown in green), confirming that $CD170^{hi}$ B cells are the predominant IL-10 producers, and that $CD170^{hi}$IL-10^{+}B cells clusters separately from

$CD170^{int/low}IL\text{-}10^{-}$ B cells (Figure 6.2D). Furthermore, after CpGC stimulation tSNE analysis shows that $CD19^{+}CD170^{hi}$ B cells are not confined to either immature, mature or memory B cell subsets (Figure. 6.2E).

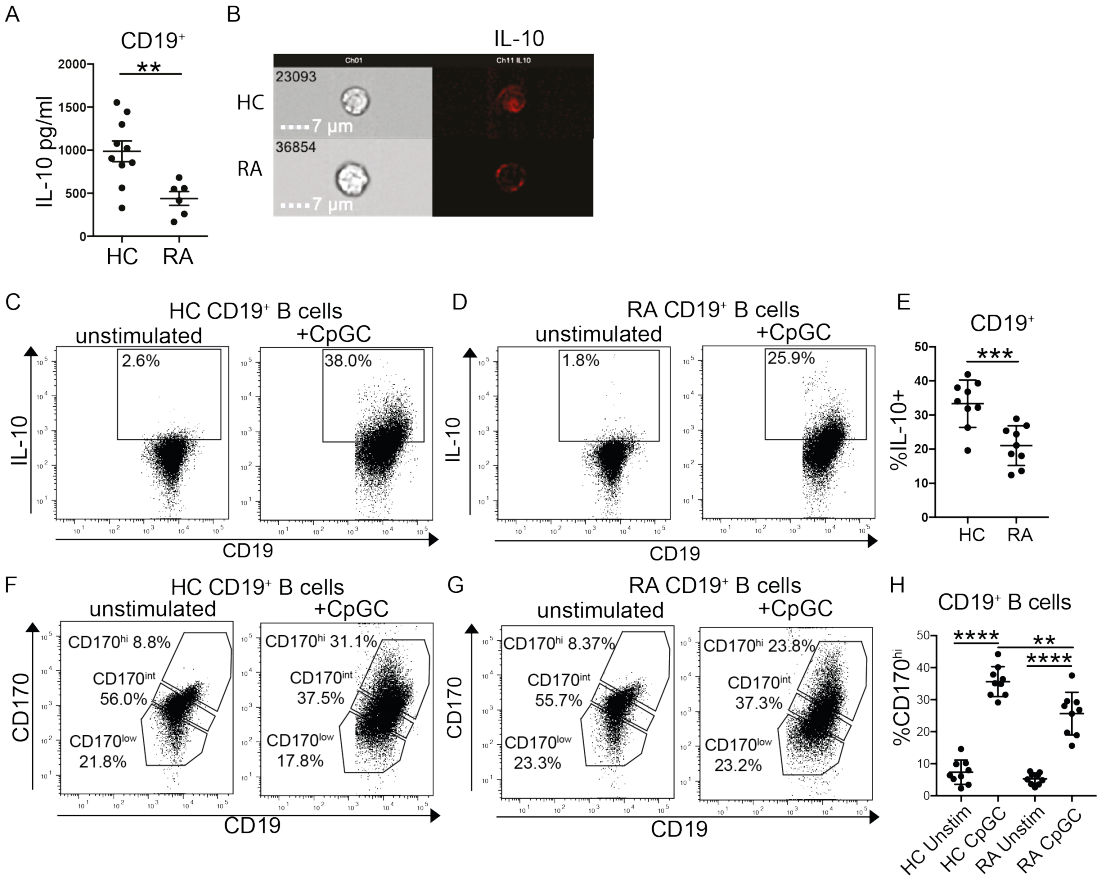

Figure 6.1 Reduced expansion of CD19^{hi}CD170^{hi} B cells in RA compared to HC. B cells isolated from PBMCs from HCs (n=9) and RA patients (n=9) were stimulated for 72h with 1µM CpGC, and 0.05µg/ml PMA, 0.25µg/ml Ionomycin and 5µg/ml Brefeldin for final 5h of culture. Supernatants were collected for cytokine analysis and cells were stained for flow cytometry. (A) IL-10 (pg/ml) measured by ELISA in supernatants. (B) ImageStream analysis of IL-10 stained intracellularly, representative images. C-E) Percentage of IL-10^{+} B cells with/without stimulation with CpGC for 72h, measured by flow cytometry, for HC versus RA patients. F-H) Frequency of CD170^{hi} B cells unstimulated or CpGC stimulated, in HC versus RA patients. Representative flow cytometry plots. Bar charts mean ±SEM, *t*-test as indicated, **p<0.01, ***p<0.001, **** p<0.0001.

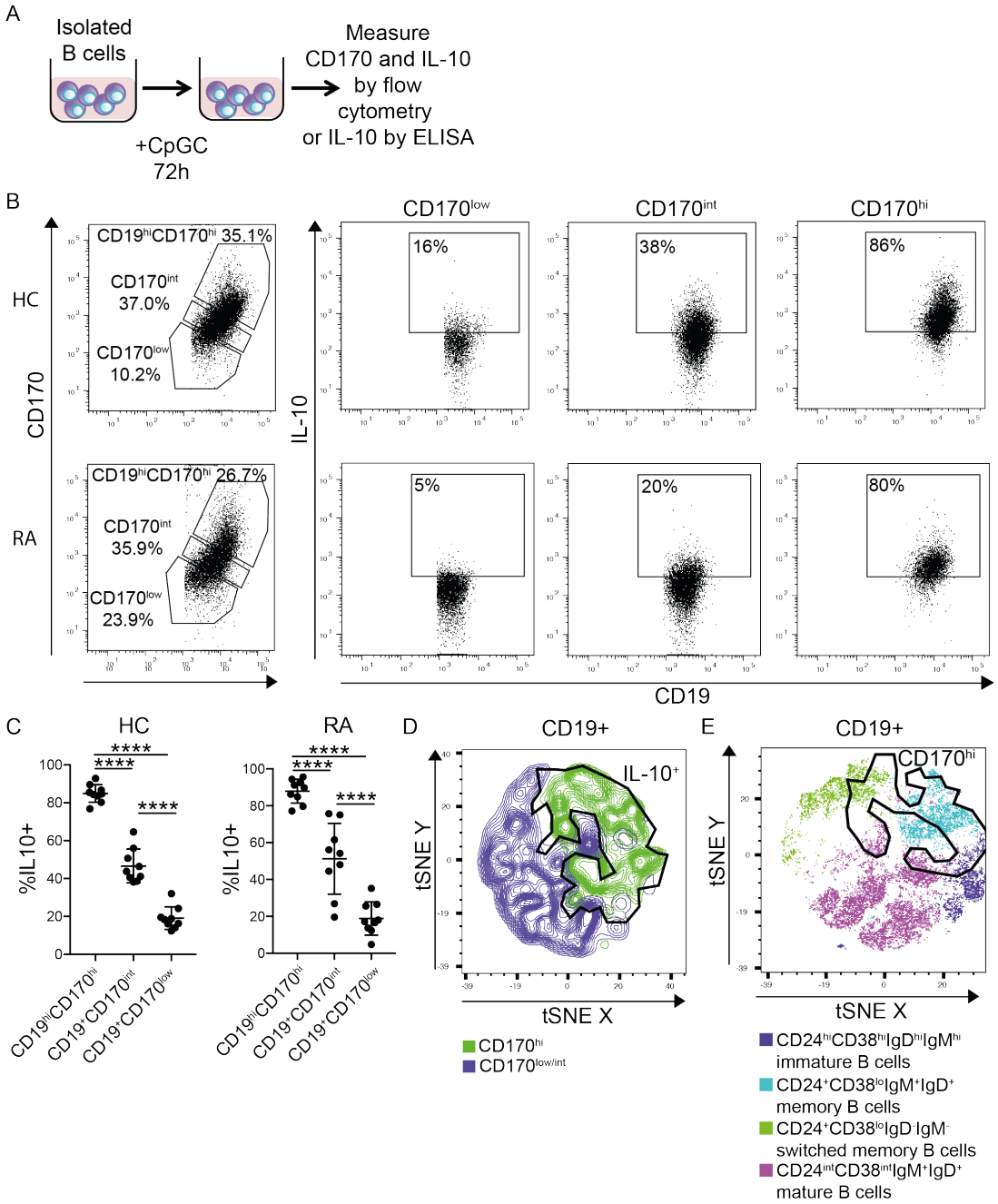

Figure 6.2. CD170^{hi} B cells are the highest producers of IL-10.
B cells isolated from PBMCs from HCs (n=9) and RA patients (n=9) were stimulated with 1µM CpGC for 72h, and 0.05µg/ml PMA, 0.25µg/ml Ionomycin and 5µg/ml Brefeldin for final 5h of culture. Cells were stained for flow cytometry. (A) Schematic of experiment. (B) Frequency of CD19^{hi}CD170^{hi}, CD19^{+}CD170^{int} and CD19^{+}CD170^{low} B cells in HC and RA, and respective IL-10 expression for each subset, representative flow cytometry plots. (C) Summary of IL-10 expression by CD19^{hi}CD170^{hi}, CD19^{+}CD170^{int} and CD19^{+}CD170^{low} B cells in HC and RA, mean ±SEM, one-way ANOVA, ****p<0.0001. (D) vSNE plot of CD170^{hi} (green) and CD170$^{int/low}$ (purple) B cells as contour plots with IL-10^{+} gate overlaid (black line). (E) vSNE plot of B cells subsets purple CD24^{hi}CD38^{hi}IgM^{hi}IgD^{hi} immature B cells, blue CD24^{+}CD38^{lo}IgM^{+}IgD^{+} memory B cells, green CD24^{+}CD38^{lo}IgM^{-}IgD^{-} switched memory B cells, and pink CD24^{int}CD38^{int}IgM^{+}IgD^{+} mature B cells, with CD170^{hi} gate overlaid (black line).

6.2. $CD19^{hi}CD170^{hi}$ B cells suppress inflammatory cytokine production by $CD4^+$ T cells in an IL-10 and CD170 dependent manner

Having established that a $CD19^{hi}CD170^{hi}$ subset of B cells produce the majority of IL-10, I next investigated the capacity of this B cell subset to suppress inflammatory cytokine production by $CD4^+$ T cells. For this purpose, isolated B cells were cultured for 72h with CpGC, and $CD19^{hi}CD170^{hi}$ and $CD19^+CD170^{int/low}$ B cells were then sorted by flow cytometry and co-cultured with freshly sorted autologous $CD4^+CD25^-$ T cells, in the presence of an anti-CD3 antibody and CpGC for 72h (Figure 6.3A). My results show that $CD19^{hi}CD170^{hi}$ B cells suppress the differentiation of $IFN\gamma^+CD4^+$ and $IL\text{-}17^+CD4^+$ T cells, whereas $CD19^+CD170^{int/low}$ B cells were unable to inhibit the differentiation of IFNγ or IL-17 expressing $CD4^+$ T cells (Figure 6.3 B+C). Neutralization of secreted IL-10 and the IL-10 receptor in the $CD19^{hi}CD170^{hi}$ B:T cell co-culture significantly impaired the ability of these cells to suppress both IFNγ and IL-17 production (Figure 6.3D+E). Of note, I confirmed that $CD19^{hi}CD170^{hi}$ B cells produce more IL-10 than their $CD170^{int/low}$ counterparts by measuring secreted IL-10 by ELISA in sorted $CD19^{hi}CD170^{hi}$ and $CD19^+CD170^{int/low}$ B cells (Figure 6.3F). To ascertain whether CD170 plays a functional role in the suppressive capacity of $CD19^{hi}CD170^{hi}$ Bregs, I blocked CD170 and show that neutralisation of CD170-mediated signalling also prevents inhibition by $CD19^{hi}CD170^{hi}$ B cells (Figure 6.3G+H). I have also demonstrated that less IL-10 is produced overall in co-cultures of T cells with $CD19^{hi}CD170^{hi}$ B cells blocked with αCD170, than with unblocked $CD19^{hi}CD170^{hi}$ B cells as measured by ELISA (Figure 6.3I). Thus, TLR9 activated $CD19^{hi}CD170^{hi}$ B cells present all the features of a bona fide Bregs. In addition, my findings show that suppression of T cells by $CD19^{hi}CD170^{hi}$ B cells requires CD170 in addition to IL-10, suggesting that this marker is functionally involved in the mechanism of suppression of Bregs and that CD170 may be involved in the IL-10 transcriptional programme.

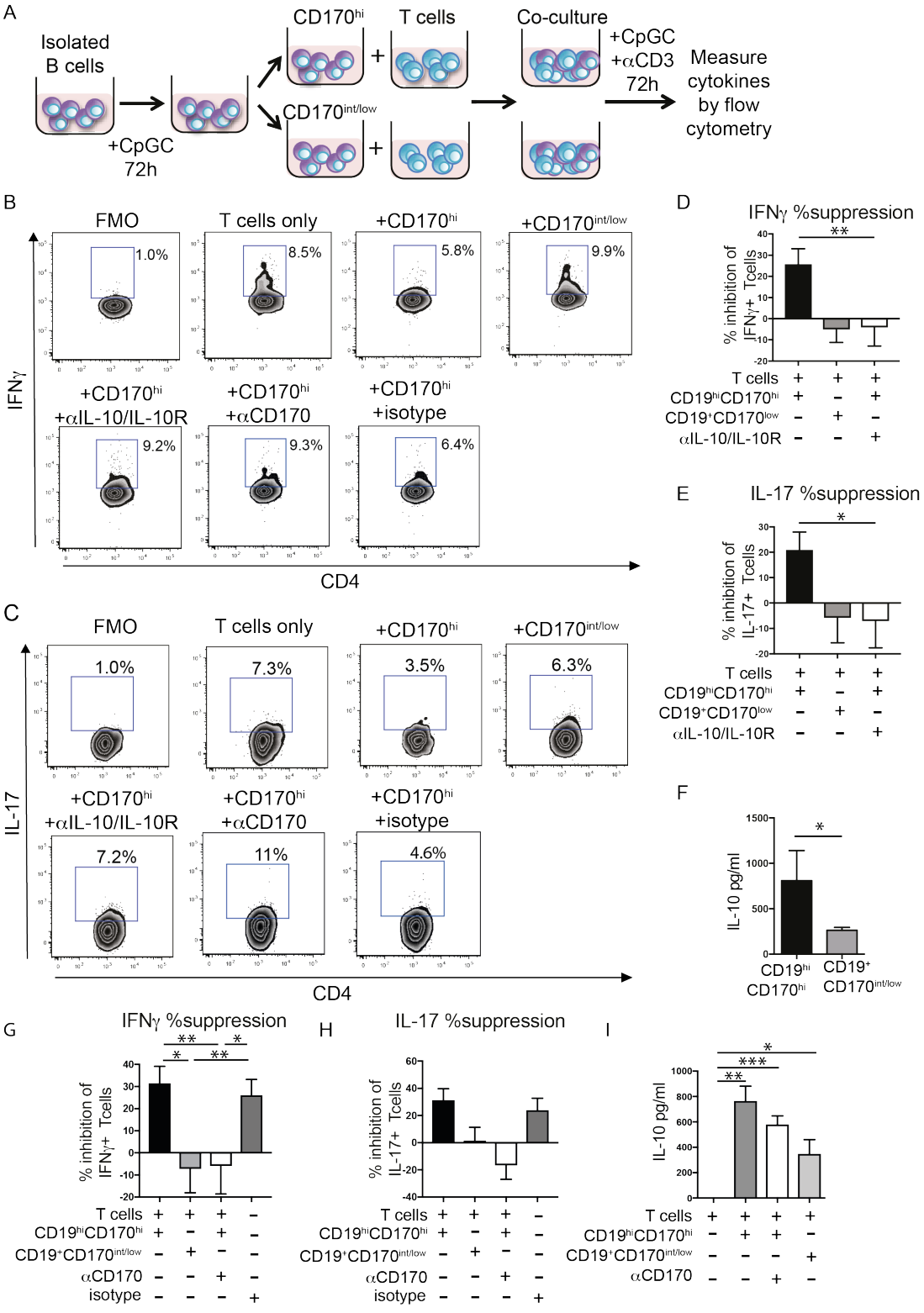

Figure 6.3. CD170^{hi} B cells suppress T cell cytokine production (IFNγ and IL-17) in an IL-10 and CD170 dependent manner.

B cells were isolated from PBMCs from HCs (n=5) and cultured with 1μM CpGC for 72h. CD19^{hi}CD170^{hi} and CD19^{+}CD170$^{int/low}$ B cells were sorted by flow cytometry. Cells were cultured 1:1 with freshly flow cytometry-sorted autologous CD4^{+}CD25^{-} T cells, and stimulated with 0.5μg/ml αCD3 and 1μM CpGC for 72

hours. 0.05μg/ml PMA, 0.25μg/ml Ionomycin and 5μg/ml Brefeldin were added to the cells for the last 5 hours of culture, followed by intracellular detection of IFNγ and IL-17 by flow cytometry. Where required cells were blocked with 10μg/ml αIL-10 and 10μg/ml αIL-10R antibodies in culture, or B cells blocked with 5μg/ml αCD170 antibody for 30 minutes prior to culture with T cells, as indicated. Supernatants were collected for analysis of cytokines. (A) Schematic of the suppression assay. B-C) Representative flow cytomtery plots and summary bar chart of IFNγ (B) and IL-17 (C) produced by $CD4^+$ T cells alone, or with B cell substes and blocking antibodies as indicated. (D-E) Bar charts for percentage inhibition of IFNγ (D) and IL-17 (E) with/without αIL-10 and αIL-10R, mean ±SEM. (F) IL-10 (pg/ml) measured by ELISA in supernatents from sorted B cells. (G-H) Bar charts for percentage inhibition of IFNγ (G) and IL-17 (H) with/without αCD170, mean ±SEM. (I) IL-10 (pg/ml) measured by ELISA in supernatants from co-cultures. One-way ANOVA or t-test as appropiate, *p<0.5, **p<0.01, ***p<0.00.1

6.3. CD170 promotes BCR-induced calcium mobilisation

Having shown that $CD19^{hi}CD170^{hi}$ B cells are able to suppress T cells in an IL-10-dependent manner, I next wanted to understand how the expression of CD170 acts to regulate the production of IL-10 by B cells. Although CD170 is known to possess an ITIM and act as an inhibitory receptor via the recruitment of SHP proteins (298), very little is known about the CD170 signalling mechanisms in immune cells.

CD22, the most well functionally characterised Siglec expressed on B cells, is a negative regulator of BCR signalling (304). To dissect whether CD170 is also a negative regulator of BCR signalling in response to antigen, I measured Ca^{2+} flux following BCR engagement via stimulation with anti-IgA+IgG+IgM $F(ab')_2$ in isolated B cells. Since this technique requires a high number of cells, and given the lack of a sufficient number of $CD19^{hi}CD170^{hi}$ B cells in PBMCs, I used total B cells and assessed how blocking CD170 affects BCR-induced Ca^{2+} mobilisation. Ca^{2+} flux acts as a measure of BCR signalling and reflects a cells capacity to subsequently differentiate into an effector B cell. Briefly, I isolated B cells from HCs and stained them with Flou-4 dye in the presence or absence of blocking αCD170 antibody. The cells were left to rest for 30 minutes before data acquisition. Baseline Ca^{2+} levels were recorded on the FACS machine for 30 seconds before addition of BCR stimulation, and recorded for a further 3 minutes to observe the variation in Ca^{2+} levels (Figure 6.4A). I showed that BCR engagement on B cells induced a robust Ca^{2+} mobilisation. Blocking CD170 signalling, however, leads to a significant reduction in Ca^{2+} mobilisation after BCR engagement (Figure 6.4B). These results suggest that CD170, unlike CD22, may promote rather than inhibit B cell signalling.

To gain more of an understanding into how CD170 may regulate and participate in regulation of signalling via the BCR, I used ImageStream to assess the co-localisation of CD170 with IgM. In ImageStream analysis, the Bright Detailed Similarity Index (log transformed Pearson's correlation coefficient of localised bright spots (i.e. staining for the protein of interest) that are 3 pixels or less in distance between them for an overlay of two images (one image for each target protein)) is used to determine co-localisation of two proteins of interest. A higher

value indicates co-localisation of two proteins. Bright Detailed Similarity analysis, indicates that CD170 does not co-localise with IgM (Figure 6.5). As expected, and in support of CD22 as a regulator of BCR signalling, ImageStream analysis shows co-localisation of CD22 with IgM. In contrast, I have observed that CD170 does not colocalise with IgM, but with CD19. CD19 is a transmembrane molecule pivotal in the early stages of BCR signalling, by associating with micro-clusters of IgM and IgD molecules that recruit Syk kinases, and leading to downstream signalling (347). Taken together these results suggest that CD170 acts indirectly to promote BCR signalling.

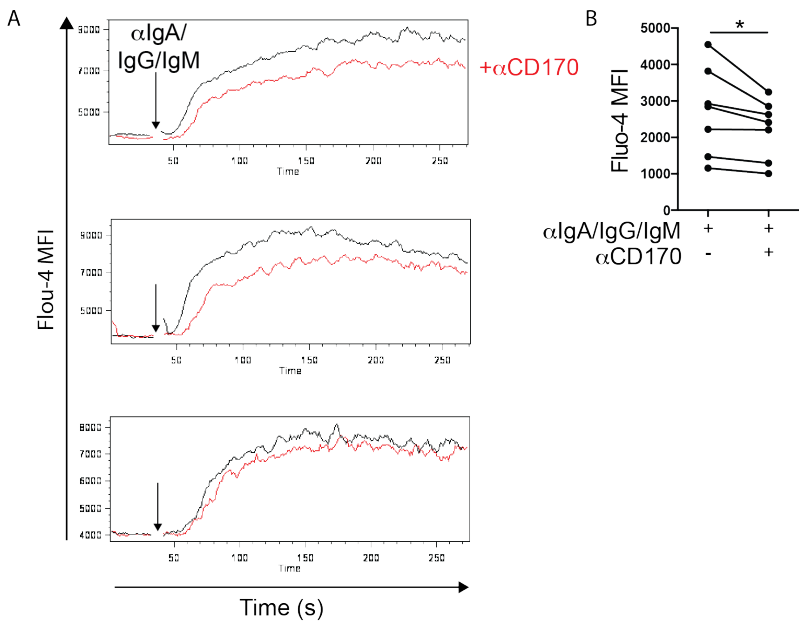

Figure 6.4. BCR-induced Ca^{2+} mobilisation is reduced in the presence of αCD170 antibody.
Ca^{2+} flux was measured by flow cytometry using Flou-4 dye following stimulation of isolated B cells from HCs (n=7) with 20μg/ml IgA +IgG +IgM $F(ab')_2$, with or without prior blocking. Samples were recorded on the FACS machine for 30 seconds before adding the BCR stimulation, and recorded for a further 3 minutes after stimulation. (A) Three representative Ca^{2+} flux kinetic plots. (B) Ca^{2+} flux measured as mean Flour-4 MFI after BCR engagement, minus mean baseline Flour-4 MFI, without and with prior blocking with αCD170, paired-*t*-test, *$p<0.05$.

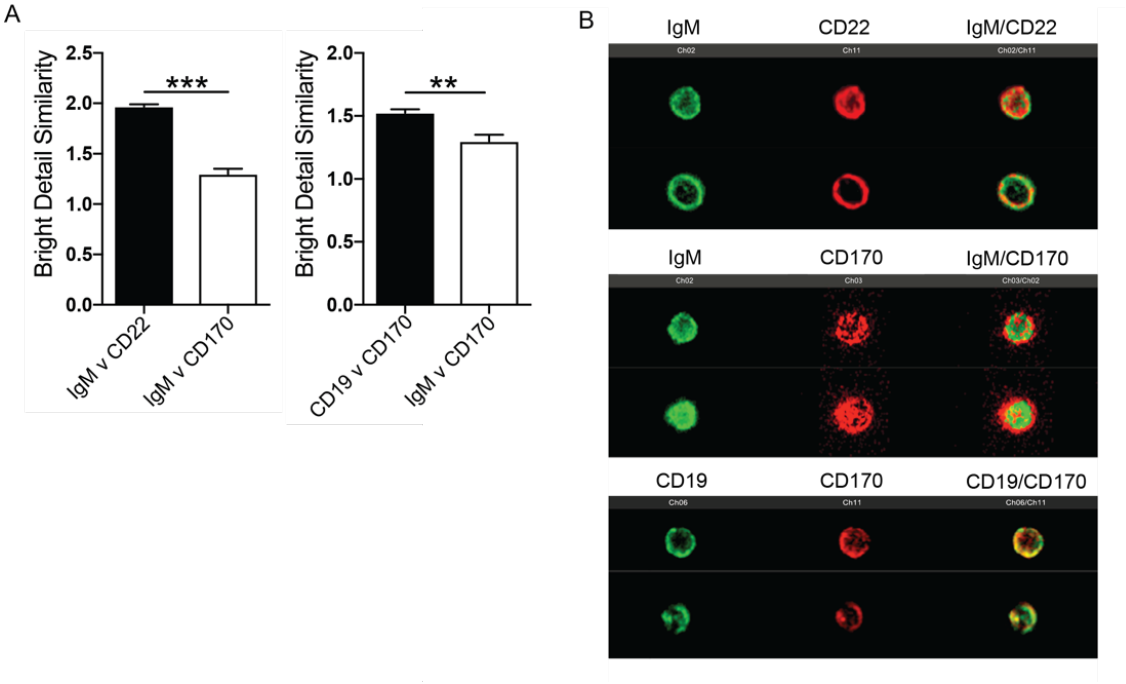

Figure 6.5. ImageStream analysis of IgM, CD22, CD170 and CD19 expression on B cells confirms co-localisation of IgM and CD22, but suggests an association of CD170 with CD19 rather than IgM.

PBMCs from HCs (n=8) were stained with conjugated antibodies against IgM, CD22, CD170 and CD19 and analysed using ImageStream. $CD19^{+}CD170^{+}$ and $CD19^{+}CD22^{+}$ cells were analysed for Bright Detailed Similarity between CD170 and IgM/CD19, and CD22 and IgM respectively. (A) Column graph showing co-localisation (Bright Detailed Similarity) of the two target proteins. Mean ±SEM, *t*-test, **p<0.01, ***p<0.001. (B) Representative ImageStream images showing IgM (green) and CD22 (red) expression, IgM (green) and CD170 (red) expression, and CD19 (green) and CD170 (red) expression as indicated, including overlay of both markers where yellow indicates co-localisation.

6.4. B cells from RA patients exhibit defective recycling of CD170

It is unknown if CD170, similarly to other Siglecs, is also an endocytic receptor that could be internalised either constitutively or following ligation (289). To determine if CD170 is recycled and to analyse the dynamic of CD170 recycling and whether a defect in CD170 internalisation and/or recycling to the surface could be the cause of the reduced frequency of $CD19^{hi}CD170^{hi}$ B cells in RA patients, B cells were purified from HCs and RA patients and surface stained with CD170. B cells were then either put on ice or incubated for 60 minutes at 37°C to allow internalisation of CD170. Internalised CD170 was detected using ImageStream. A significantly increased internalization score, after incubation for 60 minutes at 37°C compared to cells on ice (0 minutes), was observed in HCs but not in RA B cells (Figure 6.6). This suggests that whereas CD170 is constitutively internalised in HCs, this mechanism is defective in RA.

Next, I assessed the recycling dynamic of CD170 in B cells from HCs and RA patients. B cells were incubated with a saturating amount of unconjugated CD170-specific antibody to block cell surface CD170 molecules on ice for 30 minutes. B cells were then incubated at 37°C for 20 or 60 minutes to allow recycling of CD170 molecules from an intracellular location to the cell surface. Recycled intracellular CD170 molecules were not bound to the CD170 specific antibody, and therefore by staining with a PE-conjugated CD170 specific antibody I was able to assess their presence by flow cytometry. Following 20 and 60 minutes of incubation, I observed a progressive increase in surface CD170 expression, suggesting recycling of CD170 to the surface (Figure 6.7A). Comparing HC to RA, after 60 minutes of recycling, RA patients have a higher expression of CD170 (MFI) than HCs suggesting a faster rate of recycling of CD170 to the cell surface (Figure 6.7B).

Next, I addressed if CD170 is recycled via the endosomal system or is degraded in the lysosomes following internalisation. The transferrin receptor (TfR) is constitutively recycled via the endosomes and is a useful surrogate marker for the endosomes (349), while LAMP-1 has been widely used to identify the lysosomes due to its abundance in the lysosomal membrane (350). B cells from HCs were stained intracellularly with CD170, LAMP-1 and TfR and colocalization assessed by

ImageStream. The results show that CD170 co-localised with TfR but not LAMP-1, with the Bright Detailed Similarity Index significantly higher for CD170 and TfR (>2) than CD170 and LAMP-1 (<1) (Figure 6.8). Taken together, I have shown for the first time that CD170 is an endocytic receptor capable of recycling via the endosomes, similar to other members of the Siglec family. Furthermore, there is a defect in the ability of CD170 to be internalised by RA B cells, but B cells from RA patients exhibit a faster rate of recycling of CD170 to the surface.

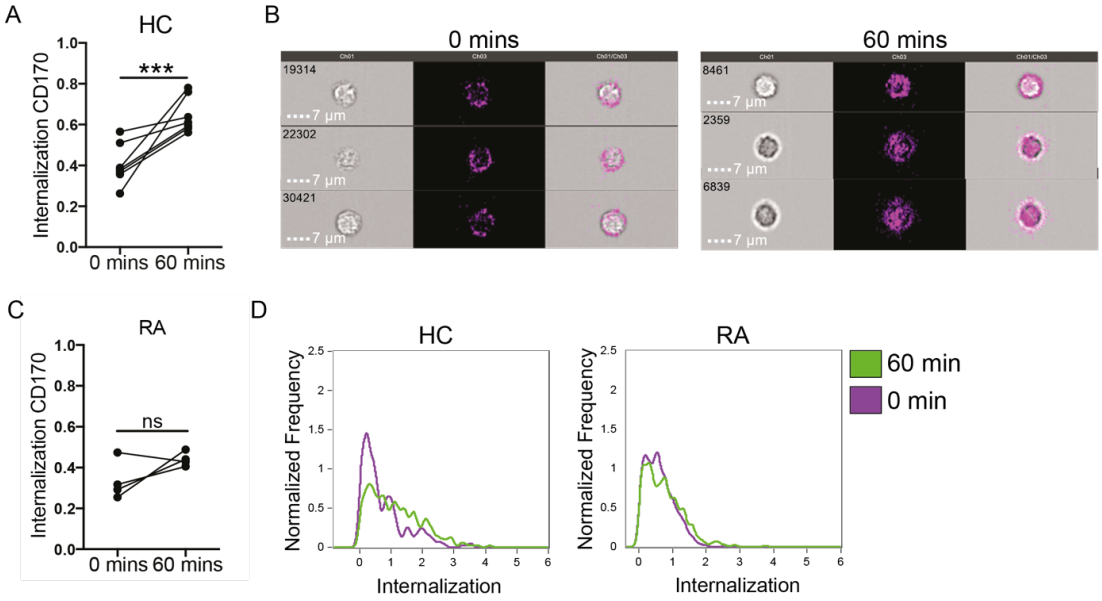

Figure 6.6. Surface CD170 is internalised in HCs, while internalisation is impaired in RA.

PBMCs from HCs (n=7) and RA patients (n=4) were stained extracellularly with CD170-PE on ice, then incubated at 37°C for 60 minutes to allow CD170 internalisation, or left on ice. Internalisation of CD170 on $CD19^+$ B cells was determined by ImageStream analysis and an Internalization Score generated for each sample. (A) Internalization of CD170 for paired samples either left on ice (0 mins) or incubated at 37°C (60 mins), t-test, *** $p<0.001$. (B) Representative ImageStream images for CD170 without (0 mins) and with (60 mins) incubation to allow internalisation; bright field image, CD170 expression (pink), and overlay. (C) As 'A' for RA patients. (D) Representative histogram overlay of frequency of internalised cells ("Internalization") of CD170 for samples without (0 mins, purple) and with (60 mins, green) incubation to allow internalisation in HC and RA patients.

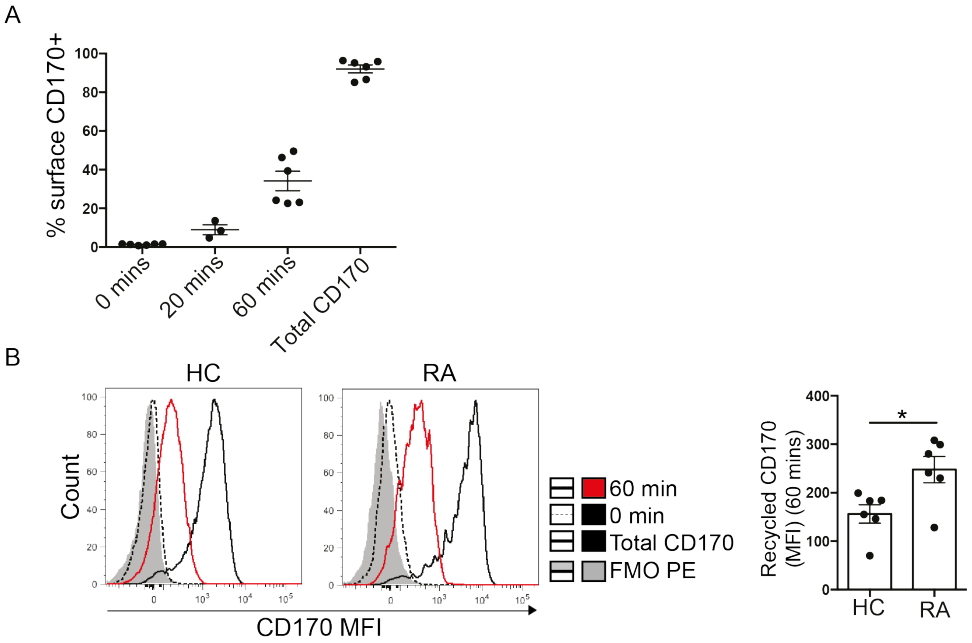

Figure 6.7. CD170 is constitutively recycled to the cell surface and this occurs at a faster rate in RA patients than in HCs.

B cells from HCs (n=6) and RA patients (n=6) were pre-incubated with an un-conjugated anti-CD170 antibody for 30 minutes on ice. B cell were then left on ice, or incubated at 37°C for 20 or 60 minutes to allow recycling of internal CD170. Cells were then stained with CD170-PE to detect recycled CD170. (A) Frequency of $CD170^+$ B cells at 0-, 20- and 60-minutes incubation following blocking with αCD170, and total CD170 without blocking. (B) Representative histogram overlays of CD170 MFI expression for HC and RA patients at 0- and 60- minutes incubation (dotted black and red line respectively) at 37°C, total CD170 (black line) and PE FMO (shaded grey), and column scatter graph showing a summary of the data; recycling calculated as CD170 MFI at 60 minutes minus MFI at 0 minutes.

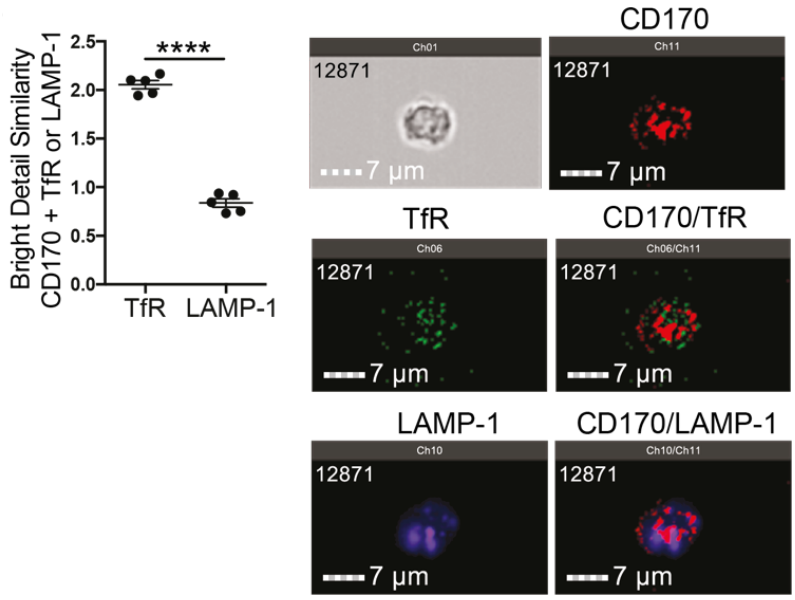

Figure 6.8. Intra-cellular CD170 colocalises with TfR suggesting recycling via the endosomes.

PBMCs from HC (n=5) were stained extracellularly for ImageStream analysis with CD19 and intracellularly with CD170-APC, transferrin receptor (TfR) and Lysosomal-associated membrane protein 1 (LAMP-1). Bright detailed similarity index was calculated between CD170 and TfR, or CD170 and LAMP-1. Column scatter graph of summary data; mean ±SEM, *t*-test, ****p<0.0001. Representative ImageStream images of CD170 staining and its expression with respect to TfR and LAMP-1.

6.5. The SIGLEC5/14 polymorphism is not associated with RA

CD170 has 99% sequence homology of the first two Ig domains with the ITAM expressing Siglec-14 (299). In some individuals Siglec-14 is absent, with the *SIGLEC5* gene having undergone conversion with the *SIGLEC14* gene resulting in a "*SIGLEC5/14*" gene whereby the *SIGLEC5* gene is under the control of the SIGLEC14 promoter, resulting in a loss of Siglec-14 expression (292) (Figure 6.2A). Furthermore, the *SIGLEC5/14* null mutation is thought to be associated with impaired immunological responses (300, 302). Therefore, it was important to evaluate if there was any association between the SIGLEC5/14 null allele and RA, and to assess if the presence of the null allele affected the expression of CD170 on B cells. Hence, I performed PCR amplification of the *SIGLEC5*, *SIGLEC14* and *SIGLEC5/14* genes, from DNA extracted from frozen PBMCs from 14 HC and 15 RA patients selected at random from our cross-sectional cohort. I ran the PCR products on a 1% agarose gel in order to visualise the presence or absence of the genes and genotype the samples (Figure 6.2B). Of note, B cells do not express siglec-14 (292), therefore I hypothesized that it is unlikely that this mutation will affect CD170 expression on B cells.

I report no incidence of individuals homozygous for the null allele and that the majority of individuals (approx. 70%) do not possess a copy of the null allele (Figure 6.2C). Furthermore, there is no difference in the frequency of the polymorphism between HC and RA, suggesting this mutation is not associated with RA. To evaluate if the genotype for the null allele affected CD170 expression by B cells I compared frequency of $CD170^{+}$ B cells in 'wild type' samples to heterozygous samples (HC and RA patients combined) (Figure 6.2D). While there was no significant difference between the two groups, interestingly all CD170 low/non-expressing individuals do not have the polymorphism, however, I cannot draw a conclusive association from our results due to the small number of these individuals.

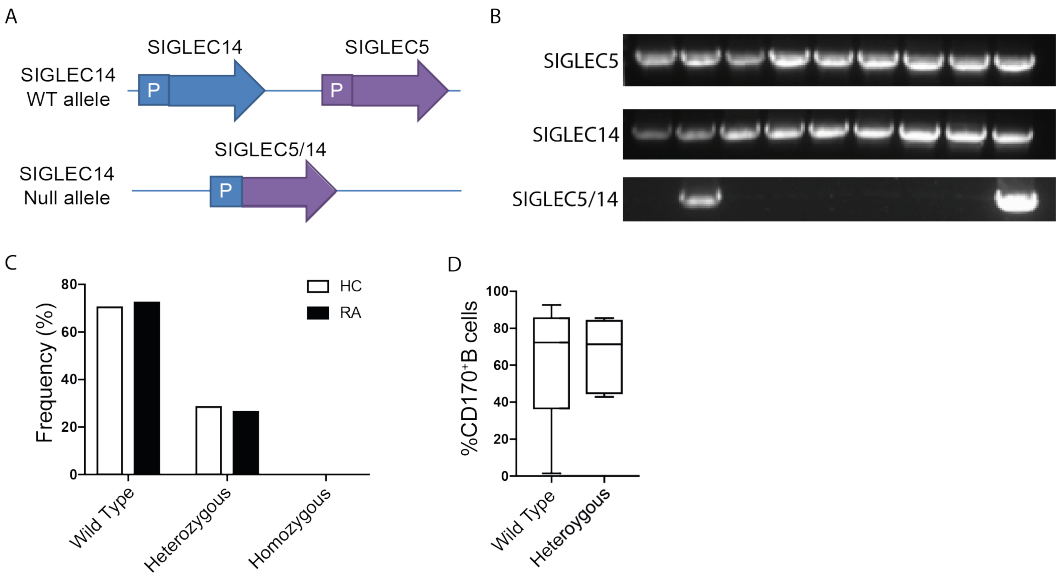

Figure 6.9. Genotyping of HC and RA for presence of SIGLEC5/14 null allele. DNA was extracted from frozen PBMC samples from HC (n=14) and RA patients (n=15); PCR amplification was performed with primers for *SIGLEC14*, *SIGLEC5* and *SIGLEC5/14* (null allele). PCR products were run on a 1% agarose gel. (A) Schematic of the WT and null alleles for the *SIGLEC5* and *SIGLEC14* genes. (B) Representative gels for the three genes (n= 9). (C) Frequency of each genotype in HC (white bars) and RA (black bars). (D) Box and whisker plot of frequency of $CD170^+$ B cells between individuals with either a 'wildtype' or a heterozygous genotype, *t*-test not significant.

6.6. Summary

I have reported a novel surface marker that uniquely identifies Bregs. The suppressive mechanism of this Breg population is dependent on the production of IL-10 and the expression of CD170. This novel Breg population is reduced in RA patients, which supports our and others work showing reduced Breg numbers in this disease. I propose that the reduced number of $CD170^{hi}$ B cells in RA could be due to a defect in the recycling of this molecule. This could have functional implications, including the reduced IL-10 production observed in B cells from RA patients. I have also reported a decrease in Ca^{2+} mobilisation after blocking CD170, suggesting that CD170 acts to regulate BCR signalling. However, I have shown that CD170 does not co-localise with the BCR, unlike CD22, but it rather it co-localises with CD19, suggesting an indirect regulation of BCR signalling that needs further exploration.

6.7. Future work

I have begun to dissect the mechanism by which CD170 may drive the transcription of IL-10 in B cells. An immediate goal is to continue to research the role of this unexplored molecule, particularly since very little is known about CD170 on B cells. I need to further unravel how CD170 may act to regulate IL-10 production. To do this I will sort $CD19^{hi}CD170^{hi}$ B cells, and $CD19^{+}CD170^{int/low}$ B cells following 72h stimulation with CpGC as described. I will initially assess by qPCR transcription factors known to regulate IL-10 production to see if any are up regulated in $CD19^{hi}CD170^{hi}$ B cells, for example, IRF4 (351) and ERK1 (352). It would also be of interest to investigate the phosphorylation of STAT3, a transcription factor involved in the transcription of IL-10 (353), using phosflow cytometry, and assess how STAT3 phosphorylation may vary with increasing CD170 expression. If this approach does not lead to the identification of a transcriptional marker, I will perform RNA-seq on the two populations from healthy individuals.

I show a possible functional association between CD170 and CD19. It has been demonstrated that cross-linking of the BCR with CD19 reduces the threshold for B cell activation. CD19 acts as a membrane adaptor protein, recruiting signalling molecules including Vav, phosphoinositide-3 kinase (PI3K), and Lyn, and resulting

in the activation of the MAP kinase signalling pathways (347). The MAP kinase signalling pathways have also been shown to be important in IL-10 production. Therefore, I will address in future experiments how disruption of CD170 impairs BCR signalling, including activation of MAP kinases, and will compare the results in HCs to RA.

Evidence has shown that Bregs in RA are not only numerically deficient but are unable to inhibit the polarisation of naïve T cells into Th1 or Th17 cells (98). Therefore, I would need to assess the suppressive capacity of $CD19^{hi}CD170^{hi}$ Bregs from RA in this setting. It could also be of interest to investigate how the recycling of CD170 may be linked to its function, for examples via interaction with TLR9 in the endosomes. I would start this investigation by assessing co-localisation of TLR9 with CD170 using ImageStream.

7. Discussion

Technological advances have enabled large-scale analyses of biological investigations, increasing the likelihood of new findings including the identification of novel biomarkers. The work presented in this thesis introduces a flow cytometry-based high-throughput immune phenotyping platform that allows identification of cell surface markers that are differentially expressed in different groups of RA patients and compared to healthy individuals. The wealth of data gathered using the LEGENDScreen™ platform was used to explore three inter-connected questions: 1. Is it possible to predict ADA development?; 2. Can novel biomarkers of RA be identified?; 3. Can a novel marker of Bregs be identified?

7.1. Large datasets as a tool for medical research, and the pros and cons of LEGENDScreen™

One of the appeals of LEGENDScreen™ beyond the wealth of data the platform is able to generate, is the possibility of identification of novel markers. The LEGENDSceen™ consists of an extensive and comprehensive set of anti-cell surface protein directed antibodies, many of which have not been studied before on B cells, or in patients with RA. One of the main challenges of this work however, was to establish the best way to analyse this wealth of unique data obtained. Devising a strategy for the analysis of these more extensive datasets is a challenge across similar technologies (354-356). In this thesis the LEGENDScreen™ is not only validated, but I also developed a systematic framework analysis in order to address several posed questions within this thesis, notably of markers that are specific to ADA or RA, and not to confounding factors including the level of inflammation.

The LEGENDScreen™ allows for extensive phenotyping of cell-surface protein expression, from which we can make direct functional inferences. This gives LEGENDScreen™ and similar 'phenotyping' platform strategies an advantage over, for example, genetic studies, where confounding factors often play a role, including SNPs identified in non-protein coding regions of the genome (357), epigenetic modifications (358), and a lack of overlap between data studies (354). The

LEGENDScreen™ is particularly unique due to its capability to measure the expression of 332 different proteins, enabling not only the assessment of novel expression patterns of cell-surface proteins, but it may also provide viable new drug targets. Such phenotypic analyses are relatively inexpensive, and the identification of cell surface proteins also lends itself better to the clinic; blood samples are already routinely taken and tested for presence of proteins such as RF and anti-CCP. Blood-based screening is easy to perform, and it can be repeated at frequent intervals. This is advantageous in comparison to, for example, biopsies that are invasive and not always routinely carried out, or imaging techniques, such as ultrasound, that require specialist equipment. Therefore, biomarkers found within the blood make the monitoring of patients more accessible. A future goal of this study is to use the RA signature as a blood test to help identify patients at greatest risk of developing RA. To our knowledge this is the first study that extensively phenotypes B cells in both healthy individuals and RA patients. Several other studies have used the LEGENDScreen™ for analysis of mucosa-associated invariant T (MAIT) cells, early activation of $CD4^+$ T cells, delta one T (DOT) cells, small-cell lung cancer (SCLC) cells, skeletal muscle progenitor cells, and DC subsets (359-363). Notably there is a dominance of T cell investigations with LEGENDScreen™, and furthermore, these studies are largely restricted to healthy individuals.

The limitations of the LEGENDScreen™ platform include the requirement of a large number of cells and the inability to analyse all 332 markers simultaneously on a single cell. While I validated our panel and the number of cells used to ensure that I collected enough events for analysis of immature cells, the requirement of a large number of cells makes the analysis of small/rare populations more challenging. This could be improved by pre-enriching for particular cell types before, although would still require a large starting number of PBMCs. Furthermore, despite the extensive list of LEGENDScreen™ markers, one remains restricted by the availability of an antibody against a target, and some targets may not be provided by the platform. The more recently established mass cytometry technique CyTOF, provides an alternative to LEGENDScreen™ that allows the analysis of a large number of proteins simultaneously on a single cell. Like LEGENDScreen™ CyTOF is also a flow cytometry technique, however, here the antibodies against target proteins are conjugated to heavy metal ions and detected by time-of-flight mass spectrometry,

mitigating the need for compensation (364, 365). CyTOF is able to detect more targets than a standard multi-colour flow cytometry panel (up to 135 compared to up to 18 colours in flow cytometry), and while LEGENDScreen™ looks at more target proteins in total (332), CYTOF has the advantage of being able to simultaneous detect multiple targeted proteins on a single cell. CYTOF may provide a potential next step for analysis for markers identified using the LEGENDScreen™.

Despite LEGENDScreen™ providing a highly comprehensive overview of the expression of cell surface proteins across multiple immune cell types, the LEGENDScreen™ provides no information on intracellular proteins such as signalling molecules and transcription factors. Where a protein has not before been described on a subset, or its functionality is relatively unknown, it is particularly important therefore to undertake follow up investigations to understand the mechanisms behind the expression of a marker, or lack thereof. Furthermore, such analysis may also uncover connections between the observed expression of multiple markers. This could be achieved by complimentary genetic analysis and/or functional analysis of individual markers; the latter of which I have done in this thesis and will discuss later. This "multi-omics" approach, has the aim of generating a complementary and global view of immune cells in health and disease and thus minimising the effects of the limitations of the individual technologies. For example, Graessel *et al.* use quantitative liquid chromatography-tandem mass spectrometry, LEGENDScreen™ and gene expression microarray to assess early activation of $CD4^+$ T cells (360).

7.2. The impact of ADA and implications within the clinic

Biological therapies are routinely used to treat autoimmune conditions such as RA, and have significantly improved the management of these diseases. However, despite their success, some patients develop immune reactions against these therapies, and develop ADA, which often leads to treatment failure (124, 126, 137-139). Different drugs possess different degrees of immunogenicity, for example adalimumab, a whole mAb, is more immunogenic than etanercept, a fusion protein that contains only the Fc portion of an antibody (138, 366). While to a certain extent, increasing drug dosage has been shown to mitigate the side-effects associated with the presence of ADA (146), there is a need to be able to predict which individuals will develop ADA. To date, there has been very little investigation of the immunological differences between ADA^+ patients and ADA^- patients that may account for why certain individuals develop antibodies against the drug. Here I aimed to identify a predictive biomarker associated with ADA. By using a UK cross-sectional cohort of adalimumab-treated RA patients, followed by validation in a European prospective cohort, I have identified that a reduced frequency of SIRPα/β^+memory B cells prior to adalimumab treatment allows the prediction of ADA development in RA patients. In addition, I show that this lower frequency of SIRPα/β^+memory B cells in ADA^+ patients remains relatively stable following treatment with adalimumab. In general, and as mentioned earlier, one of the major pitfalls in this type of study is the lack of reproducibility of a biomarker. This is often due to the absence of validation in independent cohorts (354, 367, 368). I mitigated this problem by validating our results in an independent cohort that included patients of mixed ethnicity. However, only one of the markers was reproducible in the validation cohort. This may be due to the complex nature of the development of ADA, despite our attempts to account for possible confounding factors including disease activity and treatment.

The ability to detect ADA in RA patients already on treatment would potentially deliver precision medicine to this heterogeneous disease and increase the efficiency of clinical decision-making. It is tempting to propose that the measurement of the frequency of SIRPα/β^+memory B cells could also be used as a surrogate marker for ADA in the clinic for patients already being treated with adalimumab. Currently

there is no method of measuring ADA that is reliably accurate for routine clinical use, with the many available assays varying in sensitivity (149). Using ADA titre and/or an ADA surrogate measure could also help to inform clinicians as to when to make the decision to switch treatment.

It is interesting to note that not all individuals develop ADA, suggesting the existence of specific risk factors, genetic or environmental, that predispose some individuals to develop ADA. However, despite the association of ADA with loss of response to treatment, there have been very few investigations into the immunological mechanisms that drive ADA development and/or what may predispose an individual. It has been previously reported that in RA three SNPs in the promoter region of the IL-10 gene are associated with an increased likelihood of the development of ADA against adalimumab (143). In addition, patients that were homozygous for the same IgG allotype as adalimumab (G1m17) were more likely to develop ADA (41%), and of those individuals that were homozygous for G1m3 only 10% were ADA^+ (144). In MS, which is commonly treated with an antibody therapy against interferon-β, ADA development against the drug was associated with the HLA-DR4 haplotype (145). There is currently no functional data showing the role of SIRP in B cells. Nevertheless, SIRPα has been shown to have a possible role in autoimmunity. Specifically, it was found to be a risk locus in individuals with type 1 diabetes (369), and while Crohn's disease patients have increased SIRPα/β^+CD11c^+DCs in the mesenteric LNs and in the inflamed intestinal mucosa (370). SIRP α and β are members of the signalling inhibitory receptor protein (SIRP) family, and are membrane-expressed proteins. Predominantly found on myeloid cells, they act to mediate cell-to-cell interactions by regulating the type and strength of the signal (293). The major ligand for SIRPα is CD47, which is ubiquitously expressed and has a role in apoptosis, proliferation, adhesion and migration (371), while SIRPβ has no known ligand. It would be interesting in future studies to ascertain whether for example SIRP α and β are also genetic risk loci associated to ADA development in patients with RA.

There is an increasing number of biosimilars on the market; studies investigating the efficacy of a biosimilar to the original drug have shown that the biosimilars have comparable efficacy (372). It has been demonstrated that in patients who have

developed ADA against the infliximab formulation known as Remicade, that the ADA will cross-react with the biosimilar, as shown for Remsima in IBD (373) and Inflectra in RA (374), both infliximab biosimilars. On this basis I would expect the frequency of SIRPα/β^{+}memory B cells to also be reduced in patients that lack response to or have ADA against adalimumab biosimilars however; this will be subject to future investigation.

7.3. A novel marker for the identification of Bregs

Despite on-going efforts, a unifying phenotype of Bregs remains elusive. Questions around the development and stability of Bregs remain outstanding (275, 344). Thus, the identification of a unique Breg marker would aid our understanding of the development and function of these cells. Several markers have been associated with Bregs, including CD24, CD38, CD27, Tim-1, and CD138 (249, 258, 279, 282). Unfortunately, these same markers are also expressed by B cell subsets at different stages of development. The identification of IL-10^{+} B cells by flow cytometry typically requires a brief culture with a protein transport inhibitor such as brefeldin, followed by fixation and permeabilization of cells. This negates further functional analysis of these cells and makes current studies of Bregs more difficult. While methods are available for the sorting of IL-10^{+} cells without fixation, a surface marker would better facilitate the investigation of these cells. Here I report that under Breg polarising conditions over 80% of CD19^{hi}CD170^{hi} B cells following stimulation with CpGC produce IL-10. Moreover, my results show that upregulation of CD170 parallels the induction of IL-10 production.

The identification of a unifying Breg marker could also provide a novel therapeutic target. Current treatments for multiple immune disorders typically target symptoms rather than offering a cure. Given the long-term management required with drugs this can lead to unwanted side effects, including risk of life-threatening infections or inefficacy of treatment over time. By targeting the depletion or modification of specific immune cells involved in disease pathogenesis we could provide a more successful option with less side effects. Of interest, rituximab, while depleting the inflammatory and antibody producing B cells, also depletes the beneficial Bregs (105). Bregs are an appealing target for such directed therapies. Ideally a Breg based therapeutic approach could have the potential to permanently reset immune homeostasis; harnessing the power of Bregs could lead to a more stable, pre-disease like state. We can propose two ways that Bregs could be manipulated for therapeutic intervention; firstly the depletion of Bregs, for example, for use cancer; secondly, the expansion of an individual's Bregs *in vitro* via stimulation of CD40, TLR and/or BCR engagement, followed by the adoptive transfer of their Bregs back into the

patient, for the treatment of auto-immune diseases (344). However, at the moment these strategies are limited by the lack of the ability to purify Bregs.

7.4. The role of CD170 in health and disease, and what CD170 can tell us about Bregs in RA

In this thesis I propose that high expression of CD170 by B cells defines IL-10^{+} Bregs. Very little is known about the functional role of CD170, particularly on B cells. The majority of Siglecs are inhibitory receptors, possessing an ITIM motif, and have been shown to dampen inflammatory cytokine production and signalling via the BCR. Work by Ando *et al.* showed that Siglec-9 expression on the macrophage cell line RAW264 not only inhibited TNF-α production, but also enhanced IL-10 production, thus indicating multiple pathways of regulation by Siglecs (345). To further understand the mechanism of cytokine regulation by Siglec-9, Ando *et al.* mutated the ITIM and ITIM-like motifs of Siglec-9. These mutations reversed the effects of increased Siglec-9 expression on production of IL-10 and TNFα, suggesting that signalling via the ITIMs is responsible for both phenomena. Activated ITIMs recruit SHP proteins for signalling; observations made following the siRNA knockdown of SHP-1 and SHP-2 suggest that SHPs inhibit the described enhancement of IL-10. Overall this study suggests that ITIM signalling is required for IL-10 enhancement and is regulated by SHP proteins, however, the complete mechanisms remains unclear. In line with our observations Ando *et al.* further showed that RAW264 cells expressing CD170, also produced less TNFα and exhibited an enhancement of IL-10 (345). Notably, I demonstrate that the $CD19^{hi}CD170^{hi}$ B cell population is reduced in RA patients, and this is in parallel with less IL-10^{+} B cells in RA. Although it was interesting to observe that those B cells in RA that did upregulate CD170 produced similar amounts of IL-10 compared to their HC counterparts.

To begin to understand the differential expression of CD170 between HC and RA patients I looked at the recycling of CD170. Siglecs are considered endocytic receptors, with constitutive recycling and induced endocytosis having been demonstrated (288, 323, 375, 376). I established that CD170 is able to constitutively recycle, and that the recycling of CD170 in RA patients is dysregulated. The ability of Siglecs to recycle has been demonstrated to be associated with a variety of functions, for example; the prevention of viral and bacterial spread by phagocytosis

of pathogens expressing sialic acids; antigen presentation following internalisation antigen containing sialic acids; and regulation of signalling, for example of the BCR by CD22 (289, 376, 377). It is possible that the dysregulation of CD170 recycling I have detected in RA patients could not only affect the surface expression of CD170, but directly affect the functional capacities of this molecule.

I also demonstrated that CD170 is able to recycle via the endosomes as observed for other Siglecs (378). I also show that stimulation via TLR9 induces both CD170 upregulation and the production of IL-10; within the endosome CD170 could directly interact with TLR9 which is situated in the endosomal membrane, and act to regulate signalling following TLR engagement. Multiple studies, as discussed in the introduction, have reported that KO cell lines, or cell lines over expressing Siglecs have shown changes in proliferation, activation, MHC class II expression, NF-κB transcription activity, and production of cytokines in response to stimulation via TLRs (303, 317, 318). However, none directly address the relationship between the TLR and Siglecs. Thus, future work investigating the link between CD170 and TLR9 could enhance our understanding of siglec-TLR interactions across this family of proteins.

I have already discussed how manipulation to expand or reduce the Breg population could be a useful therapeutic tool. In addition, understanding the functional mechanism of CD170 beyond a biomarker of Bregs, may open up further possibilities for therapeutic intervention. CD47 expression on cells sends a "do-not-eat-me" signal to macrophages expressing the ligand SIRPα. This system is exploited by tumour cells that overexpress CD47, and blockade of this receptor-ligand interaction has been demonstrated to lead to the inhibition of tumour growth; hence blocking other protein-protein interactions may offer viable therapeutic target options (362). Blocking the interaction of CD170 with CD19 may for example reduce B cell activation (as suggested by Ca^{2+} flux experiments).

The ability of Siglecs to recycle makes them attractive targets for therapeutic manipulation. Sialoadhesin (Siglec-1) is a macrophage specific adhesion and endocytic receptor (376). It is able to bind bacteria and viruses expressing sialic

acids limiting the spread of the bacteria/viruses, acting as a scavenger receptor, and leading to phagocytosis of the bacteria/viruses (377). This system has the potential to be exploited therapeutically using liposomes for targeted delivery into macrophages. Liposome targeted delivery of lipid antigens leads to their presentation via CD1d and the activation of iNKT (379). Similarly, this system was exploited to deliver toxins to macrophages in order to kill them (376). Having observed an impairment in recycling of CD170 in RA patients, which may be linked to IL-10 production, it is tempting to suggest a therapy whereby restoring the recycling of CD170 in B cells from RA patients could recover IL-10 production. More investigations would be required into the recycling mechanism of CD170 and how it is linked to IL-10 production.

In the literature it has been reported that as many as 70% of Chinese individuals have the Siglec-14 null mutation, which renders Siglec-14 expression absent on all cells, whereas it is much more uncommon in people of Northern European descent (only 1 in 10 had the mutation) (292). In our cohort of 29 individuals I identified no individuals homozygous for the Siglec-14 null mutation. Despite the lower frequency reported for European populations this frequency is still higher than I observed in our cohort, suggesting that the mutations may not be quite as common as documented previously. Since B cells do not express Siglec-14 one might expect that the Siglec-14 null allele is not associated with RA, a disease in which B cells play a prominent role. Furthermore, the immunological impact of this mutation is not fully appreciated (300, 302).

Overall, our initial investigations of CD170 focused on its expression on IL-10-producing B cells. This leads us to speculate that the manipulation of CD170 through therapeutic intervention could be the key to harnessing the power of Bregs and improving or promoting their function in RA, and thus facilitating the restraint of chronic inflammation that is the hallmark of RA.

7.5. Conclusion

In this thesis I have presented three main findings: a) low frequency of SIRPα/βa^{+} memory B cells predicts development of ADA against adalimumab in RA patients; b) CD97, CD170 and CD11c may predict RA onset in at-risk individuals, and c) CD170 is a novel marker of Bregs. To our knowledge this is the first extensive immune phenotyping analysis of B cells in a longitudinal cohort of RA patients treated with adalimumab. I hope that markers identified in this thesis can be used as part of a toolkit in combination with other biomarkers, genetic and other risk factors that will allow for more personalized approach to the treatment of patients with RA and similar conditions, and that CD170 may be a useful tool in future investigations of Bregs.

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List of publications

Published manuscript:

Low percentage of signal regulatory protein α/β^+ memory B cells in blood predicts development of anti-drug antibodies (ADA) in adalimumab-treated rheumatoid arthritis patients. Laura Magill, Marsilio Adriani, Véronique Berthou, Keguan Chen, Aude Gleizes, Salima Hacein-Bey-Abina, Agnes Hincelin-Mery, Xavier Mariette, Marc Pallardy, Sebastian Spindeldreher, Natacha Szely, David A Isenberg, Jessica J Manson, Elizabeth C Jury and Claudia Mauri on behalf of ABIRISK consortium.
Front Immunol. 2018 Dec 5;9:2865. doi: 10.3389/fimmu.2018.02865. eCollection 2018.

Manuscripts in preparation:

A novel Breg: $CD19^{hi}CD170^{hi}$ $IL\text{-}10^+$ B cells. Laura Magill, Diego Catalan, and Claudia Mauri.

Published abstracts:

Laura Magill, Madhvi Menon, Marsilio Adriani, William Sanderson, Jessica Manson, Elizabeth C. Jury, Claudia Mauri; BASIC SCIENCE ORAL ABSTRACTS O31. (YOUNG INVESTIGATOR AWARD WINNER) PATIENTS WITH RHEUMATOID ARTHRITIS HAVE A UNIQUE IMMUNE SIGNATURE THAT DEFINES THE DISEASE AND THEIR RESPONSE TO ADALIMUMAB, *Rheumatology*, Volume 56, Issue suppl_2, 1 April 2017, kex061.031, https://doi.org/10.1093/rheumatology/kex061.031

Appendix

A.1. ABIRISK patient consent form

UCL DIVISION OF MEDICINE
UCL

University College London Hospitals NHS
NHS Foundation Trust

Centre for Rheumatology Research
Rayne Institute
5 University Street
LONDON WC1E 6JF

Department of Rheumatology
University College Hospital
250 Euston Road
LONDON NW1 2PQ

Investigating anti-drug antibodies in autoimmune disease
Research ethics review number: 14/SC/1200 PR
Researchers: Dr. Jessica Manson. Prof. David Isenberg, Prof. Claudia Mauri, Dr. Elizabeth Jury.

CONSENT FORM

1. I confirm that I have read and understand the Patient Information Sheet (June 2015, Version 5) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that the researchers will access my medical records to obtain data that is relevant to this study. ☐

4. I understand that relevant sections of my medical notes, and data collected during the study, may be looked at by individuals from ethical and regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

5. I understand that by giving consent, I am agreeing to allow stored samples and information relevant to this study from my medical records, to be used in future research concerning autoimmune and rheumatic diseases after further regulatory and ethical body approval has been obtained. ☐

6. I understand that you will not provide any feedback or results from research analysis conducted on my sample. ☐

7. I give consent for you to contact my general practitioner. ☐

8.I agree to take part in the above study. ☐

9. I agree to part of my sample being investigated for the amount of genetic material, and some of this information being shared with a commercial third party (**OPTIONAL**). YES ☐ NO ☐

10. agree to part of my sample being sent to the Institute of Health and Medical Research in Paris for genetic analysis and I understand that you will NOT provide any feedback or results from research analysis conducted on this sample (**OPTIONAL**). YES ☐ NO ☐

Name of participant	Date	Signature
Name of person taking consent	Date	Signature

Researcher use only:

Hospital Number	Date of Birth	Patient ID Code

A.2. ABRISK patient information sheet

UCL DIVISION OF MEDICINE
UCL

University College London Hospitals NHS
NHS Foundation Trust

Centre for Rheumatology Research
Rayne Institute
5 University Street
LONDON
WC1E 6JF

Department of Rheumatology
University College Hospital
250 Euston Road
LONDON
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PATIENT INFORMATION SHEET

Investigating anti-drug antibodies in autoimmune disease:

Research Ethics Reference number: 14/SC/1200

We would like to invite you to take part in our study. Before you decide, it is important to understand what the research involves and why it is being done. Please read this information sheet and ask any questions you may have. We encourage you to take as much time as you need and to discuss it with others if you wish.

PART 1 – ABOUT THIS STUDY

What is the purpose of this research?
We are studying the drugs used to treat autoimmune diseases. They work very well in most cases, but in a minority of patients, the medicine is neutralised before it has time to take effect. This problem is caused by the patient's own antibodies, called anti-drug antibodies, and nobody knows why some people develop them while others do not. The aim of this study is to discover clinical features or blood markers that will help us to predict which medicines will work for which patients, and in the longer term, to design better medicines or ways to use these medicines, to avoid the development of anti-drug antibodies.

Why have I been invited to take part?
You have been approached for this study because you have rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) and have been treated previously with one of the drugs we want to investigate. We will also invite healthy volunteers to donate blood for comparison.

Do I have to take part?
No. It is up to you whether or not you join this study. We will describe the study and go through this information sheet. If you agree to take part you will be given this information sheet to keep and we will then ask you to sign a consent form. You can change your mind at any time without giving a reason, and this would not affect the care you receive.

What will happen to me if I take part?
You will be asked to attend the hospital once to have blood samples taken. We will ask you to donate 50mL of blood (about 3 tablespoons). This sample will be taken at the same time as your routine blood tests, so there will only be one needle and will coincide with your routine clinic visits. The procedure normally lasts 5 minutes.
We will also obtain information from your medical records that is relevant to this study.

Are there any risks?
There are no risks other than a small amount of discomfort or a small bruise that is sometimes associated with having your blood taken.

What will happen to my sample?
The blood sample will be taken to the laboratory and kept in tubes marked with a unique code, but not with any identifiable information. We will examine the surface of blood cells for features that are known to be important in the way cells communicate. In addition, we will test a substance in your cells called RNA. Your RNA carries some information about your genetic makeup. When testing the RNA, some of the information that is stored in your genes will become available to the scientists conducting the study. However, the goal of the research is to measure the amount of the different types of RNA in your cells to see how this might be related to SLE and RA. The RNA information will not be used to study your genetic makeup. The RNA tests will be done by a Contract Research Organization (CRO) and the data subsequently be shared with a commercial third party. Those parties will have no way of linking your sample to your personal information.
We will also check the levels of antibodies in the sample. It may be necessary in the future to test these samples again, with new techniques that have not yet been discovered. Also any leftover samples will be kept for future studies asking new questions relating to arthritis or SLE after obtaining the correct ethical and regulatory approval. By giving

consent, you would be prospectively agreeing to allow us to conduct this further research using your blood samples and information from your medical records relevant to this research.
If you agree to part of your sample being used for genetic analysis, it will be given a second unique code and sent to the Institute of Health and Medical Research in Paris.
We will not provide you with any feedback or results from research analysis conducted on your sample.

Will I benefit?
You will not benefit directly from taking part in this study at this time, but the information we get will help us to design better future therapies for people with autoimmune diseases.

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PART 2 – CONDUCT OF THIS STUDY

Will my involvement be kept confidential?
Yes. We follow ethical and legal codes of practice to ensure that all information about you and your involvement is handled in confidence. Some parts of your medical records may need to be accessed by authorised NHS staff and/or during a formal inspection by regulatory bodies during monitoring or auditing of the study. These staff have a duty of confidentiality to you as a research participant. All information about you will be anonymised, stored in locked cabinets and encrypted computer files, and kept strictly confidential.

What will happen to the results from this study?
Everything we discover through this research will be made as freely available as possible, through publishing in medical journals. However, these scientific results take time to collate and may not be available for several years. No personal information that could be used to identify you will ever be published or made available to anyone not directly involved in your clinical care or this research.

Can I withdraw from this study?
Yes. You can withdraw from the study at any time without giving a reason, and this would not affect the care you receive. We would then destroy any stored samples and data that had already been collected.

How is this study organised and funded?
This study is part of a collaboration between several research institutions across Europe, funded by the Innovative Medicines Initiative (www.imi.europa.eu). The research in London is organised by Dr. Jessica Manson, Prof. David Isenberg, Prof. Claudia Mauri and Dr. Elizabeth Jury. Nobody, including your doctor, receives any payment for being involved in this study.

Who has evaluated this study?
An independent committee reviews all research in the NHS in order to protect your interests. This study has been given approval by the National Research Ethics Service, following a review by a panel of healthcare professionals and volunteers from the public.

What if there is a problem or what happens if something goes wrong?
If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated by members of staff you may have experienced due to your participation in the research, National Health Service or UCL complaints mechanisms are available to you. Please ask your research doctor if you would like more information on this.
In the unlikely event that you are harmed by taking part in this study, compensation may be available.
If you suspect that the harm is the result of the Sponsor's (University College London) or the hospital's negligence then you may be able to claim compensation. After discussing with your research doctor, please make the claim in writing to Dr Elizabeth Jury who is the Chief Investigator for the research and is based at the Centre for Rheumatology Research, Rayne Building, University College London. Chief Investigator will then pass the claim to the Sponsor's Insurers, via the Sponsor's office. You may have to bear the costs of the legal action initially, and you should consult a lawyer about this.

How can I get further information or make a complaint?
If you have a concern or question about any aspect of this study, you should speak to the researchers involved for further information (jessica.manson@uclh.nhs.uk, 0203 447 9035, d.isenberg@ucl.ac.uk, 0203 447 9143). General information about this study, and the wider collaboration of which it is part, can be found on the ABIRISK website (www.abirisk.eu). If you wish to make a formal complaint, this can be done through the UCLH Patient Advice and Liaison Service; ask any member of staff or make contact by email (pals@uclh.nhs.uk).

This study is covered by Insurance Z6364106/2014/07/64. No Fault Compensation for Clinical Trials and/or Human Volunteer Studies

Please ask any questions you may have.

Dr Jessica Manson: Centre for Rheumatology & Principal Investigator for this study

A.3. Full list of LEGENDScreen™ markers.

Plate location, maker name, antibody clone, and antibody isotype.

Plate 1

	Marker	Clone	Isotype
A1	Blank		
A2	CD1a	HI149	Mouse IgG1, κ
A3	CD1b	SN13 (K5-1B8)	Mouse IgG1, κ
A4	CD1c	L161	Mouse IgG1, κ
A5	CD1d	51.1	Mouse IgG2b, κ
A6	CD2	RPA-2.10	Mouse IgG1, κ
A7	CD3	HIT3a	Mouse IgG2a, κ
A8	CD4	RPA-T4	Mouse IgG1, κ
A9	CD5	UCHT2	Mouse IgG1, κ
A10	CD6	BL-CD6	Mouse IgG1, κ
A11	CD7	CD7-6B7	Mouse IgG2a, κ
A12	CD8a	HIT8a	Mouse IgG1, κ
B1	CD9	HI9a	Mouse IgG1, κ
B2	CD10	HI10a	Mouse IgG1, κ
B3	CD11a	HI111	Mouse IgG1, κ
B4	CD11b	ICRF44	Mouse IgG1, κ
B5	CD11b (activated)	CBRM1/5	Mouse IgG1, κ
B6	CD11c	3.9	Mouse IgG1, κ
B7	CD13	WM15	Mouse IgG1, κ
B8	CD14	M5E2	Mouse IgG2a, κ
B9	CD15 (SSEA-1)	W6D3	Mouse IgG1, κ
B10	CD16	3G8	Mouse IgG1, κ
B11	CD18	TS1/18	Mouse IgG1, κ
B12	CD19	HIB19	Mouse IgG1, κ
C1	CD20	2H7	Mouse IgG2b, κ
C2	CD21	Bu32	Mouse IgG1, κ
C3	CD22	HIB22	Mouse IgG1, κ
C4	CD23	EBVCS-5	Mouse IgG1, κ
C5	CD24	ML5	Mouse IgG2a, κ
C6	CD25	BC96	Mouse IgG1, κ
C7	CD26	BA5b	Mouse IgG2a, κ
C8	CD27	O323	Mouse IgG1, κ
C9	CD28	CD28.2	Mouse IgG1, κ
C10	CD29	TS2/16	Mouse IgG1, κ
C11	CD30	BY88	Mouse IgG1, κ
C12	CD31	WM59	Mouse IgG1, κ
D1	CD32	FUN-2	Mouse IgG2b, κ
D2	CD33	WM53	Mouse IgG1, κ
D3	CD34	581	Mouse IgG1, κ
D4	CD35	E11	Mouse IgG1, κ
D5	CD36	5-271	Mouse IgG2a, κ
D6	CD38	HIT2	Mouse IgG1, κ
D7	CD39	A1	Mouse IgG1, κ
D8	CD40	HB14	Mouse IgG1, κ
D9	CD41	HIP8	Mouse IgG1, κ
D10	CD42b	HIP1	Mouse IgG1, κ
D11	CD43	CD43-10G7	Mouse IgG1, κ
D12	CD44	BJ18	Mouse IgG1, κ
E1	CD45	HI30	Mouse IgG1, κ
E2	CD45RA	HI100	Mouse IgG2b, κ
E3	CD45RB	MEM-55	Mouse IgG2b, κ
E4	CD45RO	UCHL1	Mouse IgG2a, κ
E5	CD46	TRA-2-10	Mouse IgG1
E6	CD47	CC2C6	Mouse IgG1, κ
E7	CD48	BJ40	Mouse IgG1, κ
E8	CD49a	TS2/7	Mouse IgG1, κ
E9	CD49c	ASC-1	Mouse IgG1, κ
E10	CD49d	9F10	Mouse IgG1, κ
E11	CD49e	NKI-SAM-1	Mouse IgG2b, κ
E12	CD49f	GoH3	Rat IgG2a, κ
F1	CD50 (ICAM-3)	CBR-IC3/1	Mouse IgG1, κ
F2	CD51	NKI-M9	Mouse IgG2a, κ
F3	CD51/61	23C6	Mouse IgG1, κ
F4	CD52	HI186	Mouse IgG2b, κ
F5	CD53	HI29	Mouse IgG1, κ
F6	CD54	HA58	Mouse IgG1, κ
F7	CD55	JS11	Mouse IgG1, κ
F8	CD56 (NCAM)	HCD56	Mouse IgG1, κ
F9	CD57	HCD57	Mouse IgM, κ
F10	CD58	TS2/9	Mouse IgG1, κ
F11	CD59	p282 (H19)	Mouse IgG2a, κ
F12	CD61	VI-PL2	Mouse IgG1, κ
G1	CD62E	HAE-1f	Mouse IgG1, κ
G2	CD62L	DREG-56	Mouse IgG1, κ
G3	CD62P (P-Selectin)	AK4	Mouse IgG1, κ
G4	CD63	H5C6	Mouse IgG1, κ
G5	CD64	10.1	Mouse IgG1, κ
G6	CD66a/c/e	ASL-32	Mouse IgG2b, κ
G7	CD66b	G10F5	Mouse IgM, κ
G8	CD69	FN50	Mouse IgG1, κ
G9	CD70	113-16	Mouse IgG1, κ
G10	CD71	CY1G4	Mouse IgG2a, κ
G11	CD73	AD2	Mouse IgG1, κ
G12	CD74	LN2	Mouse IgG1, κ
H1	CD79b	CB3-1	Mouse IgG1, κ
H2	CD80	2D10	Mouse IgG1, κ
H3	CD81	5A6	Mouse IgG1, κ
H4	CD82	ASL-24	Mouse IgG1, κ
H5	CD83	HB15e	Mouse IgG1, κ
H6	CD84	CD84.1.21	Mouse IgG2a, κ
H7	CD85a (ILT5)	MKT5.1	Rat IgG2a, κ
H8	CD85d (ILT4)	42D1	Rat IgG2a, κ
H9	CD85g (ILT7)	17G10.2	Mouse IgG1, κ
H10	CD85h (ILT1)	24	Mouse IgG2b, κ
H11	CD85j (ILT2)	GHI/75	Mouse IgG2b, κ
H12	CD85k (ILT3)	ZM4.1	Mouse IgG1, κ

Plate 2

	Marker	Clone	Isotype
A1	Blank		
A2	CD86	IT2.2	Mouse IgG2b, κ
A3	CD87	VIM5	Mouse IgG1, κ
A4	CD88	S5/1	Mouse IgG2a, κ
A5	CD89	A59	Mouse IgG1, κ
A6	CD90 (Thy1)	5.00E+10	Mouse IgG1, κ
A7	CD93	VIMD2	Mouse IgG1, κ
A8	CD94	DX22	Mouse IgG1, κ
A9	CD95	DX2	Mouse IgG1, κ
A10	CD96	NK92.39	Mouse IgG1, κ
A11	CD97	VIM3b	Mouse IgG1, κ
A12	CD99	HCD99	Mouse IgG2a, κ
B1	CD100	A8	Mouse IgG1, κ
B2	CD101 (BB27)	BB27	Mouse IgG1, κ
B3	CD102	CBR-IC2/2	Mouse IgG2a, κ
B4	CD103	Ber-ACT8	Mouse IgG1, κ
B5	CD104	58XB4	Mouse IgG2a, κ
B6	CD105	43A3	Mouse IgG1, κ
B7	CD106	STA	Mouse IgG1, κ
B8	CD107a (LAMP-1)	H4A3	Mouse IgG1, κ
B9	CD108	MEM-150	Mouse IgM, κ
B10	CD109	W7C5	Mouse IgG1, κ
B11	CD111	R1.302	Mouse IgG1, κ
B12	CD112 (Nectin-2)	TX31	Mouse IgG1, κ
C1	CD114	LMM741	Mouse IgG1, κ
C2	CD115	9-4D2-1E4	Rat IgG1, κ
C3	CD116	4H1	Mouse IgG1, κ
C4	CD117 (c-kit)	104D2	Mouse IgG1, κ
C5	CD119 (IFN-g R α chain)	GIR-208	Mouse IgG1, κ
C6	CD122	TU27	Mouse IgG1, κ
C7	CD123	6H6	Mouse IgG1, κ
C8	CD124	G077F6	Mouse IgG2a, κ
C9	CD126 (IL-6Rα)	UV4	Mouse IgG1, κ
C10	CD127 (IL-7Rα)	A019D5	Mouse IgG1, κ
C11	CD129 (IL-9 R)	AH9R7	Mouse IgG2b, κ
C12	CD131	1C1	Mouse IgG1, κ
D1	CD132	TUGh4	Rat IgG2b, κ
D2	CD134	Ber-ACT35 (ACT35)	Mouse IgG1, κ
D3	CD135	BV10A4H2	Mouse IgG1, κ
D4	CD137 (4-1BB)	4B4-1	Mouse IgG1, κ
D5	CD137L (4-1BB Ligand)	5F4	Mouse IgG1, κ
D6	CD138	DL-101	Mouse IgG1, κ
D7	CD140a	16A1	Mouse IgG2a, κ
D8	CD140b	18A2	Mouse IgG1, κ
D9	CD141	M80	Mouse IgG1, κ
D10	CD143	5-369	Mouse IgG1, κ
D11	CD144	BV9	Mouse IgG2a, κ
D12	CD146	SHM-57	Mouse IgG2a, κ
E1	CD148	A3	Mouse IgG1, κ
E2	CD150 (SLAM)	A12 (7D4)	Mouse IgG1, κ
E3	CD152	L3D10	Mouse IgG1, κ
E4	CD154	24-31	Mouse IgG1, κ
E5	CD155 (PVR)	SKII.4	Mouse IgG1, κ
E6	CD156c (ADAM10)	SHM14	Mouse IgG1, κ
E7	CD158a/h	HP-MA4	Mouse IgG2b, κ
E8	CD158b (KIR2DL2/L3, NKAT2)	DX27	Mouse IgG2a, κ
E9	CD158d	mAb 33 (33)	Mouse IgG1, κ
E10	CD158e1 (KIR3DL1, NKB1)	DX9	Mouse IgG1, κ
E11	CD158f	UP-R1	Mouse IgG1, κ
E12	CD161	HP-3G10	Mouse IgG1, κ
F1	CD162	KPL-1	Mouse IgG1, κ
F2	CD163	GHI/61	Mouse IgG1, κ
F3	CD164	67D2	Mouse IgG1, κ
F4	CD165	SN2 (N6-D11)	Mouse IgG1, κ
F5	CD166	3A6	Mouse IgG1, κ
F6	CD167a (DDR1)	51D6	Mouse IgG3, κ
F7	CD169	7-239	Mouse IgG1, κ
F8	CD170 (Siglec-5)	1A5	Mouse IgG1, κ
F9	CD172a (SIRPα)	SE5A5	Mouse IgG1, κ
F10	CD172b (SIRPb)	B4B6	Mouse IgG1, κ
F11	CD172g (SIRPg)	LSB2.20	Mouse IgG1, κ
F12	CD178 (Fas-L)	NOK-1	Mouse IgG1, κ
G1	CD179a	HSL96	Mouse IgG1, κ
G2	CD179b	HSL11	Mouse IgG1, κ
G3	CD180 (RP105)	MHR73-11	Mouse IgG1, κ
G4	CD181 (CXCR1)	8F1/CXCR1	Mouse IgG2b, κ
G5	CD182 (CXCR2)	5E8/CXCR2	Mouse IgG1, κ
G6	CD183	G025H7	Mouse IgG1, κ
G7	CD184 (CXCR4)	12G5	Mouse IgG2a, κ
G8	CD193 (CCR3)	5.00E+08	Mouse IgG2b, κ
G9	CD195 (CCR5)	T21/8	Mouse IgG1, κ
G10	CD196	G034E3	Mouse IgG2b, κ
G11	CD197 (CCR7)	G043H7	Mouse IgG2a, κ
G12	CD200 (OX2)	OX-104	Mouse IgG1, κ
H1	CD200 R	OX-108	Mouse IgG1, κ
H2	CD201 (EPCR)	RCR-401	Rat IgG1, κ
H3	CD202b (Tie2/Tek)	33.1 (Ab33)	Mouse IgG1, κ
H4	CD203c (E-NPP3)	NP4D6	Mouse IgG1, κ
H5	CD205 (DEC- 205)	HD30	Mouse IgG1, κ
H6	CD206 (MMR)	15-Feb	Mouse IgG1, κ
H7	CD207 (Langerin)	1.00E+03	Mouse IgG1, κ
H8	CD209 (DC- SIGN)	9E9A8	Mouse IgG2a, κ
H9	CD210 (IL- 10 R)	3F9	Rat IgG2a, κ
H10	CD213a2	SHM38	Mouse IgG1, κ
H11	CD215 (IL- 15Rα)	JM7A4	Mouse IgG2b, κ
H12	CD218a (IL-18Rα)	H44	Mouse IgG1, κ

Plate 3

	Marker	Clone	Isotype
A1	Blank		
A2	CD220	B6.220	Mouse IgG2b, κ
A3	CD221 (IGF-1R)	1H7/CD221	Mouse IgG1, κ
A4	CD226 (DNAM-1)	11A8	Mouse IgG1, κ
A5	CD229 (Ly-9)	HLy-9.1.25	Mouse IgG1, κ
A6	CD231 (TALLA)	SN1a (M3-3D9)	Mouse IgG1, κ
A7	CD235ab	HIR2	Mouse IgG2b, κ
A8	CD243	UIC2	Mouse IgG2a, κ
A9	CD244 (2B4)	C1.7	Mouse IgG1, κ
A10	CD245 (p220/240)	DY12	Mouse IgG1, κ
A11	CD252 (OX40L)	11C3.1	Mouse IgG1, κ
A12	CD253 (Trail)	RIK-2	Mouse IgG1, κ
B1	CD254	MIH24	Mouse IgG1, κ
B2	CD255 (TWEAK)	CARL-1	Mouse IgG3, κ
B3	CD257 (BAFF, BLYS)	T7-241	Mouse IgG1, κ
B4	CD258 (LIGHT)	T5-39	Mouse IgG2a, κ
B5	CD261 (DR4, TRAIL-R1)	DJR1	Mouse IgG1, κ
B6	CD262 (DR5, TRAIL-R2)	DJR2-4 (7-8)	Mouse IgG1, κ
B7	CD263 (DcR1, TRAIL-R3)	DJR3	Mouse IgG1, κ
B8	CD266 (Fn14, TWEAK Receptor)	ITEM-1	Mouse IgG1, κ
B9	CD267 (TACI)	1A1	Rat IgG2a, κ
B10	CD268 (BAFF-R, BAFFR)	11C1	Mouse IgG1, κ
B11	CD270 (HVEM)	122	Mouse IgG1, κ
B12	CD271	ME20.4	Mouse IgG1, κ
C1	CD273 (B7- DC, PD-L2)	24F.10C12	Mouse IgG2a, κ
C2	CD274 (B7- H1, PD-L1)	29E.2A3	Mouse IgG2b, κ
C3	CD275 (B7- H2, B7-RP1, ICOSL)	9F.8A4	Mouse IgG1, κ
C4	CD276	MIH42	Mouse IgG1, κ
C5	CD277	BT3.1	Mouse IgG1, κ
C6	CD278 (ICOS)	C398.4A	Arm. Hamster IgG
C7	CD279 (PD-1)	EH12.2H7	Mouse IgG1, κ
C8	CD282 (TLR2)	TL2.1	Mouse IgG2a, κ
C9	CD284 (TLR4)	HTA125	Mouse IgG2a, κ
C10	CD286 (TLR6)	TLR6.127	Mouse IgG1, κ
C11	CD290	3C10C5	Mouse IgG1, κ
C12	CD294	BM16	Rat IgG2a, κ
D1	CD298	LNH-94	Mouse IgG1, κ
D2	CD300e (IREM-2)	UP-H2	Mouse IgG1, κ
D3	CD300F	UP-D2	Mouse IgG1, κ
D4	CD301	H037G3	Mouse IgG2a, κ
D5	CD303	201A	Mouse IgG2a, κ
D6	CD304	12C2	Mouse IgG2a, κ
D7	CD307	509f6	Mouse IgG2a, κ
D8	CD307d (FcRL4)	413D12	Mouse IgG2b, κ
D9	CD314 (NKG2D)	1D11	Mouse IgG1, κ
D10	CD317	RS38E	Mouse IgG1, κ
D11	CD318 (CDCP1)	CUB1	Mouse IgG2b, κ
D12	CD319 (CRACC)	162.1	Mouse IgG2b, κ
E1	CD324 (E- Cadherin)	67A4	Mouse IgG1, κ
E2	CD325	8C11	Mouse IgG1, κ
E3	CD326 (Ep- CAM)	9C4	Mouse IgG2b, κ
E4	CD328 (Siglec-7)	6-434	Mouse IgG1, κ
E5	CD334 (FGFR4)	4FR6D3	Mouse IgG1, κ
E6	CD335 (NKp46)	9.00E+02	Mouse IgG1, κ
E7	CD336 (NKp44)	P44-8	Mouse IgG1, κ
E8	CD337 (NKp30)	P30-15	Mouse IgG1, κ
E9	CD338 (ABCG2)	5D3	Mouse IgG2b, κ
E10	CD340 (erbB2/ HER-2)	24D2	Mouse IgG1, κ
E11	CD344 (Frizzled-4)	CH3A4A7	Mouse IgG1, κ
E12	CD351	TX61	Mouse IgG1, κ
F1	CD352 (NTB-A)	NT-7	Mouse IgG1, κ
F2	CD354 (TREM-1)	TREM-26	Mouse IgG1, κ
F3	CD355 (CRTAM)	Cr24.1	Mouse IgG2a, κ
F4	CD357 (GITR)	621	Mouse IgG1, κ
F5	CD360 (IL- 21R)	2G1-K12	Mouse IgG1, κ
F6	β2- micro- globulin	2M2	Mouse IgG1, κ
F7	BTLA	MIH26	Mouse IgG2a, κ
F8	C3AR	hC3aRZ8	Mouse IgG2b
F9	C5L2	1D9-M12	Mouse IgG2a, κ
F10	CCR10	6588-5	Arm. hamster IgG
F11	CLEC12A	50C1	Mouse IgG2a, κ
F12	CLEC9A	8F9	Mouse IgG2a, κ
G1	CX3CR1	2A9-1	Rat IgG2b, κ
G2	CXCR7	8F11-M16	Mouse IgG2b, κ
G3	δ-Opioid Receptor	DOR7D2A4	Mouse IgG2b, κ
G4	DLL1	MHD1-314	Mouse IgG1, κ
G5	DLL4	MHD4-46	Mouse IgG1, κ
G6	DR3 (TRAMP)	JD3	Mouse IgG1, κ
G7	EGFR	AY13	Mouse IgG1, κ
G8	erbB3/HER-3	1B4C3	Mouse IgG2a, κ
G9	FcεRIα	AER-37 (CRA-1)	Mouse IgG2b, κ
G10	FcRL6	2H3	Mouse IgG2b, κ
G11	Galec n-9	9M1-3	Mouse IgG1, κ
G12	GARP (LRRC32)	7B11	Mouse IgG2b, κ
H1	HLA-A,B,C	W6/32	Mouse IgG2a, κ
H2	HLA-A2	BB7.2	Mouse IgG2b, κ
H3	HLA-DQ	HLADQ1	Mouse IgG1, κ
H4	HLA-DR	L243	Mouse IgG2a, κ
H5	HLA-E	3D12	Mouse IgG1, κ
H6	HLA-G	87G	Mouse IgG2a, κ
H7	IFN-g R b chain	2HUB-159	Hamster IgG
H8	Ig light chain k	MHK-49	Mouse IgG1, κ
H9	Ig light chain λ	MHL-38	Mouse IgG2a, κ
H10	IgD	IA6-2	Mouse IgG2a, κ
H11	IgM	MHM-88	Mouse IgG1, κ
H12	IL-28RA	MHLICR2a	Mouse IgG2a, κ

Plate 4

	Marker	Clone	Isotype
A1	Blank		
A2	Integrin α9β1	Y9A2	Mouse IgG1, κ
A3	integrin β5	AST-3T	Mouse IgG2a, κ
A4	integrin β7	FIB504	Rat IgG2a, κ
A5	Jagged 2	MHJ2-523	Mouse IgG1, κ
A6	LAP	TW4-6H10	Mouse IgG1, κ
A7	Lymphotoxin b Receptor (LT-bR)	31G4D8	Mouse IgG2b, κ
A8	Mac-2 (Ga- lec n-3)	Gal397	Mouse IgG1, κ
A9	MAIR-II	TX45	Mouse IgG1, κ
A10	MICA/MICB	6D4	Mouse IgG2a, κ
A11	MSC (W3D5)	W3D5	Mouse IgG2a, κ
A12	MSC (W5C5)	W5C5	Mouse IgG1, κ
B1	MSC (W7C6)	W7C6	Mouse IgG1, κ
B2	MSC and NPC (W4A5)	W4A5	Mouse IgG1, κ
B3	MSCA-1 (MSC, W8B2)	W8B2	Mouse IgG1, κ
B4	NKp80	5D12	Mouse IgG1, κ
B5	Notch 1	MHN1-519	Mouse IgG1, κ
B6	Notch 2	MHN2-25	Mouse IgG2a, κ
B7	Notch 3	MHN3-21	Mouse IgG1, κ
B8	Notch 4	MHN4-2	Mouse IgG1, κ
B9	NPC (57D2)	57D2	Mouse IgG1, κ
B10	Podoplanin	NC-08	Rat IgG2a, λ
B11	Pre-BCR	HSL2	Mouse IgG1, κ
B12	PSMA	LNI-17	Mouse IgG1, κ
C1	Siglec-10	5G6	Mouse IgG1, κ
C2	Siglec-8	7C9	Mouse IgG1, κ
C3	Siglec-9	K8	Mouse IgG1, κ
C4	SSEA-1	MC-480	Mouse IgM, κ
C5	SSEA-3	MC-631	Rat IgM, κ
C6	SSEA-4	MC-813-70	Mouse IgG3, κ
C7	SSEA-5	8.00E+11	Mouse IgG1, κ
C8	TCR g/d	B1	Mouse IgG1, κ
C9	TCR Vβ13.2	H132	Mouse IgG1, κ
C10	TCR Vβ23	oHUT7	Mouse IgG1, κ
C11	TCR Vβ8	JR2 (JR.2)	Mouse IgG2b, κ
C12	TCR Vβ9	MKB1	Mouse IgG2b, κ
D1	TCR Vδ2	B6	Mouse IgG1, κ
D2	TCR Vg9	B3	Mouse IgG1, κ
D3	TCR Vα24- Jα18	6B11	Mouse IgG1, κ
D4	TCR Vα7.2	3C10	Mouse IgG1, κ
D5	TCR α/β	IP26	Mouse IgG1, κ
D6	Tim-1	1D12	Mouse IgG1, κ
D7	Tim-3	F38-2E2	Mouse IgG1, κ
D8	Tim-4	9F4	Mouse IgG1, κ
D9	TLT-2	MIH61	Mouse IgG1, κ
D10	TRA-1-60-R	TRA-1-60-R	Mouse IgM, κ
D11	TRA-1-81	TRA-1-81	Mouse IgM, κ
D12	TSLPR (TSLP-R)	1B4	Mouse IgG1, κ
E1	Ms IgG1, κ ITCL	MOPC-21	Mouse IgG1, κ
E2	Ms IgG2a, κ ITCL	MOPC-173	Mouse IgG2a, κ
E3	Ms IgG2b, κ ITCL	MPC-11	Mouse IgG2b, κ
E4	Ms IgG3, κ ITCL	MG3-35	Mouse IgG3, κ
E5	Ms IgM, κ ITCL	MM-30	Mouse IgM, κ
E6	Rat IgG1, κ ITCL	RTK2071	Rat IgG1, κ
E7	Rat IgG2a, κ ITCL	RTK2758	Rat IgG2a, κ
E8	Rat IgG2b, κ ITCL	RTK4530	Rat IgG2b, κ
E9	Rat IgM, κ ITCL	RTK2118	Rat IgM, κ
E10	AH IgG, ITCL	HTK888	Arm. Hamster IgG
E11	Blank		
E12	Blank		
F1	Blank		
F2	Blank		
F3	Blank		
F4	Blank		
F5	Blank		
F6	Blank		
F7	Blank		
F8	Blank		
F9	Blank		
F10	Blank		
F11	Blank		
F12	Blank		
G1	Blank		
G2	Blank		
G3	Blank		
G4	Blank		
G5	Blank		
G6	Blank		
G7	Blank		
G8	Blank		
G9	Blank		
G10	Blank		
G11	Blank		
G12	Blank		
H1	Blank		
H2	Blank		
H3	Blank		
H4	Blank		
H5	Blank		
H6	Blank		
H7	Blank		
H8	Blank		
H9	Blank		
H10	Blank		
H11	Blank		
H12	Blank		

A.4. LEGENDScreen™ ADA results.

Differentially expressed markers (*t*-test, $p<0.005$) on B cells (total) and mature, immature and memory B cell subsets from ADA^+ vs. ADA^- adalimumab treated RA patients.

Total B cells

	P value	Mean ADA^-	Mean ADA^+	Fold change
CD158f	0.002	14.4	3.6	4.0
Notch 4	0.003	10.3	4.5	2.3
CD105	0.007	50.6	20.3	2.5
CD158d	0.009	16.6	6.7	2.5
CD1a	0.009	7.6	3.1	2.4
CD138	0.010	13.6	4.2	3.2
CD226	0.010	25.4	6.8	3.7
DR3	0.016	17.5	6.7	2.6
CD1c	0.019	86.6	67.9	1.3
CD111	0.022	39.6	5.5	7.2
CD338	0.024	10.3	5.4	1.9
CD275	0.024	80.5	61.7	1.3
CD87	0.026	33.0	11.0	3.0
CD172a	0.027	22.1	8.5	2.6
Nectin-2	0.031	31.5	11.1	2.8
CD324	0.037	35.4	3.7	9.7
CD276	0.038	22.1	6.5	3.4
CD167a	0.039	21.1	11.6	1.8
CD262	0.042	18.5	11.8	1.6
CD165	0.043	81.2	67.2	1.2
CD178	0.046	6.8	2.7	2.5
FcRL6	0.048	3.4	13.0	0.3

Mature B cells

	P value	Mean ADA^-	Mean ADA^+	Fold change
CD158f	0.010	7.3	2.0	3.6
CD167a	0.012	17.5	9.4	1.9
CD138	0.018	6.9	2.7	2.5
CD1c	0.021	82.8	73.2	1.1

Immature B cells

	P value	Mean ADA^-	Mean ADA^+	Fold change
IL-7Ra	0.005	7.8	1.9	4.1
CD138	0.005	13.3	3.8	3.4
DR3	0.022	16.8	7.4	2.3

CD87	0.022	32.9	11.0	3.0
CD158f	0.027	15.5	5.5	2.8
CD324	0.036	34.5	3.0	11.6
CD105	0.046	49.5	27.2	1.8

Memory B cells

	P value	Mean ADA^-	Mean ADA^+	Fold change
CD158d	0.004	24.6	9.4	2.6
CD87	0.010	37.8	13.8	2.7
CD172a	0.012	30.2	10.3	2.9
CD111	0.013	42.8	4.7	9.1
CD167a	0.017	24.9	14.4	1.7
CD275	0.020	63.4	48.6	1.3
CD226	0.021	31.6	13.2	2.4
CD138	0.021	18.4	5.4	3.4
CD59	0.024	99.7	98.8	1.0
CD158f	0.028	19.3	5.6	3.4
CD1a	0.028	11.3	5.3	2.2
Notch 4	0.034	13.0	5.8	2.2
CD324	0.040	35.6	4.6	7.8
CD63	0.040	99.6	98.8	1.0
CD109	0.042	17.3	5.9	2.9
CD1c	0.044	89.4	80.9	1.1
CD178	0.044	10.7	3.5	3.0
CD338	0.048	17.4	9.8	1.8
CD97	0.048	74.1	54.2	1.4

A.5. LEGENDScreen™ results; markers significantly correlated with DAS28, on total B cells and B cell subsets (p>0.05). RA patients.

B cells

	r	P value
BTLA	-0.521	0.006
CD102	-0.432	0.019
CD11a	-0.544	0.002
CD11c	0.470	0.013
CD134	0.497	0.006
CD135	0.398	0.033
CD152	0.404	0.033
ADAM10	-0.523	0.004
CD180	-0.418	0.024
CD184	-0.444	0.016
CD196	-0.434	0.019
CD200	-0.460	0.012
CD22	-0.416	0.025
CD229	0.401	0.034
CD231	-0.525	0.003
CD245	-0.450	0.014
CD252	0.381	0.046
CD26	0.382	0.041
CD267	0.425	0.022
CD268	-0.373	0.046
CD270	-0.443	0.018
CD275	-0.410	0.027
CD319	0.539	0.003
CD35	-0.415	0.025
CD45RA	-0.410	0.027
CD47	-0.529	0.003
CD48	-0.484	0.008
CD53	-0.436	0.018
CD55	-0.380	0.042
CD74	-0.413	0.026
CD99	-0.387	0.038
HLA-A2	0.413	0.036
HLA-DQ	-0.425	0.024
HLA-DR	-0.419	0.027
IgD	-0.412	0.026

Mature B cells

	r	P value
CD102	-0.505	0.005
CD11a	-0.516	0.004
CD11c	0.458	0.016
CD134	0.499	0.006
CD152	0.415	0.028
ADAM10	-0.443	0.016
CD166	-0.387	0.038
CD196	-0.383	0.040
CD229	0.425	0.024
CD231	-0.519	0.004
CD24	-0.486	0.008
CD245	-0.434	0.019
CD270	-0.432	0.022
CD319	0.529	0.004
CD44	-0.480	0.008
CD47	-0.500	0.006
CD48	-0.443	0.016
CD55	-0.375	0.045
CD99	-0.411	0.030
HLA-A2	0.425	0.031

Immature B cells

	r	P value
β2 microglobulin	0.427	0.029
BTLA	-0.436	0.023
CD11a	-0.418	0.024
CD11c	0.422	0.028
CD134	0.408	0.028
CD148	-0.447	0.015
ADAM10	-0.405	0.030
CD158d	-0.375	0.049
CD184	-0.510	0.006
CD200	-0.424	0.022
CD209	0.376	0.044
CD215	0.374	0.050
CD229	0.448	0.017
CD231	-0.488	0.007
CD252	0.435	0.021

CD275	-0.389	0.037
CD31	-0.424	0.022
CD319	0.602	0.001
CD360	-0.385	0.047
CD44	-0.545	0.002
CD48	-0.417	0.027
CD49d	-0.403	0.034
CD53	-0.382	0.041
CD55	-0.428	0.023
CD6	0.375	0.045
CD84	-0.372	0.047
CD86	0.446	0.017
CD9	-0.412	0.027
Ig light chain λ	-0.435	0.024
IgD	-0.399	0.035
IL-28RA	0.602	0.001
MICA-MICB	0.381	0.042

Memory B cells

	r	P value
β2 microglobulin	0.419	0.033
BTLA	-0.466	0.014
CCR10	-0.432	0.022
CD11a	-0.398	0.033
CD134	0.475	0.009
ADAM10	-0.449	0.015
CD196	-0.403	0.030

CD200R	-0.421	0.023
CD22	-0.515	0.004
CD231	-0.533	0.003
CD245	-0.454	0.013
CD270	-0.386	0.047
CD275	-0.368	0.050
CD307d	-0.380	0.042
CD314	-0.439	0.017
CD338	-0.442	0.018
CD35	-0.391	0.036
CD352	-0.424	0.025
CD39	-0.425	0.024
CD45	-0.382	0.041
CD47	-0.506	0.005
CD48	-0.424	0.025
CD49d	-0.417	0.027
CD50	-0.478	0.009
CD52	-0.371	0.048
CD53	-0.408	0.028
CD55	-0.484	0.009
CD71	-0.516	0.004
CD81	-0.390	0.036
HLA-DQ	-0.449	0.017
HLA-E	-0.377	0.048
NKp80	-0.432	0.019
Notch 1	-0.382	0.045

A.6. Differentially expressed markers from LEGENDScreen™ analysis for HC vs. RA. Differentially expressed markers for T cells, B cells and B cell subsets, following removal of non-expressed markers. Significant markers only; fold change (ratio of RA:HC) and p value (*t*-test).

T cells

	Fold Change	P Value
CD97	0.056	0.000
CD48	0.000	0.006
CD52	0.002	0.024
CD164	1.081	0.028
CD43	1.081	0.033
CD49c	1.435	0.038
CD134	1.733	0.042
CD53	0.897	0.042
	Total	**9**

Total B cells

	Fold Change	P Value
CD97	1.910	<0.001
CD150	2.084	<0.001
CD162	1.657	0.001
CD158d	3.122	0.002
Ig light chain κ	1.177	0.002
CD107a	1.285	0.004
CD231	1.887	0.004
CD275	1.454	0.005
CD62L	1.111	0.007
CD105	2.599	0.008
CD335	6.908	0.008
CD23	1.222	0.009
CD166	1.228	0.012
CD123	1.929	0.012
NKp80	4.510	0.013
CD183	0.708	0.017
CD307	1.631	0.018
Notch 1	1.605	0.020
CD2	1.684	0.021
Notch 4	1.968	0.022
Galectin-9	0.402	0.022
CD164	1.037	0.023
CD172a	2.005	0.024
CD314	2.214	0.035
CD148	1.219	0.037
HLA-E	1.051	0.038
CD245	1.375	0.042
CD15	3.697	0.043
CD16	2.589	0.043
CD61	2.031	0.044
CD317	1.127	0.045
CD94	3.824	0.048
Notch 2	1.456	0.049
CD11b	0.761	0.050
	Total	**34**

Immature B cells

	Fold Change	P Value
Ig light chain k	1.213	<0.001
CD71	1.414	<0.001
Ig light chain λ	1.365	<0.001
β2 microglobulin	0.531	0.001
CD23	1.239	0.001
CD170	1.784	0.001
CD307	1.953	0.001
CD166	1.487	0.001
CD243	1.383	0.001
CD317	1.364	0.001
CD107a	1.547	0.002
CD181	0.333	0.002
TRA-1-81	0.374	0.002
Notch 2	1.964	0.002
CD197	1.270	0.002
CD162	1.499	0.003
CD1d	1.103	0.003
CD55	1.039	0.003
CD231	1.914	0.003
CD245	1.607	0.003
CD164	1.326	0.004
CD97	2.165	0.004
CD150 (SLAM)	2.239	0.004
CD290	1.395	0.004
Siglec-8	0.344	0.005
CD84	1.184	0.006
CD119 (IFNgR chain)	1.235	0.009
CD158d	3.216	0.009

Galectin-9	0.379	0.010
CD74	1.248	0.012
CD184	1.188	0.015
CD63	1.193	0.016
NKp80	7.331	0.019
NPC	0.429	0.020
CD39	1.110	0.023
CD11a	1.008	0.024
CD8a	2.469	0.026
CD62L	1.194	0.027
CD123	1.554	0.030
CD1c	1.086	0.034
CD132	1.261	0.034
CD275	1.251	0.037
CD335	3.982	0.039
CD99	1.122	0.040
CD3	1.826	0.046
CD172a	1.628	0.047
CD49f	1.164	0.047
SSEA-5	0.476	0.049
CD16	2.294	0.050
	Total	**49**

Mature B cells

	Fold Change	P value
Ig light chain κ	1.258	<0.001
CD150	2.215	<0.001
CD97	2.152	<0.001
CD107a	1.448	<0.001
CD62L	1.186	0.001
CD162	1.671	0.002
CD158d	3.640	0.002
CD307	2.261	0.002
CD23	1.160	0.002
CD231	2.028	0.002
CD71	1.144	0.002
CD166	1.409	0.002
CD275	1.483	0.003
CD148	1.437	0.005
CD119	1.269	0.007
CD243	1.230	0.008
CD84	1.203	0.008
CD245	1.608	0.008
CD290	1.465	0.012
CD74	1.176	0.015
Ig light chain λ	1.300	0.015
CD317	1.223	0.015
CD2	2.050	0.017
Notch 1	1.844	0.017
CD11c	1.982	0.017
CD335	10.038	0.018
CD164	1.123	0.018
NKp80	7.062	0.021
Notch 2	1.669	0.022
CD124	1.253	0.022
CD123	1.936	0.024
CD8a	2.339	0.025
TLT-2	1.234	0.026
CD21	0.982	0.031
CD172a	2.586	0.032
CD3	1.916	0.035
CD183	0.656	0.037
CD61	2.432	0.045
CD15	4.565	0.046
CD226	2.478	0.049
	Total	**40**

Memory B cells

	Fold Change	P value
CD97	2.003	<0.001
Ig light chain κ	1.281	<0.001
CD11c	1.692	<0.001
Ig light chain λ	1.472	<0.001
CD162	2.243	<0.001
CD158d	5.174	0.001
CD71	1.218	0.001
CD107a	1.390	0.001
CD62L	1.182	0.001
CD335	9.595	0.002
CD88	2.368	0.002
CD307	2.022	0.002
CD231	2.049	0.002
CD290	1.402	0.003
CD116	1.901	0.003
CD226	2.541	0.004
CD23	1.736	0.004
CD2	1.694	0.004
CD184	1.251	0.005
CD275	1.663	0.005
CD338	2.640	0.005
CD150	2.520	0.006
CD55	1.017	0.006

CD245	1.637	0.006
NKp80	4.337	0.006
CD124	2.031	0.007
CCR10	1.605	0.007
IgM	1.186	0.007
CD262	1.798	0.008
CD243	1.205	0.008
CD276	3.305	0.011
CD123	2.674	0.011
CD80	1.606	0.011
CD261	1.707	0.011
Notch 2	1.738	0.011
CD166	1.226	0.013
CD114	4.642	0.013
CD164	1.136	0.015
CD220	2.328	0.017
Notch 4	2.643	0.018
CD14	3.726	0.018
CD314	2.345	0.018
CD172a	2.245	0.018
CD84	1.157	0.019
CD49f	1.183	0.020
Nectin-2	3.166	0.022
CD26	1.799	0.022
CD74	1.124	0.022
CD307d	1.994	0.023
CD141	2.220	0.027
CD148	1.203	0.028
CD5	1.710	0.028
CD200R	1.338	0.030
CD108	1.349	0.031
CD134	5.136	0.031
CD16	2.937	0.031
CD131	1.991	0.032
CD66a-c-e	1.557	0.032
CD143	2.153	0.033
CD193	0.498	0.035
CD83	2.080	0.036
CD202b	4.071	0.038
CD15	3.884	0.039
CD197	1.128	0.039
Integrin β5	2.905	0.041
CD45	1.137	0.042
CD61	2.013	0.042
CD195	1.946	0.042
CD115	2.176	0.044
CD1d	1.061	0.047
CD158f	2.533	0.049
CD138	3.476	0.050
	Total	**72**

Table A.7. LEGENDScreen™ results for HC vs. RA treated with cDMARDs (RA-DMARDs) and HC vs. RA treated with adalimumab (RA-Adalimumab), for each cell subset. Marker and p value given (multiple *t*-test).

Total B cells - HC vs. RA-DMARDs

Marker	P value
CD97	<0.0001
CD150 (SLAM)	0.000895
CD32	0.002278
CD45	0.00239
CD82	0.002767
CD18	0.002997
CD172a	0.010581
CD81	0.010982
CD47	0.016066
CD21	0.016491
CD35	0.018505
CD270	0.022873
CD11c	0.023862
CD307	0.025101
CD53	0.027614
Integrin alpha9 beta1	0.032312
CD158d	0.042852
CD57	0.045233
CD131	0.049928

Total B cells - HC vs. RA-adalimumab

Marker	P value
CD162	0.00009
CD231	0.000744
CD158d	0.00134
CD150 (SLAM)	0.001495
CD57	0.001566
CD97	0.00328
NKp80	0.003309
CD226	0.004921
CD2	0.004944
CD105	0.005777
CD335	0.00605
beta2 microglobulin	0.006387
CD314	0.006656
CD94	0.007913
CD15	0.011078
CD6	0.011447
Jagged2	0.012755
Integrin alpha9 beta1	0.013646
CD109	0.013661
CD275	0.016113
CD245	0.01854
CD172a	0.018806
CD338	0.019309
CD16	0.020713
CD66b	0.021727
Ig light chain lambda	0.02239
CD112 (Nectin-2)	0.023109
CD183	0.023225
CD14	0.024567
CD61	0.025375
CD88	0.025923
CD108	0.028843
Ig light chain k	0.030476
CD107a	0.035731
CD213a2	0.037165
delta-Opioid Receptor	0.03802
CD229	0.04022
Notch 4	0.044316
CD114	0.045636

Mature B cells- HC vs. RA-DMARDs

Marker	P value
CD97	0.0006
CD19	0.0006
CD150 (SLAM)	0.0038
CD11c	0.0047
CD158d	0.0134
CD307	0.0182
CD172a	0.0211

CD62L	0.0225
Ig light chain k	0.0290
CD41	0.0499

Mature B cells- HC vs. RA-adalimumab

Marker	P value
CD162	0.0002
CD231	0.0003
CD226	0.0003
Ig light chain k	0.0005
CD158d	0.0006
CD150 (SLAM)	0.0007
CD107a	0.0011
CD8a	0.0011
CD2	0.0013
CD307	0.0019
CD245	0.0020
Ig light chain lambda	0.0021
CD148	0.0025
CD275	0.0026
CD166	0.0028
CD108	0.0038
CD97	0.0055
CD338	0.0067
NKp80	0.0075
CD11c	0.0091
CD74	0.0118
CD335	0.0128
CD314	0.0131
CD172a	0.0152
CD16	0.0179
CD15	0.0183
CD3	0.0189
CD119 (IFNgR a chain)	0.0199
CD229	0.0213
CD61	0.0237
CD6	0.0246
CD290	0.0255
CD317	0.0256
CD14	0.0293
CD23	0.0304
CD62L	0.0309
CD36	0.0314
Notch 1	0.0318
CD71	0.0327
CD82	0.0330
Notch 2	0.0369
TCR a-b	0.0420
CD21	0.0438
CD243	0.0453
CD112 (Nectin-2)	0.0470
CD88	0.0484
CD49c	0.0495

Immature B cells - HC vs. RA-DMARDs

Marker	P value
CD11c	0.0036
CD97	0.0045
CD150 (SLAM)	0.0105
CD27	0.0108
CD172a	0.0120
CD267	0.0122
CD32	0.0181
Notch 2	0.0195
CD9	0.0212
CD10	0.0236
CD307	0.0291
CD170	0.0306
CD104	0.0366
CD81	0.0423
FceRIalpha	0.0468
CD170	0.0135

Immature B cells - HC vs. RA-adalimumab

Marker	P value
beta2 microglobulin	0.0000
Ig light chain k	0.0002
CD162	0.0003
CD231	0.0004

Ig light chain lambda	0.0005
CD23	0.0009
CD66b	0.0009
CD158d	0.0013
CD245	0.0014
CD307	0.0018
CD226	0.0025
Jagged2	0.0036
CD166	0.0038
CD181	0.0050
CD170	0.0059
TRA-1-81	0.0065
CD275	0.0067
CD317	0.0069
CD71	0.0073
Integrin alpha9 beta1	0.0075
Notch 2	0.0085
CD107a	0.0086
CD150 (SLAM)	0.0088
CD15	0.0092
CD290	0.0101
NKp80	0.0108
CD39	0.0143
CD243	0.0153
CD119 (IFNgR a chain)	0.0195
CD8a	0.0196
CD74	0.0212
CD11a	0.0219
CD16	0.0233
CD6	0.0246
CD84	0.0248
CD63	0.0255
CD1c	0.0257
NPC (57D2)	0.0259
CD89	0.0272
CD229	0.0272
Notch 4	0.0297
CD88	0.0314
CD1d	0.0316
CD123	0.0355
CD205	0.0368
CD44	0.0380
CD335	0.0395
CCR10	0.0398
CD213a2	0.0400
CD55	0.0401
CD183	0.0406
delta-Opioid Receptor	0.0418
Galectin-9	0.0423
Siglec-8	0.0432
IL-28RA	0.0460
CD105	0.0467
CD314	0.0492
CD197	0.0492

Memory B cells - HC vs. RA-DMARDs

Marker	P value
CD11c	0.0001
CD97	0.0029
CD335	0.0057
CD150 (SLAM)	0.0160
CD47	0.0180
CD44	0.0184
CD226	0.0186
CD172a	0.0205
CD158d	0.0209
CD42b	0.0226
CD307	0.0287
CD114	0.0311
CD152	0.0327
CD57	0.0341
CD202b	0.0363
Integrin beta5	0.0381
Integrin alpha9 beta1	0.0415
CD134	0.0439
Ig light chain k	0.0442
HLA-A2	0.0453

Memory B cells - HC vs. RA-adalimumab

Marker	P value
CD226	0.0000

CD338	0.0000
CD158d	0.0000
CD162	0.0001
CD108	0.0003
Integrin alpha9 beta1	0.0004
CD231	0.0004
Ig light chain lambda	0.0004
CD57	0.0005
CD245	0.0007
CD2	0.0010
CD11c	0.0011
CD14	0.0012
CD314	0.0014
NKp80	0.0014
Ig light chain k	0.0016
CD88	0.0017
CD307	0.0018
CD220	0.0018
CD275	0.0021
CD97	0.0022
CD66b	0.0026
CD107a	0.0027
CD200R	0.0033
CCR10	0.0041
CD112 (Nectin-2)	0.0043
CD150 (SLAM)	0.0043
CD116	0.0044
CD202b	0.0046
Integrin beta5	0.0048
CD335	0.0049
CD71	0.0057
CD15	0.0071
CD114	0.0073
CD290	0.0090

CD276	0.0091
CD83	0.0101
CD123	0.0117
Notch 2	0.0119
CD124	0.0122
CD23	0.0126
beta2 microglobulin	0.0129
CD138	0.0142
CD74	0.0153
CD16	0.0163
CD158f	0.0174
CD66a-c-e	0.0181
CD307d	0.0194
CD163	0.0202
IgM	0.0208
Notch 4	0.0232
CD172a	0.0251
CD166	0.0267
CD55	0.0291
CD36	0.0296
CD205	0.0306
CD325	0.0315
CD42b	0.0356
CD62L	0.0366
CD80	0.0371
CD105	0.0372
CD5	0.0378
CD262	0.0409
CD61	0.0425
CD243	0.0435
CD141	0.0440
CD261	0.0441
CD184	0.0483