An Investigation into the Role of FcRL4^{+} B Cells in Rheumatoid Arthritis

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Abstract

Rheumatoid Arthritis (RA) affects approximately 1% of the world population and is associated with pain, fatigue, progressive disability and eventual joint destruction if left untreated. The last decade has revealed the importance of B cells in the pathology of RA through the success of B cell depletion therapy. What has also become clear in recent years is the importance of the microbiota in autoimmunity, with dysbiosis being associated with many conditions, including RA. FcRL4^{+} B cells have found in a number of situations of chronic antigenic stimulation and described as having the potential for an intriguing link between innate and adaptive immunity, and between the pathology of the rheumatoid joint and mucosal immunity. This work investigated the presence of FcRL4^{+} B cells in the tonsil, the joint of patients with active RA and peripheral blood, and their presence in the age-associated B cell population, with which they share a number of characteristics. The ability of FcRL4^{+} B cells to bind IgA was also assessed. Furthermore, the regulation of this population was probed. Finally, the reactivity of monoclonal antibodies, derived from joint resident FcRL4^{+} B cells was tested against the human microbiota.

Contents

Abbreviations

ABC – Age/Atypical B cell

ACPA – Anti–citrullinated protein antibody

ACR – American College of Rheumatology

AMC – Anti-modified Citrulline

Anti-CCP – Anti-cyclic citrullinated peptide

APC – Antigen Presenting Cell

BAFF – B cell Activating Factor

BALT – Bronchus Associated Lymphoid Tissue

BAFF – B cell Activating Factor of the TNF Family

BCA – Bicinchoninic acid assay

BCDT – B cell depletion therapy

BCR – B cell Receptor

BSA – Bovine Serum Albumin

CCP – Cyclic citrullinated peptides

CD – Crohn's Disease

CDR – Complementarity Determining Region

CIA – Collagen Induced Arthritis

CRP – C-Reactive Protein

CSR – Class Switch Recombination

DMARDs – Disease-modifying antirheumatic drugs

DNA – Deoxyribonucleic Acid

EAE – Experimental Autoimmune Encephalitis

ECL – Enhanced Chemiluminescence

ECM – Extra-Cellular Matrix

EDTA – Ethylenediaminetetraacetic acid

EULAR – European League Against Rheumatism

FAE – Follicle Associated Epithelium

FACS – Fluorescence Activated Cell Sorting

FCS – Foetal Calf Serum

FMF – Familial Mediterranean Fever

FLS – Fibroblast-Like Synoviocytes

FMOs – Fluorescence Minus One's

FWR – Framework Region

GALT – Gut Associated Lymphoid Tissue

GI – Gastrointestinal Tract

GPS – L-Glutamine–Penicillin–Streptomycin

GWAS – Genome Wide Association Studies

HA – Heat Aggregated

HLA – Human Leukocyte Antigen

HKLM – Heat-killed Listeria monocytogenes

HMW – High Molecular Weight

IC – Immune Complex

ILCs – Innate Lymphoid Cells

IECs – Intestinal Epithelial Cells

IFN-γ – Interferon Gamma

Ig – Immunoglobulin

IL – Interleukin

LMW – Low Molecular Weight

LPS – Lypopolysaccharide

MACS – Magnetic Activated Cell Sorting

mAb – Monoclonal Antibody

MALT – Mucosa Associated Lymphoid Tissue

MTX – Methotrexate

MHC – Major Histocompatibility Complex

mi-RNA – Micro Ribonucleic Acid

MLN – Mesenteric Lymph Node

MOI – Multiplicity of Infection

NALT – Nasal Associated Lymphoid Tissue

NETs – Neutrophil Extracellular Traps

NSAIDS – Non-Steroidal Anti-Inflammatory Drugs

OA – Osteoarthritis

OCs – Osteoclasts

OCPs – Osteoclast Precursor Cells

OPG – Osteoprotegerin

PAD – Peptidyl Arginine Deiminase

PAGE – Polyacrylamide Gel Electrophoresis

PB – Peripheral Blood

PBMC – Peripheral Blood Mononuclear Cells

PCs – Plasma Cells

PBS – Phosphate Buffered Saline

PsA – Psoriatic Arthritis

pSS – Primary Sjogren's Syndrome

PPs – Peyer's Patches

PVDF – Polyinylidene difluoride

QC – Quality Control

RA – Rheumatoid Arthritis

RANK – Receptor activator of nuclear factor kappa-β

RANKL – Receptor activator of nuclear factor kappa-β ligand

RF – Rheumatoid Factor

RNA – Ribonucleic Acid

RPMI-1640 – Roswell Park Memorial Institute-1640

s.d. – Standard Deviation

SDS – Sodium dodecyl sulfate

SEM – Standard Error of the Mean

SF – Synovial Fluid

SFB – Segmented Filamentous Bacteria

SFMCs – Synovial Fluid Mononuclear Cells

SLE – Systemic Lupus Erythematosus

SHM – Somatic Hypermutation

T1D – Type 1 Diabetes

TBS – Tris-Buffered Saline

TLR – Toll-like Receptor

TMCs – Tonsil Mononuclear Cells

TRAPS – Tumour Necrosis Factor Receptor-Associated Periodic Syndrome

UA – Undifferentiated Arthritis

UC – Ulcerative Colitis

QC – Quality Control

List of illustrations

List of Tables

1 Introduction

1.1 Rheumatoid Arthritis

1.1.1 Inflammation

Inflammation is a common response to injury involving redness, pain, and swelling, associated with an increase in temperature and loss of function. Inflammation is an important process for wound healing and prevention of infection by which the body responds to tissue injury or infection. It involves the generation of signaling cascades in affected tissue resident cells and the subsequent secretion of cytokines, chemokines and other factors such as prostaglandins which leads to the recruitment of cells of the innate immune system to the point of insult[1]. These cells, the first responders of which are typically neutrophils and macrophages, are then able to act against the infectious agent and present the relevant antigens to the adaptive immune system if necessary or maintain the area free from infection whilst it heals[1]. When this process goes awry as in autoimmune disease, these actions are well understood for their role in which either local or systemic inflammation can lead to pathology[2].

1.1.2 Autoimmunity

Autoimmunity was initially believed to be an impossibility according to Paul Ehrlich's theory of "*horror autotoxicus"*. This was defined as the "innate unwillingness of the organism to endanger itself through the formation of an immune response against the host tissues"[3]. Ehrlich would later qualify this statement by suggesting that a self-directed immune response is prevented "by certain contrivances" from causing harm[4]. This view became dogma for decades in the face of increasing countervailing evidence. For example, an autoantibody response in paroxysmal cold hemoglobinuria

(PCH) was described as early as 1904[3], and further evidence from many other diseases collected over the years, including sympathetic ophthalmia, phacoanaphylaxis, acquired hemolytic anemias, "allergic" encephalomyelitis, thrombocytopenic purpura, and aspermatogenesis[45]. However, it was not until 1956 that Ernest Witebsky published on the presence of thyroid autoantibodies present in an experimental thyroiditis that the field moved beyond Ehrlich's theory and the modern field of autoimmunity began to take shape[6]. This eventually led to our modern definition of autoimmunity as 'a break in the normal mechanisms of immune tolerance leading to self-directed inflammation mediated, predominantly through an adaptive immune response in which the self is recognised as foreign'[7–10].

There are over 80 autoimmune diseases currently diagnosed, all characterised by the presence of an immune response mounted against self- or auto- antigens[11]. Autoimmune diseases are typically defined primarily by the organ or anatomical compartment targeted, for example in multiple sclerosis (MS) the nerves of the brain and nervous system are stripped of their myelin sheaths which enable nervous signals to be transduced, leading to progressive disability and eventual death. In primary Sjogren's Syndrome (pSS), the salivary glands are targeted leading to dryness amongst a host of other symptoms. In rheumatoid arthritis (RA) it is the small joints of the hands which are initially afflicted by inflammation and eventual bone erosion, leading to progressive disability. One characteristic these diseases share is an unknown cause or aetiology. Whilst common antigens have been described in several of the diseases, they are not universal and there is a lack of understanding as to causation. Coeliac Disease is potentially an exception since the antigen, tissue transglutaminase, is known and the response against it well characterised[12].

Much of the understanding of the processes behind autoimmunity is due to work carried out in murine models, much of it in animals in which certain genes have been knocked-out or

downregulated. Since this would be fundamentally unethical to carry out in humans, primary immunodeficiencies have provided vital examples necessary to illuminate our understanding. Immunodeficiencies typically result from a mutation in a gene involved in an immunological process leading to a deficiency in that specific cell/cytokine/process. Therefore, immunodeficiencies have been incredibly useful in understanding autoimmunity as they have provided many of nature's experiments, enabling the understanding of certain genes and factor's roles in humans as these can often differ markedly from animal models[13–18].

Key to understanding autoimmune disease is appreciating the differences between autoimmunity and autoinflammation. Whilst inflammation plays a role in autoimmunity it is not its sole defining characteristic. The defining characteristic of autoimmunity is the role that immune tolerance plays. Immune tolerance refers to the ability of the immune system of an organism to 'tolerate' the host tissues, ignoring any self-antigens, but remain highly sensitive to exogenous antigens. Autoimmunity involves a loss, or break in tolerance, leading to an adaptive immune response against host tissues involving T and B cells, which leads to the symptoms of chronic inflammation and the local tissue destruction, although the innate immune system can also be implicated[7]. In contrast, autoinflammatory disorders are characterised by systemic inflammation caused by triggering of an overzealous innate immune system by either endogenous or exogenous factors[7,10]. However, the distinctions between innate and adaptive immunity are increasingly blurred with the supposedly defining feature of adaptive immunity, its' memory of past immune reactions, also being reported in natural killer cells and even in fibroblasts[19,20].

These diseases also differ in the degree of genetic involvement, with autoinflammatory diseases typically the result of monogenic mutations in contrast to the more multi-gene mutations behind many autoimmune diseases[7,10]. Examples of autoinflammatory diseases include tumour necrosis

factor (TNF) receptor–associated periodic fever syndrome (TRAPS) and familial Mediterranean fever (FMF), amongst others[7]. It is also possible to divide autoinflammatory and autoimmune diseases based on the pathways implicated, which result from dysregulated production of IL-1β and IL-18 or the type I IFN pathways respectively[2]. Organ specificity is another characteristic used to differentiate autoimmune disease. Some affect only specific organs; such as type I diabetes mellitus (T1D) and Hashimoto's thyroiditis in which the beta cellsand thyroid of the pancreas are targeted respectively; and diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) in which autoantibodies against a number of antigens are detected and the symptoms are systemic[21].

1.1.3 Immune Tolerance

As described previously, immune tolerance is the process by which the immune system tolerates self-antigens whilst remaining sensitive to the presence of non-self-antigens. There are several views as to how the process of immune tolerance, or failures thereof, can lead to autoimmunity. The first theories to address this problem suggested that central tolerance was infallible, by which any autoreactive T and B cells are eliminated during their maturation in the thymus and bone marrow and foetal liver respectively. This process is known as negative selection and involves any maturing T or B lymphocyte which reacts with self-antigens being eliminated by one of several processes including, receptor editing, anergy, ignorance, and deletion[22]. Receptor editing involves repeated journeys through the developmental process that generates receptor arrangements in the first place, this is primarily a process which B cells experience, it is less clear if it is a central mechanism for T cells[23]. Anergy involves low level, continuous stimulation via a cells' T or B cell receptor in the absence of any co-stimulatory signals, through CD28 or CTLA-4 for example. In this situation a cell enters an 'anergic' state in which it displays a reduced ability to become activated following stimulation[24–26]. Ignorance is another explanation for why cells which have autoreactive receptor

arrangements fail to become activated and generate an autoimmune response, this theory relies upon the idea of immune privilege, which involves certain anatomical sites of the body remaining immunologically segregated from the rest[27,28]. Examples of immunologically privileged sites include the brain, testes, and eyes[27,28]. However, these are not truly impenetrable to immune cells, and particularly following tissue damage, lymphocytes can enter these tissues and mount a response against previously hidden self-antigens[27]. This can also occur at the cellular level with proteins and nucleic acids usually hidden from the view of the surveilling immune system and absent from the thymic education of T cells becoming exposed during inflammation, or infection associated necrosis, or improperly regulated apoptosis. This follows into the idea of neo-antigens, those newly generated epitopes created via post-translational modifications, such as citrullination in rheumatoid arthritis, mutations, or molecular mimicry by which foreign antigens with high levels of sequence similarity to self-antigens can result in epitope spreading and autoimmunity[29–31].

However, many studies have demonstrated significant failures of this central process, through the detection of autoreactive T and B cells in the periphery of healthy individuals[32,33]. The percentage of autoreactive B cells in the periphery of humans is estimated to be approximately 40% for newly emigrated B cells to the blood, which drops to 20% in antibodies produced by mature B cells[33]. In work investigating the frequency of autoreactive T cells in healthy individuals, investigators found that autoreactive $CD8^+$ T cells were present at a comparable frequency to those $CD8^+$ T cells specific for non-self-antigens[32]. Insight from a primary immunodeficiency is provided by Autoimmune Poly-Endocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED), also called Autoimmune polyendocrine syndrome type I (APS-I), a disease caused by a rare autosomal mutation in *AIRE*, the autoimmune regulator gene[34] . Symptoms include T cell-mediated destruction of multiple endocrine organs and is due to failures in the process of negative selection of T cells, resulting in the presence of autoreactive

clones in the periphery of affected individuals[2,34].

The presence of autoreactive lymphocytes in the periphery of healthy individuals is not pathogenic in and of itself, and suggests that, if not a function of their presence, autoimmunity may be a function of their abundance, their regulation, or their proneness to activation. However, it also demonstrates that central tolerance, whilst playing a vital role insomuch as when it is lacking disease such as APECED develops, does not explain the entirety of autoimmunity. For this, further hypotheses suggest that there are numerous mechanisms in the periphery which normally prevent autoimmunity but, in their absence or dysregulation, can cause it. These include the role of inhibitory molecules present on the surface of lymphocytes, the presence of circulating regulatory T lymphocytes (T_{regs}), the influence of our microbiota, and the function of innate signals.

A range of inhibitory molecules are expressed on the surface of mature circulating lymphocytes to dampen excessive activation during normal immune responses, they also serve to reduce the chances that an autoreactive clone is activated. These include molecules such as CTLA-4, PD-1, and numerous members of the Siglec family. These inhibitory molecules serve as useful targets in treatment of autoimmunity and cancers[35–38]. Among these are immune-checkpoint blockers, preventing certain interactions between cells, for example the targeting of CTLA-4, a key negative regulator of T cell activation, has enabled efficient treatment of many cancers[36,37,39]. However, these therapies have also initiated symptoms of autoimmunity in patients as demonstrated by the many autoimmune events which have occurred post-treatment[40,41]. Treatment with a PD-1 targeting monoclonal antibody has proven highly successful in treating previously untreatable melanoma but has also led to the initiation of multiple arthritides and inflammatory bowel disease[40].

Regulatory T cells, and more recently, regulatory B cells, have been demonstrated to be vital in

humans and many animal models of experimental autoimmunity since their discovery and provide a vital role in peripheral tolerance[42]. Both of these regulatory lymphocyte populations control immune responses through the secretion of the immunomodulatory cytokines such as IL-10, IL-35, and TGF-β, although a host of other processes and functions have been described for these populations[43–49]. In addition, deficiency in FOXP3, a transcription factor responsible for the differentiation of regulatory T cells leads to autoimmunity, further strengthening the argument that regulatory T cells role in peripheral tolerance is important[50]. A deficiency in regulatory B cells has been demonstrated to be responsible for chronic inflammation in many models of autoimmunity in mice[43,44,51]. Regulatory B cells can also exert their effects through their regulation of regulatory T cells, promoting T_{reg} development and suppressing the differentiation of T_H1 and T_H17 cells via IL-10 production, and through the inhibition of dendritic cell's ability to generate autoreactive T cells[52,53].

The microbial inhabitants of our bodies and in particular, our gastrointestinal tracts, are increasingly appreciated as playing a role in the development of autoimmune disease[54]. In the context of autoimmunity, taxonomic shifts in the composition of the microbes of the gut have been detected in the time between the detection of autoantibodies in the peripheral blood and diagnosis of disease. Expansions of proinflammatory microbes have been implicated in skewing towards a proinflammatory T_H17 response and contractions in immunomodulatory *B. fragilis* and *Clostridium* subspecies have been detected prior to disease onset[55–61]. Work in animal models has demonstrated the importance of our gut microbes for the development of autoimmunity, with colonisation of germ-free animals with a single species of bacteria, sufficient for protection against the development of autoimmunity[62,63]. In humans, one study focused on infants in different geographic regions, in which the prevalence of early-onset type 1 diabetes (T1D) varies, demonstrated a correlation between developing disease and exposure to LPS derived from different species[64]. These infants were determined to be predominantly colonised by either *E.coli* or *Bacteroides* and the LPS which

these species secrete differs in its structure and appeared to have immune-stimulatory stimulatory or immune-inhibitory respectively[64]. This was associated with a six-fold higher incidence of T1D in the Bacteroides-colonised infants in which the innate immune system is suppressed[64]. Work following this in a mouse model of diabetes found that intraperitoneal injection of E. coli LPS lead to a reduced incidence of T1D suggesting that stimulation of the innate immune system might dampen the predisposition towards developing autoimmunity, providing a putative mechanistic explanation for the "hygiene hypothesis" of autoimmunity[64,65]. Given its obvious direct link to the microbiota, diet has also been shown to have effects upon autoimmunity, and meta-analyses have shown probiotics to be effective in treatment of certain gastrointestinal diseases[66,67].

Each of these commonly proposed mechanisms by which autoimmunity can arise is supported by experimental and observational evidence. However, it should be noted that none provides a unifying explanation for autoimmunity. It is likely the contribution of a combination predisposing genetic backgrounds and environmental factors which lead to development of autoimmune diseases. They are outlined in further detail for rheumatoid arthritis in latter sections.

1.1.4 Rheumatoid Arthritis – A Definition

Rheumatoid arthritis (RA) is a common chronic inflammatory syndrome with an undefined aetiology[68]. RA affects approximately 0.5-1% of the population worldwide, and is primarily associated with progressive joint pain and bone erosion, leading to increased stiffness and progressive disability[11,69,70]. However, the effects of RA are systemic and go beyond the joints with increased incidences of heart disease, depression, fatigue, lung inflammation, and early death all associated with the disease[71–76].

The prevalence of RA appears to partly depend upon geographical location with decreasing incidences from north to south in the northern hemisphere and with higher incidences in the developed world[11,69,77,78]. The disease is more common in females than in males but this difference narrows with age[77]. Hormonal differences are suggested to explain this difference between sexes but percentages of smokers and access to health services are also implicated in this imbalance[79].

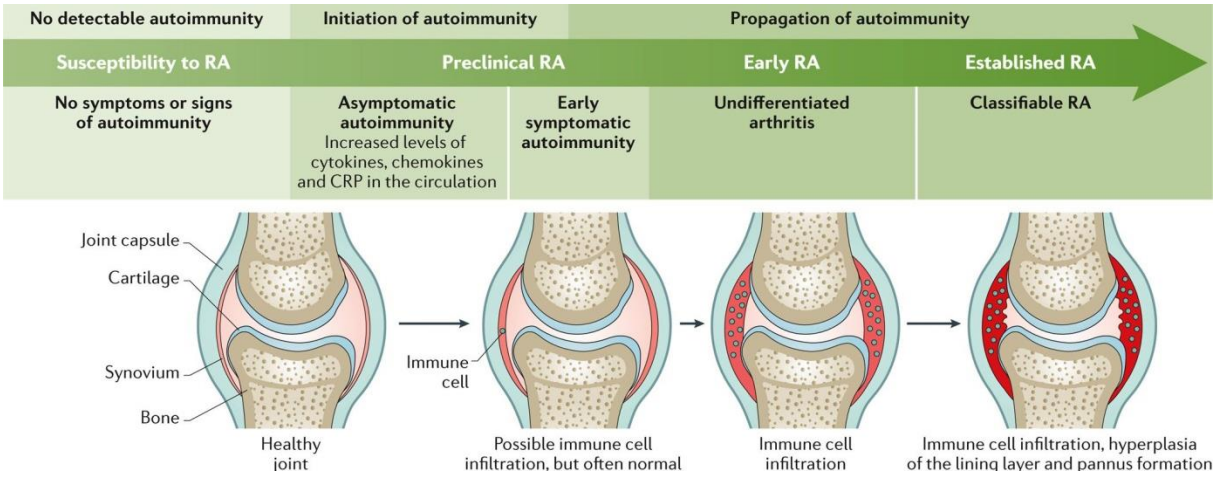

Figure 1 – A diagram detailing the development and progression of Rheumatoid Arthritis. *From a healthy asymptomatic joint with a thin synovial lining layer and organised anatomical compartments; individuals develop a background of asymptomatic systemic autoimmunity with elevated circulating inflammatory markers such as C-reactive protein (CRP) and autoantibodies such as Anti-Citrullinated Protein Antigen (ACPA) antibodies and Rheumatoid Factor (RF). Still without the presence of joint pain or inflammation, infiltrating immune cells make their way into the synovium, their continued infiltration and activation, combined with inflammation of the joint and hyperplasia of the lining layer known as a pannus lead to a diagnosis of established rheumatoid arthritis. Figure adapted from Yamamoto, K., et al., 2018[80].*

RA is typified by both localised (Figure 1) and systemic features. Local features include inflammation and hyperplasia of the synovium, leading to swollen and tender joints[68,70,81]. These features are eventually accompanied by cartilage destruction and bone erosion leading to deformity[68,70]. Systemic features include the presence of antibodies in the serum directed against self-antigens, these are known as autoantibodies and have also been found in the synovial fluid (SF). In the case of RA these autoantibodies include Rheumatoid Factor (RF) which is directed against the Fc portion of immunoglobulins and anti-citrullinated protein antibodies (ACPAs) directed against citrullinated

proteins[82,83]. Other systemic features also include cardiovascular, pulmonary, psychological, and bone disorders (in particular generalised osteoporosis)[84–87].

1.1.5 Rheumatoid Arthritis – Diagnosis

RA is diagnosed on the basis of a wide range symptoms and signs including those mentioned above, and laboratory assays including; blood tests for autoantibodies and inflammation including ACPA, RF, and C-reactive protein (CRP); and morphological assessment of the joints via X-ray[88,89]. For research studies and clinical trials, classification criteria for RA are applied, ensuring a consistent approach to the definition of RA. The 1987 American Rheumatism Association classification criteria were updated in 2010 in collaboration with the European League Against Rheumatism (EULAR) to reflect the improved understanding of the disease, which now have a higher sensitivity for the earlier stages of disease[88–91].

The traditional characterisation of RA as solely a disease of the joints involving local inflammation and bone erosion has given way to a molecular definition of at least two major disease subsets. These subsets are defined by the presence or absence of ACPA autoantibodies in the peripheral blood, referred to as ACPA-positive and ACPA-negative RA respectively[92–95]. These subdivisions of ACPA-positive and ACPA-negative disease appear to denote diseases with very different genetic backgrounds and clinical courses, but many other divisions can be made including; the response to therapy, environmental determinants, and clinical features[8,96–103]. However, despite these molecular distinctions between these two forms of RA, at present the aetiology of either has not been pinpointed but and is best understood to arise from the complex interplay between many genetic and environmental factors[68,104]. Genome wide association studies (GWAS) point towards the

disturbance of key immunomodulatory factors as regulators of disease[105,106].

1.1.6 Rheumatoid Arthritis Mechanisms – Genes

RA has been demonstrated to have a level of heritability associated with it and many studies have elucidated single nucleotide polymorphisms (SNPs) and other mutations as increasing the risk of individuals developing the disease. Family-based heritability studies in the UK, Finland, and Japan have demonstrated that the heritability of RA is between 53-68%, with just over 100 genetic loci associated with heritability of RA[107–113]. These loci contribute ~ 15% of the phenotypic variance seen in RA. However, analysis of a Swedish patient register demonstrated clear differences in the heritability of seropositive and seronegative arthritis, with an observed familial risk of ∼50% for ACPA-positive RA and ∼20% for ACPA-negative RA[114]. Studies in monozygotic twins have demonstrated that they share RA on 12-15% of occasions which, whilst dramatically higher than that of the general population (~1%) and first degree relatives (2-5%), is still relatively low and strongly implicates other factors beyond genetics[11,69,108–110,114,115].

The best described and most significantly contributing individual genetic risk factor for developing RA is that of genetic variants in the MHC class II or Human Leukocyte Antigen (HLA)-DR region, which is thought to account for more than 60% of the genetic heritability of RA[116,117]. This region encodes for over 250 functional genes involved in antigen processing, cytokines, complement factors, and HLAs[116–118].

HLA-DRβ1 is regarded as one of the key HLA alleles associated with RA, and especially ACPA-positive RA, in particular a common amino acid motif (QKRAA, QRRAA, or RRRAA) at positions 70-74 in the third hypervariable region (HVR3) of the DRβ1 chain[119,120]. This is an inwardly facing region of the

antigen presenting binding groove of the MHC II involved in antigen presentation to T cells and is referred to as the shared epitope or susceptibility epitope (SE)[119,120]. The SE suggests that a common antigen could be shared between individuals carrying the 'at risk' HLA-DRβ1 alleles and that the SE could play a role in the disease process by shaping the T cell receptor repertoire or by presenting a microbial or auto-antigenic peptide to self-reactive T cells, inducing a form of molecular mimicry[121].

Citrullinated autoantigens have been proposed as the shared antigen since the 'at risk' HLA-DRβ1 alleles are reported to lead to a higher avidity for citrullinated vimentin compared to the un-citrullinated peptide[122]. Despite the disease-relevant suggested molecular mechanism for the involvement of the SE, it is not the most significant genetic linkage present in the HLA-DR alleles. Using MHC SNP data from European populations the most significant association for RA-risk mapped to amino acid position 11 in HLA-DRβ1, followed by positions 71 and 74 from within the SE, and amino acid position 9 in both HLA-B and HLA-DPβ1[113]. Further weaker associations have also been found through-out the HLA genes[123–127].

Not all HLA alleles are associated with a predisposition towards developing RA, mutations in the HLA-DRB1*13 allele are associated with protection against developing the disease via a cross-reacting mechanism involving vinculin-derived and microbial-derived DERAA epitopes[128–130]. These epitopes which would otherwise predispose towards developing disease lead to the deletion of autoreactive T cells in the thymus in individuals carrying the HLA-DRB1*13 allele, thus leading to a protective effect[130].

Many other non-MHC genetic factors of susceptibility have been described for RA including *PTPN22*, *BACH2*, and *CTLA4*, genes which are involved in the differentiation, activation and function of T- and B-cells[131–133]. Further gene linkages include *ELMO1*, *STAT4*, *GTF-2I*, and *TRAF1-C5* which are involved

in immune regulation, peptidylarginine deiminase (*PADI*), responsible for post-translationally citrullinating proteins, and *RAD51B* which is involved in DNA repair[132,134–138]. Additionally the involvement of micro-RNAs (mi-RNAs)has increasingly been demonstrated to be important to the disease process with many novel linkages described[139].

PTPN22 is another RA risk locus and has been implicated in a variety of autoimmune diseases including type-1 diabetes (T1D), Crohn's disease (CD), and systemic lupus erythematosus (SLE)[140–143]. *PTPN22* encodes for a tyrosine phosphatase but its precise role in disease pathogenesis is not fully understood. However, the R620W allele of *PTPN22* has been shown to be a gain-of-function mutation in which the threshold for T cell activation is lowered through increased phosphorylation of the signaling proteins Lck and ZAP70[144]. This could lead to an increase in the number of self-reactive T cell clones present in the thymus and periphery, which could increase the likelihood of a break in immune tolerance[68].

PADI is particularly interesting in the context of the strong environmental link between citrullination and RA, since *PADI* encodes for a citrullinating enzyme, peptidylarginine deiminase (PAD)[145,146]. There are five highly conserved PAD enzymes (~50% between isoforms) in humans, with tissue specific expression patterns and varying subcellular localisations[145,147,148]. For example, PAD4 is primarily expressed in the cells of the immune system[145,148–150]. An elongated *PADI4* allele has been associated with increased mRNA stability and thus increased translation and protein citrullination[151].

The genetic factors described so far are common to seropositive RA, i.e. those patients testing positive for rheumatoid factor (RF) and antibodies specific for citrullinated protein antigens (ACPA)[96]. Genetic factors for seronegative RA patients have been less well described in the literature but include different HLA-DRβ1 alleles, interferon regulatory factors, and lectin-binding proteins[96]. An

obstacle in defining the genetic factors involved in ACPA-negative disease is the lack of a conclusive diagnostic test. Since this subset of the disease is defined by the absence of autoantibodies rather than the presence of any particular factor, it is possible for many seropositive patients to fall below the clinical threshold of APCA positivity due to the lack of sensitivity in the commercial anti-cyclic citrullinated peptide antibody (anti-CCP2) test. One study has illustrated this fact clearly, with more than 10% of seronegative patients tested using a more sensitive method demonstrated to be producing ACPAs[152]. A further issue is the inclusion of a higher proportion of $ACPA^+$ than $ACPA^-$ patients in genetic studies, this reflects the population but complicates the discovery of genetic factors in $ACPA^-$ RA as these studies might not achieve sufficient statistical power to analyse the $ACPA^-$ population. It is also possible for other immune diseases to be falsely diagnosed as seronegative RA, further complicating the genetic understanding of this disease[152].

Outside of the traditional genetic risk factors for RA, the field of epigenetics has illuminated a host of epigenetic signatures which correlate with disease pathology[105]. Studies have implicated global DNA hypomethylation in T cells, and methylation of a single SNP in *IL-6* in RA, as well as the MHC locus; suggesting that a proportion of the genetic risk associated with the MHC locus may in fact be a consequence of epigenetic mechanisms[153–155].

Much work in the fibroblast-like synoviocytes (FLS) of the joints has also implicated a role for epigenetics[156–162]. In one study the authors hypothesise that the reason for the symmetrical presentation of RA which focusses on the small joints of the hands is due to the underlying epigenetic variations in the FLS of these joints compared to those in the hips, which are generally only involved later in the disease progression, or the distal interphalangeal joints which are rarely involved in disease[163]. Evidence for this theory comes from the differing DNA methylation statuses of the FLS from the aforementioned locations, many of which correlated with pathways involved in

inflammation and extracellular matrix (ECM) degradation[163]. Other studies have suggested that epigenetics could explain the differences in developing RA seen in monozygotic twins[164].

These data implicating genetics in the aetiology of RA, whilst hinting towards many potential mechanisms in immune dysfunction, leave much of the risk of developing the disease unexplained, and suggest and important role for environmental factors.

1.1.7 Rheumatoid Arthritis Mechanisms – Environment

Many gene-environment interaction studies have shed light on environmental factors associated with an increased risk of RA, with a great deal of evidence from epidemiological studies pointing towards a mucosal origin of the disease pathogenesis[165]. Several mucosal sites have been strongly associated with RA including the lungs, gut, and periodontium[166–168].

The lungs have been implicated in epidemiological studies which demonstrated that smoking and other pulmonary stressors such as textile dust, and silica are significant risk factors for RA, especially seropositive RA (Figure 2)[149,169–172]. Much of this risk is restricted to patients positive for the mutations in the MHC class II region, particularly those carrying an SE allele[173].

Smoking is the most common environmental risk factor for seropositive RA[169,173]. Cigarette smoke interferes with both innate and adaptive aspects of mucosal immunity; generating damage-associated molecular patterns (DAMPS), through direct damage to the delicate structures of the alveoli of the lungs; and pathogen-associated molecular patterns (PAMPS), via the increased ease of viral infection[174,175]. These PAMPS and DAMPS lead to the subsequent activation of Toll-like receptors (TLRs), particularly TLR4, on the lung epithelium and the activation of local immune cells including

macrophages, dendritic cells, and neutrophils[176–178]. Smoking does not simply alter the immunological environment of the lungs but has been demonstrated to lead to an increase in the expression of citrullinated proteins in the lungs of healthy smokers as compared to non-smokers[179]. Other stressors of pulmonary and other mucosal tissues have also been demonstrated to increase the level of post-translational modification of proteins such as acetylation, carbamylation, and citrullination, all of which have been detected at elevated levels in RA patients[180–182].

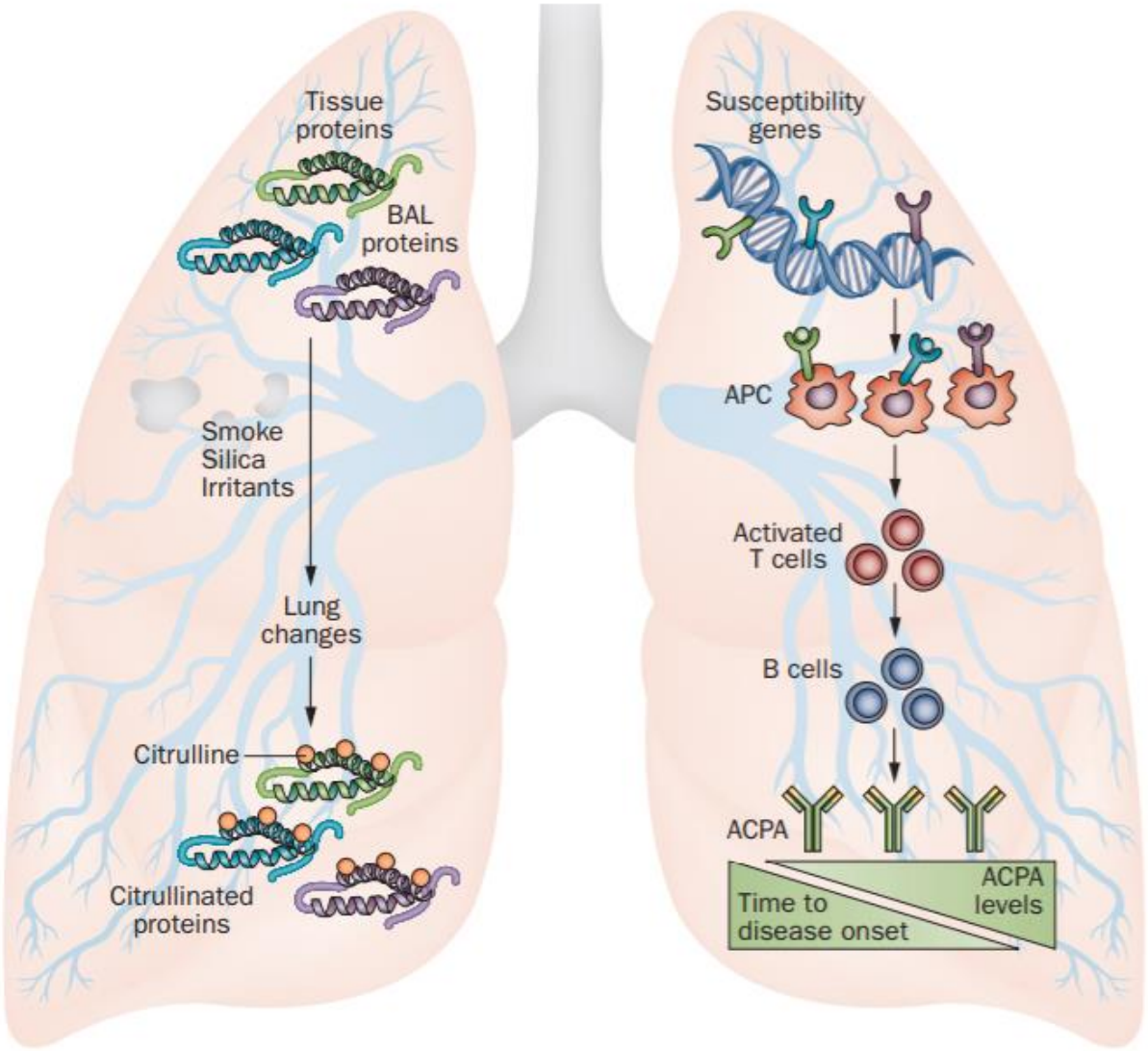

Figure 2 – A schematic of the lungs demonstrating the impact of environmental factors on aspects of immunity implicated in the pathogenesis of RA. *Damage to the lungs from smoking, silica, and textile dust causes molecular changes such as an increase in citrullinated proteins (BAL - bronchoalveolar lavage). Antigen-presenting cells (APCs) such as dendritic cells and B cells expressing MHC of a susceptible genetic background can then present these modified proteins leading to the activation of specific B- and T- cells, leading to the activation and maturations of these cells. This results in the production of Anti-citrullinated protein antibodies long before disease onset. These ACPAs are subject to epitope spreading and maturation of specific clones. Serum concentrations of these ACPAs increase as disease onset approaches in at risk individuals. Figure from Klareskog, L., et al., 2014*[183].

The initial epitope specificity in individuals with RA is unknown, although citrullination is a common modification in the disease population. This suggests that it is not a specific epitope which is recognised but rather citrullinated epitopes in general[122]. This leads to the hypothesis that smoking

and other lung damage leads to presentation of citrullinated auto-antigens to T cells by activated antigen presenting cells (APCs) leading to a break in immune tolerance, particularly in those individuals with a genetic predisposition such as mutations in the Shared Epitope (SE)[120,122].

The process of citrullination is well understood and occurs when peptidyl arginine deiminase (PAD) enzymes replace the primary ketimine group (=NH) of a peptide with a ketone group (=O)[145]. This de-imination of arginine converting it to citrulline, leads to a loss of charge on the amino acid, as arginine is positively charged at a neutral pH, whereas citrulline has no net charge[145]. This altered charge can increase a protein's hydrophobicity which can have implications for protein folding and structure[145,148]. In the immune system, citrullination is known to be carried out primarily by neutrophils via PAD4[184]. One of the consequences of citrullination in neutrophils is the release of neutrophil extracellular traps (NETs), which are lattices of DNA, thought to have an antibacterial function[185]. The positively charged arginine and lysine residues on histones have been shown to be highly anti-bacterial and citrullination is necessary for the release of these NETs[185]. The loss in charge is thought to enable the neutrophils to secrete the NETs more easily[185]. It has been demonstrated that PAD4 is released into the synovial cavity when neutrophils are subjected to chronic inflammatory stress or when they release NETs[184,186]. Both lead to increased citrullination of synovial proteins including α-enolase, keratin, fibronectin, fibrinogen, collagen, and vimentin as well as novel proteins such as Apo E, MNDA, β-actin, and cyclophilin A[184]. A subsequent loss of tolerance to these new citrullinated proteins results in these epitopes eliciting an ACPA response[8,95].

In ACPA positive patients, antibodies to the immunodominant citrullinated α-enolase CEP-1 epitope were detected in 43–63% of the population, and this was strongly associated with HLA-DRB1*04, risk alleles in PTPN22, and smoking; similar interactions have also been reported for citrullinated vimentin and fibrinogen epitopes[119,187]. RA also appears to be associated with periodontal disease[188–

[190]. One potential explanation for this link is the presence of *Porphyromonas gingivalis* in the inflamed gums, to which protein citrullination is linked[168,189,191] *P. gingivalis*, also expresses PPAD an enzyme that is also able to citrullinate proteins[189]. However, the type of citrullination detected in RA is associated with PADI4 rather than PPAD, and clear epidemiological evidence linking periodontitis with RA is lacking[191]. Another link is the presence of *P. gingivalis*-specific antibodies in seropositive RA patients[191].

Other infectious agents and pathogen-associated molecular patterns (PAMPs) have also been associated with RA. Whilst an individual organism has yet to be identified, it is suggested that the process of molecular mimicry could be implicated[120,130,191]. And even the generation of immune complexes during infection, be it viral or bacterial, can lead to the production of rheumatoid factor (RF)[192,193]. One study has linked microbial DERAA epitopes and vinculin cross-reactivity, suggesting a bacterial peptide may be responsible for disease development and demonstrate that in its absence disease is less likely[130]. In the case of RF and the link between the mucosa and joints, IgA RF have been strongly associated with adverse disease outcomes[194,195].

Another mucosal site implicated in the development of autoimmunity is the gut, and several defined bacterial 'signatures' have been found to be associated with seropositive RA[147,194,196]. These include expansion of the population of *Prevotella copri* and *Lactobacillus* in early rheumatoid arthritis, and reduced microbiota diversity[197–202]. Despite these strong associations, there is currently no clear direct link in humans between the gut mucosa and RA.

A lack of Vitamin D synthesis is suggested to explain the increasing incidence of RA and autoimmune diseases in general at higher latitudes, at least in the Northern Hemisphere[203,204]. However, this could also link with the negatively correlated levels of hygiene and exposure to helminths in these areas,

the hygiene hypothesis[205].

1.1.8 Rheumatoid Arthritis Mechanisms – Joint Specific Disease

The above descriptions of predisposing genetic- and epigenetic-backgrounds and involvement of environmental factors in developing systemic autoimmunity provide evidence of the mechanisms and processes of disease. What they do not do, at least yet, is to explain the primary localisation of the disease to the joints. These factors outlined above contribute to disease, to a lesser or greater extent, but none is enough on its own to break tolerance and lead to the joint specific disease seen in patients. Autoantibodies are seen in patients, years before symptoms develop, and are also detected in healthy individuals[206]. Mucosal dysbiosis is seen in some patients preceding disease onset, but the responsible bacteria are also present at lower levels in healthy individuals[200]. A strong genetic predisposition may exist in an individual, but it is no guarantee of disease[113]. A person may smoke and have high detectable levels of citrullinated proteins in their lungs, but it is not certain that they will develop RA[86]. In all these cases the transition from this state of at risk or pre-RA to clinical diagnosis is regarded as requiring a 'second strike'.

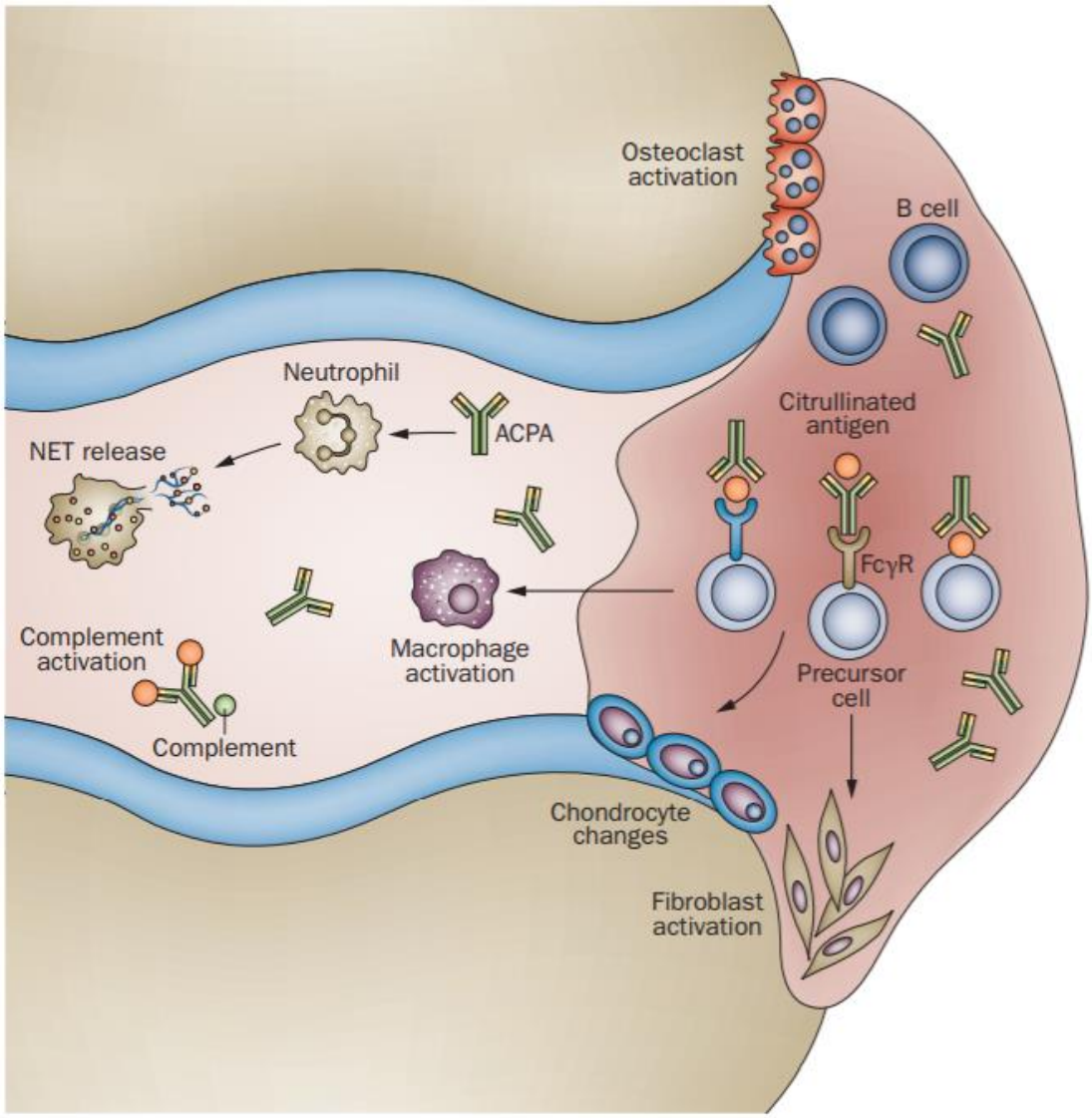

Figure 3 – A diagram detailing the various roles which ACPA can play in the pathogenesis of RA. *ACPA are capable of contributing to the inflammation of the synovium directly through their binding to Fcγ receptors on monocytic precursors leading to the activation of inflammatory macrophages and fibroblasts, and indirectly through binding to membrane bound (via TLR4 for example) or membrane expressed citrullinated proteins and binding to citrullinated proteins present in neutrophil extra-cellular traps (NETs). Subsequent binding of complement can further contribute to synovial inflammation. Klareskog, L., et al., 2014*[183]*.*

One intriguing theory which links the circulating autoantibodies which predate clinical disease and joint symptoms is that of ACPA-mediated activation of osteoclasts. Because the autoantibodies are not a joint specific event, given that they also occur in healthy individuals who never go on to develop RA, this hypothesis posits that it is not the autoantibodies which are unique to disease but

the joints, and in particular the presence of osteoclasts present in the joints which express surface citrullinated proteins during their development, such as cit. vimentin. In one study, the ability of ACPAs directed against citrullinated vimentin to induce OC-mediated bone erosion suggests that this may provide a link between systemic autoimmunity and the pathology of the RA joint[207]. However, whilst this theory goes a long way to explaining the joint localisation it still does not explain the jump from a erosive, bone-directed pathology to an inflammatory, whole joint oriented pathology. Importantly the formation of APCA can happen without the presence of autoimmunity, with this break in tolerance only occurring later in the disease course following this negative feedback loop. The various roles of ACPA in the pathology of RA are outlined in (Figure 3).

It is also possible that this 'second strike' takes the form of a random immunological insult such as viral infection or physical damage to the joint; for example, Epstein-Barr virus has been suggested to trigger disease in the right context[208]. The joint is regarded as poorly defended against such diseases, lacking any of the structural barriers which the epithelium or gut have, such as tight junctions or organised basement membranes. The joint is also highly porous to cells and secreted cytokines, with a well-established vasculature[209].

1.1.9 Synovial Biology

The synovium encapsulates the joints and functions to provide structural support, lubrication, and nutrients to the articular cartilage[209]. The synovial joint lining is divided into two compartments based on anatomical and functional differences, the intimal lining layer and the sub-lining layer, although these are not separated by a basement membrane, as in the gut for example[209]. The intimal lining layer is about 2-3 cells deep, in contact with the intra-articular cavity, and responsible for the production of lubricating synovial fluid[209]. The composition of the intimal lining layer is approximately

half macrophage-like synoviocytes and half fibroblast-like synoviocytes (FLS) [209]. The FLS play a number of physiological roles including enabling free movement of the joints, accomplished through the secretion of hyaluronic acid for lubrication and other proteins to the cartilage. FLS also play a role in the continuous remodelling of the extracellular matrix (ECM) of the joint, both producing ECM components such as collagen and hyaluronan and the matrix metalloproteinases (MMPs) which degrade them. The intimal lining layer is loosely attached to the stroma which contains blood vessels, the lymphatics, and nerves. The sub-lining layer is composed of immune cells, fibroblasts, adipocytes, and vasculature, with an underlying layer of innervation.

1.1.10 The Synovium in Rheumatoid Arthritis

The typical synovial pathology of RA involves hyperplasia of the lining layer, normally around 2-3 cells deep, expanding to a 10-12 cell deep pannus in disease[210]. Under this layer is a infiltration of lymphocytes, angiogenesis, and formation of tertiary lymphoid organs (Figure 4)[81].

Whilst the adaptive immune system is vital to the pathogenesis of RA, the stroma has often been relegated to a supporting role, but recent work has suggested that far from this, the stroma plays a leading role in the pathology. In 1996 Gay, et al. demonstrated that synovial fibroblasts maintain an aggressive phenotype for 60 days following removal from the patient and subsequent grafting onto SCID mice[211]. Continued elevated expression of cellular adhesion molecule VCAM-1 and the matrix metalloprotease enzymes, cathepsins, was seen suggesting this phenotype is independent of inflammation[211]. This led to the suggestion that some form of imprinting of the synovial fibroblasts, for example epigenetically, can lead to disease initiation or exacerbation.

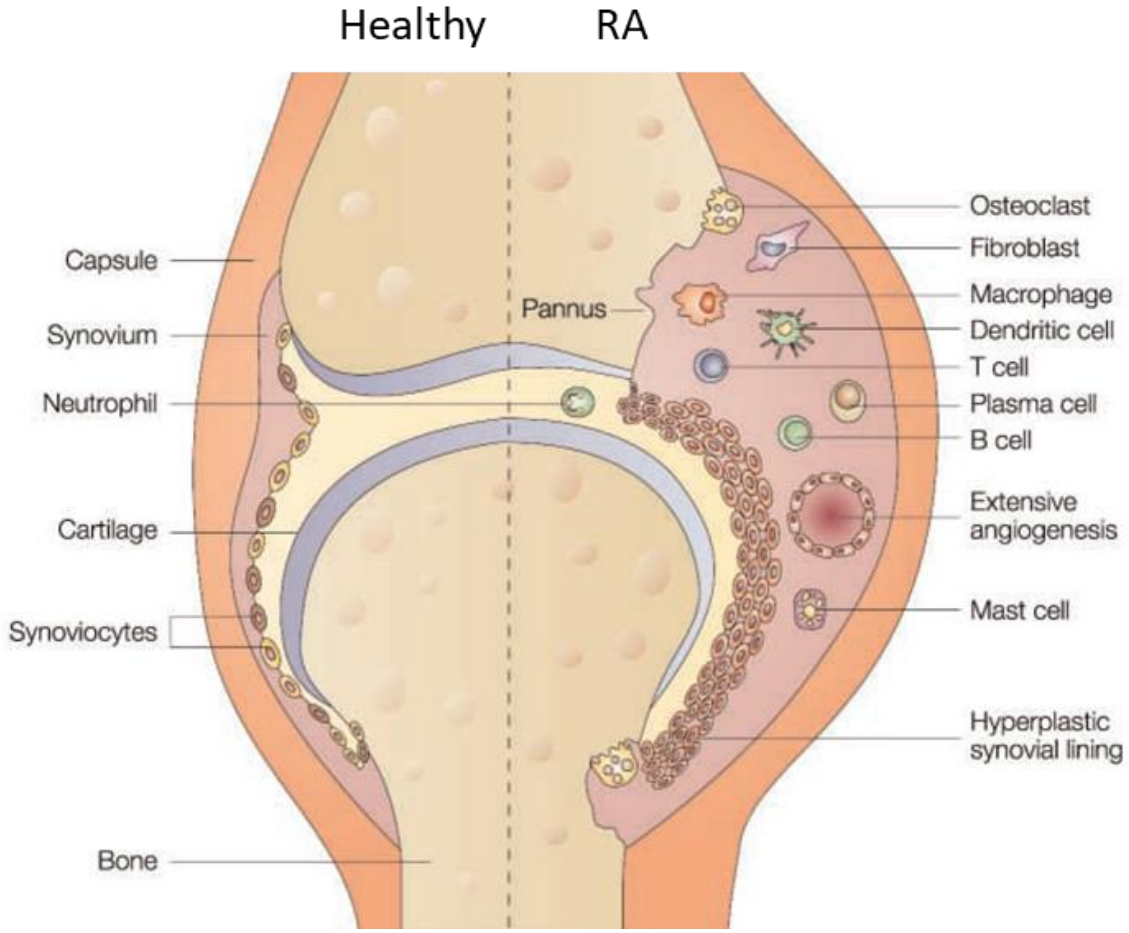

Figure 4 – A schematic of joint in a healthy individual (left) and of a patient with established arthritis (right). *This schematic shows the pathological anatomical features associated with RA and the involvement of both joint and immune cells in the pathology (right) and compares this with the anatomy of the healthy joint (left). In the diseased (right) diagram the infiltrating, hyperplastic synovial lining (pannus) can be seen to destructively invade the joint space and bone. Furthermore, inflammatory signals realesed by the cells of the joint in response to this, results in the recruitment of immune cells into the synovium and angiogenesis, allow for further recruitment. These activated immune cells include neutrophils which, along with inflammatory synoviocytes and chondrocytes, secrete destructive enzymes leading to cartilage degradation; and osteoclasts which lead to bone erosion, a signature of RA. Figure adapted from Steiner, G., et al., 2003*[212].

Various other roles of synovial fibroblasts have also been demonstrated in addition to that of remodelling the microenvironment of the joint; including cytokine secretion, co-stimulatory interactions, and even antigen presentation, subsequently altering the immune reaction[9,157,213–219]. Fibroblasts are not a homogeneous population of cells but vary depending upon their anatomical location with site-specific gene expression patterns and functions[162,215,217]. Their identity is regulated

by their microenvironment, with inflammatory cytokines capable of transiently altering their gene expression[215,219]. It is not just the FLS which participate, the synovial macrophages are highly inflammatory, characterised by an M1-like phenotype, and release a copious quantity of cytokines (IL-6, TNF-α, IL-1), chemokines, and MMPs into synovium[219].

1.1.11 Treatment of Rheumatoid Arthritis

RA has proved to be a difficult disease to cure partly because its causes are not fully understood and partly because the mechanisms behind the persistence of synovial inflammation in established disease are not completely understood. Underlying pathologies can be present for years prior to the onset of symptoms, as evidenced by the presence of autoantibodies such as RF and ACPA years before the onset of clinically apparent disease; suggesting that the pathogenesis of RA originates outside of the joints. Another issue is that many of the initial symptoms of RA are non-specific; including fatigue, morning stiffness, and flu-like symptoms, as well as a small number of swollen joints, which can present asymmetrically[73,90]. It is only later in the disease progression that the traditional symmetrical polyarthritis is present[90].

Despite these difficulties, the diagnosis and treatment of RA has evolved greatly in the past few decades and a positive diagnosis is no longer a guarantee of progressive joint erosion and serious disability. Treatment of RA nowadays means for most patients the possibility of managing the disease symptoms and achieving a level of remission[220,221]. Whilst a genuine cure is lacking, a number of studies suggest that there is a 'window of opportunity' after the onset of clinically apparent disease during which treatments are dramatically more successful than when administered later in the disease course[222–224].

According to EULAR recommendations, patients are initially treated with small molecule disease modifying anti-rheumatic drugs (DMARDs) such as Methotrexate (MTX) plus low dose glucocorticoids, with biologic drugs reserved for those who fail to respond[225]. Non-steroidal anti-inflammatory drugs (NSAIDs) can also be included as adjunct therapies[225]. The biologic drugs are typically monoclonal antibodies directed against targets of populations known to participate in the immune response; for example, rituximab targets CD20, thus depleting circulating B cells, and abatacept blocks costimulatory interactions between CD28 and CD80/CD86 expressed by T Cells and APC respectively[226–228]. Biological drugs can also target inflammatory mediators such as TNF-α or IL-6 in the case of adalimumab and tocilizumab, respectively[229,230].

1.2 Mucosal Origins of Autoimmunity in Rheumatoid Arthritis

1.2.1 The Human Microbiota

The human body is densely populated by commensal and symbiotic microbes which occupy varied habitats such as the mouth, skin, lungs, and gut[231,232]. Not only does the variety and abundance of microbes differ between locations, but also between individuals[231–234]. The combination of these microorganisms' and their ecosystem constitute the microbiota, although the term microbiome is used somewhat interchangeably, it is more generally used to describe the total genetic material of these microorganisms[235].

The complex ecology of the microbiota has evolved in co-existence with humans in a mutually beneficial relationship, providing us with immunological, metabolic, and physiological benefits in exchange for nutrients[236–239]. This leads us to a conception of humans and their microbiotas as a collective 'super-organism' comprised of human and (rather more) non-human cells[54,240]. Until relatively recently, the positive influence of the microbiota on human health had been neglected, but

since the advent of large-scale sequencing studies its place in health and disease has become better appreciated[54,240].

Next generation sequencing technologies have enabled culture-independent assessments of the microorganisms present in the various niches of the human body; some estimates suggest that 50% of the human microbiota has not been cultured in a laboratory setting[241,242]. Until the complex interactions of our microbial ecology are unravelled, sequencing studies remain the best option to understand these complex systems[243]. These phylogenetic sequencing studies rely upon the fact that bacteria share the 16S ribosomal subunit[244,245]. Many regions of the 16S subunit remain highly conserved, enabling unbiased sequencing using universal primers for these regions[244–246]. However, in addition to these highly conserved regions there are also unique, species-specific hypervariable regions also present in the sequences which enables identification of the bacterial species present[244,245].These phylogenetic 16S rRNA sequencing studies provide a wealth of information about the identities of the microorganisms which colonise our bodies which has enabled us to define the diversity of the microbiota at different sites of the human body[244–247]. What 16S rRNA sequencing fails to tell us is what the microbiota is doing, such as the metabolic or enzymatic pathways in use, and how these change over time. For this information, whole genome and transcriptome sequencing is required. Efforts to replicate what the Human Genome Project set out to achieve for our genome are underway to characterise the microbiome, our "second genome"[54,240].

1.2.2 The role of the mucosal immune system in health

Our ability to coexist with the enormous antigenic burden of bacteria present in our microbiota is largely thanks to the numerous defence mechanisms of our mucosal immune system. How this is balanced between maintaining homeostasis of our commensal microbiota whilst remaining alert to

pathogens is the topic of active research.

The mucosal immune system or Mucosa Associated Lymphoid Tissue (MALT) is comprised of an integrated system of lymphoid tissues found in the various submucosal membranes such as those in the gut (GALT), and in the nasopharyngeal tract (NALT), and various other sites including; the salivary glands, breast, lungs, eyes, and skin[248]. The GALT will be focussed on given its complexity and the large proportion of the MALT which it comprises; the NALT will also be discussed briefly due to its epidemiologically demonstrated relevance to Rheumatoid Arthritis.

The anatomical structure of the gut reflects its function at the interface between microbiota and self. A layer of intestinal epithelial cells (IECs) divides the luminal gut contents of bacteria, mucus, etc. from the lamina propria, in which the gut associated lymphoid tissues (GALT) reside[249]. The GALT includes the mesenteric lymph nodes (MLN), solitary lymphoid nodules in the gastrointestinal tract (GI), and the Peyer's Patches (PP)[249]. These layers each have a vital role in maintaining the stratification of anatomical compartments. It should be noted that the GALT also includes other lymphoid tissues, extending to the tonsillar ring and lymphoid aggregates in the appendix and large intestine.

The barrier function of the mucous lining of the gut is the first line of defence. It is comprised of a thick layer of mucus containing antimicrobial peptides and secreted IgA produced by specialised intestinal goblet cells in the gut epithelium[249,250]. The primary function of this layer is to minimise contact between the commensal microbiota of the gut lumen and the intestinal epithelial cells lining the gut wall[251]. These epithelial cells then form the second layer of defence, thanks to the tight junctions formed between them, providing a physical barrier to bacterial entry. These epithelial cells

are not inert bystanders though, as they are able to respond to the presence of bacteria through their Toll-like Receptors (TLRs) which recognise PAMPs (pathogen associated molecular patterns). Downstream of TLR signaling, MyD88 activation leads to the secretion of antimicrobial proteins and peptides including defensins, cathelicidins, and C-type lectins. These "structural defences" serve to stratify the gut contents, limiting direct contact between the gut epithelium and the intestinal bacteria. However; given the size of the gut, the intestinal surface area is approximately $200m^2$ in humans; the numbers of bacteria present, $\geq 10^{12}$ per cm^3 of intestinal contents; and the thickness of the epithelial layer, approximately 10µm, it is all but guaranteed that despite the physical barriers in place, bacteria will penetrate into the lamina propria[248,252]. Thus, in addition to the structural defences, the various cells of gut associated lymphoid tissue (GALT) provide vital roles in defending against pathogenic bacteria and minimising the immune response against commensal bacteria[251,253].

Bacteria that do penetrate the epithelial barrier encounter lamina propria resident macrophages and dendritic cells, which phagocytose the bacterial invaders[254]. These cells will either digest the bacteria within intracellular organelles containing reactive oxygen species and digestive enzymes or process them and transport antigens to the mesenteric lymph nodes (MLNs) respectively, although DCs have also been demonstrated to carry them alive to the MLNs[255]. DCs act as antigen-presenting cells (APCs) in this context, resulting in the engagement of the adaptive immune system through MHC-II dependent antigen presentation to antigen specific T cells, and subsequent B cell activation. These activated B cells can subsequently class switch to go on to produce secretory IgA which is then transcytosed across the IEC barrier into the mucus of the gut lumen[254,256,257]. This IgA can be of either isotype, the reasons for switching to either IgA1 or IgA2 are not yet known but recombination is usually direct, despite the downstream location of the IgA2 locus[258]. As the IgA2:IgA1 ratio increases in the areas of the intestine associated with higher levels of bacterial load, it is possible that the antigen presented may affect the switch to a particular IgA isotype[258,259]. The T cell response is

complex, with T_H1, T_H2, T_{H17}, and T_{reg} cells all produced in response to differing stimuli[62,260–262]. T_H1 cells have been predominantly associated with responses against intracellular pathogens and macrophage activation, whereas T_H2 and T_{H17} cells mainly respond to the detection of extracellular pathogens. These pro-inflammatory cell types are inhibited by the presence of IL-10 secreting T_{reg} cells[262,263]. In mice, lamina propria resident type 3 innate lymphoid cells (ILC 3s) have been demonstrated to be required for the containment of commensal bacteria to the GALT via an IL-22 dependent mechanism[264]. These ILC 3s share a similar cytokine profiles to T_H cells and secrete IL-22 (and/or IL-17) in response to IL-23 produced by DCs & macrophages in response to the microbiota[264].

Although there are many mechanisms dedicated to maintaining barriers between the gut lumen and the GALT and preventing interactions between the intestinal immune system and the luminal bacteria; there are several specialised cells and mechanisms which allow safe interaction between the two. Microfold (M) cells (Figure 5) and CX3CR1+ DCs are key examples. M cells are specialised intestinal epithelial cells which are located in the follicle associated epithelium (FAE) of the gut, i.e. the epithelium which covers the Peyer's Patches and other organized GALT structures[253,265]. M cells lack the microvilli of other IECs on their luminal membrane and instead have 'microfolds', short, fold-like structures visible by microscopy, on their surface[253,265]. Another contrasting feature with that of the surrounding IECs is M cells unique pocket in their basal membrane[265]. Thanks to a very thin cytoplasm in M cells, this pocket forms a large envelope for DCs, macrophages, and other lymphocytes to reside[253,265]. M cells actively sample the intestinal lumen, phagocytosing and transcytosing bacteria into this pocket of immune cells where mucosal immune responses can be initiated[265]. However, M cells are not the only route by which the healthy immune system samples the intestinal lumen contents. Dendritic cells (DCs) are key antigen-presenting cells (APCs) and crucial modulators of the immune response, due to their ability to process and present antigens to T cells[266]. Intestinal DCs are essential for the activation of inflammatory pathways and maintaining local

immunological tolerance[266]. The chemokine receptor CX3CR1 appears to demarcate a tissue resident subset of intestinal DCs able to extend dendritic processes through the intestinal epithelium in order to sample the luminal bacteria[267]. This is dependent on CX3CR1, and maintains the barrier function of the epithelium as these DCs express tight junction proteins[267]. These cells thus provide an M cell independent method by which the intestinal immune system can sample the lumen and maintain appropriate responses to the bacteria present.

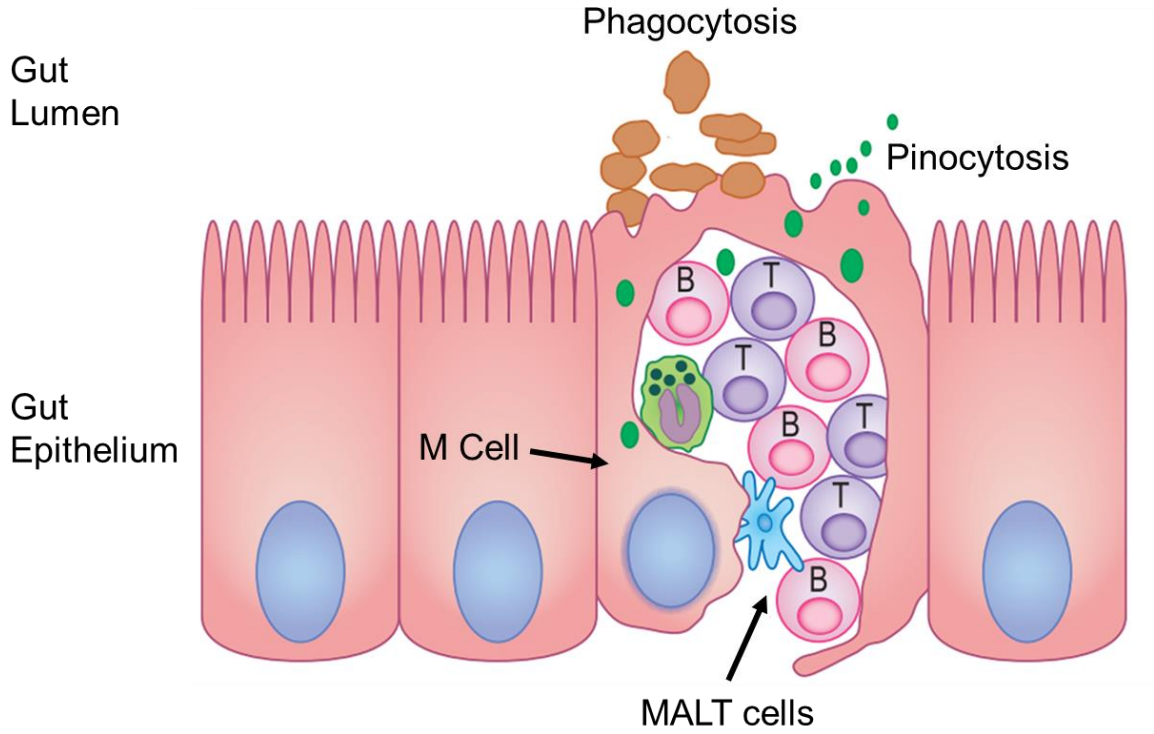

Figure 5 – A diagram showing the vital functions of M cells in gut immunity. *M cells form a unique pocket in the membrane of the intestinal epithelium, allowing the memory lymphocytes of the MALT to come into close contact with the gut lumen and allow sampling of soluble antigens via active pinocytosis and phagocytosis of viral and bacterial particulates [248]. Figure adapted from Fujihashi, K., et al 2012[248].*

The above mechanisms all detail the ways in which the human immune system and gut anatomy achieve homeostasis with our microbiota, but this is not a one-way line of communication. Recent work in mice has suggested that individual bacterial species may have dramatic effects upon the microbiota and the immune system. The specific introduction of segmented filamentous bacteria

(SFB) to the small intestine induced the formation and activation of T_H17 cells in the lamina propria leading to secretion of IL-17[268,269]. This colonisation was also linked to increased expression of inflammation and anti-microbial associated genes in the terminal ileum[270]. To the contrary, *Bacteroides fragilis* has been demonstrated to selectively activate IL-10 producing T_{reg} cells via a symbiosis factor, capsular polysaccharide A (PSA) binding to TLR2 expressed on IECs, leading to immune suppression[63,271]. *Clostridium* has also been demonstrated to induce T_{regs} via upregulation of transforming growth factor– β (TGF- β) by intestinal epithelial cells in the colon[272].

The lymphocytes of the GALT enter from the blood via specialised high endothelial venules (HEV) which are present in the interfollicular T cell zones[273]. In the GALT, the HEV express mucosal addressin cell adhesion molecule-1 (MAd-CAM-1), intercellular adhesion molecule-1 (ICAM-1), and CCR7, all of which are vital for lymphocyte homing to the gut[273–279]. Naïve lymphocytes express L-selectin, $\alpha_4\beta_7$ integrin, and leukocyte function associated antigen-1 (LFA-1), which interact with the HEV ligands in order to roll, bind, activate, arrest, and subsequently extravasate into the GALT[274,275,279]. Memory lymphocytes express higher levels of $\alpha_4\beta_7$ integrin and LFA-1. Chemokines also play a role in the mucosal homing of lymphocytes as evidenced by the specific homing of memory T cells expressing CCR9 the receptor for thymus-expressed chemokine (TECK) to the lamina propria of the GALT of mice[280].

1.2.3 The role of IgA in the mucosa

The microbiome has vital roles in development and homeostasis of the immune system in healthy humans, with the Immunoglobulin A (IgA) shown to be the most abundant isotype of immunoglobulin in human secretions, some estimates suggest that over 1 gram of IgA is secreted every day[281]. IgA plays a fundamental role in mucosal immunity through the coating and trapping of

bacteria in the secreted mucus layer lining the mucosal sites[282]. This IgA-mediated trapping and subsequent immobilisation results in the trapped bacteria downregulating flagella-related genes, further contributing to their immobilisation[283]. IgA coating also enables translocation of non-invasive bacteria into the intestinal lumen and antigen presentation resulting in an adaptive immune response and production of antigen-specific IgA[284].

The production of IgA is exquisitely sensitive to the presence of the gut microbiome. Mice raised in germ-free environments are devoid of mucosal IgA, but upon bacterial colonisation, production of mucosal IgA is rapidly induced[282]. Subsequent work looking at IgA's effect upon the microbiota demonstrated the importance of two-way communication in this system[284]. Knock-out mice lacking the enzyme activation-induced cytidine kinase (AID), and thus the ability to diversify their immunoglobulin repertoire via somatic hypermutation (SHM) and class switch recombination (CSR), suffered from an expansion of the microbiota in their lower intestine[285]. This showed that in addition to the microbiota regulating IgA production, the converse is also true; with IgA production regulating the microbiota. A follow-up study built upon this work using a mouse strain capable of Class-Switch Recombination (CSR) but not SHM, thus allowing the production of IgA but not its affinity maturation[286]. This resulted in production of low affinity IgA as opposed to a total absence of IgA as in the previous study[285,286]. An expansion of the microbiota was detected and an increased susceptibility to oral challenge with cholera toxin compared to wild-type mice led the authors to conclude that the process of IgA SHM and production of high affinity IgA was critical in maintaining intestinal homeostasis and efficient mucosal defence[286].

It is not just the presence or absence of a microbiota which affects the IgA response; colonisation with different microbes can also lead to differing IgA responses. This is due to a variety of factors including; the pathway of IgA induction, either T-dependent or T-independent; the involvement of

follicular helper T cells and follicular regulatory T cells; and the role of Type 3 ILCs[282,284,287–289]. For example, the quality of T follicular helper cell T_{FH} – B cell interactions have been demonstrated to affect IgA mediated microbiota regulation[290,291]. Mice with a T cell specific deletion of PD-1 and MyD88 have shown hypersensitivity to colitis and expansion of the microbiota[292].

The induction of IgA is also dependent upon cytokines such as TGF-β and IL-10 [293–296]. TGF-β has been demonstrated to induce IgM expressing B cells to switch to expressing the IgA isotype. In mice, addition of TGF-β to B cell cultures which have been stimulated with LPS led to an increase in the IgA synthesis[297]. In addition to cytokines, co-stimulatory factors have been demonstrated to be important. Blockade of the CD40-CD40L interaction, has been demonstrated to lead to IgA class switching via endogenous TGF- β production[298]. In humans, the CD40-CD40L pathway has been implicated in work on tonsillar B cells. In these experiments, B cells were stimulated via the CD40 receptor in the presence of TGF-β and IL-10, which led to IgA production[293,299]. The B cell survival factors, B-cell activation factor of the TNF family (BAFF) and, a proliferation-inducing ligand (APRIL) are also potent inducers of IgA class switching, as is transmembrane activator and CAML interactor (TACI) which is a receptor for both BAFF and APRIL[251]. TACI has been illustrated to play a vital role in CD40-independent IgA class switching in both mouse studies and in humans, and mutations in TACI has been shown to result in a selective IgA deficiency although this is contentious[300–303].

IgA exists as two isotypes in humans, IgA1 and IgA2, with differing patterns of expression and induction[298,304]. The two isotypes share a great deal of similarity in their sequences but differ in their hinge regions, which is elongated in IgA1 (Figure 6)[304,305]. This hinge links the two Fab regions and the Fc portion of the antibody. The hinge region is comprised of a proline-, serine-, and threonine- rich repeating sequence of 16 amino acids, allowing an uncommon degree of flexibility[281,305]. However, a high degree of O-linked glycosylation has been detected on the hinge serine residues which may

decrease its conformational variability[305,306]. The reasons for the evolution of IgA1's elongated hinge region are not certain, however suggestions include that the added length enables IgA1 to interact with antigens spaced far apart from each other[305,306]. This purported gain-of-function is associated with a cost, as a number of pathogenic bacteria present in the mucosa, including *Neisseria meningitidis*, *Haemophilus influenza*, and *Streptococcus pneumonaie* have evolved proteases specific for this hinge region, thus compromising its function in defence[305]. Of the two IgA isotypes found in humans and the great apes, IgA1 is unique to this group; having evolved later than the IgA's found in other mammals. IgA2 is most similar to the IgA expressed in other mammals. In humans the majority of serum IgA is IgA1 produced in the bone marrow and it exists in a monomeric form, whereas the secreted IgA comprises of proportionally more IgA2 and is predominantly polymeric[307].

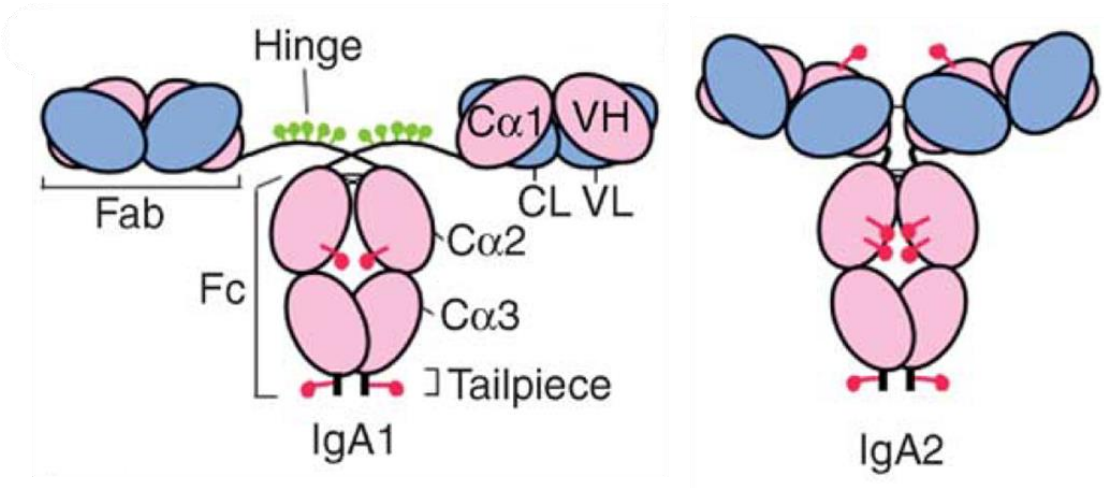

Figure 6 – Schematic of the structure of human IgA1 and IgA2. *Heavy-chain domains are shown in pink, light-chain domains in light blue, - and O-linked oligosaccharides are shown in red and green, respectively[304]. Adapted from Russell, M. W., et al. 2011[304].*

There is also an apparent division in the isotype of IgA secreting cells in the different areas of the mucosa, with IgA1-secreting cells prevalent in most human mucosal tissues, particularly in the small intestine and the respiratory tract[304]. In contrast the colon and genital tract are enriched in IgA2 secreting cells. Most secreted IgA is produced locally at mucosal sites, requiring the polymeric Ig receptor (pIgR) expressed on the basal surface of the intestinal epithelial cells to carry the IgA as a

dimer across the epithelial barrier. When secreted into the mucosa, a portion of the pIgR remains bound to the IgA dimer following cleavage, this is described as the secretory component (Figure 7). This secretory component is heterogeneous in size, with molecular masses between 50 and 90kDa having been reported. IgA itself can in fact be produced in a variety of sizes from monomers and dimers, to tetramers, and even larger polymeric forms. Studies of individual plasma cells reported that the varying sizes can be produced by a single clone, this has also been suggested to be linked to the anatomical site in which the IgA is produced[308].

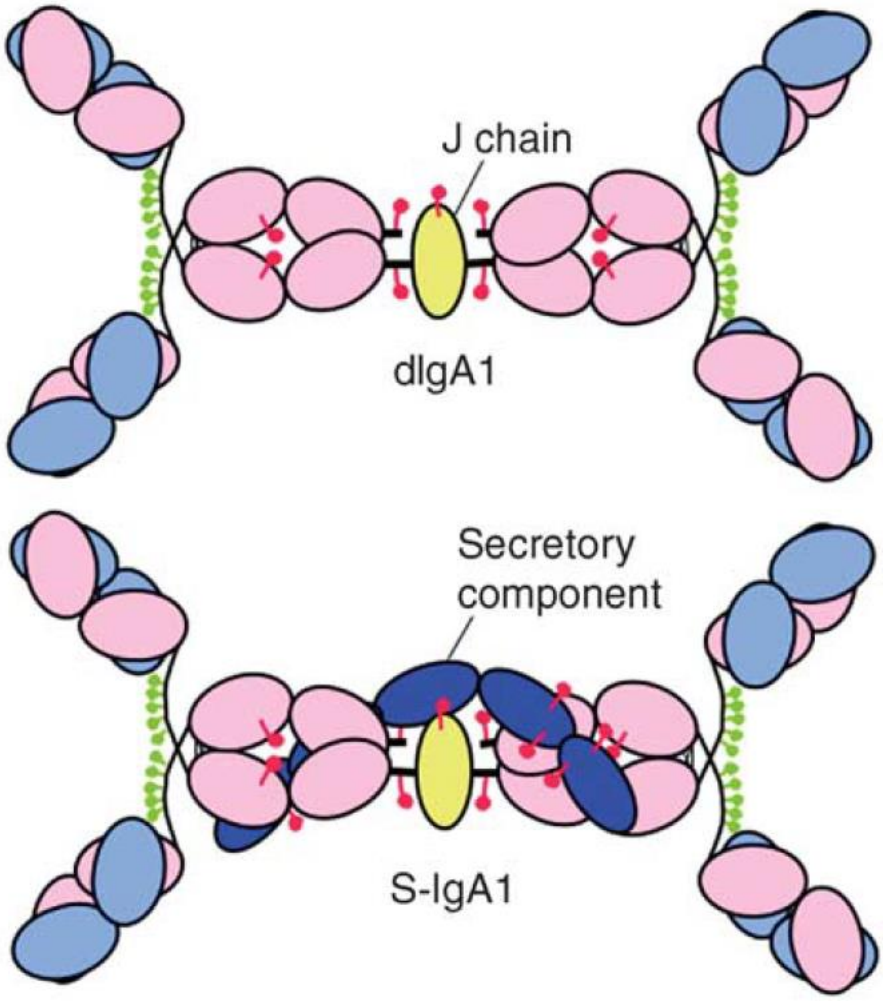

Figure 7 – Schematic of the structure of dimeric and secretory human IgA1. *IgA heavy-chain domains are shown in pink, light-chain domains in light blue, J chain in yellow, and pIgR domains (D1 – D5) in dark blue. N- and O-linked oligosaccharides are shown in red and green, respectively[304]. Adapted from Russell, M. W., et al. 2011[304].*

Despite its importance to mucosal immunology, selective IgA deficiency does not lead to significant problems in humans, although there is a slight increase in susceptibility to autoimmunity, and mouse models describe a mild phenotype in which compensatory IgM and IgG levels comprise the most notable changes[309,310]. In the context of RA, autoreactive IgA has been demonstrated to delineate between individuals at high risk of developing damaging disease, in particular the ratio of IgA to IgG plasmablasts have been observed in the antibody positive group compared to other groups[311]. The IgA plasmablast dominance in the seropositive individuals suggests that a subset of RA-related autoantibodies may arise from mucosal immune responses and may be involved in early disease pathogenesis in individuals who are at risk of developing RA[311]. And importantly, IgA is not able to bind complement due to a lack of appropriate residues in the Fc portion.

1.2.4 The role of the microbiota in autoimmune disease

Mounting evidence from both mouse and human studies points towards an involvement of immune dysregulation and dysbiosis at mucosal sites during the initiation and progression of autoimmune diseases. The links between Crohn's Disease (CD), Ulcerative Colitis (UC), Inflammatory Bowel Disease (IBD), Type I Diabetes (T1D), Autoimmune Liver Disease (ALD) and the microbiota have been extensively investigated[188,200,231,252,312–314]. Perhaps less obvious but with increasing evidence suggesting a link are the systemic autoimmune diseases such as Multiple Sclerosis (MS), and Rheumatoid Arthritis (RA)[315–318]. In these diseases the candidate sites of origin include the oral, lung, and gastrointestinal mucosa, and supporting data for this hypothesis exists for each location[59,149,316].

Hypotheses generally focus on a disruption or dysbiosis of the mucosa or microbiome leading to epitope spreading and disease eventually converging on similar antigens, in the nervous system –

MS, or joints – RA (Figure 8)[318,319]. Involvement of the microbiome has also been implicated in a pro-inflammatory skewing of the immune repertoire caused by mucosal inflammation, as evidenced by T_H17 polarisation[269]. T_H17 cells are most abundant in the lamina propria of the gut where they are responsible for the secretion of many pro-inflammatory cytokines which have been implicated in being vital for maintaining separation and compartmentalisation of the gut lumen and lamina propria[320]. T_H17 cells have also been demonstrated to have roles in exacerbating disease with a key hypothesis for autoimmunity being that an imbalance in inflammatory and regulatory T cell subsets leads to autoimmunity[270]. These hypotheses are independent of environmental considerations, as much of the risk from smoking is due to its disruption of the mucosa in the lungs, either through increased levels of citrullination or through direct disruption to the alveolar membranes[86].

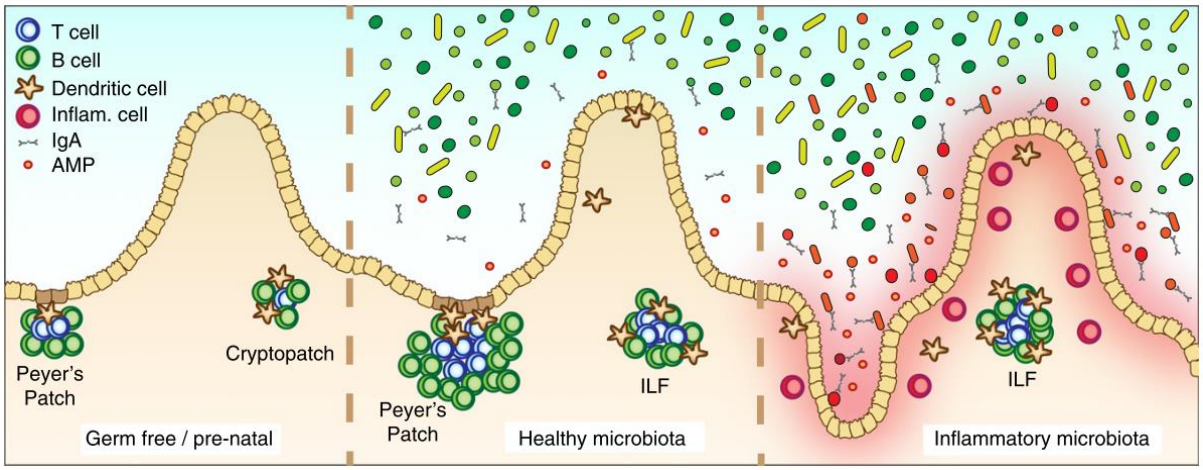

Figure 8 – A diagram detailing the interactions between the immune system of the gut and the microbiota. *Under germ free or pre-natal conditions (left), the Gut associated lymphoid tissues (GALT) are underdeveloped and fewer in number, with small Peyer's Patches and unstimulated cryptopatches as opposed to organised lymphoid follicles. Following colonisation with a healthy microbiota, the GALT is stimulated and expands. The secretion of anti-microbial peptides (AMPs) and IgA serves to segregate the anatomical sites, as does the secretion of a mucus layer from the specialised epithelial goblet cells. The microbiota is sampled via specialised follicle-associated epithelial cells (FAEs) known as M cells, adjacent to the Peyer's Patches, which enable the GALT to experience microbiota associated antigens resulting in the development of a healthy intestinal immune reaction. Dysbiosis leads to disruption of these processes (right). The infiltration of the normally sterile lamina propria by inflammatory commensals and other bacteria causes a strong IgA response, and the influx of inflammatory immune cells such as Th17, Th1, neutrophils and inflammatory monocytes. Figure adapted from Flavell, R. A., et al., 2015*[231].

Striking evidence for a role of the microbiota in autoimmunity, affecting a relatively immune privileged distal site, is provided by multiple sclerosis (MS)[321]. In a mouse model of MS, experimental autoimmune encephalomyelitis (EAE), the symptoms were significantly diminished in animals raised in a germ-free environment compared to those housed conventionally[322]. Animals then colonised solely with Segmented Filamentous Bacteria (SFB) went on to develop symptoms driven by a T_H17 imbalance[262].

In humans, patients with MS display a variety of microbiome imbalances[323]. These alterations correlate with variation in the gene expression of pathways involved in dendritic cell maturation, IFN and NF-kB signalling pathways in circulating T cells and monocytes[323].,It has been demonstrated that when human microbiota from MS patients is introduced into transgenic mice prone to developing disease symptoms, they developed disease symptoms at a far higher rate than when given healthy microbiota from healthy monozygotic twins[324]. Collectively, these findings point us towards the potential for microbiota dysbiosis involvement in autoimmunity, even at sites distal or protected from the mucosa.

1.2.5 The role of the microbiota in Rheumatoid Arthritis

The idea that the cause of RA originates in the mucosa is not a new one. Hypotheses which implicate bacteria present in the joint as responsible for the pathogenesis of rheumatoid arthritis stretch back over 100 years [325]. More recent hypotheses suggest that the gut flora and oral microbiome are responsible[326–328]. The use of sulfasalazine as a treatment for RA in the 1940s originated from the hypothesis that streptococci present in milk were a causative agent of RA[316,329–331]. Initial results were considered positive but use of sulfasalazine fell from standard rheumatological practice for several decades before more recent studies demonstrated that in conjunction with hydroxychloroquine it

was comparable in efficacy with methotrexate, all three of which are disease modifying anti-rheumatic drugs (DMARDs) in common use today[332,333].

Evidence that the microbiota is involved in rheumatic joint disease can be found in prospective observational studies which have reported expansions and contractions of certain bacteria in humans prior to disease onset[59,165,167,168]. A large percentage of patients suffering from autoimmune diseases of the gut such as Crohn's Disease (CD), and Ulcerative Colitis (UC), suffer from articular manifestations, i.e. joint pain and inflammation, suggesting that inflammation of the mucosa is capable of spreading to the joints[334–338]. The pathogenesis of reactive arthritis is also thought to be bacterial in origin[339].

Gnotobiotic animal studies have also provided strong evidence for a role of the microbiota in RA. Gnotobiotic animals are raised in a germ-free environment and colonised with either specific bacteria or a pre-defined microbiota at specific stages in their life[241]. These experiments have revealed the absence of certain bacteria to be protective of arthritis and the presence of certain bacteria to be responsible for the development of disease.

Early experiments conducted using adjuvant-induced arthritis in germfree, specific-pathogen-free, and conventionally raised rats suggested that susceptibility was dependent upon the presence of a microbiota[340]. Every rat raised in the germ-free environment developed symptoms of arthritis, whereas the conventionally housed rats developed only low-level disease[340]. This suggests that, whilst not essential for disease development, the microbiota exerts a huge immunomodulatory effect. This also suggests that the adaptive immune system is not required for disease development since germ-free rats do not produce specific autoantibodies against heat shock protein 65 but do

develop disease[341]. Another murine model which is strongly affected by germ-free housing is that of the SKG mouse, a T cell-driven model of arthritis in which autoreactive T cells are selected for during thymic selection in order to test the hypothesis that high self-reactivity and resulting thymic production of pathogenic autoimmune T cells may be a primary cause of and also a predisposing factor for RA in humans. In this model there is strong T_H17 cell driven joint inflammation[269,342]. When raised in germ-free conditions, the SKG phenotype is again diminished[57,269].

Mice in which the IL-1 receptor antagonist has been knocked out (*Il1rn*$^{-/-}$) spontaneously develop a T cell-mediated arthritis when housed in normal conditions, but do not develop disease when raised in a germ-free environment[343]. However the colonisation of these *Il1rn*$^{-/-}$ mice with the commensal bacterium *L. bifidus* lead to the rapid onset of disease symptoms comparable with the normally housed mice[343]. The mechanism was determined to be an expansion of T_H17 cells and corresponding contraction in the T_{reg} cell population mediated via TLR2-TLR4 signaling[343].

In the experimental collagen induced arthritis (CIA) model disease can be immunised against via oral administration of *Bacteroides fragilis* expressing colonisation factor I[63]. This leads to an upregulation of IL-35 which subsequently activates IL-10 secreting $CD39^+$ T_{reg} cells leading to amelioration of disease[63]. The K/BxN transgenic mouse strain, expressing both the T cell receptor (TCR) transgene KRN and the MHC class II molecule Ag7, develop a severe inflammatory arthritis due to T cell mediated production of autoantibodies recognizing glucose-6-phosphate isomerase (GPI)[344]. Germ-free animals were largely free of disease, which was thought to be linked to a corresponding deficiency of T_H17 cells[269]. Introduction of SFB, a commensal microbe, led to disease symptoms comparable to normally housed mice of this genotype[269]. Prophylactic use of SFB-specific antibiotics was found to attenuate disease symptoms[269]. However, this picture was complicated by results demonstrating that in a different genetic background the requirement for IL-17 was in fact

dispensable and that T_{FH} cells were responsible for driving disease. This still supports the notion of the gut influencing arthritis but has implications for IL-17 targeting therapeutics[345].

The composite human/microbiota 'superorganism' is initiated by the vertical transmission of components of the mother's microbiota at birth. Normally, intestinal colonization of neonates is dominated by transmission of bacteria of the maternal vaginal flora, which is less diverse than that of the lower intestinal tract. The 'pioneer' species received from the mother appear to be important, as infants born by Caesarean section and initially colonized by bacterial species of epidermal, rather than vaginal, origin may be predisposed to development of immune disorders later in life[346–350].

Although B cells directed against citrullinated autoantigens are a common feature of rheumatoid arthritis, there has been little work characterising the reactivity of B cells in RA patients against mucosal antigens. The hypothesis being that RA involves a process of molecular mimicry in which a response is mounted against an antigen which appears to the cell to be very similar to a host antigen present in a joint restricted manner which leads to disease and joint symptoms. One example has been the link between periodontal disease, particularly that resulting from *Porphyromonas gingivalis*, and rheumatoid arthritis[351]. Cross-reactivity between bacterial antigens and citrullinated human epitopes from antibodies cloned from gingival-tissue resident B cells has been demonstrated, but there is a lack of data in which other sites and a broader range of microbial antigens are assessed[319].

1.3 B cells

1.3.1 A Brief History of B cells

Bone-marrow derived or bursa-derived (B) cells are an essential column of the adaptive immune system, primarily responsible for the production of immunoglobulin (Ig) directed against microbial

invaders[352]. Since this central role was defined, further roles for B cell subsets, with functions in both adaptive and innate immune responses, as well as playing a role in regulating T cell immunity, have been described.

The role of the B cell was initially described in parallel with that of the T cell during the 1960s and 1970s in work from Max Cooper and Robert Good[353,354]. This work focussed on the role of two lymphoid organs in the chicken, the bursa and the thymus. They described that surgical removal of the thymus followed by a sub-lethal dose of X-rays led to an abrogation of cellular immunity: graft versus host reactions, responses of delayed hypersensitivity and homograft rejection[353,354]. These thymectomised animals also grew far more slowly and lacked the "small lymphocytes of the circulation", which we now describe as T lymphocytes[353,354]. If on the other hand, the bursa was surgically removed and the chickens treated with the same X-ray dose, these animals were shown to lack the gamma globulins, the "large lymphocytes of the circulation", and were unable to produce antibodies in response to repeated immunisation with bovine serum albumin, or *Brucela abortus*[353,354]. In all other aspects of immunity it was demonstrated that they were normal[353,354].

This work demonstrated that there were two complementary systems of immunity, with those Bursa-derived, or B-cells, responsible for the production of antibodies and those cells requiring an intact thymus responsible for the delayed-type hypersensitivity response. Subsequent work in murine bone-marrow transplant models went on to demonstrate that the cells responsible for the antibody response were derived from the bone marrow in mice[355,356].

These cells were eventually named "B cells" which coincided both with the site of their discovery and the site of their development in mammals. Following this, the description of immunoglobulins as unique surface markers of B cells enabled the discrimination of B cells from other lymphocytes and

the description of phenotypic differences between healthy and lymphoblastic cells[357,358]. This is a key diagnostic parameter still in use today[359]. It took several years however, before the precursors to the B cell was described in humans and the development of these cells was unravelled[360].

1.3.2 B cell Precursors

One important caveat to our current understanding of B cell development is that much of the work has been carried out in murine models, however cells with similar phenotypes to those developmental stages seen in mice have also been found in humans, suggesting that much of the pathway of B cell development is conserved.

B cells derive from haemopoietic stem cells (HSCs), initially produced in the human foetal liver during gestation by 7.5 weeks and in the bone marrow by weeks 14-17[360]. These HSCs are regarded as 'noncommitted' as they have the potential to become any lymphoid cells such as T cells, Natural Killer (NK) cells, and B cells; or indeed blood cell, from a mast cell to an erythrocyte.[361–363]. It is for their potential to develop into any lymphoid cell means that they are known as Common Lymphoid Progenitors (CLPs)[361–363]. During their development these CLPs then have their differentiation potential sequentially narrowed over a number of developmental intermediates until they give rise to a lymphoid cell[361–363]. These developmental intermediates are restricted by the tightly regulated, sequential expression of transcription factors[361–363]. The transcription factors which have been demonstrated to be most important for B cell development include PU.1, Ikaros, E2A, Early B cell Factor (EBF), and Pax5, however there are many other transcription factors which act together to give rise to the mature B cell.

1.3.3 Transcriptional Regulation of B cell Development

PU.1's importance to B cell development has been charted through mouse model experiments which have demonstrated that knock-out of the gene encoding PU.1, *Sfpi1*, leads to an absence of lymphoid cells[364,365]. This severe phenotype results in very early death, some days after birth or earlier. That PU.1 controls many of the events in early HSC to B cell development such as expression of IL-7Rα (necessary only in mice) and B220 is demonstrated by deletion of the *Sfpi1* gene by the CLPs stage showing no effect on B cell development but arresting the cells at the CLP stage[364]. However, downregulation of PU.1 is also critical for a B cell's development as higher levels lead progenitors down a path of myeloid development[366]. The DNA-binding proteins EBF1 and E2A have been shown to be essential for the expression of B cell lineage determining genes and the suppression of other genes which would lead precursors down a different route of development[365]. These transcription factors bring the CLP to an early B cell progenitor stage, with EFB1 positively regulating many further transcription factors important for this, including expression of Igα, VpreB, and Pax5[365]. These follow from PU.1 developmentally, deletion of the DNA-binding proteins VpreB, and Pax5 prevents cells from progressing beyond the pro-B cell stage[365].

Once these cells are expressing Pax5 they are restricted to becoming a B cell precursor, for this reason Pax5 is known as the master transcriptional controller of B cell development[365,367]. Pax5's key function is to limit the expression of any factors which may lead down a different developmental pathway and to activate genes which lead to becoming a B cell[367]. For example, it has been shown in mice that Pax5 antagonises the expression of Notch1, required in T cell development and that Pax5 prevents expression of receptors for macrophage colony-stimulating factor (M-CSF)[365]. Pax5 also has a role in the process of immunoglobulin (Ig) loci rearrangement which leads to the surface expression of a unique Ig, and thus a functional B cell receptor (BCR) and mature B cell[365].

1.3.4 Immunoglobulin Rearrangement

The process of Ig rearrangement serves to generate an Ig molecule comprised of a unique pairing of two heavy chains and two light chains, so-called due to their relative size differences[352]. This Ig molecule is then expressed at the surface of B cells and functions as their BCR, enabling them to react to antigens, or as secreted Ig or antibody, should they differentiate into plasma cells[352]. The rearrangement of Ig genes occurs in the bone marrow (BM) and involves the error-prone process of rearrangement of the genes encoding the heavy and light chains of the Ig through the action of two enzymes, RAG1 and RAG2[368]. The RAG proteins are involved in DNA cleavage and joining, and have been demonstrated to be essential to Ig rearrangement in numerous mouse models and in humans, Ku is a DNA-repair protein which is also vital to this procedure[368–370]. The process is sequential and mechanistically very similar for both light and heavy chains, however key differences exist.

The first set of rearrangements occurs at the locus of the heavy chain gene which is located on chromosome 14 and consists of a number of variable (VH), diversity (DH), joining (JH), and constant (CH) gene segments each separated from the next by an intron[371]. The process is complex but follows clear rules which dictate how each segment interacts with the others, there also appears to be biases between which DH, JH, and VH gene segments interact despite it being technically feasible for completely random recombination.[372–376].

Firstly, a DH region is juxtaposed with a JH region and the signal sequences between them are excised, this occurs during the CLP stage[377]. Next, this DHJH segment is joined to a VH segment and again a signal sequence is excised, this now occurs during the pro-B cell stage[377]. This newly generated VHDHJH segment, including the C region still separated by an intron, is then transcribed[377]. Following transcription, the intron separating the VHDHJH segment and the C region is deleted, and the complete heavy chain protein is translated and expressed, containing the IgM constant region (Cμ) which is the constant region closest to the variable regions on the chromosome[377]. The cell now

has a newly rearrange IgH locus but still maintains the germline configuration of its light chain loci[377]. Given that each pro-B cell has two Ig heavy chain alleles it might be expected that they would express both, however, only one is responsible for the expressed heavy chain, this process is known as allelic exclusion and is still the subject of active research.

At this point a pre-BCR is expressed at the surface of the cell consisting of the rearranged IgH, called the μ-heavy chain protein due to its μ constant region and the surrogate light chain formed of a λ5 and Vpre-B protein; in a complex with the Igα-Igβ dimer[377]. This pre-BCR represents a critical stage for the cells development and demonstrates its transition from a pro-B cell to a pre-B cell. Any developing B cells which have non-productive IgH rearrangements remain arrested at this stage of development[377].

The next stage of B cell development involves the rearrangement of the light chain loci. This occurs in a similar manner to that of the IgH chain, under the control of the RAG1 & RAG2 proteins, but there are only two light chain encoding genes, kappa and lambda, since the light chain gene loci lack the D regions seen in the heavy chain loci[361,377,378]. This results in the final light chain protein being translated from a VLJLC transcript. Prior to this rearrangement there is a round of proliferation of pre-B cells, each expressing the same IgH, but following the light chain rearrangements, different IgL[371,377]. Once this process is complete a population of B cells exist, sharing their heavy chain sequence but each with a uniquely rearranged light chain, this forms a complex with the Igα-Igβ dimer to form a mature IgM BCR[377].

The heavy chains of the BCR have relatively short intracellular regions which limit their ability to directly signal, however the Igα-Igβ dimer both contain an intracellular tyrosine activating motif (ITAM) which enables signal transduction following antigen binding to the BCR. This signal

transduction is dependent on a great number of intracellular proteins and results in, amongst other signals, the influx of calcium. This calcium influx signals downstream mediators including mitogen-activated protein (MAP), kinase cascade ERK, and transcription factors, including nuclear factor of activated T cells (NFAT) and nuclear factor kappa-B (NFκB) resulting in transcriptional changes and signaling cascades leading to changes in proliferation, differentiation, and survival[379]. The initial expression of this newly generated BCR leads to allelic exclusion of the light chain loci and downregulation of RAG1 and RAG2[380].

1.3.5 Immature B cells

Before departing from the bone marrow these immature B cells are subject to negative selection or repertoire censoring in which cells expressing a strongly self-reactive BCR are removed or attenuated. This is achieved through a variety of mechanisms including receptor editing, deletion, and anergy, and is in part determined by the strength of signal through a BCR upon antigen binding[381]. It is due to the great number of combinations of different antibodies that can be generated during Ig rearrangement that all healthy individuals are known to produce self-, or auto-reactive B cells in which the BCR recognises host antigens[382,383]. It is estimated that >75% of immature B cells are self-reactive, however once they reach the periphery as naïve B cells that percentage drops to an estimated 30%[33].

The process of receptor editing involves giving cells with a strongly self-reactive BCR another chance to rearrange their Ig loci in order to produce a less self-reactive combination[382,383]. If this is not successful then the cell has two choices, either this B cell can be deleted, or it can be rendered anergic[384,385]. Deletion was long believed to be the primary mechanism by which organisms managed the problem of central tolerance, however it is now seen as one option of many. If a B cell fails at receptor rearrangement and still expresses a self-reactive BCR then it can be deleted via signaling

through Fas and Bcl-2[383]. Any B cells which have productive BCR rearrangements and avoid the above fates then leave the bone marrow and enter the circulation[381]. However, as mentioned previously, this process is not infallible and autoreactive clones do 'escape' into the periphery.

1.3.6 Naïve B cells

In the mouse, in order to continue their development, these circulating transitional B cells migrate to the B cell follicles of the spleen where they pass through a number of transitional stages before developing into follicular B cells[386]. In humans this site of maturation is not well understood[371,381]. It must be stressed again here that much of the work elucidating the development and roles of B cells has been carried out in mice and whilst a great deal of the biology is conserved between the two, differences do exist. This is most obviously illustrated in the subsets of circulating naïve B cells for which there are often no direct homologs between mice and man.

Two states of transitional B cells have been described in mice, distinguished by their expression of IgD, with the transitional 1 (T1) B cells lacking its expression[23,371]. This stage of B cell development is highly important for the selection of self-reactive clones, negatively during the T1 stage particularly against blood-borne antigens, and positively during the T2 stage via BCR signaling[371,387]. At this point B cells are highly dependent on survival factors such as B cell activating factor of the tumour necrosis family (BAFF), without which they will fail to develop beyond the T1 stage[388]. BAFF is a highly important cytokine produced by cells of the innate immune system and stromal cells and has been implicated in autoimmunity[389–393]. Mice with mutations leading to excessive production of BAFF have higher numbers of activated autoreactive B cells compared to controls[394]. Higher levels of BAFF in the serum have been detected in patients with primary Sjogren's Syndrome (pSS), Systemic Lupus Erythematosus (SLE), and Rheumatoid Arthritis (RA)[389,392,393].

In mice the T2 B cells are then presented with two fates, either to become a Marginal Zone B cell so-called because they localise to the splenic marginal zone, or to become circulating follicular (FO) B cells[386,387]. These two subsets have clear phenotypic and functional differences with MZ B cells able to rapidly respond to T -independent antigens and FO B cells to antigens requiring the help of T cells, or T-dependent antigens[371]. The signals which lead mature naïve B cells down these paths are not fully understood, however Notch2 signaling, NF-κB, and the BCR are vitally important for this[386].

In humans approximately 60% of the circulating B cells are naïve B cells with a heterogeneous population of 'MZ-like' equivalents present, with a similar phenotype to that found in mice, however the development and localisation remain different[395]. These 'MZ-like' B cells are distinguished by their surface expression of CD27, CD21, CD1c, and IgM and low levels of IgD[395]. It is not yet clear if these circulating 'MZ-like' B cells are memory B cells, which given their expression of CD27 they might be considered[395,396]. In patients with genetic mutations leading to CD40L deficiency, which would thus disqualify T cell help, these cells are still present suggesting that they may be T-independent antigen experienced[371]. Whilst in humans the spleen has a very different structure, lacking a marginal zone, cells of this phenotype can be found and in splenectomised individuals have been shown to be more susceptible to encapsulated bacteria[397]. This is thought to be related to a deficiency in the antibody response to the T-independent antigens of the capsular polysaccharides and thus implicates a lack of MZ-like B cells. This is also seen in infants of up to 2 years of age who remain susceptible to infection from capsulated bacteria despite the presence of MZ-like B cells, this implicates a level of development required or a lack of cooperation between antigen recognition and presentation[371,398]. However, other work has implicated the role of an immunosuppressive $CD71^{+}$ erythroid population[399].

The naïve follicular B cells in mice leave the spleen and recirculate to the secondary lymphoid organs where they undergo further development[371,381]. These organs include the lymph nodes (LN), the spleen, and the Peyer's Patches of the intestines. Mature follicular B cells mediate the majority of the T helper cell-dependent humoral immune responses, resulting in immunological memory[400]. During an infection, BCRs which recognise antigen are internalised and processed by the B cell[401]. This processed antigen peptide is then presented by the B cell in an MHC dependent fashion to CD4+ T cells, which have been primed by the same antigen presented by dendritic cells (DCs)[402]. This interaction leads to the production of plasma cells (PCs) via two routes; an extrafollicular route which does not require the germinal centre (GC) and requires less time, and the longer formation of a germinal centre in which the processes of somatic hypermutation (SHM), class-switch recombination (CSR), and clonal expansion take place[402]. The extrafollicular route leads to production of low affinity IgM in order to provide a rapid response to infection[402]. The follicular response leads to the production of long-lived plasma cells expressing BCRs with a higher affinity for their antigen and the subsequent isotype switching increases the number of antibody effector mechanisms available to clear the infection[403,404].

1.3.7 The Germinal Centre

The GC reaction involves the action of activation-induced cytidine deiminase (AID), the role of AID is to introduce mutations in the variable regions of the Ig locus the region responsible for antigen binding, thus leading to increases in affinity of that Ig/BCR for its cognate antigen (Figure 9) [405]. AID achieves this through catalysing the conversion of the DNA base cytosine (C) to uracil (U) via deamidation[406]. These uracils are then recognised by further enzymes, either the mismatch recognition MutSα, comprised of two enzymes MSH2 and MSH6, or by uracil-DNA glycosylase (UNG)[406]. This leads to several outcomes including replacement of the uracils by another base

inducing SHM or double-stranded breaks in the DNA which are required for CSR, or repair of the uracil to its original cytosine[377,405–407]. Given its role in altering DNA, AID is implicated in B cell lymphomas[408]. CSR involves deletions in the germline DNA encoding the VDJ region of the IgH and subsequent re-ligation of the VDJ to a C region gene such as Cγ1, which would lead to the IgG1 isotype being expressed[406]. Each of the C region genes has a different role and provides the antibodies produced with a specialised effector function. CSR has been demonstrated to be negatively regulated by the aryl hydrocarbon receptor[409].

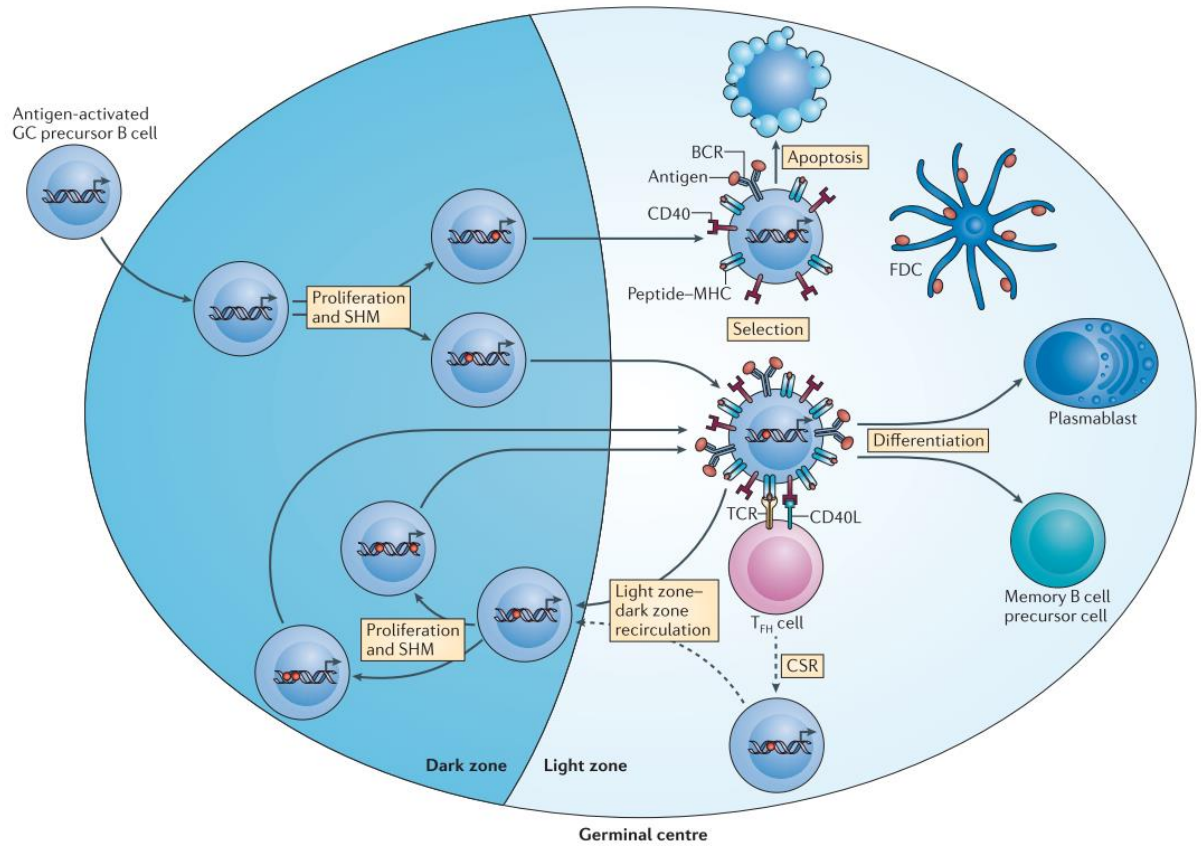

Figure 9 – A schematic of the Germinal Centre and its' processes. *The anatomical compartments of the Light and Dark Zones are shown and the sequential processes which take place in the GC, leading to the affinity maturation and clonal selection of B cells and the differentiation of Plasmablasts and Memory B cell are outlined. Figure adapted from Ulf Klein, U., et al., 2015*[410].

These GC centred processes involve help from the other cells present, such as the T follicular helper (TFH) cells and Follicular Dendritic Cells (FDCs), and associated secreted factors, and relies on the histological structure of the GC with its light zone (LZ) and Dark Zone (DZ) through which the maturing B cells move through in cycles as the higher affinity clones are selected for through successive rounds of SHM[410–413]. The DZ consists almost entirely of proliferating B cells and the LZ in which these B cells interact with TFH, FDCs, and macrophages (Mϕ)[413].

Following the GC reaction these somatically hypermutated, isotype switched B cells then differentiate in memory B cells (Bmem) or long-lived plasma cells (PCs) that migrate to the bone marrow[396,404]. Memory B cells are retained in peripheral lymphoid tissues for the lifetime of the host enabling faster and higher affinity responses to secondary microbial challenges[396].

1.3.8 Autoantibodies

One fundamental feature of autoimmune disease is the presence of auto-reactive antibodies due to a loss of peripheral tolerance, in which B cells are obviously implicated. This is the case in Systemic Lupus Erythematosus (SLE) in which antibodies specific for nucleic acids are detected and for Rheumatoid Arthritis (RA) in which antibodies targeting citrullinated proteins are often detected[104,195,414,415]. Antibodies targeting the Fc portion of immunoglobulin G, known as Rheumatoid Factor (RF), are another common self-directed antibody response seen in autoimmunity, common to both RA and SLE[416]. However, RFs are not unique to autoimmune diseases, they can occur in the elderly, and immunised but otherwise healthy individuals[417].

The presence of immune complexes (ICs) formed of autoantigens and autoantibodies is also a signature of many autoimmune diseases, including RA, SLE, and primary Sjögren's syndrome[414,418].

These agglomerations of antibody can lead to inflammation via a variety of routes including the activation of complement, and various functions dependent on Fc receptors[419,420]. The classical complement cascade involves the binding of an Fc portion of an antibody by C1q, this triggers activation of a series of processes leading to active inflammatory components such as C5a and C3a[419]. Combined, these components attract and activate a wide range of effector cells such as natural killer cells, mast cells, and neutrophils and stimulate them to release proteolytic enzymes and cytokines[421]. These Ics have been demonstrated to accumulate in the kidneys of patients with SLE and lupus nephritis and contribute to damage involved in these diseases[420]. Ics are also able to bind directly to Fc receptors which can lead to antibody-dependent-cell-mediated cytotoxicity (ADCC)[419].

The precise role of these autoantibodies in RA is not known but they have been detected years prior to the onset of symptoms[8,95]. This could suggest a level of step-wise decline of immune tolerance or that a threshold of circulating autoantibody levels must be passed before disease develops. However, not all patients have these autoantibodies which strongly suggests that they do not completely define RA's aetiology[98]. The presence of these ACPA does appear to prognose a more aggressive form of disease but is not required for disease diagnosis; contrastingly RF has not been demonstrated to provide utility as a prognostic tool but is relied upon as a diagnostic[98,101,422].

Given their role in antibody production it would be reasonable to assume that treatment with Rituximab, a B cell depleting biologic, would ameliorate disease through reduction of autoreactive B cells and thus the auto-antibodies they produce. However rituximab only targets $CD20^{+}$ cells, thus leaving the $CD20^{lo}$ B cell populations intact, such as short-lived plasmablasts, bone marrow resident B cells, and $CD79^{+}$ plasma cells[423,424]. It has been demonstrated that in many rituximab treated patients, extended periods of clinical remission are achieved independently of serum autoantibody reduction[227]. This suggests that B cells can contribute to disease pathogenesis through antibody

independent mechanisms.

1.3.9 Memory B cells

Retaining a memory of the antigens experienced over a lifetime is vital for the host and it relies upon the adaptive humoral immune response to do so[425]. The humoral immune system consists of constitutive and adaptive arms, the constitutive response is comprised of pre-existing antibodies secreted by long-lived plasma cells[425,426]. The adaptive response enables for an enhanced response to a subsequent infection and it does so through memory lymphocytes, including memory B (Bmem) cells. Upon activation with a previously recognised antigen these Bmem are capable of differentiating into antibody producing plasma cells and rapidly proliferating in a CD40 dependent manner, enabling the host to produce a large amount of high affinity class switched antibodies to prevent and fight infection[412,425–427]. This process can be subverted in the case of autoimmunity in which an adaptive immune response is mounted directed against the host tissues[428,429].

Signaling through innate receptors such as the TLR in addition to BCR activation can affect the outcome of memory B cell responses. Despite the fact that this secondary adaptive response primarily consist of somatically hyper-mutated and class switched antibodies, it has been demonstrated in mice that not all of the memory B cells are derived from the germinal centre reaction.[430,431]. IgM^+ memory B cells which are not class switched have been found to be produced in mice in response to strong CD40 signaling, the levels of SHM are heterogeneous within the population with CD27, a traditional memory B cell marker seeming to discriminate those with higher levels of SHM[430]. In addition, “natural effector” B cells and $CD27^-$ IgA^+ memory B cells have been described in humans, which are also generated outside of the GC reaction[432]. These natural effectors are also CD40/CD40L independent as they have been seen in patients with mutations in CD40/CD40L

leading to deficiency in these molecules, this suggests that these cells are generated free of T cell help[395,433,434].

B cell memory is long-lived, a striking example being survivors of the 1918 flu pandemic having circulating memory B cells, able to secrete neutralising antibodies against the virus, 90 years after initial infection[435]. In mice this has also been demonstrated using phycoerythrin as a model antigen, whereupon antigen specific memory B cells were detected up to 450 days after initial stimulation[436]. This work also revealed a differential response from IgM^+ and IgG^+ memory B cells with IgM^+ memory B cells outlasting the IgG+, with the causes not certain but suggested to be a combination of factors including responsiveness to survival factors and differential colocalization to survival niches[411,437].

Memory B cells are also able to mount more effective immune responses than naïve B cells when challenged with a pre-recognised antigen[425]. This is dependent partially on the help they receive from the TFH and FDCs in the secondary lymphoid tissue enabling secondary GC formation and further affinity maturation; partially on the differences between the unswitched IgM and antigen experienced, switched IgG; and partially on the previous experience of the BCR of the antigen in question[438,439,448–450,440–447]. The increased numbers of antigen-specific cells produced during an immune response is also a factor. Very different memory B cell responses can form depending upon the antigen involved in their generation, for example, T dependent antigens leading to the classic GC response[451]. In the case of chronic antigenic stimulation, for example during viral infection or in many autoimmune conditions an atypical memory B cell response can be generated[452].

1.3.10 Atypical B cells

Atypical memory B cells (ABCs) have been variously described as “anergic”, “exhausted”, and “atypical”[385,452]. A common discriminator of these cells is their expression of low levels of the Complement receptor CD21, and elevated expression of the integrin CD11c[452,453]. The population has been described in primary Sjögren’s syndrome (pSS), Common Variable Immunodeficiency (CVID), Hepatitis C virus (HCV) which is associated with the B-cell lymphoproliferative disorders mixed cryoglobulinemia (MC), Human Immunodeficiency Virus (HIV), malaria, and in Rheumatoid Arthritis (RA)[452–461].

Although there are variations between the features of this population described in the different disease contexts common features include; unresponsiveness to CD40/BCR stimulation as determined by calcium flux, proneness to apoptosis, whilststill remaining responsive to TLR-9 and TLR 7 agonists. This ‘exhausted’ description, so-called due to its similarities with exhausted T cells, has been used to primarily describe the $CD27^{-}$ $CD21^{lo}$ atypical memory B cells[462]. The subset has also been correlated with lymphoproliferation and autoimmunity. Whilst they are able to differentiate into antibody secreting cells in vitro the corresponding antibodies are absent from patients serum, they express a variety of characteristic inhibitory receptors, and that interfering with the inhibitory signaling of these receptors leads to an increase in the cells proliferation upon BCR stimulation [463,464].

From a phenotypic perspective atypical B cells express a number of molecules on their surface associated with anergy and the inhibition of signals including $CD21^{lo}$, $CD11c^{+}$, $FCRL4^{hi}$, $IL\text{-}4R^{lo}$ and highly upregulated genes such as SOX5, ITGAX, Galectin-1, and FGR[465]. An enrichment of autoreactive B cell receptors (BCRs) have also been described in this population using the variable heavy chain genes VH1–69 and VH4–34 which are often associated with autoimmunity[466]. T-bet has also come to light as an important transcriptional marker of this subset, capable of driving the formation of MBCs

which are phenotypically similar to atypical MBCs. Certain atypical MBCs have been described lacking the classical memory B cell marker CD27 in addition to expressing low or negative levels of CD21[467]. As of yet the requirements for the generation of atypical MBCs are unknown beyond their association with chronic infectious disease and antigenic exhaustion[468].

1.3.11 B cells in Autoimmune Disease

In addition to their role as autoantibody producers, B cells with an activated phenotype have been demonstrated to secrete a number of pro-inflammatory cytokines and chemokines including IL-6, IL-12, TNF-α, IFN-γ, and RANKL, all of which have been implicated in the inflammatory cascade of autoimmune pathology[469–476]. In addition, antigen presentation by autoreactive B cells is seen in autoimmune disorders such as Type I Diabetes (T1D), and RA and can promote pathology independent of antibody production[477–479].

The effectiveness of B cells as antigen presenting cells (APCs) is demonstrated by their specificity in antigen capture, in comparison with other APCs such as macrophages and dendritic cells who internalise antigens via pinocytosis, B cells capture specific antigens via their BCRs[401]. This BCR mediated process is highly specific and has been demonstrated to be many times more efficient than pinocytosis and enables the internalisation and subsequent presentation of antigens which are present at very low concentrations, as is often the case in autoimmune diseases[480–484]. B cells' role as APCs have been documented in a number of autoimmune diseases including RA, and in animal models, their APC function has been demonstrated to be vital for the development of disease[485]. In one study, the authors demonstrated that transgenic mice deficient in total immunoglobulin (Ig) expression, both surface and secreted, did not develop proteoglycan-induced arthritis, and those with expressing it only on their surface failed to develop disease[482]. However, when antigen was

directly targeted to the BCRs, T cells were activated, and the mice developed arthritis[482]. The transfer of autoantibody containing serum from these mice to others only induced a mild arthritis[482]. This suggests that B cells are required for the development of experimental arthritis and that it is not simply expression of surface BCRs which enable them to respond, as the T cells were not adequately primed, it also requires Ag-specific B cells[482]. It further suggests that the presence of autoantibodies in the serum is not sufficient to generate disease and that a synergistic effect of the antigen specific B cells inducing autoreactive T cells and autoantibodies are required to recapitulate the symptoms of arthritis[482].

It is clear that B cells both amplify and suppress immune responses by antibody independent mechanisms, including pro/anti-inflammatory cytokine secretion and through their functions as professional antigen presenting cells (APCs).

1.3.12 B Cell Depletion Therapy

B cell depletion therapy (BCDT) has been applied in several autoimmune diseases such as Multiple Sclerosis and primary Sjogren's syndrome, with varying levels of success and trials ongoing[486–489]. However, the paradigmatic shift in using BCDT in rheumatic diseases came not from these diseases but from RA, a disease which, at the time, was thought to be driven by macrophages and the inflammatory milieu of the synovium. The first application involved the treatment of five of these first patients all suffering from RA which was deemed refractory to all other treatments[227]. Surprisingly, all five of the patients achieved a ≥50% improvement in disease activity as determined by the American College of Rheumatology criteria[227]. Following this success, a clinical trial involving 22 RA patients further demonstrated the safety and efficacy of BCDT[490]. These studies utilised Rituximab, a chimeric mouse-human monoclonal antibody which specifically targets CD20, and its

success as a treatment in RA underlines the importance of B cells in the disease pathology[490,491].

However, it should be noted that not all B cells express CD20, notably the bone marrow resident B cell precursors and mature plasma cells, and that it is not B cells alone which express CD20 as a population of $CD20^{+}$ T cells has also been described[403,423,492–495]. It is also not clear whether BCDT is capable of total depletion of the B cells present in the other immunological compartments of patients with RA, however there is some evidence that this does occur but with varying degrees of efficacy[496,497]. Furthermore, following BCDT, a decrease in the levels of circulating autoantibody levels is detected and after approximately 6 months B cell numbers are recovered. However, it should be noted that the decrease in autoantibody levels doesn't correlate directly with the improvement in symptoms seen in patients, most likely as a function of the plasma cells being spared[403]. This implicates a function beyond autoantibody production in disease pathology for B cells.

Of particular note in this context is the reduction of RANKL in the synovium after rituximab treatment, suggesting that B cells present in the synovium are responsible either directly or indirectly for the production of this cytokine which is known to play a role in secondary lymphoid organ development and bone erosion, both of which are highly important in the diseased state[496,498]. Work has indicated that the B cells were largely responsible for the production of RANKL as determined by mRNA levels in the respective leukocyte populations of the SF[499]. Further analysis demonstrated that this RANKL production was in fact largely limited to a sub-population of B cells with a unique phenotype, most notably so by their expression of the Fc-like Receptor 4 (FcRL4). These cells express high levels of CD20 and depletion of these cells by Rituximab is could be an explanation for the loss of RANKL[474,496].

1.3.13 FcRL4

Fc-receptor like (FcRL) receptors are a commonly described feature of atypical memory B cell subsets, with upregulated FcRL3, FcRL4, and FcRL5 particularly common[500–502]. FcRL4^{+} memory B cells, originally described in the tonsils, were suggested to delineate the atypical memory B cells found in malaria[461,465,503]. More recent studies have questioned this and suggest that it is FcRL5 which discriminates the functionally impaired memory B cells in malaria, and that the previous results were obtained using an FcRL4/FcRL5 cross-reactive antibody[500]. What is clear however is that these malarial atypical B cells are able to produce protective antibodies which indicates a productive role for these cells and suggests their phenotype is a by-product of chronic antigenic stimulation[504].

Fc receptors play important roles for a wide array of immune responses. In contrast to the well-defined roles for Fcγ and Fcε receptors, the structural and functional characteristics of Fc-like receptors remain incompletely understood. There are presently six members of the Fc Receptor-Like protein family that have been described, homologous to FcγRI and predominantly expressed on B cells [505,506]. Fc Receptor-Like 4 (FcRL4) is notably absent from mice and expressed solely in humans and other higher primates [507,508]. FcRL1-6 are thought to have varying stimulating and inhibiting effects due to the presence of Intracellular Activating- and Inhibiting- Motifs (ITAMS and ITIMS respectively) in their intra-cellular portions[509]. FcRL4 encodes a receptorcharacterized by 4 extracellular Ig-type domains and 3 potential ITIM motifs in its intracellular domain[509].

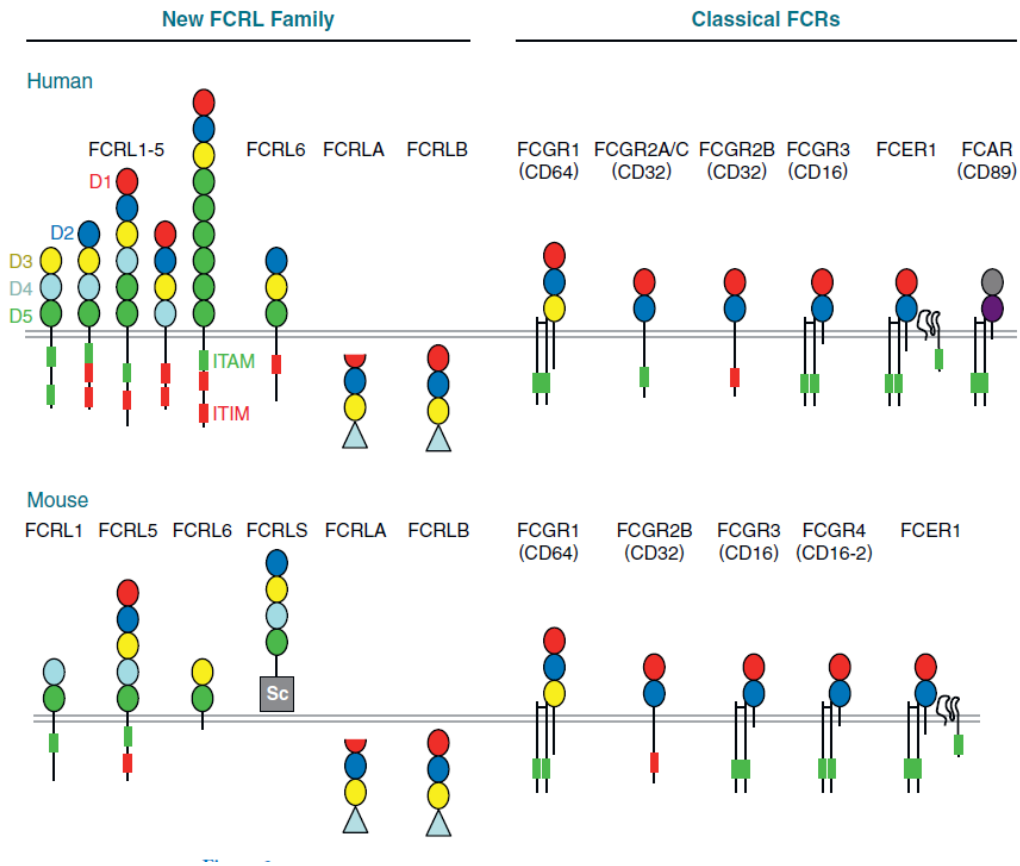

Figure 10 – A diagram detailing the characteristics of the Fc Like Receptor family and comparing them to the Classical Fc Receptor family in human and mouse. *Extracellular Ig domains are color-coded according to phylogenetic relationships as determined previously in comprehensive family Ig domain amino acid sequence alignments*[508,510]*. Cytoplasmic ITIM or ITAM are represented by red or green boxes, respectively. The FCRLS type B-SRCR domain is shown as a grey rectangle. FCRLA possesses a partial Ig domain. Both FCRLA and FCRLB contain C-terminal mucin-like regions (blue triangles) and are expressed intracellularly. Activating FCR family members are shown in complex with the FCERG1 adaptor subunit, and FCER1A also associates with FCER1B/MS4A2. Figure from Davis, R., 2007*[509]*.*

Experiments using chimeric proteins, consisting of the extracellular and transmembrane domains of FcγRIIb fused to the FcRL4 cytoplasmic domain and co-ligating these to the BCR have indicated an inhibitory capability greater than that of FcγRIIb, the classical, inhibitory B cell receptor[511]. The intracellular domain of FcRL4 in the chimeric protein has been found to associate with SHP-1/2 and almost entirely abrogate BCR mediated signaling[511]. Other experiments utilising transfected B cell lines expressing FcRL4 have suggested that FcRL4 acts as an inhibitor to the formation of a stable immune synapse (IS), preventing the BCR from interacting by spatially displacing it from the centre of

the IS[511]. These data also point towards an additional role in sensitizing B cells to TLR9 mediated NFκB activation which suggests that the consequences of its ligation are context dependent[511]. This initial work in which FcRL4 has been reported as an inhibitory receptor, through phosphorylation of SHP-1 leading to inhibition of BCR signaling, did not use FcRL4's ligand, instead relying upon an FcRL4 specific mAb[512]. However, further work used IgA aggregates demonstrated that FcRL4's immunomodulatory activity is highly dependent upon the co-expression of src-family kinases HCK and FGR[513]. The authors reported that FcRL4 had inhibitory activity in cells co-expressing FGR but an activating function in cells co-expressing HCK p59[513]. Although this paper did not show the data, they suggest that binding of IgA to FcRL4 alone was not sufficient to detect any increase in tyrosine phosphorylation, and that this depended upon subsequent BCR cross-linking[513]..

FcRL4 was originally described as discriminating a subset of B cells, first identified by Falini, et al. and further described by Ehrhardt et al., found to be preferentially expressed on a subset of memory B cells ($IgD^-/CD38^-$) found in the MALT, perifollicular and sub-epithelial regions of the tonsil epithelium, and dome epithelium of Peyer's patches[503,514,515]. In addition to the tonsil they have since been described in other mucosal sites such as the human penis[516].

$FcRL4^+$ B cells are morphologically distinct; being relatively large, possessing prominent nucleoli and a mitochondrion rich cytoplasm[503,514]. They share a number of characteristics with interfollicular B cells such as the large size and having undergone somatic hypermutation, albeit at a higher level[514,517]. However, other characteristics of $FcRL4^+$ cells, such as expression of CD21, CD23, and the CD80 and CD86 activation markers, are not shared by the interfollicular B cells.[517]

A defining characteristic of tonsillar $FcRL4^+$ memory B cells compared to their $FcRL4^-$ counterparts is the reciprocal expression of RUNX2 and RUNX1 transcription factors, respectively[518]. There was very

little bound RUNX2 detected bound to the promoter sequences of FcRL4 in FcRL4^{+} cells, however there were significantly higher levels of RUNX1 present in the promoter sequences of the FcRL4^{-} cells, suggesting that RUNX1 playing a functional role in the regulation of FcRL4^{-} cells[465]. Elevated expression of the transcription factor SOX5 has also been detected in tonsillar FcRL4^{+} memory B cells but attempts to test the function of SOX5 in the activation of FcRL4 promoters did not reveal any significant functional influence of SOX5 in the regulation of the FCRL4 gene expression[465].

Further discriminating markers include expression of CD11c and low levels of CD11b which, through hetero-dimerisation with CD18, both function as complement receptors and adhesion molecules, with expression of CD11c potentially contributing to the localisation of FcRL4^{+} B cells along the mucosal borders[503,514]. A number of similarities between Age-associated B cells (ABCs) and the tonsillar and RA SF FcRL4+ subset include low expression of CD21, and elevated expression of CD11c, CD80, CD86. The increased expression levels of FGR and HCK by tonsillar FCRL4^{+} B cells are also noteworthy in this context, as these src family kinases have been implicated in the regulation of signaling induced by macrophage inflammatory protein 1α, one of the ligands for CCR1 and CCR5[513]. Tonsillar FcRL4+ B cells have strongly upregulated mRNA levels for CCR1 and CCR5, which could contribute to the lympho-epithelial tissue localization of FcRL4+ cells, as epithelial cells and their stromal cell neighbours release these chemokines in inflammatory responses[465,499]. Furthermore, tonsillar FcRL4+ cells also have increased levels of RANKL and DLL1 expression[465]. Of note in the mucosal context of the tonsil, is work suggesting that FcRL4 is a low affinity receptor for heat aggregated IgA[519].

Recent reports have emphasized the presence of lower percentages of total memory B cells in peripheral blood of RA patients compared to controls, in addition to enhanced percentages of IgD^{-} CD27^{-} memory B cells in the SF compared to the peripheral blood of RA patients. One study has

detected FcRL4^{+} transcript in the peripheral blood of healthy controls at a higher level than in RA patients. This perhaps suggests that these cells are migrating from the peripheral blood into the SF of RA patients, or since this work merely quantified mRNA, that B cells are transcribing but not expressing FcRL4 in the periphery[520].

FcRL4 has also been detected on a subset of B cells found in the SF of patients with RA[474]. These cells have been described as having a memory B cell phenotype and whilst unable to produce antibody they have been shown to be proliferative via presence of Ki-67 at both the mRNA and protein levels (unpublished data). Cytokine profiling of these cells identified a pro-inflammatory phenotype with upregulated mRNA expression of TNFα and RANKL, which have roles in bone erosion and inflammation and the potential for lympho-organogenesis[499]. Cytokine profiling also revealed the upregulated expression of CD80/86 and CCR1/5, co-stimulatory molecules and molecules involved in T cell homing respectively[499]. This transcriptomic profile suggested that these cells could play a role in B-T cell interactions such as priming, presenting antigen, or even in the formation of TLOs or ectopic germinal centres. The evidence that FcRL4 binds IgA and the initial localisation of FcRL4+ B cells to the MALT suggests that these cells may play a role in linking mucosal immunity and RA.[503,514,521,522].

1.3.14 Hypothesis

Based on the literature discussed above, the work contained in this study sought to provide information with regards the hypothesis that FcRL4^{+} B cells provide a novel link between the mucosal immune system and the immune reaction of the joint in rheumatoid arthritis.

1.3.15 Aims

This work had three aims, addressed in the following chapters in the order below:

- To investigate the presence of FcRL4$^+$ B cells in the joint, mucosa, and periphery and regulation of the FcRL4$^+$ B cell population.
- To test the ligand binding properties of the FcRL4 receptor itself and of FcRL4$^+$ B cells from the anatomical sites described above.
- To probe the reactivity of RA SF FcRL4$^+$ B cells, particularly against mucosal antigens.

2 Methods

2.1 Patients and Patient Material

Synovial fluid and blood samples were predominantly obtained from patients with established RA, who fulfilled 1987 or 2010 American College of Rheumatology (ACR) classification criteria, although disease durations varied between individuals. Some patients were newly presenting and not on disease modifying anti-rheumatic drugs, (DMARDs) whilst others were on DMARDs including biologic therapies. Synovial fluid was also obtained from patients with other forms of inflammatory arthritis including Psoriatic Arthritis (PsA), Osteoarthritis (OA), or undifferentiated arthritis (UA) which can be reclassified later if criteria are met. All patients have active disease when synovial fluid is taken. Mononuclear cells were also isolated from mechanically dissociated tonsil tissues from patients undergoing tonsillectomies. Control peripheral blood was obtained from healthy donors. This study was conducted in compliance with the Helsinki declaration and ethical approval was obtained from the local ethics committee. All subjects gave informed written consent. When available for a patient, clinical data is included (Appendix).

Table 1 – Solutions used in assays

Solution	**MACS buffer**	**Acid Wash**	**Culture Medium**
Reagents	100ml PBS	0.7507g Glycine	445ml RPMI
	2g BSA	0.5844g NaCl	50ml FCS
	1.6ml 0.5M EDTA	100ml Deionised water	5ml GPS
pH	7	3	-

2.2 Cell Culture

Cells were maintained in complete medium (RPMI, 10% FCS, 1% PenStrep) at 37°C/5%CO_2 and passaged every 2-3 days.

2.3 Synovial Fluid/Peripheral Blood Mononuclear Cell (SF/PBMC) Isolation

Mononuclear cells (MCs) were isolated from synovial fluid (SF), peripheral blood (PB), and Tonsil preparations using density gradient centrifugation. SF was incubated with 1000U/ml endotoxin free hyaluronidase (Wockhardt UK) at 37°C for 15 minutes. PB was diluted 1:1 in PBS. The resulting solution was layered onto Ficoll at a 3:1 ratio of sample to Ficoll before being centrifuged at 300g for 30 minutes with slow acceleration and no brake applied. The interface between the two fluids containing the MCs was removed carefully using a Pasteur pipette before cells were counted.

2.4 Tonsil preparation

Tonsils were received and cut into 1mm^3 pieces in a Petri dish using a pair of scalpels. 10ml complete medium was added before the samples were digested mechanically using a Biomaster Stomacher 80 at normal interval and speed for 6 minutes. The resulting cell suspension was passed through a 100µm filter before being layered upon Ficoll and processed as described above.

2.5 Flow cytometry

Cells were stained with BD Zombie Aqua Viability Dye™ in PBS before being washed in 200µl PBS and stained in MACS buffer (Table 1) containing mouse monoclonal antibodies. Isotype, concentration, species, and label-matched control antibodies were also used (Table 2), as well as "Fluorescence minus one's" (FMOs) in which all the fluorochromes in a panel, except for the fluorochrome that is being measured, are included. After staining, cells were washed in 200µl PBS and centrifuged at 300g

for 5 minutes. Once labelled with antibody the samples were fixed in 0.1% paraformaldehyde. Data were acquired using a Dako Cyan ADP or a BD Fortessa flow cytometer and analysed using the SUMMIT or FlowJo software.

Table 2 – Antibodies and concentrations for flow cytometry experiments

Colour	antigen	antibody	dilution	Isotype control	Isotype dilution
BV421	CD19	Biolegend IgG1 Clone: 302234	1/50	Biolegend Clone: 400158	1/100
FITC	IgD	Biolegend IgG2zκ, Clone: IA6-2	1/100	eBioscience, IgG1 control Clone: 11-4714-42	2.5/100
APC-Cy7	CD27	Biolegend Anti-Human Clone: 302818	5/100	BioLegend IgG1 Isotype Control Clone: MOPC-21	5/100
APC	CD21	Mouse anti-human, Clone: HBS	1/50	BioLegend IgG1 Isotype Control Clone: MOPC-21	1/50
PE	CD11c	Immunotools, Clone: BU15 IgG1	1/50	eBioscience, Clone: P3-62-81	1/100
PE-Cy7	FcRL4	Biolegend CD307d (FcRL4) Clone: 413D12	1/50	Biolegend, IgG2b Isotype Control, Clone: MDC- 11	1/100
PE	FcRL4	Biolegend, Clone: 340204	1/200	Biolegend, Clone: 400312	1/200
APC	FcRL4	Biolegend, Clone: 340204	1/200	Biolegend, Clone: 400312	1/200
APC	IgA	MACs 130-099-220 IgG1 11ug/ml	1/200	Biolegend, Clone: 400312	1/100
FITC	IgA	Miltenyi Biotec, Clone: IS11-8E10	1/100	eBioscience, IgG1 control Clone: 11-4714-42	2.5/100
PE	IgG	Biolegend, clone HP6017	1/100	eBioscience, Clone: P3-62-81	1/100
V2	Live/Dead	Zombie Aqua	1/100	N/A	N/A

2.6 IgA Acid Wash and IgA Incubation

Human IgA from colostrum (Sigma), serum (Serum I – Sigma & Serum II – Jackson Research), or myeloma derived IgA1 or IgA2 (Abcam) was heat aggregated for 30 minutes at 63°C unless indicated in otherwise in figure legends. SFMCs were incubated for approximately 1 min in pH3 acid wash (Table 1) to remove surface bound proteins and washed in 20ml RPMI 1640 at 4°C before being centrifuged at 300g for 5 minutes. HA-IgA at 1mg/ml in RPMI 1640 was added to test wells, unless indicated in otherwise in figure legends, for 30 minutes on ice before being washed in 200µl PBS and centrifuged at 300g for 5 minutes. After centrifugation, samples were stained per the Flow Cytometry staining protocol.

2.7 Fluorescent Activated Cell Sorting for Culturing Assays

Cells were stained with mouse monoclonal antibodies against CD19 (Biolegend), IgD (Biolegend), FcRL4 (Biolegend) and sorted using an Astrios cell sorter, gating on CD19+ IgD- cells. Sorted populations had a purity of >95%.

2.8 Stimuli and Downregulation Assay

Sorted $IgD^-/CD19^+$ tonsil cells were cultured overnight in 96-well plates at a density of $1x10^5$cells/well in 200µl complete medium (Table 1). To each well, stimuli were added at the following concentrations (Table 3). After incubation, they were stained per the Flow Cytometry staining previously described.

Table 3 – Stimuli and concentrations for culture assays

Stimulus	**Concentration**
TLR 1 agonist – Pam3CSK4 (Invivogen)	1µg/ml
TLR 2 agonist – HKLM (Invivogen)	10^8cells/ml
TLR 3 agonist LMW (low molecular weight) – Poly(I:C) LMW (Invivogen)	10µg/ml
TLR 3 agonist HMW (high molecular weight)- Poly(I:C) (Invivogen)	10µg/ml
TLR 4 agonist – LPS (Invivogen)	10µg/ml
TLR 5 agonist – Flagellin (Invivogen)	10µg/ml
TLR 6 agonist – FSL-1 (Invivogen)	1µg/ml
TLR 7 agonist – Imiquimod (Invivogen)	10µg/ml
TLR 8 agonist – ssRNA40 (Invivogen)	10µg/ml
TLR 9 agonist – ODN2006 (Invivogen)	5µM
Recombinant human IL-6 (Sigma)	0.5µg/ml
Recombinant human IL-10 (Sigma)	0.1µg/ml
Recombinant human IL-21 (Sigma)	0.5µg/ml
Recombinant human IL-33 (Sigma)	0.1µg/ml
Recombinant human TNF-α (Sigma)	100µg/ml
Recombinant human IFN-γ (Sigma)	0.1µg/ml
Recombinant human BAFF (R&D Systems)	1µg/ml
Recombinant human TGF-β (R&D Systems)	1µg/ml
Heat-aggregated IgA (human colostrum) (Sigma)	1mg/ml

2.9 Cell Transfection

Approximately 12 hours prior to transfection Raji cells were passaged in order to ensure correct cell densities and healthy cell populations. Raji cells were transfected approximately 18 hours prior to usage in assays. 2.0 x 10^6 cells were taken from culture and centrifuged at 100g for 5 minutes, supernatant was carefully aspirated before 5µg of FcRL4-GFP containing plasmid was added, followed by 100µl transfection solution. This cell suspension was then transferred to a transfection cuvette. Programme Raji ATCC was used on a Lonza Nucleofector. Following electroporation, 1ml antibiotic-free complete medium was added to the cells and they were transferred to a 6-well plate, a further 1ml antibiotic-free complete medium was then added before the cells were placed into a 37^{o}C/5%CO_2 incubator until use in assays.

2.10 Lentivirus Transduction

An FcRL4 containing lentiviral construct (Figure 11) was transduced into Raji cells. Multiplicity of Infections' (MOI's) of 1, 5, & 10 were used and 1mg/ml Polybrene was included in transducing culture to reduce electrostatic repulsion between cell membranes and viral particles. Expression of FcRL4 was based on selection by FACS of GFP^+ cells and cultured according to the conditions described above.

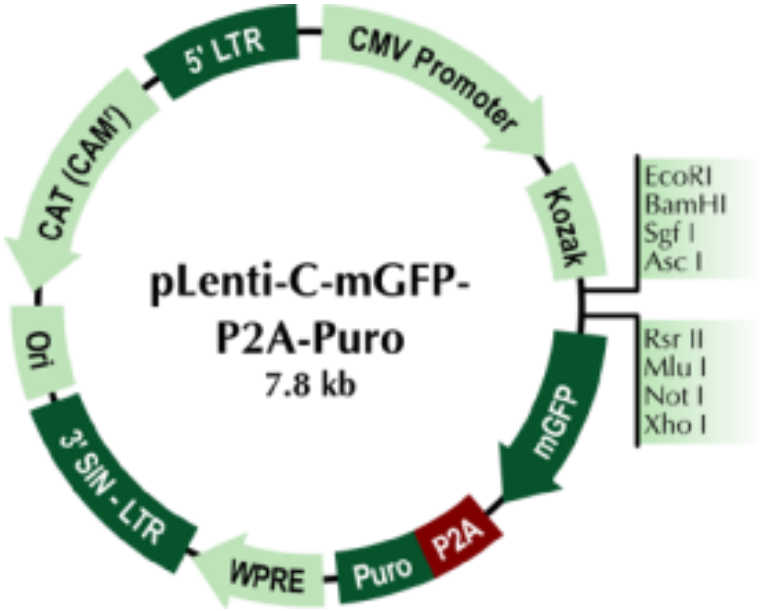

Figure 11 – Schematic of the lentiviral vector used to generate stably expressing FcRL4$^+$ Raji cell lines, pLenti-C-Myc-DDk-P2A-Puro. This lenti viral vector has been designed to express FcRL4 with a myc-DDK tag tag at its carboxy terminus that can be detected using antibodies. The fusion protein is expected to be transmembrane in its expression, although some intracellular cycling is also anticipated. Within the plasmid there are several cloning sites: EcoRI, BamHI, Sgf I, Asc I, Rsr II, MluI, NotI, Xho I and Pme I. The FcRL4 gene has been cloned into the vector using the Sgf I and the MluI sites. It contains a Chloramphenicol selection marker for use with E.Coli cells, and Puromycin selection marker for selection in mammalian cells to generate a stably transfected cell line. It contains a CMV promoter and a Kozak sequence to enhance expression of the open reading frame in mammalian cells. Lentivirus production will involve a third-generation self-inactivating system in which the genes required for viral replication are not packaged into the infective particles. Consequently, the resulting particles are replication deficient.

2.11 Single Cell Fluorescent Activated Cell Sorting for BCR Analysis

SFMCs were thawed and re-suspended in 20ml complete medium to dilute out DMSO. Samples were centrifuged at 300g/4^{o}C for 5 minutes and subsequently re-suspended in FACS buffer containing mouse monoclonal antibodies against CD19 (Biolegend), IgD (Biolegend), CD27 (Biolegend), FcRL4 (Biolegend), and IgA (Miltenyi Biotec). Single CD19$^+$, IgD$^-$, CD27$^{+/-}$ cells were sorted into four populations based on the following markers; FcRL4$^+$ IgA$^+$, FcRL4$^+$ IgA$^-$, FcRL4$^-$ IgA$^+$, and FcRL4$^-$ IgA$^-$ using a BD Biosciences FACS Aria™ Fusion. Cells were sorted into FACS buffer and frozen at -80^{o}C until further analysis.

2.12 BCR Analysis

From the single sorted B cells, the Ig heavy chain genes were amplified by PCR using universal primers for these regions and subsequently sequenced using next-generation sequencing, before the framework regions (FWR) and complementarity determining regions (CDRs) were assessed for somatic replacement (non-synonymous) mutations, comparing against germline sequences.

2.13 Stool Sample Pool Preparation

1-3g fresh human stool was resuspended in 1ml ice-cold PBS and centrifuged at 13000g for 5 minutes before being washed and centrifuged twice using 2ml ice-cold PBMS and 13000g for 5 minutes. Following the wash steps the suspension was passed through a 70µm filter and diluted approximately 1:40. An OD600 measurement was taken and appropriate dilution to 0.08 was made. An aliquot was subsequently analysed for DNA content using the ZymoBIOMICS 96 MagBead Kit, according to the manufacturer's instructions to provide an analysis of the microbiome prior to storage. Samples from a minimum of 6 donors were then pooled, centrifuged at 13000g and resuspended in glycerol, before being frozen at -80^{o}C until use.

2.14 Monoclonal Antibody Labelling

Monoclonal antibodies (mAbs) derived from the sequences of single B cells isolated from the joints of patients with active RA were labelled with either APC or FITC using the Expedion Lightening Link kit according to the manufacturer's instructions. Briefly, mAbs were diluted or concentrated to 1mg/ml and 100µl taken for conjugation. 10ul of LL-Modifier was added to the mAb solution and mixed thoroughly, the entire mixture was then mixed thoroughly with the lyophilised fluorophore and incubated overnight at room temperature in the dark. Following the incubation, the newly labelled mAb solution was mixed thoroughly with 10µl of LL-Quencher and left for 30 minutes at room temperature in the dark before use.

2.15 Bacterial Labelling

Pooled stool samples were thawed on ice and washed twice using 2ml sterile, ice-cold PBS, centrifuging each time at 13000g for 5 minutes, these wash conditions were repeated throughout the protocol. Following the wash steps, samples were blocked with labelled Infliximab for 30 minutes

on ice in the dark, unless otherwise indicated in the figure legend or results section. Following blocking/labelling with infliximab, samples were washed, samples were then stained with either the appropriate mAb clone or an anti-human IgA mAb. Following labelling, samples were washed and incubated for 10 minutes in the dark with 20ul of the appropriate anti-fluorophore magnetic beads, either anti-FITC or anti-APC, in 180µl sterile, ice-cold PBS. The bead labelled samples were then passed though prepared MACS columns, 1.8ml sterile, ice-cold PBS was passed through the columns to wash the non-specifically labelled bacteria through. The column was then removed from the magnet and a fresh collection tube placed underneath the MACS column. 2ml sterile, ice-cold PBS was pushed through the column using the plunger to release the specifically bound bacteria. This enriched sample was then centrifuged at 13000g for 5 minutes before being resuspended in 5mM Hoechst or 1:200 SYBR Green solution for a minimum of 30 minutes prior to sorting via FACS.

2.16 Bacterial FACS

Bacterial samples were sorted via FACS using a BD Influx Fluorescence Activated Cell Sorter. Appropriate FMOs were used including DNA-binding dye alone, and DNA-binding dye + Infliximab. Events were gated on FSC/SSC, DNA-binding dye^{+}, Clone^{+}/anti-IgA^{+}. Positively and negatively labelled bacteria were sorted, and purity checks carried out.

2.17 16S rRNA Sequencing

Sorted fractions were amplified using 5µM specific primers (Table 4) for the non-variable V3 and V4 regions of the 16S subunit of the bacterial ribosome, KAPA HiFi DNA Polymerase and 35 cycles of PCR. Following amplification, Illumina index barcodes (Nextera XT index Kit V2 Set D (96 samples 192 samples), Cat no.: 15052166. (illumina, US, San Diego)) were added to the bacterial sequences according to the manufacturer's instructions the amplification products were subject to another 8 rounds of PCR using the previously used primers (Table 4). Following this, the DNA content was

analysed via Qubit using the High-Sensitivity DNA settings. 1µl of index-PCR product was incubated with 199µl of 1:200 DNA binding dye. Following Qubit measurements, index PCR products were diluted and analysed using the Advanced Analytics Fragment Analyser to provide information on the size and purity of the index PCR products. The index PCR products were then diluted to 2nM and pooled before next-generation sequencing using an Illumina MiSeq.

Table 4 – Primers used in bacterial 16S rRNA sequencing

16s AMPLICON-PCR-forward	TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG
16s AMPLICON-PCR-reverse	GTC ZCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA

3 Results

FcRL4$^+$ B cells: Expression & Regulation

3.1 Introduction

FcRL4$^+$ B cells are the subject of heterogeneous descriptions in the literature. In these experiments the presence of FcRL4$^+$ B cells was investigated in various locations, including the tonsil where the population was initially described, the synovial fluid where recent work has described them as a RANKL-producing subset involved in the autoimmune response of rheumatoid arthritis, and in the periphery due to phenotypic similarities with the 'Age-associated B Cell' population described in the literature. In addition to this, the expression of FcRL4 was characterised, both in response to stimuli and in standard culture conditions, in order to better understand regulation of the receptors' expression, and the proneness to apoptosis of tonsil FcRL4$^+$ and FcRL4$^-$ B cells was assessed.

3.2 FcRL4$^+$ B cells in the synovial fluid

3.2.1 FcRL4$^+$ B cells are present in patients' synovial fluid from a range of arthritides

Mononuclear cells were isolated from the synovial fluid of arthritis patients with three different diagnoses, stained for CD19 and FcRL4, and analysed by flow cytometry. The FcRL4 population is largely limited to the CD19$^+$ B cells detected in the synovial fluid of patients with all of the tested arthritides, confirming and extending findings in by our group (Figure 12)[474].

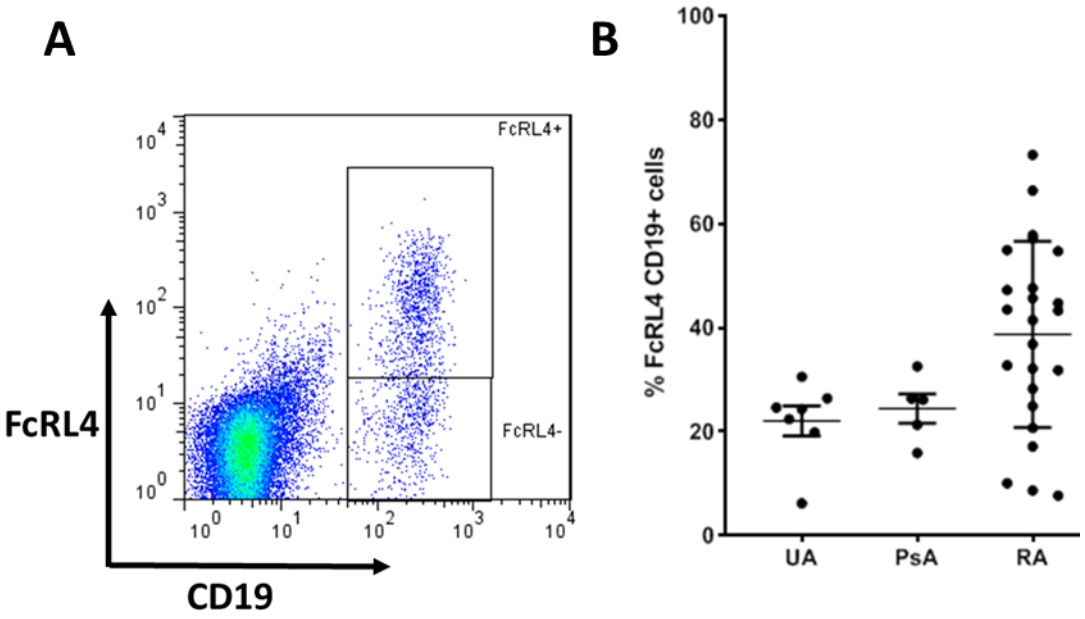

Figure 12 – FcRL4⁺ B cells are present in the synovial fluid of various arthritides. (A) *Flow cytometry plot showing the presence of FcRL4⁺ B cells in RA SF and detailing the gating strategy used in (B), synovial fluid mononuclear cells were gated according to their FSC/SSC profile, Singlets, and dead cell exclusion by Zombie⁻; CD19⁺ and FcRL4⁻ and FcRL4⁺ gates were determined after protocol set up using Fluorescence minus ones.* ***(B)*** *Scatter dot plot showing the proportion of FcRL4⁺ cells within the CD19⁺ population across several arthritides. (UA – Undifferentiated Arthritis, n=7; PsA – Psoriatic Arthritis, n=5; RA – Rheumatoid Arthritis, n=23, 35 independent experiments). Mean and SEM are shown. Each point represents one patient.*

3.3 FcRL4 in the Periphery

3.3.1 FcRL4$^+$ is enriched in the Age-Associated B cell population present in the periphery of healthy controls

Recent work has indicated that a population of B cells are present in the peripheral blood with a similar transcriptional and phenotypic profile to the atypical memory B cell subsets described in the literature with elevated levels of CD11c and low levels of CD21[523,524]. They also demonstrated an association with autoimmune diseases and correlate with increasing age and being female[525]. These cells have consequently been described as "Age-associated B Cells" (ABCs)[525]. There are many similarities described in publications discussing ABCs and the FcRL4$^+$ population found in the joints, these experiments set out investigate if FcRL4 was expressed on the surface of this population, and if it might provide a phenotypic marker for it. An observation of FcRL4 expression on ABC was also made by collaborators in Newcastle (Pedrola, et al., unpublished observation) and independently, and in salivary glands of primary Sjögren's syndrome patients[526].

Peripheral blood mononuclear cells were isolated from age-matched healthy controls and RA patients with active disease and stained for CD19, CD27, IgD, CD21, CD11c, and FcRL4, then analysed by flow cytometry. The surface markers CD21 and CD11c were used to discriminate the ABC or atypical population, with low surface of expression of CD21 and high surface expression of CD11c considered a signature of the population.

These experiments demonstrated a comparable percentage of ABCs detected in the periphery of the healthy controls to that reported in the literature, comprising approximately 5% of the memory B cell (CD27$^+$ IgD$^-$) compartment (Figure 13). The majority of samples tested had a slightly lower percentage; however, one patient sample was much higher, 11.56%, (Figure 13). In looking at the

percentage of ABCs which stained positive for FcRL4 it was possible to discern a significant increase of these cells present in the ABC population when compared to the “non-ABC” ($CD21^+$ $CD11c^-$) population, with a mean of 23% of the ABCs positive for FcRL4 (Figure 13). However, it was not a universally expressed marker of the population (Figure 13).

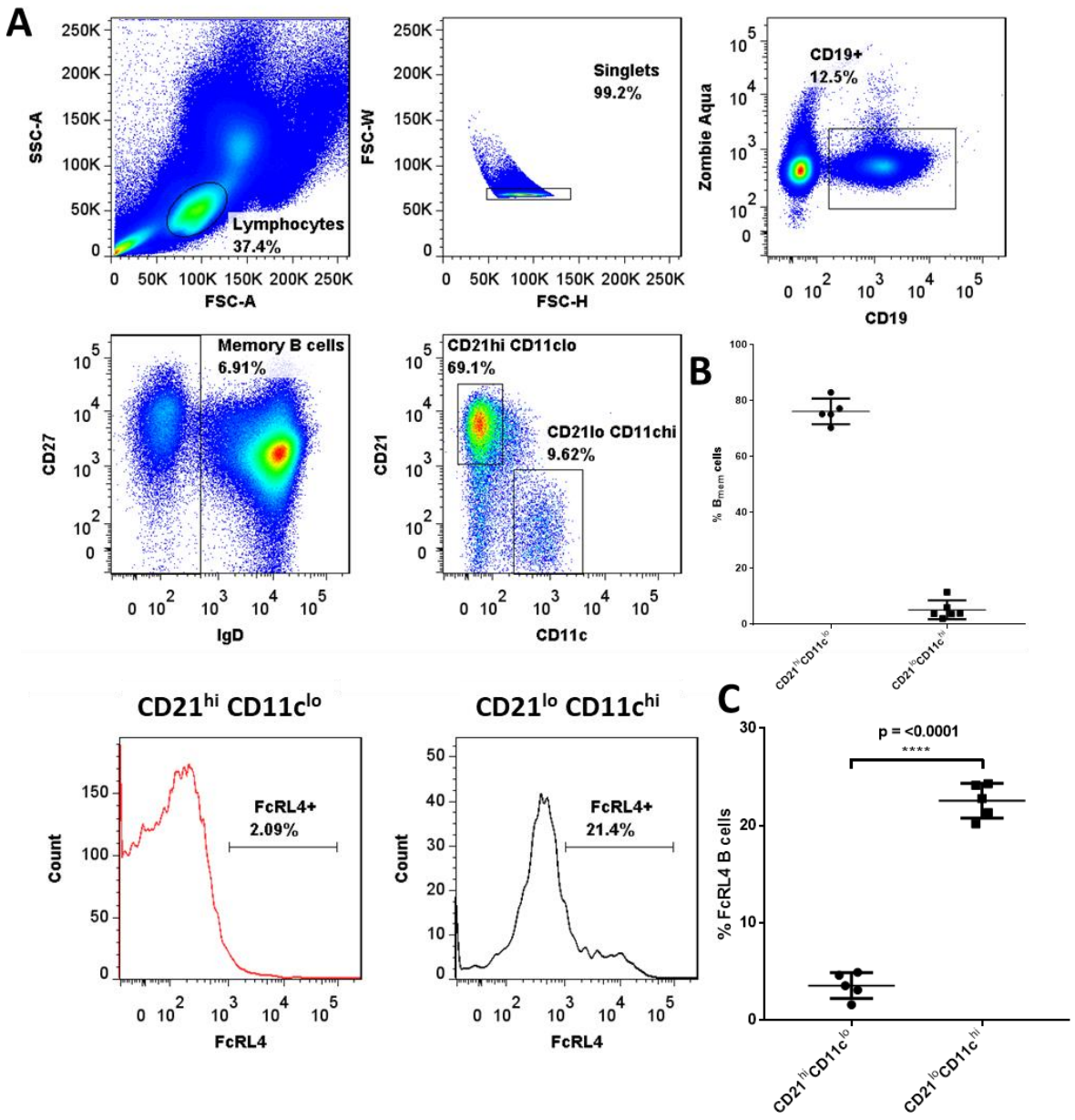

Figure 13 – FcRL4⁺ B cells are over-represented in the ABC population in the periphery of healthy controls. (A) *Flow cytometry dot plots showing the gating strategy used in (B) and (C) showing the CD21 vs. CD11c MFI, ABCs were gated on FSC/SSC, Singlets, Zombie⁻, CD19⁺, CD27⁺, IgD⁻, and either CD21^{lo}, CD11c^{hi}, FcRL4⁺ or CD21^{hi}, CD11c^{lo}, FcRL4⁺.* ***(B)*** *Dot plots showing the %ABC for the population gated upon in (A) for each of the samples. (n=6; 6 experiments). Statistics were carried out using the paired Wilcoxon signed ranked test, mean and SEM shown.* ***(C)*** *Dot plots showing the %FcRL4⁺ for the for the population gated upon in (A). (n=6; 6 independent experiments). Statistics were carried out using the paired Wilcoxon signed ranked test, mean and SEM shown. Each point represents one donor.*

3.4 FcRL4 Expression

3.4.1 Dynamics of FcRL4 expression

Although it is possible to detect FcRL4 on the surface of synovial fluid B cells, it is not known how stable the expression of FcRL4 is at the cell surface, or whether it requires certain stimuli. In these experiments mononuclear cells were isolated from human tonsils by mechanical dissociation followed by density centrifugation. As tonsils are comprised of approximately 60% $CD19^+$ lymphocytes, this provided a rich source of B cells. The cells were then stained and $CD19^+$ IgD^- cells sorted by fluorescent activated cell sorting (FACS), then either fixed prior to analysis, or cultured. Following 20 hours culture in complete medium, the cells were stained with antibodies against CD19, IgD, and FcRL4 before being analysed by flow cytometry and comparisons of the surface levels of FcRL4 made.

After 20 hours in culture the surface levels of FcRL4 expression had dropped significantly suggesting that expression of FcRL4 is not a stable event and that it requires a stimuli in order to maintain levels of surface expression (Figure 14). It is also possible that surface expression is a cyclical event and that this time point measured a lower level of expression, or that the $FcRL4^+$ B cells died in culture at a faster rate than the $FcRL4^-$ which would lead to a decrease in the population size and level of expression.

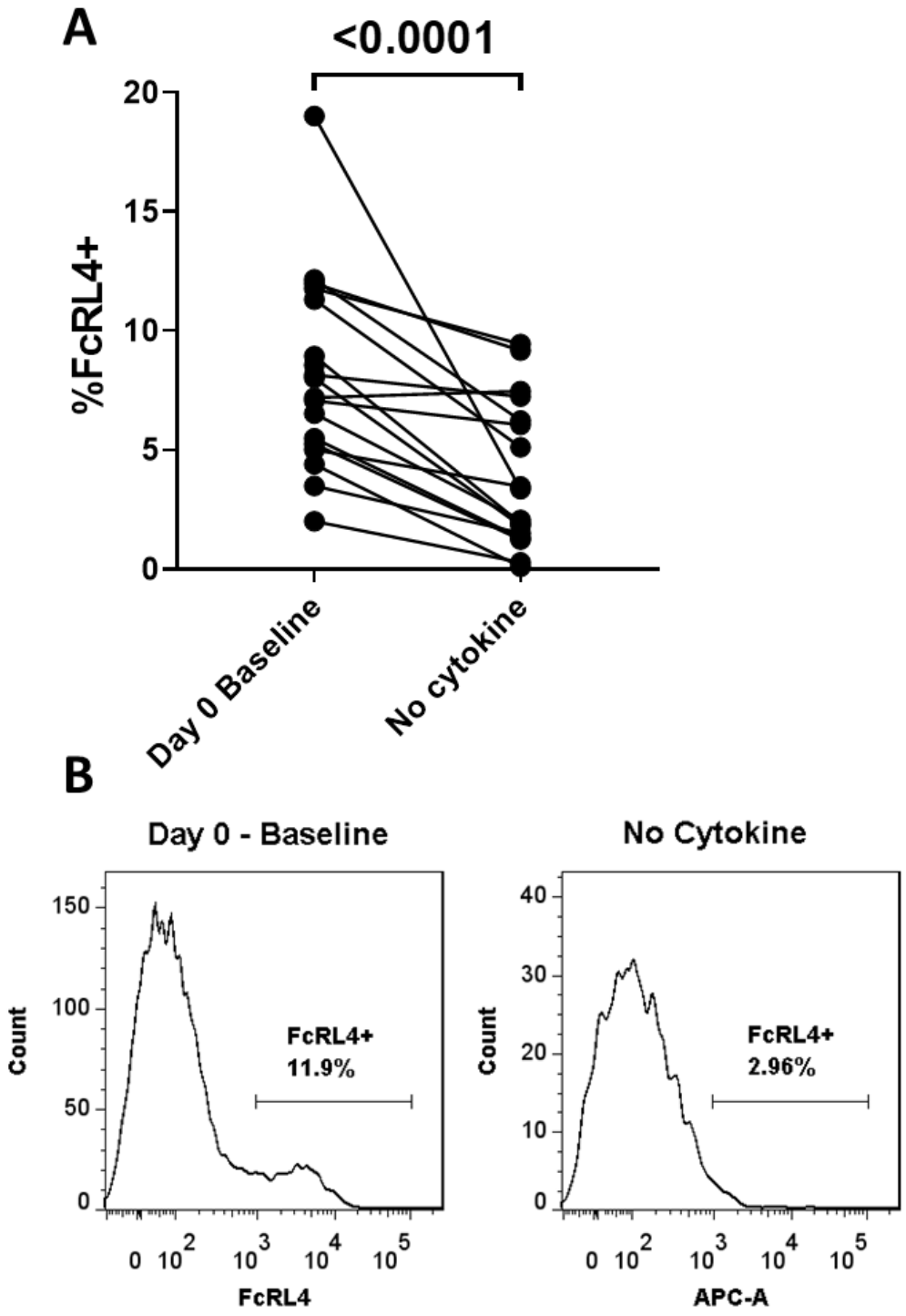

Figure 14 – FcRL4 expression is not a stable aspect of a cells' phenotype. (A) *A dot plot showing the level of of FcRL4⁺ expression as determined by MFI on (CD19⁺IgD⁻) memory B cells sorted from human tonsils and cultured in complete medium overnight. Each dot represents one donor.* ***(B)*** *A flow cytometry dot plot detailing the gating strategy used in (A), events were gated on FSC/SSC, Singlets, Zombie⁻, CD19⁺ and FcRL4⁺ (n=11, 11 independent experiments) Wilcoxon paired ranked test was carried out. Mean and SEM shown.*

Given the disparity in levels of detected surface expression for FcRL4 and to avoid the potential stress to the cells during FACS, further experiments using unsorted tonsil mononuclear cells (TMCs) analysed earlier time points to understand the dynamics of FcRL4 expression in culture. Tonsil mononuclear cells (TMNCs) were isolated by density centrifugation from mechanically digested human tonsils from patients undergoing tonsillectomies and cultured up to 24 hours, staining and analysing at every hour for 12 hours and at 24 hours. Following a period of time in culture the cells were recovered and stained for CD19 and FcRL4.

These experiments showed a decrease occurring in both the level of expression of surface FcRL4 and the percentage of FcRL4 positive cells over 24 hours (Figure 15). Much of the decrease in the MFI of $FcRL4^{+}$ $CD19^{+}$ cells occurred over the first 5 hours of culture, with a significant difference in the first 3 hours and subsequent slowing in the rate of decline (Figure 15). There was little difference in the level of surface FcRL4 expression between the first timepoint and after 1 hour in culture. After 24 hours the level of surface expression of FcRL4 was not significantly different to $FcRL4^{-}$ $CD19^{+}$ cells at the first hour (Figure 15). The percentage of $FcRL4^{+}$ $CD19^{+}$ cells also demonstrated a decrease over the timepoints measured, almost halving over 12 hours but not changing significantly between 12 and 24 hours (Figure 15).

From these experiments it can be concluded that FcRL4 is not a stable cell surface marker. However, these experiments are subject to caveats, discussed further in the summary but including the location from which the cells originated being an inflamed site, the impurity of the cultured population leading to unpredictable cell-cell interactions, and the use of a single method to assess the expression levels. However, notwithstanding these considerations, these experiments raise the possibility that these cells require a stimulus to maintain surface expression over the time measured.

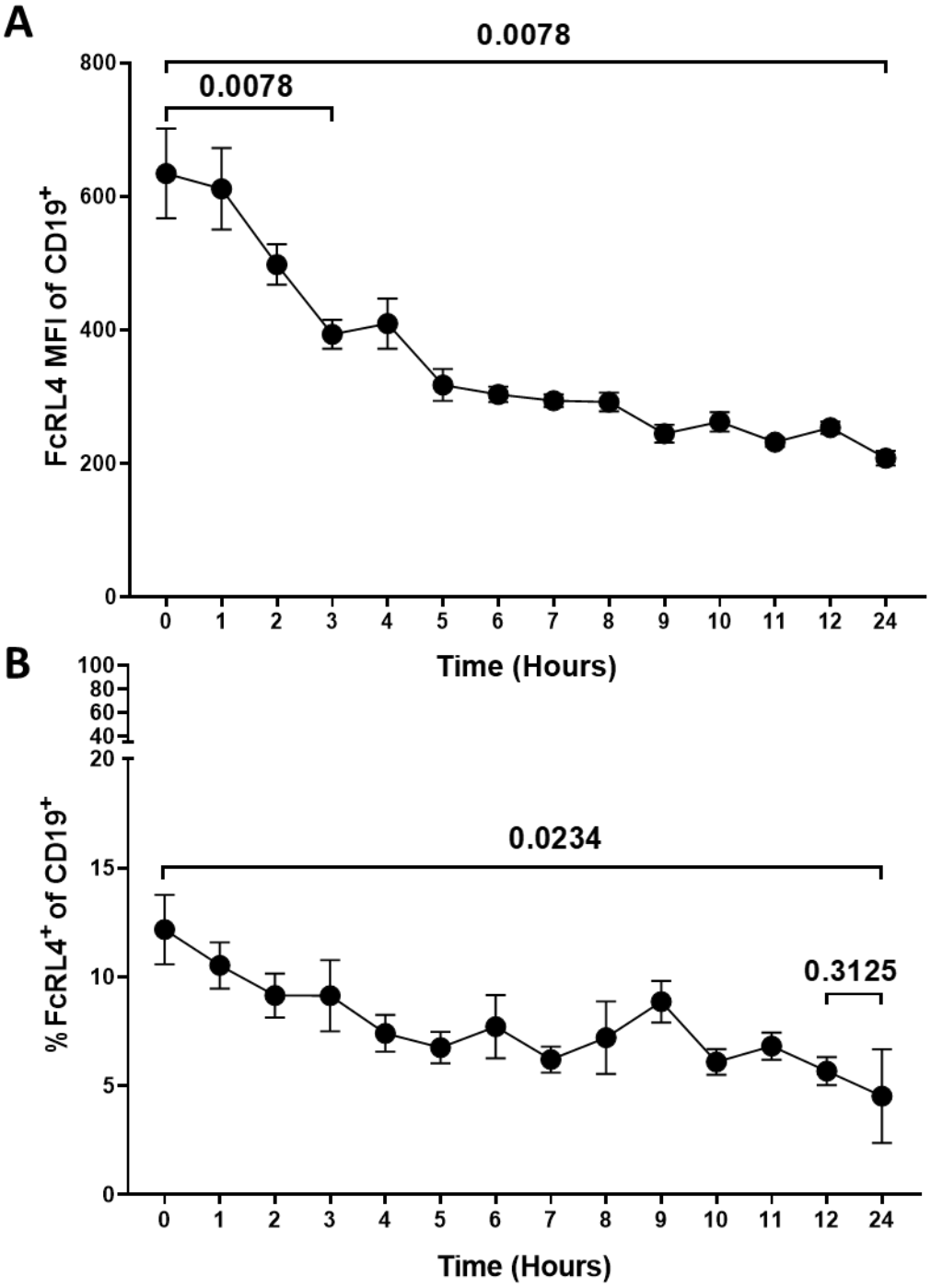

Figure 15 - FcRL4 expression decreases steadily over 24 hours. (A) *Dot plot showing surface FcRL4 MFI over time on cultured TMCs. Events were gated on FSC/SSC, Singlets, Zombie$^-$, CD19$^+$, and FcRL4$^+$.* ***(B)*** *Dot plot showing the percentage FcRL4$^+$ cells in the CD19$^+$ population over time. Events were gated on FSC/SSC, Singlets, Zombie$^-$, and CD19$^+$. (n=6 separate donors, 4 independent experiments) Wilcoxon paired ranked test was carried out. Mean and SEM are shown.*

3.4.2 FcRL4 Regulation

The regulation of FcRL4's expression is not well documented in the published literature, and although the HIV envelope protein gp120 has been reported to cause its upregulation in memory B cells and complex culture mediums involving many cytokines and TLR agonists have been applied in similar situations, it is not known how individual signals may affect it[468,501,527]. These experiments set out to test a range of stimuli for their effects on the surface expression of FcRL4. These stimuli included cytokines, chemokines, and Pathogen Associated Molecular Patterns (PAMPS) and Damage Associated Molecular Patterns (DAMPS) from the Toll-like Receptor (TLR) agonist family. In part, this was to determine if the inflammatory milieu of the joint contributed to the surface expression or if other factors were involved, perhaps including those in the mucosa.

In these experiments, memory B cells ($CD19^+$, IgD^-) derived from human tonsils, were sorted by fluorescent activated cell sorting (FACS) and cultured overnight in complete medium, either in the presence or absence of stimuli, the list and concentrations of which are listed in (Table 3). Although tonsils are comprised of approximately 60% $CD19^+$ cells and the proportion of $FcRL4^+$ $CD19^+$ cells are approximately 10%, it proved too time-consuming and negatively impacted upon cell viability when attempting tosort sufficient numbers of a pure $FcRL4^+$ $CD19^+$ population to culture and subsequently analyse (data not shown). For this reason, memory B cells, as determined by their lack of surface IgD and expression of CD19, were sorted and cultured. Following overnight culture, the cells were stained with antibodies against CD19, IgD, and FcRL4, and a viability dye, before being analysed by flow cytometry and the surface levels of FcRL4 compared across treatments.

A number of conclusions can potentially be drawn from these culturing experiments. As described previously, the expression of FcRL4 is significantly lower after culture with no stimuli present than at baseline (Figure 16). These data suggested that none of the cytokines tested individually, led to the

upregulation of FcRL4 (Figure 16). The same was true for the TLR agonists (Figure 16). However, the TLR 9 agonist, ODN 2006 and TGFβ were both separately able to maintain the surface expression of FcRL4 in line with the baseline level of expression (Figure 16 & 17). IgA also maintained FcRL4 expression throughout the culture time (Figure 17).

Furthermore, combinations of stimuli were used with a strong synergistic effect demonstrated on expression by the combination of TLR9 agonist and TGF-β (Figure 17). This suggested that FcRL4 expression depends on regulation through external signals. Furthermore, despite the significant decrease in the level of FcRL4 detected at the surface of these cells after 20 hours in culture without stimuli, certain stimuli displayed a trend of reducing the percentage of $FcRL4^{+}$ cells further. These stimuli included IL-6, IL-10, IL-21, IL-33, and TLR agonists 1-8 (Figure 16). However, caveats further elaborated upon in the discussion, including a lack of anatomical structure, circulation, complex stimuli, and cell numbers applied in this context as well.

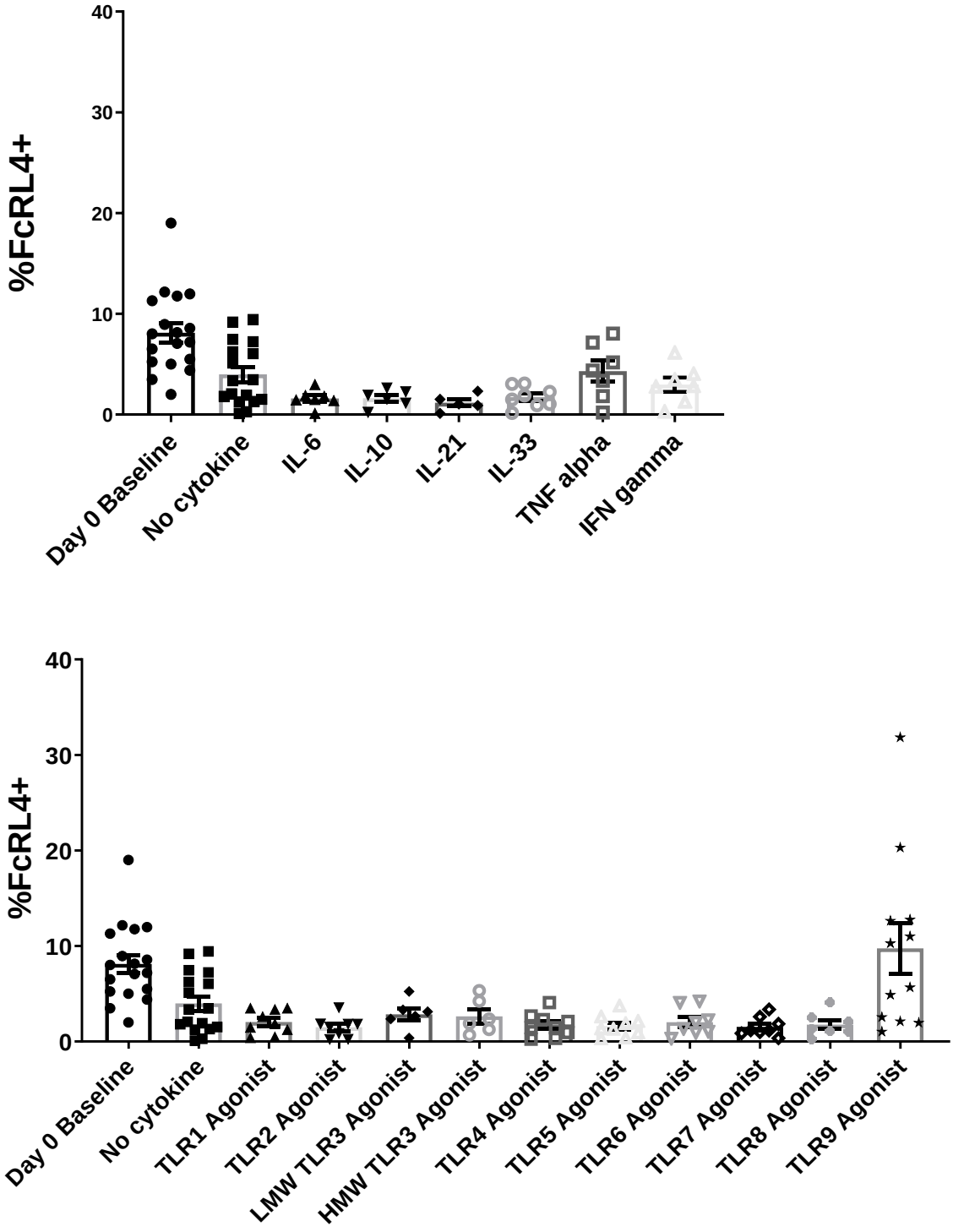

Figure 16 – FcRL4⁺ expression appears to be negatively regulated by cytokines and members of the TLR agonist family but maintained by TLR9 agonist. *%FcRL4⁺ cells in a sorted population of memory B cells (CD19⁺, IgD⁻), following overnight culture in the presence of a variety of stimuli. Sorted TMCs were gated on FSC/SSC, Singlets, CD19⁺, IgD⁻ and FcRL4⁺ and the percentage of Zombie⁻ events quantified. (Day 0 Baseline, n=11; No cytokine, n=9; TLR1 Agonist, n=6; TLR2 Agonist, n=6; TLR3 Agonist, n=6; TLR4 Agonist, n=6; TLR5 Agonist, n=6; TLR6 Agonist, n=6; TLR7 Agonist, n=6; TLR8 Agonist, n=6; TLR9 Agonist, IL-6, n=6; IL-10, n=6; IL-21, n=6, IL-33, n=6; TNFα, n=5; IFNγ, n=6; BAFF, n=4; TGFβ, n=6; heat-aggregated IgA, n=4; BAFF + IgA, n=3; TLR9 Agonist + TGFβ, n=3; 11 independent experiments). Each dot represents one donor.*

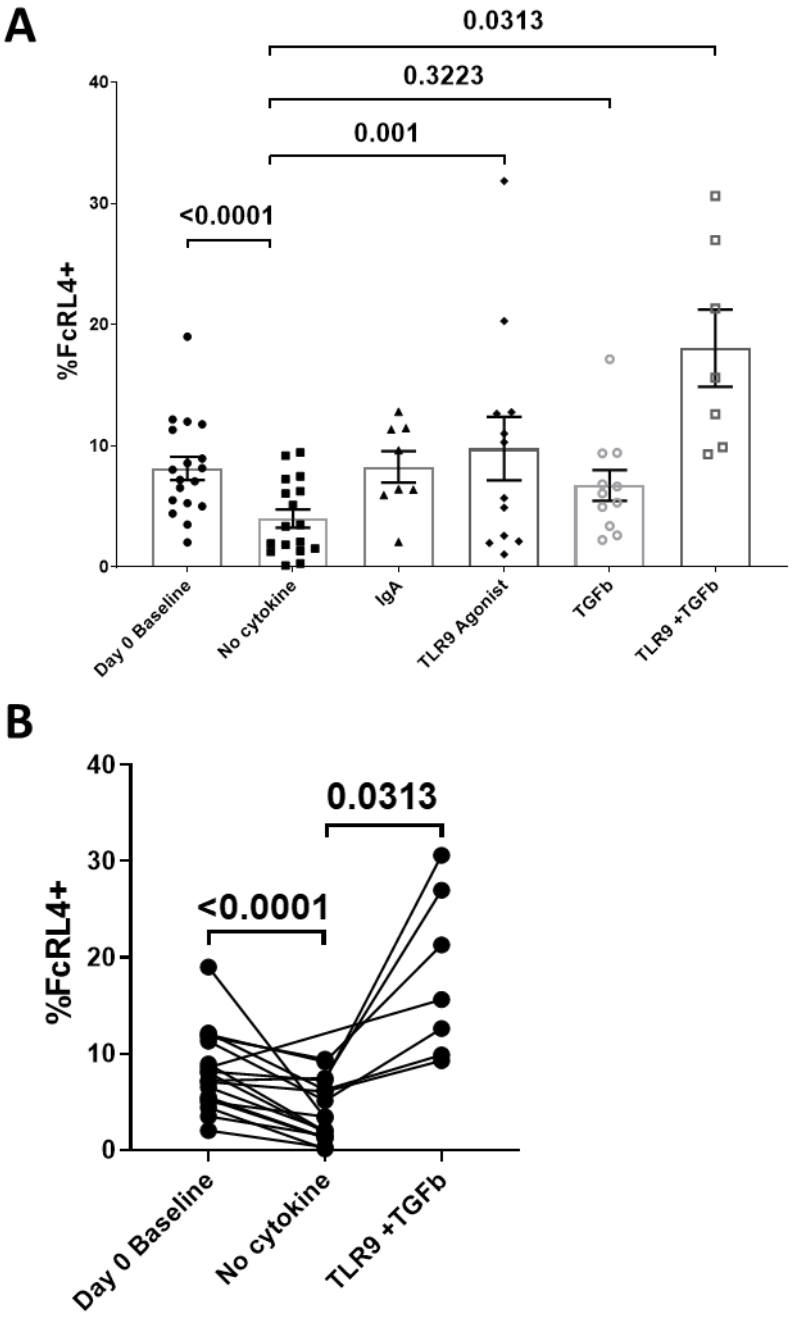

Figure 17 – FcRL4⁺ expression is positively regulated by TLR9 agonist in conjunction with TGF-β and maintained by several other stimuli. (A) *%FcRL4⁺ cells in a sorted population of memory B cells (CD19⁺, IgD⁻), following 20 hours culture in the presence of a variety of stimuli. Sorted TMCs were gated on FSC/SSC, Singlets, CD19⁺, IgD⁻ and FcRL4⁺ and the percentage of Zombie⁻ events quantified. (Day 0 Baseline, n=11; No cytokine, n=9; heat-aggregated IgA, n=8; TLR9 agonist, n=12; TGF-β, n=6; TLR9 agonist+ TGF-β, n=7; 11 experiments). Wilcoxon paired ranked test was carried out. Mean and SEM are shown.* ***(B)*** *Aligned dot plot of linked experiments showing %FcRL4⁺ cells in a sorted population of memory B cells (CD19⁺, IgD⁻), following overnight culture in the presence of a variety of stimuli. Sorted TMCs were gated on FSC/SSC, Singlets, CD19⁺, IgD⁻ and FcRL4⁺ and the percentage of Zombie⁻ events quantified. (Day 0 Baseline, n=11; No cytokine, n=9; TLR9 agonist+ TGF-β, n=7; 11 independent experiments). Wilcoxon paired ranked test was carried out. Mean and SEM are shown. Each dot represents one donor.*

3.4.3 FcRL4$^+$ B cells and Apoptosis

From the data generated in the previous experiments in which different stimuli were tested for their ability to alter the surface expression levels of FcRL4, it was also possible to investigate the susceptibility to apoptosis of FcRL4$^+$ B cells when compared to FcRL4$^-$ B cells. Gating on FcRL4$^+$ and FcRL4$^-$ B cells before and following overnight culture and quantifying the percentage of those positive for a dead cell dye, it was possible to compare the progression towards apoptosis between FcRL4$^+$ and FcRL4$^-$.

At Day 0, before the samples were cultured, there was already a significant difference between the numbers of FcRL4$^+$ cells staining positive for dead cell dye (Figure 18). This suggested that FcRL4$^+$ B cells were more prone to apoptosis than their FcRL4$^-$counterparts *ex vivo*, with the limitation that these cells may be losing FcRL4 expression prior to entering into apoptosis, clouding analysis, and complicating conclusions.

Following from this initial comparison, the differences between the FcRL4$^+$ and FcRL4$^-$ populations after culturing with no cytokine present demonstrated the same pattern, with more apoptotic FcRL4$^+$ than FcRL4$^-$ B cells following 20 hours in culture (Figure 18). Comparing the percentages of live cells in the populations between the two time-points it was possible to discern significant differences between Baseline and following overnight culture for each (Figure 18) However, it was again, possible that these cells down-regulated FcRL4 prior to entering into apoptosis, and so these data could represent an underestimate of their progression to apoptosis.

It was possible to extend this analysis to the susceptibility to apoptosis which FcRL4+ B cells exhibit under the influence of stimuli, in particular TLR9 agonism which was previously demonstrated to maintain the FcRL4$^+$ population. Analysis of these data suggests that TLR9 agonism narrows the

difference between the FcRL4^{+} and FcRL4^{-} populations after culture, with the difference between these two populations no longer significant (Figure 18). This was in contrast to the untreated cells, where FcRL4^{+} B cells were seen to be more susceptible to apoptosis (Figure 18). However, FcRL4^{+} cells treated with the TLR9 agonist were not significantly less susceptible to apoptosis than their untreated counterparts (Figure 18). TLR9 agonism narrowed the difference between FcRL4^{+} and FcRL4^{-} populations, but not between treated and untreated samples.

However, when the level of expression is also considered, this could suggest that this signal is capable of maintaining the current FcRL4+ population, rather than stimulating new expression of FcRL4 (data not shown). These data suggest that surface expression of FcRL4 can be regulated by a number of extracellular signals common to the inflammatory milieu of the rheumatoid joint and by DAMPS common to autoimmune diseases such as SLE.

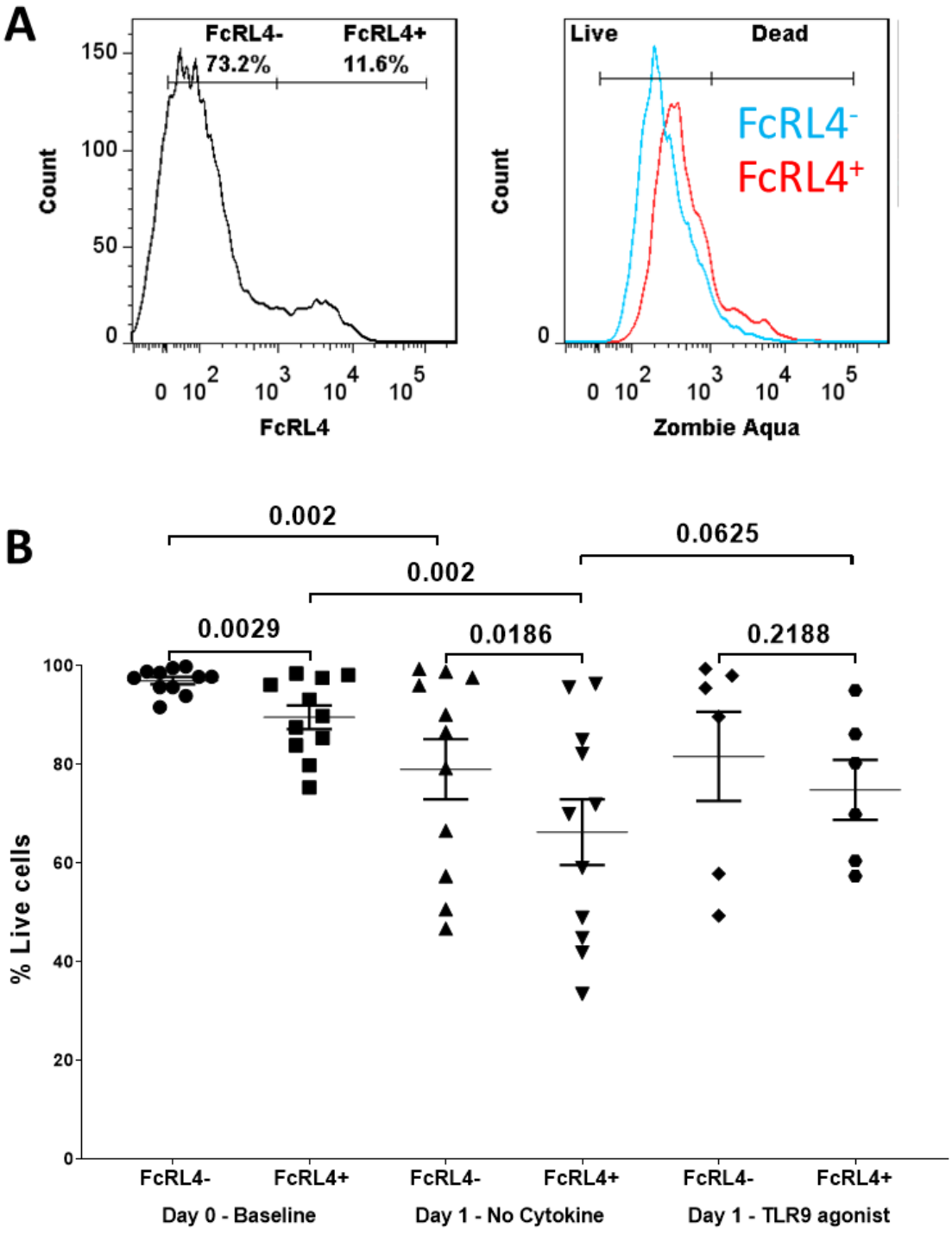

Figure 18 – FcRL4⁺ B cells are more susceptible to apoptosis than FcRL4⁻ B cells both ex-vivo and over time in the absence of stimuli. ***(A)*** *Flow cytometry dot plot showing the percentage of live cells at baseline, prior to culture for FcRL4⁺ (top) and FcRL4⁻ (bottom) B cells. tonsil mononuclear cells were gated on FSC/SSC, Singlets, CD19⁺ and either FcRL4⁻, or FcRL4⁺ and the percentage of Zombie⁻ events quantified for each.* **(B)** *Dot plot showing the percentage of live cells at "Day 0 - Baseline" prior to culture, at "Day 1 – No Cytokine", following overnight culture, and at "Day 1 – TLR9 agonist", following overnight culture in the presence of ODN2006. TMCs were gated on FSC/SSC, Singlets, CD19⁺ and either FcRL4⁻, or FcRL4⁺ and the percentage of Zombie⁻ events quantified (Day 0 – Baseline, and Day 1 – No Cytokine, n=11; Day 1 – TLR9 agonist, n=6; 11 independent experiments). Mean and SEM shown. Wilcoxon paired ranked test carried out. Each dot represents one donor.*

The next experiments were carried out over a 24-hour time-course investigated the expression of FcRL4 on unsorted TMCs and provided an opportunity to assess the temporal dynamics of this population's susceptibility to cell death. In these experiments, unsorted TMCs were cultured in complete medium and stained for CD19, FcRL4, and dead cells, and analysed every hour for 12 hours, and after 24 hours in culture. These experiments provided insight into how the cells survive in culture without the stimuli and showed a high level of similarity between the $FcRL4^+$ and $FcRL4^-$ populations in their dynamics of cell death, but also showed some significant differences.

As demonstrated before, the level of cell death initially seen in the two populations showed some significant differences, with a higher level of $Zombie^+$ $CD19^+$ $FcRL4^+$ cells compared to $Zombie^+$ $CD19^+$ $FcRL4^-$ cells (Figure 19). However, this initial difference narrowed over the first 3 hours (Figure 19). The percentage of dead $CD19^+FcRL4^-$ cells remained the same at around 10%, whilst over this time the percentage of dead $CD19^+$ $FcRL4^+$ cells decreased from ~25% to ~15% (Figure 19). Between the 2 and 3-hour timepoints, there was a significant increase in the percentage of dead cells detected in both $CD19^+FcRL4^+$ and $CD19^+FcRL4^-$ populations, although a high level of variability was seen across the repeats (Figure 19). This peak in cell death subsequently dropped at the 4-hour timepoint for both populations, although the change was not determined to be statistically significant to that seen at 3 hours, due to the high level of variability seen at these timepoints in comparison with those earlier (Figure 19). This level of cell death at 4 hours steadily increased for both populations, peaking at 70-90% for the $FcRL4^-$ population and 50-80% for the $FcRL4^+$ population by 12 hours (Figure 19). This difference in the percent $Zombie^+$ $FcRL4^+$ and $Zombie^+$ $FcRL4^-$ cells was not statistically significant but the trend did not agree with data shown in earlier experiments with approximately 65% of $FcRL4^+$ B cells alive at 20 hours (Figure 18).

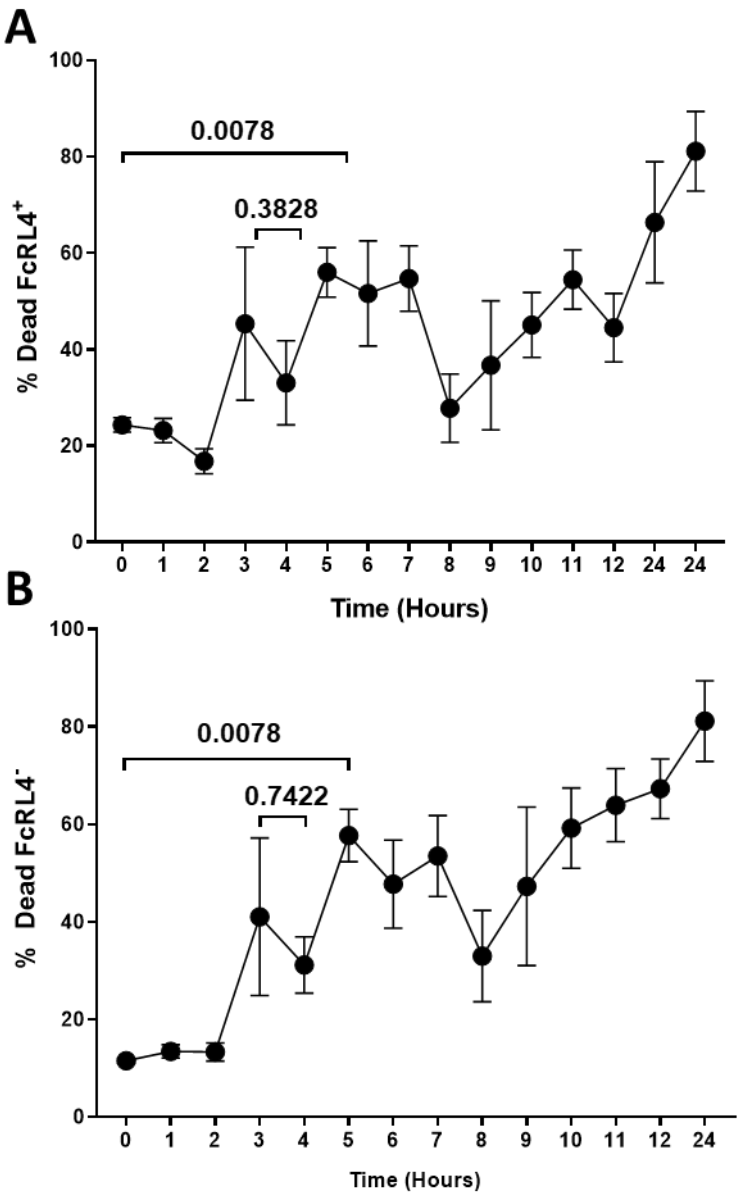

Figure 19 – FcRL4⁺ B cells are less prone to apoptosis when cultured in unsorted cell suspension from tonsil. (A) *Dot plot showing the percentage Zombie⁺ FcRL4⁻B cells over time. Tonsil mononuclear cells (TMCs) were gated on FSC/SSC, Singlets, CD19⁺ and either FcRL4⁻and the percentage of Zombie⁻ events quantified (n=6, 4 experiments). Mean and SEM shown.* ***(B)*** *Dot plot showing the percentage Zombie⁺ FcRL4⁺ B cells over time. TMCs were gated on FSC/SSC, Singlets, CD19⁺ and FcRL4⁺ and the percentage of Zombie⁻ events quantified. (n=6 separate donors, 4 independent experiments). Mean and SEM shown.*

3.5 Summary – FcRL4$^+$ B cells: Expression and Regulation

These experiments investigated the presence and distribution of FcRL4$^+$ B cells in the joints and periphery and the regulation of this population. They show that FcRL4$^+$ B cells can be found in the joints of patients with a number of different arthritides and in the periphery of healthy controls, over-represented here in the Age-associated B cell population. They also demonstrate that FcRL4 is an unstable surface marker which can be regulated by a combination of different exogenous signals.

3.5.1 Experimental Limitations and Discussion – FcRL4$^+$ B cells: Expression and Regulation

When analysing the proportion of FcRL4$^+$ CD19$^+$ cells in first experiment in which the synovial fluid of different arthritides were analysed, it is important to note that the numbers of patient samples from across the diseases varies dramatically which complicates comparisons between different diseases, compounding this is the difference in fluorophores used across these experiments. Although the same monoclonal antibody clone was used in all of the experiments to stain for FcRL4, the fluorophores varied. This may affect the resolution, or ability to discriminate different populations of cells based on their expression of the markers, which in the case of FcRL4 is of particular note, given that it rarely shows up as a clear population but as a graduated level of expression. With these caveats noted, the firmest conclusion which can be drawn from these data is that FcRL4$^+$ B cells are present in the synovial fluid of various arthritides.

Looking at FcRL4 in the periphery on ABCs it was clear that FcRL4 is expressed by a sizable proportion, approximately 25% of the population but would not suffice as a unique marker. One of the samples which recorded a value of 11.56% of their CD19$^+$ cells as ABCs may suggest that this patient was of a significantly greater age than the others, female, or had recently received a vaccination. Unfortunately, none of this information was available as these blood samples originated

from the anonymised blood donation services.

In the experiments investigating the surface expression of FcRL4 over time on cultured Tonsil mononuclear cells (TMCs), there are a number of considerations which are important to note. As the cells in this experiment were not sorted or enriched there is the potential that the B cell populations of interest may come into contact with many other lymphocytes or mononuclear cells. This may alter their phenotype or functional characteristics enabling them to maintain expression of FcRL4 for longer, or simply to survive for longer, or even lead to their activation. It is highly likely that this would have led to a different outcome had the cells be isolated prior to culture. To determine whether or not secreted factors from other cell types or indeed other B cell populations could have been responsible for the effects seen pre- and post- measurement ELISA's could be carried out on the supernatant to determine if this had occurred and may provide useful information on the cytokine milieu which leads to FcRL4 expression or its' downregulation. The number of cells included in each well may have also had an effect on the expression of FcRL4, it could be the case that in response to fewer or greater numbers of cells that FcRL4 expression varies as a result of various factors being present at lower or higher levels which could impact on both the likelihood of cell-cell interactions and the concentration of secreted factors. The numbers included in these experiments would have been higher than found in the circulation at any one point but also potentially lower than found in areas of the tissues of the tonsil. The anatomical structure of the tonsil is also lacking, which may have a similar influence, not just based on the different cells exposed to but the exposure to a different circulation and mechanical forces which have been demonstrated to be important to cells. It is possible to culture whole tissue or tissue sections in complete medium to potentially bypass some of these effects, however this would not completely recapitulate the environment in vivo as, whilst cell populations may be anatomically segregated, at least more so than when in a cell suspension, it would not provide the circulation necessary to bring various factors required for the

cells' health and stimulation and equally fails to remove those capable of damaging the cells. This could perhaps be solved through the use of reperfusion equipment, or more simply through cell culture inserts which would allow for frequent changes of medium. The complete medium in which these cells were cultured in may have lacked the essential stimuli required for the survival of other cells present, whose entering into apoptosis may have provided signals which lead to the active downregulation of FcRL4, or processes which lead to this including the moving of resources away from maintaining expression rather than a direct effect. The fact that these cultures also took place in the "standard cell culture conditions" of 37°C/5% CO_2 could have also had an effect on the cells' survival and expression of surface markers, given the potential for the *in vivo* situation to be more hypoxic, although normothermic preservation has been demonstrated to improve liver transplant success rates, perhaps suggesting that this may in fact provide help to the cells[528]. Another caveat is the fact that individuals will not have their tonsils removed for no reason, which means that all these cells are derived from a site which has experienced inflammation, either chronically or recurrently. This could lead to the interpretation of these data that removal from the inflammation milieu of the tonsil, leads to down-regulation of FcRL4.

A further criticism might be made of the singularity of experimental methods used, these experiments used flow cytometry and only quantified the external, not internal, levels of FcRL4. It is conceivable that this receptor is internalised or flipped in when the cell no longer perceives any requirement for it. In the case of the tonsil could feasibly be the presence of IgA, which could be internalised via FcRL4, or the presence of TGFβ which is known to be involved in the mucosa-associated class switching to the IgA B cell receptor, both weredemonstrated to maintain the surface expression of FcRL4[293,529–531]. These experiments did not assess the levels of transcript over time, this would have potentially clarified the apparent ability of IgA to maintain the expression levels of FcRL4 being due to maintaining receptor occupancy and retaining FcRL4 protein at the surface or whether a

signaling event took place which led to upregulated transcription and translation of FcRL4 protein. However, whilst this would have enabled a greater understanding it would have required the cells to be sorted by either magnetic-activated cell sorting (MACS) or fluorescent-activated cell sorting (FACS). FACS is a time-consuming process given the size of the $FcRL4^+$ $CD19^+$population, approximately 6% of total lymphocytes in the tonsil. It is also relatively expensive, requiring relatively large quantities of antibodies for the numbers of cells used in these experiments. Furthermore, the use of an FcRL4 specific antibody may affect the expression levels of FcRL4 by retaining it at the surface, bound with the fluorophore labelled mAb, or induce unpredictable intracellular signaling events which could have unknown downstream effects. Alternatively, the $FcRL4^+$ $CD19^+$ cells could be enriched for using MACS but since no specific antibody exists for FcRl4 for MACS this was also not possible, the added caveat being that if this was possible, the same considerations for FACS must be made. In the case of the culturing experiments in which different stimuli were tested for their ability to alter FcRL4 expression $CD19^+$ IgD^- cells were sorted from tonsils. Not only does this represent a larger population than the $FcRL4^+$ $CD19^+$ and thus make it more feasible to sort the numbers of cells required, but it is a population known to contain $FcRL4^+$ B cells, as a predominantly memory B cell population. It also avoids any issues around directly binding antibody to FcRL4 and altering its' expression in such a way.

A number of interpretations can be made from the culturing experiments. As described previously, the expression of FcRL4 is significantly lower after 24 hours in culture with no stimuli present than before going into culture. Given the low level and percentage of FcRL4 expression detected at 12 and 24 hours it is also possible that these cells represent a population of low but stable expressing cells and that other $CD19^+$ cells may be capable of transiently upregulating FcRL4. However, that brings to the fore one of the key drawbacks of these experiments, that these did not utilise a pure population of $FcRL4^+$ $CD19^+$ cells and thus drawing a distinction between upregulation and maintenance of

expression is not possible, or differences in proneness to apoptosis. Although in light of the other data it might be expected that this is not the case. The reasons for doing so are detailed above, but primarily concern avoiding unwanted effects from the direct binding of antibody to the marker of interest and avoiding time which may negatively impact on the cells' survival.

Notwithstanding the above considerations these experiments suggest that none of the cytokines or TLR agonists tested were able to upregulate FcRL4 alone, although several of the stimuli appeared to be able to maintain the level of expression seen at the baseline, time 0. These included TLR9 agonist, TGFβ, and IgA. To speculate as to why these stimuli appeared to maintain expression could implicate a variety of mechanisms. In the case of the TLR9 agonist it suggests firstly that these cells express the necessary receptor, TLR9, which it should be noted is an intracellular receptor, and secondly that stimulation is not dependent on nucleic acid alone, since TLR7 and TLR8 recognise ssRNA and TLR3 is stimulated by dsRNA. This suggests that FcRL4 is regulated by a pathway directly linking to TLR9 agonism. As TLR9 agonists consist of bacterial DNA with repeating motifs, these are also present in the mitochondrion and in the synovial fluid of RA patients. Thus, it is possible that in the synovial fluid these stimuli are present are interacting with $FcRL4^{+}$ B cells, either causing them to maintain expression of FcRL4 or with cells which are then driven express FcRL4. Experiments investigating this are highly possible. In light of this, not testing the synovial fluid of RA patients was oversight, but the idea of the experiments initially was to determine if any singular stimuli was capable of altering FcRL4 expression and so this was not included. Although the literature has demonstrated that TLR9 agonists are present in the synovial fluid, the presence of TGFβ is less well reported but in the mucosa TGFβ is an important cytokine for a multitude of functions and TLR9 agonists could potentially derive from the bacteria present.

It is unlikely given the lack of IgA receptors expressed on the surface B cells that IgA is signaling through another receptor and so it could be assumed that it is acting directly via FcRL4. Although other cells such as neutrophils are capable of interacting with IgA via receptors such as Fcα, these will have been screened out in the sorting process[532]. It is possible that IgA binding to FcRL4 causes the recruitment of more FcRL4 molecules to the surface of the cell, it is also possible that the binding of IgA aggregates at the cell surface results in the retention of FcRL4 molecules at the surface, unable to internalise. The size of the IgA complexes was not assessed but they are certainly larger than 180kDa, the size of an individual monomer[533]. However, a consideration must be made from the location from which these cells are derived and information from latter experiments, which is that these tonsil-derived FcRL4$^+$ B cells may already have IgA bound to their surface and so the addition of more IgA would not necessarily lead to further binding. If this is accepted then there is the potential for some level of competition between the receptor bound IgA and the added IgA, which might reach to a higher threshold of receptor occupancy otherwise not seen *in vivo*.

TGFβ is an important pleiotropic cytokine, particularly in the mucosa associated lymphoid tissue (MALT) as it is involved in the class switching of B cell receptors to IgA, but it also has roles in cell growth, proliferation, differentiation, and apoptosis[48,293,531,534,535]. The route by which TGFβ could lead to FcRL4 expression is not known but if it acts as a survival factor in this case it could explain why a higher level of expression is maintained[536].

Later combinations of stimuli were used with a strong additive effect demonstrated on expression of FcRL4$^+$ by the combination of TLR 9 agonist and TGF-β. Suggesting that either these induce a positive feedback loop or that they regulate different pathways which regulate FcRL4. Although the number of repeats (n=3) of this particular condition prevents any definitive conclusions being made from these particular experiments, it does suggest an intriguing effect taking place. Interestingly, addition

of the TLR7 agonist to the culture medium did not result in an upregulation/maintenance of FcRL4 expression. TLR7 has been described as an important signal in the formation of the Age-associated B Cell population in mice[523].

Despite the significant decrease in the level of FcRL4 detected at the surface of these cells after 20 hours in culture without stimuli, and the ability of certain stimuli to individually maintain or in combination, upregulate the expression of FcRL4, others appeared capable of reducing the percentage of detected FcRL4$^+$ cells further. These stimuli included IL-6, IL-10, IL-21, IL-33, and TLR agonists 1-8. This could be attributed to either direct effects negatively regulating the expression or potentially due to effects leading to the apoptosis of the FcRL4$^+$ cells at a higher rate than the FcRL4$^-$ cells. The lack of effect of IL-10 and IL-6 are particularly interesting in light of their respective regulatory and inflammatory effects on many immune cells, suggesting that FcRL4 might not be relevant as a marker of chronic inflammation and also that FcRL4 expression is not affected by the immunomodulator IL-10. This conclusion is reinforced in light of analysis of patient data in which no correlation between the percentage of FcRL4$^+$ B cells and inflammation was found (data not shown).

Ideally it would be possible to sort a pure population of FcRL4$^+$ CD19$^+$cells from a non-inflamed tissue in a manner which does not risk stimulating them as FACS can. A “checkerboard” of concentrations and combinations of stimuli would be used. In this work, these stimuli focussed on inflammatory cytokines given the reports of FcRL4 as having similarities with populations of chronically antigenically stimulated B cells. Future work could broaden the selection to include other important B cell signals such as CD40L, APRIL, TACI, etc. Analysis would include both phenotypic and transcriptomic methods to unravel regulatory pathways. Following this, different populations of naïve and memory B cells would be sorted and stimulated using the combinations demonstrated to lead to FcRL4 expression in order to better describe their possible ontogeny.

Another improvement for future work could be inclusion of reagents which would allow the calculation of cell numbers following culture with the different stimuli. Although a predetermined number of cells were counted before addition to the plate wells for culture, not all cells are recovered afterwards. Methods could include defined numbers of counting beads added prior to the data acquisition step, with the potential for the same issues with recovering fewer or greater numbers of cells depending on cell death and adherence that were experienced in these experiments. Or perhaps more usefully would be the inclusion of a cell dye, which following internalisation prior to culturing, would allow the quantification of the number of cell divisions the cells have experienced. This would provide information as to the proliferation stimulating properties of a number of stimuli tested, and if used in pure populations of $FcRL4^+$ $CD19^+$ and $FcRL4^-$ $CD19^+$ cells allow the determination of whether new cells are being driving to express FcRL4 by any of the stimuli or if the $FcRL4^+$ $CD19^+$ and $FcRL4^-$ $CD19^+$ populations differ in proliferative capacity.

In the experiments investigating the dynamics of apoptosis of the $FcRL4^+$ $CD19^+$ and $FcRL4^-$ $CD19^+$ populations, there is a relatively high degree of inter-experiment variability between the two populations in the experiments in which $CD19^+$ IgD^- cells were sorted by FACS and cultured, and those in which unsorted TMCs were cultured. In the sorted experiment, the $FcRL4^+$ $CD19^+$ population was determined to be more prone to apoptosis, whereas in the unsorted experiment the opposite was seen. This could potentially be attributable to the tonsil donors which will be different across these experiments, but this is unlikely given the reproducibility of the effect seen. The background signal seen could be factor which would be greater in the $FcRL4^-$ population than the $FcRl4^+$ population due to the other B cells being included which may be more prone to apoptosis. One potential confounder could be the difference in size of the $FcRL4^+$ and $FcRL4^-$ populations, as the $FcRL4^+$ B cells have been described as larger and more dendritic in structure[503,515]. This could lead to an artificially high level of

apoptosis being recorded as the dead cell dye, Zombie Aqua™, binds to amine groups, thus the higher level of apoptosis could simply reflect a higher level of dead cell dye on a larger cell. Further clarification of these data could be generated by using further markers of cell cycle such as DNA intercalating dyes like Draq7 and propidium iodide, or proliferation markers like Ki-67, which would enable a more detailed analysis of these populations' activity. Clarification would also be needed about the impact of purifying these cell populations as the mixture of cell population is a potential confounder, as well as also being, perhaps, more relevant to the *in vivo* situation.

It should be noted that whilst these latter experiments show a significant proportion of the cells dying during culture, that the earlier experiments detailing the various effects of a number of stimuli on the surface expression of FcRL4 are all gated on live cells and so avoid any direct confounding effects in the analysis, such as an increase of autofluorescence signal on dying cells providing a false positive for expression levels. However, they do provide an indirect effect by which the earlier experiments will have been affected by which is the presence of a high proportion of dead cells in culture which may have released signals capable of altering the remaining live cells' activity. Given the impact of TLR9 agonist, the repeating motif of double stranded bacterial DNA, upon FcRL4 expression, it might be thought that the apoptosis of cells would lead to mitochondrial DNA being present in the culture medium and thus leading to an increase in the level of surface FcRL4 detected. However, clear and statistically significant differences were detected between the various treatments and the addition of TLR9 agonist in conjunction with TGFβ lead to a significant difference to the addition of TGFβ alone. This would not be expected if the presence of dead and dying cells lead to the release of TLR9 agonists from their mitochondria.

This enrichment of FcRL4 in the ABC population is interesting in light of the literature on "atypical" memory B cells which are described as having a relatively similar phenotype to both the ABCs

present in the periphery of elderly patients and in the FcRL4^{+} population of the RA joints[474,523]. That FcRL4 is detectable on the surface of B cells in the periphery suggests that these cells do not necessarily require an inflammatory stimulus in order to express FcRL4, or that this is present at high enough levels in the periphery to drive expression. It is also possible that this population represents a stably expressing population of cells which have already responded to stimuli and do not require further stimulation. However, the literature on ABCs and "atypical" memory B cells suggests that these cells are the result of chronic antigenic stimulation. This could be the case with regards to these peripheral FcRL4^{+} ABCs. The fact that these cells are a memory B cell population as per their expression of CD27 and lack of IgD, strongly suggests that this population is antigen experienced. It would be of interest to compare the antigen specificity of FcRL4^{+} and FcRL4^{-} ABCs and determine the proportion of self-reactive clones within these populations. Whether the population described here is truly the ABC population described in other literature is a point of contention. Many papers describe the use of the transcription factor T-bet as a marker for ABCs, but it is not limited to ABCs and is also found in other subsets, and as a marker of activation. The combination of CD21^{-} CD11c^{hi} is accepted as an approximation of the ABC population for most, although additional staining for T-bet would indeed reinforce confidence in this description.

3.6 Conclusion – FcRL4^{+} B cells: Expression and Regulation

Collectively these data suggest that FcRL4 is a marker of a memory B cell subset present in the synovial fluid of patients with different arthritides. Expression of FcRL4 is unstable and is rapidly downregulated over a 12-hour period in culture. This surface expression is responsive to certain individual stimuli and combinations of these, primarily the TLR9 agonist ODN2006 in conjunction with TGFβ. It is also possible to detect FcRL4 in the periphery of healthy controls in contrast with the reported literature, and it appears to be enriched in the ABC or "atypical" B cell population.

4 FcRL4^{+} B cells and IgA

4.1 FcRL4^{+} B cells interact with IgA

IgA is the most abundant immunoglobulin produced in the human body and has a number of receptors including the transferrin receptor (TfR), which binds the secretory chain of polymeric IgA, and the myeloid-cell-specific type I Fc receptor for IgA (FcαRI). It has also been reported that FcRL4's natural ligand is IgA[513,537]. It should also be noted that the poly-Ig chain receptor (pIgR) is capable of binding the joining chain which links IgA dimers. This is important for enabling the translocation of IgA across the mucosal membranes from the lamina propria where it is produced, into the lumen where it can interact with bacteria. In this chapter the links between IgA and FcRL4 are investigated and further work was carried out in *ex vivo* SFMCs, TMCs, and PBMCs following up reports that FcRL4 may be an IgA receptor.

4.1.1 Synovial fluid FcRL4^{+} B cells have a higher level of surface IgA than FcRL4^{-} B cells

Isolated synovial fluid mononuclear cells (SFMCs) from patients with active rheumatoid arthritis (RA) at the time of sampling were stained for CD19, FcRL4, and IgA. Gated upon live B cells (Zombie^{-}, CD19^{+}) cells it was possible to distinguish a strong association between surface levels of FcRL4 and IgA (Figure 20). There was a significant difference in the IgA MFI between FcRL4^{+} and FcRL4^{-} cells suggesting there is more IgA present on the surface of FcRL4^{+} B cells than on FcRL4^{-} (Figure 20). However, from these plots, it was not possible to determine whether this signal derived from IgA expressed by the cells on their membrane as BCR or from IgA bound to surface receptors.

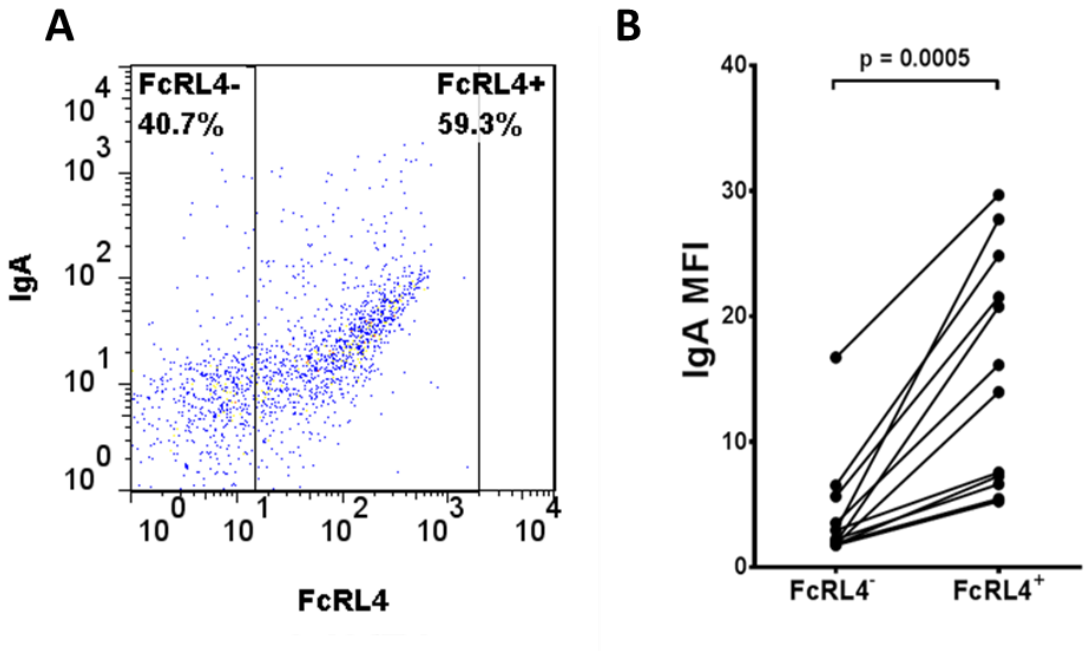

Figure 20 – FcRL4⁺ SF B cells have a higher IgA MFI than FcRL4⁻ B cells. (A) *Flow cytometry dot plot detailing the gating strategy used in (B), RA synovial fluid mononuclear cells were gated on FSC/SSC, Singlets, Zombie⁻, CD19⁺.* ***(B)*** *Linked dot plot showing the respective IgA MFI for paired populations of FcRL4⁻ and FcRL4⁺ B cell populations from RA patient synovial fluid. (n=12, independent 12 experiments) Statistics were carried out using the paired Wilcoxon signed ranked test. Each dot represents a patient.*

4.1.2 Surface IgA can be removed and re-bound by synovial fluid FcRL4$^+$ B cells

To determine whether the surface bound IgA can be removed from FcRL4$^+$ B cells and if they can subsequently bind it again, SFMC samples from patients with RA were initially divided into two fractions. One fraction was left untreated. The other fraction was incubated briefly with pH3 buffer to remove the surface bound proteins, washed, centrifuged and then divided into a further two fractions. One acid treated fraction was not treated further and the other incubated with heat-aggregated IgA (HA-IgA). At the end of this there were three fractions, one untreated, one acid-treated from which surface proteins had been stripped, and a final fraction which had been acid-treated to remove its' surface bound proteins and then incubated with heat-aggregated IgA. Following these treatments, the samples were stained for CD19, FcRL4, and IgA, and Zombie Aqua to distinguish between live and dead cells. This allowed for the determination of whether there was any surface IgA on the cells *ex vivo*, and whether they could bind aggregated IgA following this removal.

Gating on the live CD19$^+$ cells, the initial level of IgA detected on the FcRL4$^+$ B cells compared to the FcRL4$^-$ varied significantly again demonstrating the presence of significantly more IgA on the surface of FcRL4$^+$ B cells compared to FcRL4$^-$ B cells. Following the 60 second incubation in the pH3 buffer, far lower levels of surface IgA were detected on the FcRL4$^+$ B cells compared to the FcRL4$^-$, suggesting that bound IgA had been stripped off (Figure 21). After incubation with heat aggregated IgA (HA-IgA) a significantly increased level of IgA was detected on the FcRL4$^+$ cells compared to the pH3 treated cells, at a level comparable with that seen initially, suggesting that the FcRL4$^+$ cells had bound IgA (Figure 21). Although there was a degree of non-specific binding seen on the FcRL4$^-$ cells it was significantly lower that seen on the FcRL4+ cells (Figure 21). These results suggest that FcRL4$^+$ cells are capable of binding IgA aggregates.

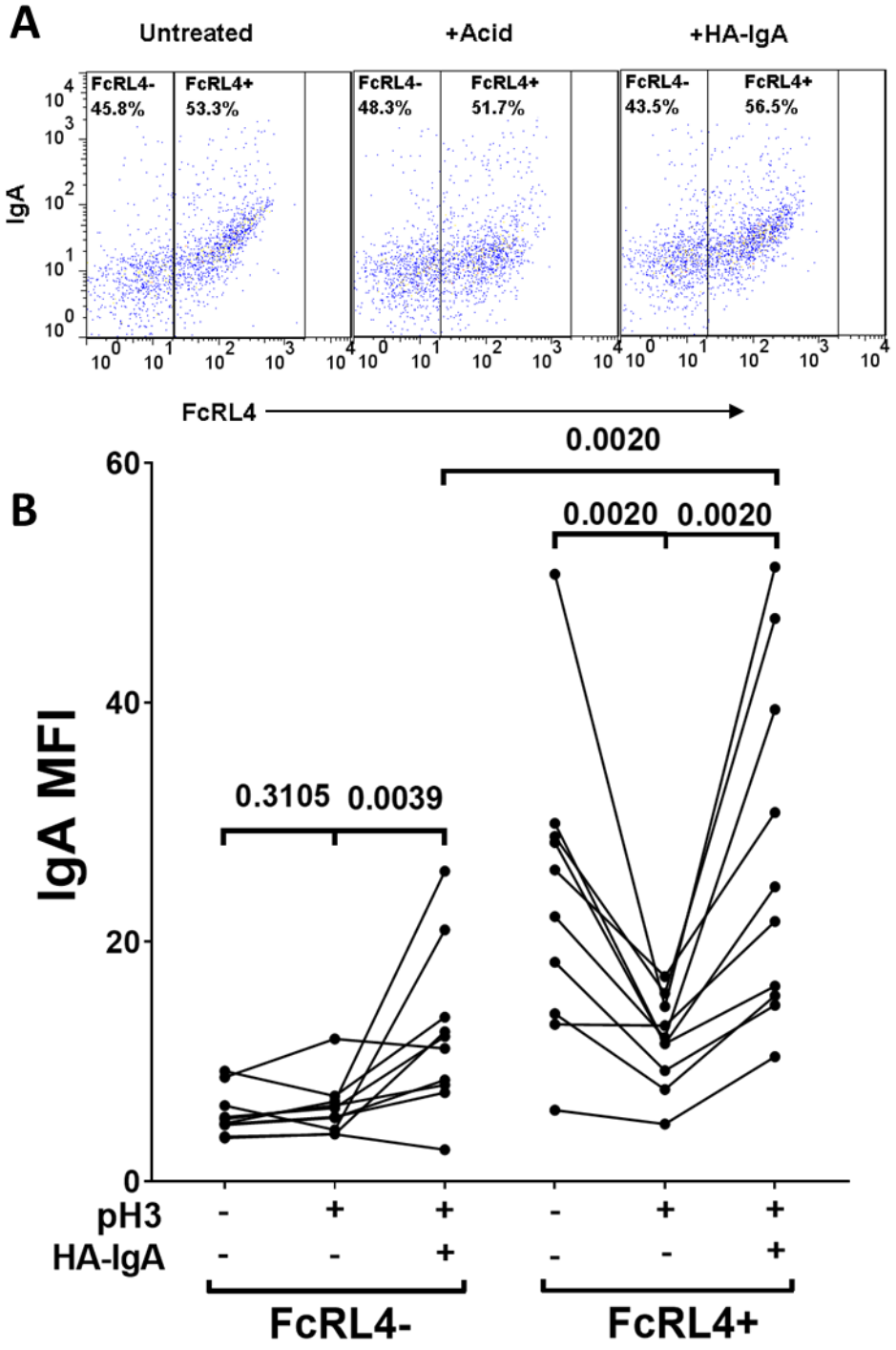

Figure 21 – FcRL4⁺ RA SF B cells are capable of binding IgA aggregates. (A) *Flow cytometry dot plot detailing the gating strategies used in (B) for the three linked values (untreated, +acid, +HA-IgA) shown for each population (FcRL4⁺ & FcRL4⁻). RA SFMCs were gated on FSC/SSC, Singlets, Zombie⁻, & CD19⁺.* ***(B)*** *Linked dot plot showing the respective IgA MFI for paired populations of FcRL4⁻ and FcRL4⁺ B cell populations from RA patient SF prior to acid wash, following acid washing, and following acid washing and subsequent addition of HA-IgA. (n=10, 8 independent experiments) Statistics were carried out using the paired Wilcoxon signed ranked test.*

4.1.3 Surface IgA can be removed and re-bound by MALT-derived FcRL4$^+$ B cells

Following these assays to determine the ability of synovial fluid FcRL4$^+$ B cells to bind IgA the focus shifted to the site at which FcRL4$^+$ B cells had been first described, the tonsil. In agreement with the data showing FcRL4$^+$ B cells having a higher initial surface level of IgA than FcRL4$^-$ B cells and the stripping and re-binding of IgA occurring in *ex vivo* synovial fluid, FcRL4$^+$ B cells in tonsils showed the same pattern (Figure 22). A titration of HA-IgA was also carried out, demonstrating that maximal binding was achieved roughly at 0.5 mg/ml and that there was not a clear difference above this (Figure 22).

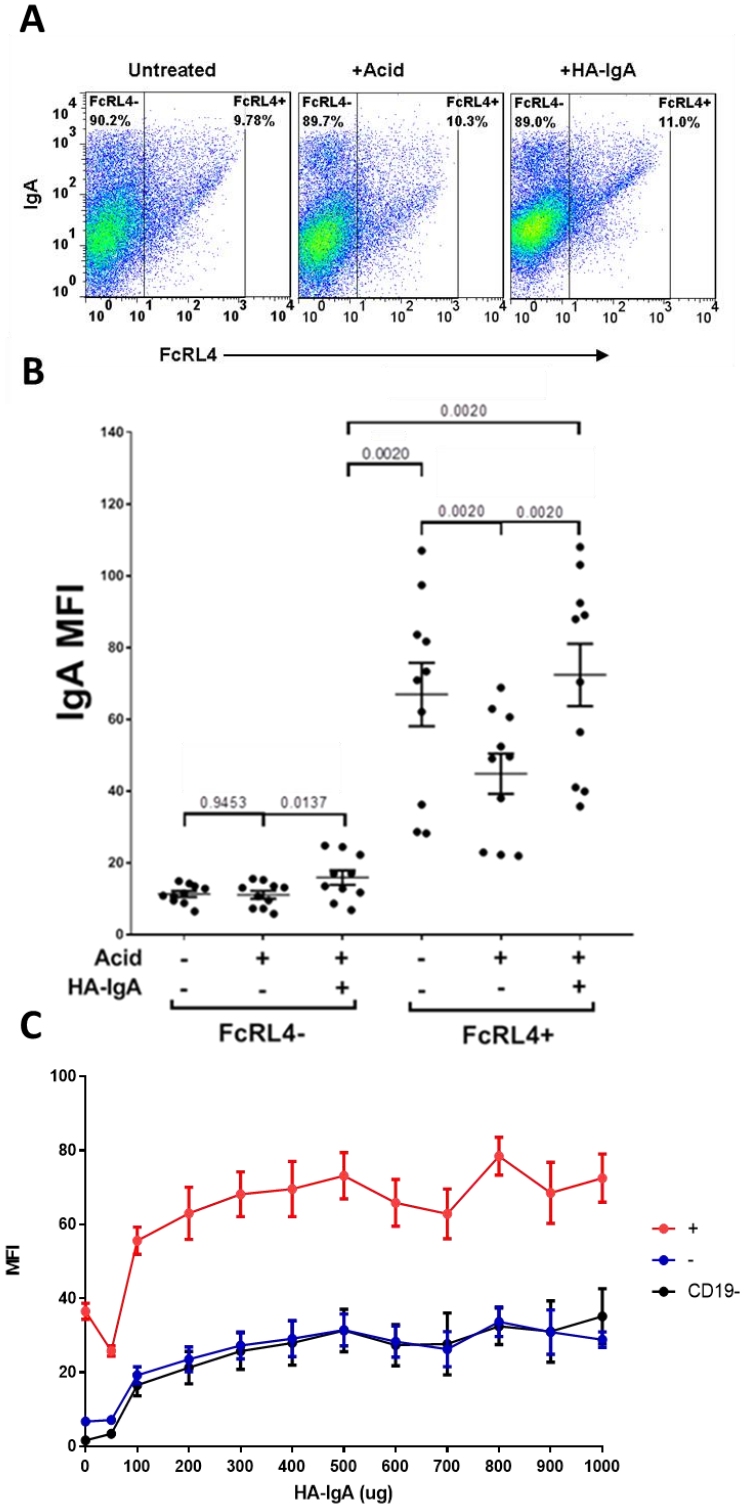

Figure 22 – FcRL4⁺ Tonsil B cells are capable of binding IgA aggregates. (A) *Flow cytometry dot plot detailing the gating strategy used in (B), TMCs were gated on FSC/SSC, Singlets, Zombie⁻, CD19⁺.* ***(B)*** *Linked dot plot showing the respective IgA MFI for paired populations of FcRL4⁻ and FcRL4⁺ B cell populations from tonsillectomy patient TMCs. (n=11, 11 experiments) Statistics were carried out using the paired Wilcoxon signed ranked test.* ***(C)*** *Linked dot plot showing the IgA MFI for the FcRL4⁺, FcRL4⁻ and CD19⁻ populations present in TMC samples following incubation in a pH3 buffer and subsequent addition of increasing amounts of HA-IgA. +: FcRL4⁺, -: FcRL4⁻, CD19+: All B cells. (n=5, 5 independent experiments). Mean and SEM are shown. Statistics were carried out using the paired Wilcoxon signed ranked test*

4.1.4 SF FcRL4+ B cells are enriched for IgA BCRs compared to SF FcRL4- B cells

Once the synovial fluid cells have been treated with pH3 acid wash and the surface-bound proteins stripped away, it was possible to assess the proportion of B cells with IgA BCRs present in the $FcRL4^-$ and $FcRL4^+$ populations. These data were generated from further analysis of the data generated in 4.1.2.

Once gated on $CD19^+$ and then subsequently the $FcRL4^+$ and $FcRL4^-$ populations it was possible to see a significant overrepresentation of IgA BCR expressing cells in the $FcRL4^+$ population when compared to the $FcRL4^-$ population (Figure 23). This conclusion is supported by published data from our group in which individual FcRL4+ and FcRL4- CD19+ B cells were sorted for analysis of their immunoglobulin subclass by PCR amplification and sequencing of the constant regions at the single cell level (Figure 23)[521].

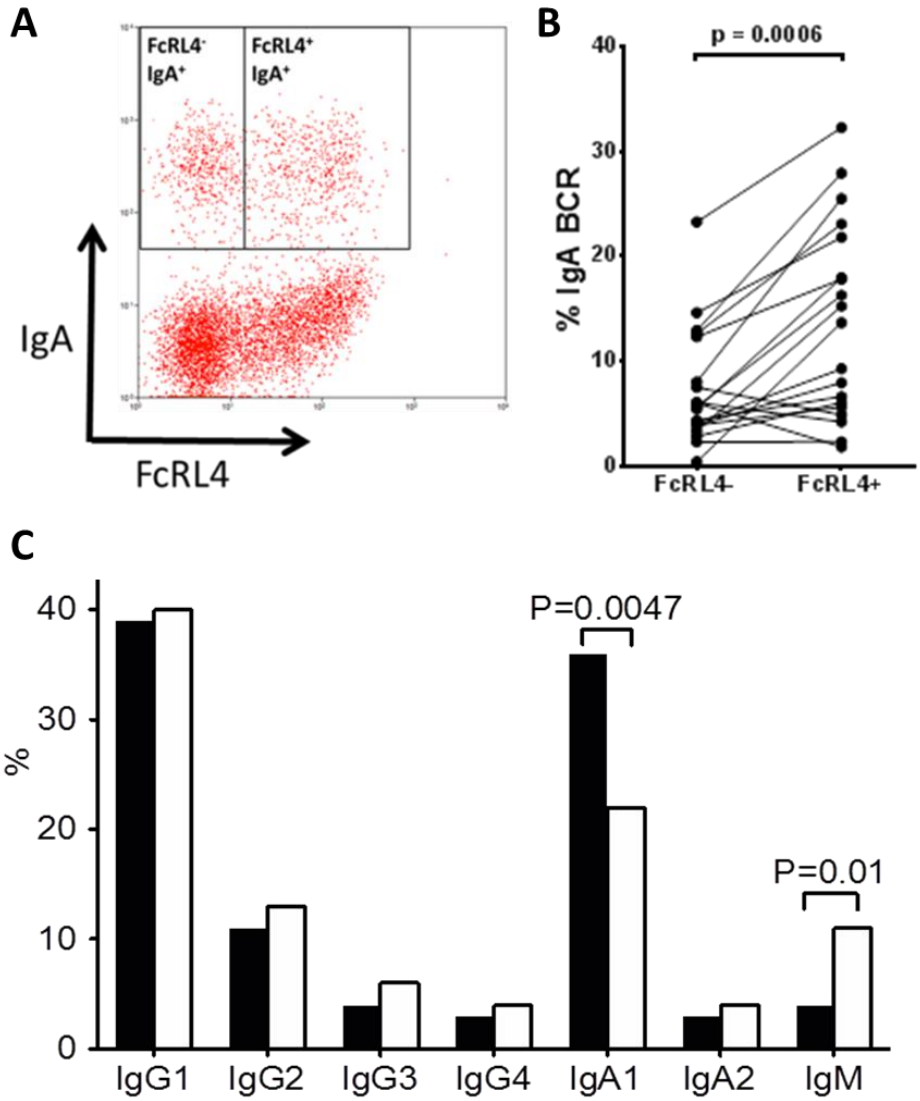

Figure 23 – IgA BCRs are over-represented in the FcRL4⁺ population. (A) *Flow cytometry dot plot detailing the gating strategy used in (B), RA SFMCs were gated on FSC/SSC, Singlets, Zombie⁻, CD19⁺, and either FcRL4⁻ IgA ⁺, or FcRL4⁺ IgA⁺.* ***(B)*** *Linked dot plot showing the respective %IgA⁺ for paired populations of FcRL4⁻ and FcRL4⁺ B cell populations from RA patient SF. (n=20, 20 independent experiments). Each paired dot represents a patient.* ***(C)*** *%Isotype B cell receptor. Statistics were carried out using the paired Wilcoxon signed ranked test. VH genes were amplified by PCR. For full methods see Amara, et al., 2017*[521]*.*

4.2 FcRL4 is a specific IgA receptor

4.2.1 Transfected B cells expressing FcRL4 bind heat-aggregated IgA (HA-IgA)

The experiments thus far have suggested that FcRL4^{+} B cells are capable of binding IgA, to determine whether it was FcRL4 which was responsible for binding IgA or whether another molecule present on the FcRL4^{+} B cells present in the mucosa and synovial fluid was binding IgA, a cell line was utilised. The Raji B cell line was transiently transfected with a plasmid encoding FcRL4 and GFP which was expressed on the intracellular N-terminus of the FcRL4 molecule (Figure 11).

These transiently transfected cells were then incubated with heat-aggregated IgA (HA-IgA) derived from human colostrum and a significant increase of IgA MFI was detected on the FcRL4^{+} compared to the FcRL4^{-} cells (Figure 24). This suggested that FcRL4 was a receptor for HA-IgA and that it is possible that this process was taking place in both the mucosa and in the synovial fluid of rheumatoid arthritis patients.

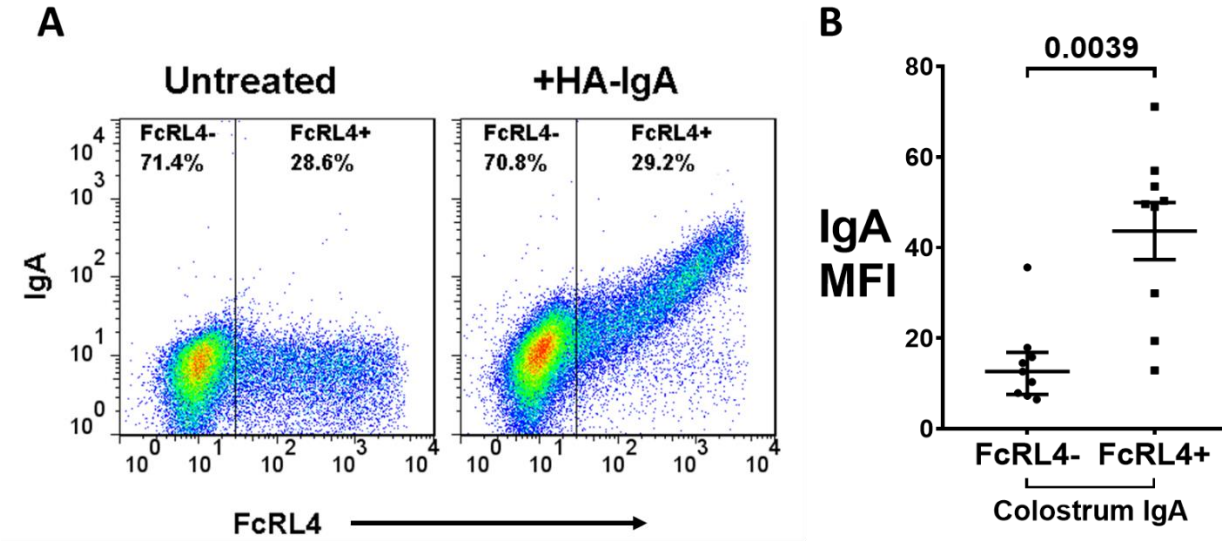

Figure 24 – FcRL4 is an IgA receptor. (A) *Flow cytometry dot plot detailing the gating strategy in (B), showing the FcRL4 vs. IgA MFI Raji cells were gated on FSC/SSC, Singlets, Zombie$^-$, and either FcRL4$^-$ IgA $^+$, or FcRL4$^+$ IgA$^+$.* ***(B)*** *Dot plots showing the IgA MFI for the populations gated upon in (A) following the addition of either colostrum or serum HA-IgA to the cells for 30 minutes. (n=6, 6 independent experiments). Mean and SEM are shown. Statistics were carried out using the paired Wilcoxon signed ranked test.*

4.2.2 pH3 treatment does not affect binding capacity of FcRL4 for IgA

In light of the ability of pH3 buffer to remove all surface proteins it also might have potential effects on the receptor itself. To test this, transiently transfected cells were incubated with the pH3 buffer prior to the addition of IgA, and either "rested" on ice for 30 minutes prior to the addition of HA-IgA, or immediately incubated with HA-IgA across a titration of concentrations.

These experiments suggest that the brief incubation in the pH3 buffer, whilst having little effect on the viability of the cells, did alter their surface as to lead to an increase in binding on both $FcRL4^+$ and $FcRL4^-$ cells (Figure 25). Resting these cells for 30 minutes on ice following the pH3 acid treatment appeared to reduce the level of signal detected on the $FcFL4^-$ B cells, without visibly reducing the level of IgA binding seen on the $FcRL4^+$ cells (Figure 25). The clear differences between $FcRL4^+$ and $FcRL4^-$ B cells suggest furthermore, that IgA binding is specific to $FcRL4^+$. However, firm inferences cannot be drawn from this experiment, as despite that a range of HA-IgA concentrations were used, only 1 repeat was carried out.

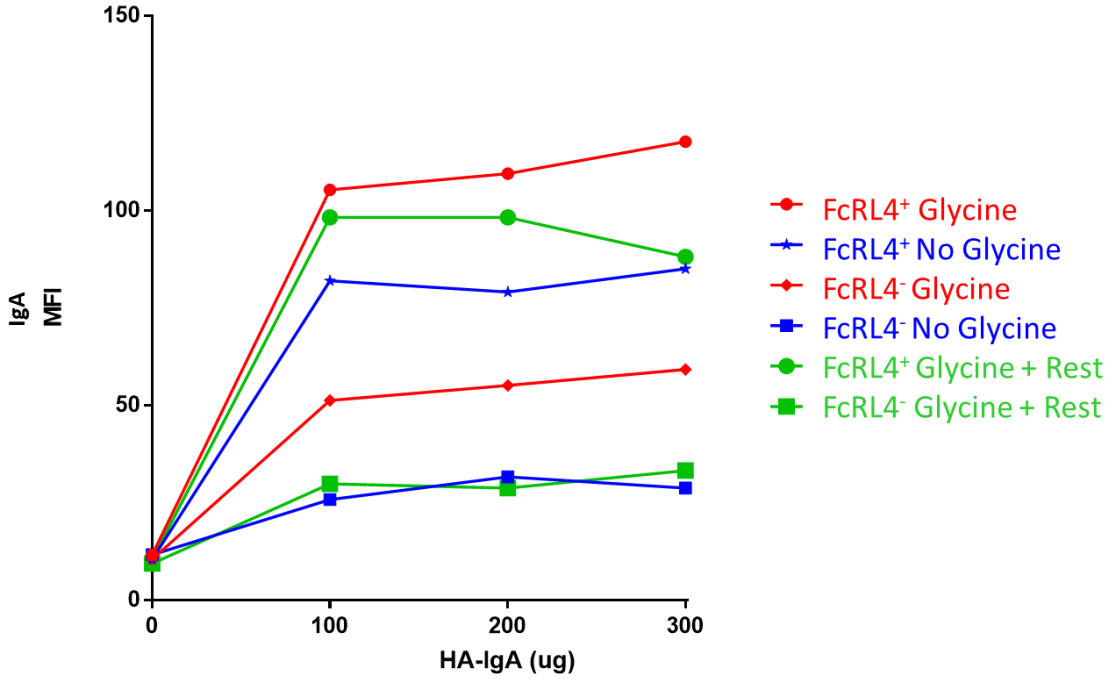

Figure 25 – Incubation in a pH3 buffer alters the IgA binding of FcRL4⁺ and FcRL4⁻ cells. *Graph showing the IgA MFI of transiently transfected Raji cells following incubation in pH3 buffer and subsequent binding of IgA with or without a rest period on ice. Data shown are untreated, pH3 treated, and pH3 treated and rested for 30 minutes on ice. Raji cells were gated on FSC/SSC, Singlets, Zombie⁻, and either FcRL4⁻ IgA ⁺, or FcRL4⁺ IgA. (n=3, 1 experiment).*

4.2.3 Transfected B cells expressing FcRL4 specifically bind IgA present in the RA synovial fluid

Although the previous experiments demonstrated the ability of FcRL4 to act as an IgA receptor they did so in a relatively artificial context, in which IgA was the sole ligand added to the cells' incubation medium. To test if the FcRL4-HA-IgA binding demonstrated in *ex vivo* SFMCs and TMCs could take place in a more biologically relevant situation, Raji cells were transiently transfected with the FcRL4 containing viral vector and then incubated with synovial fluids from seropositive RA patients. Samples were subsequently washed and stained for both IgG and IgA binding.

A significant difference in the level of IgA bound to the surface of the FcRL4+ cells compared to the FcRL4- cells was detected (Figure 26), further strengthening the case that this process is occurring *in vivo* and that FcRL4 acts as a receptor for IgA immune complexes in the joints of RA patients and in the tonsil.

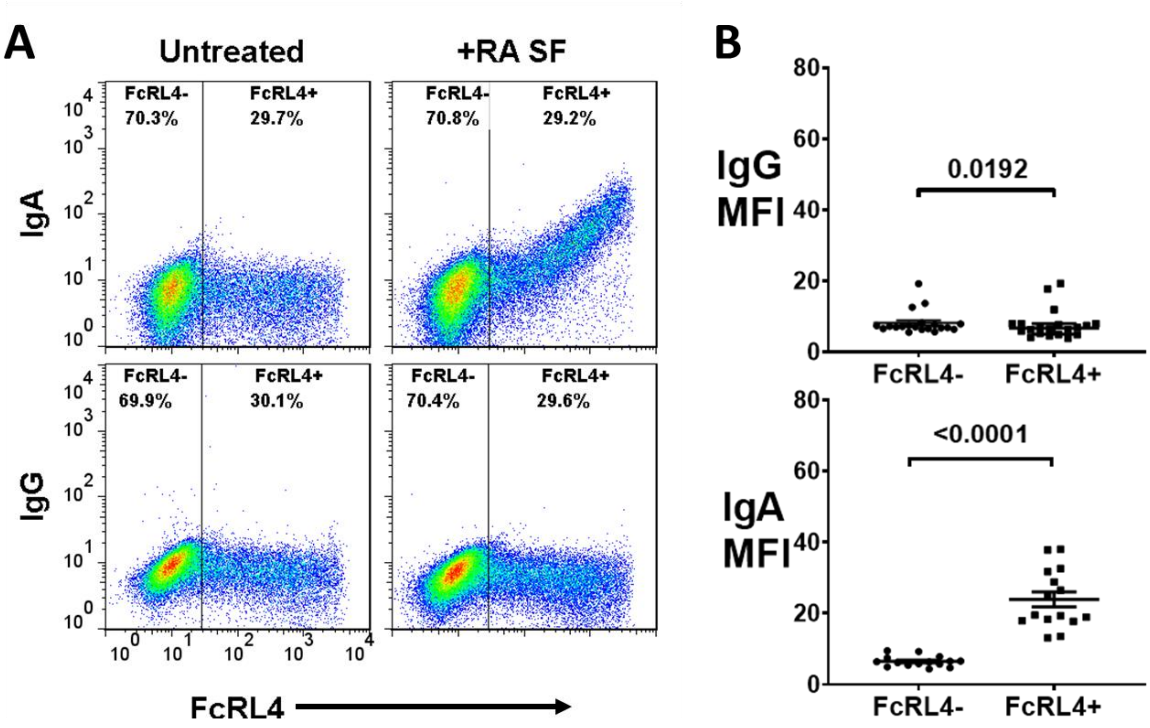

Figure 26 – FcRL4⁺ specifically binds IgA aggregates present in the RA SF. (A) *Flow cytometry dot plot detailing the gating strategy in (B), showing the FcRL4 vs. IgA MFI Raji cells were gated on FSC/SSC, Singlets, Zombie⁻, and either FcRL4⁻ IgA ⁺, or FcRL4⁺ IgA⁺.* ***(B)*** *Dot plots showing the IgA and IgG MFI for the populations gated upon in (A) following the addition of RA SF to the cells for 30 minutes. Statistics were carried out using the paired Wilcoxon signed ranked test, (n=15, 3 independent experiments).*

4.2.4 FcRL4 is capable of binding IgA ICs present in the synovial fluid of patients with different arthritides

Using a stably transduced B cell line expressing FcRL4 generated using a lentivirus, the IgA binding capacity of FcRL4 was assessed using the SF from a number of different arthritides in collaboration with Ian McInnes's group at Glasgow University. A stably expressing cell line was used to reduce the potential for inter-experiment variability and enable more reliable direct comparisons to be made between diseases. These diseases vary in their involvement of the adaptive and innate immune system, and in their localisation. Osteoarthritis (OA) is a primarily degenerative inflammatory condition with no defined autoimmune component and thus an involvement of the adaptive immune system and autoantibodies is unlikely. Psoriatic arthritis (PsA) is an autoimmune disease which primarily affects the skin and involves inflammation of the joints, it is considered a seronegative spondyloarthropathy, although the literature suggests that autoantibodies may be feature of some patients. Rheumatoid arthritis is a systemic inflammatory autoimmune disease of which autoantibodies are a diagnostic feature and which primarily affects the joints.

Synovial fluid from each of these diseases was incubated with stably expressing FcRL4^{+} Raji cells and Raji cells not expressing FcRL4. There was a significant change in the IgA MFI seen in the FcRL4^{+} population for all the diseases suggestive of IgA binding, although the positive control of heat-aggregated IgA appeared to bind at a higher level than any of the SFs (Figure 27). No significant change occurred in the Raji cells which did not express FcRL4, and no change in the IgG MFI was detected in any sample, it should be noted that Raji cells express IgM and no IgG expression is expected on their surface (Figure 27). However, this conclusion suffered from the same drawback of the previous experiment which is a lack of a positive control for IgG. IgA but not IgG binding was detected with SF from all of the disease groups.

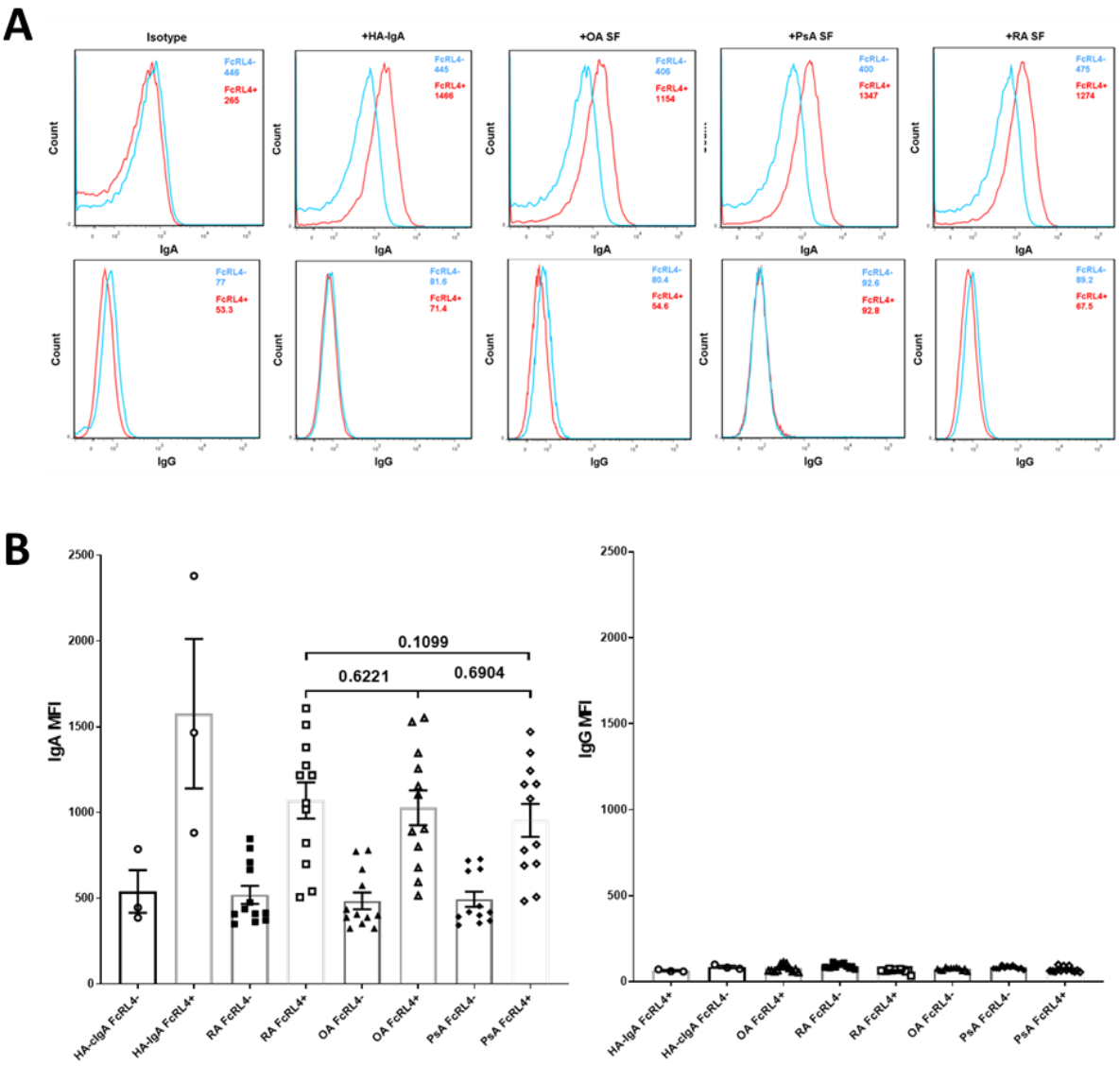

Figure 27 – FcRL4 is capable of binding IgA aggregates present in the SF of different arthritides. (A) *Flow cytometry histogram plots the gating strategy in (B), showing the IgA or IgG MFI for either FcRL4⁻ or FcRL4⁺ Raji cells. These were gated on FSC/SSC, Singlets, Zombie⁻, and either FcRL4⁻, or FcRL4⁺.* ***(B)*** *Dot plots with bars, showing the IgA and IgG MFI for the populations gated upon in (A) following the addition of either colostrum HA-IgA or RA, OA, or PsA SF to the medium for 30 minutes. Statistics were carried out using the paired Wilcoxon signed ranked test and mean and SEM are shown, (SFs n=12, HA-IgA n=3, 3 independent experiments).*

4.2.5 FcRL4 expresses a preference for colostrum IgA over serum IgA

To test whether any differences were seen in FcRL4 binding IgA between different sources of IgA, transiently transfected cells were used to assess any preference between the IgA samples. In these experiments a significant difference was seen between the FcRL4^{+} and FcRL4^{-} populations for both sources of IgA, however, the increase seen was markedly higher upon the addition of colostrum IgA compared to serum IgA (Figure 28).

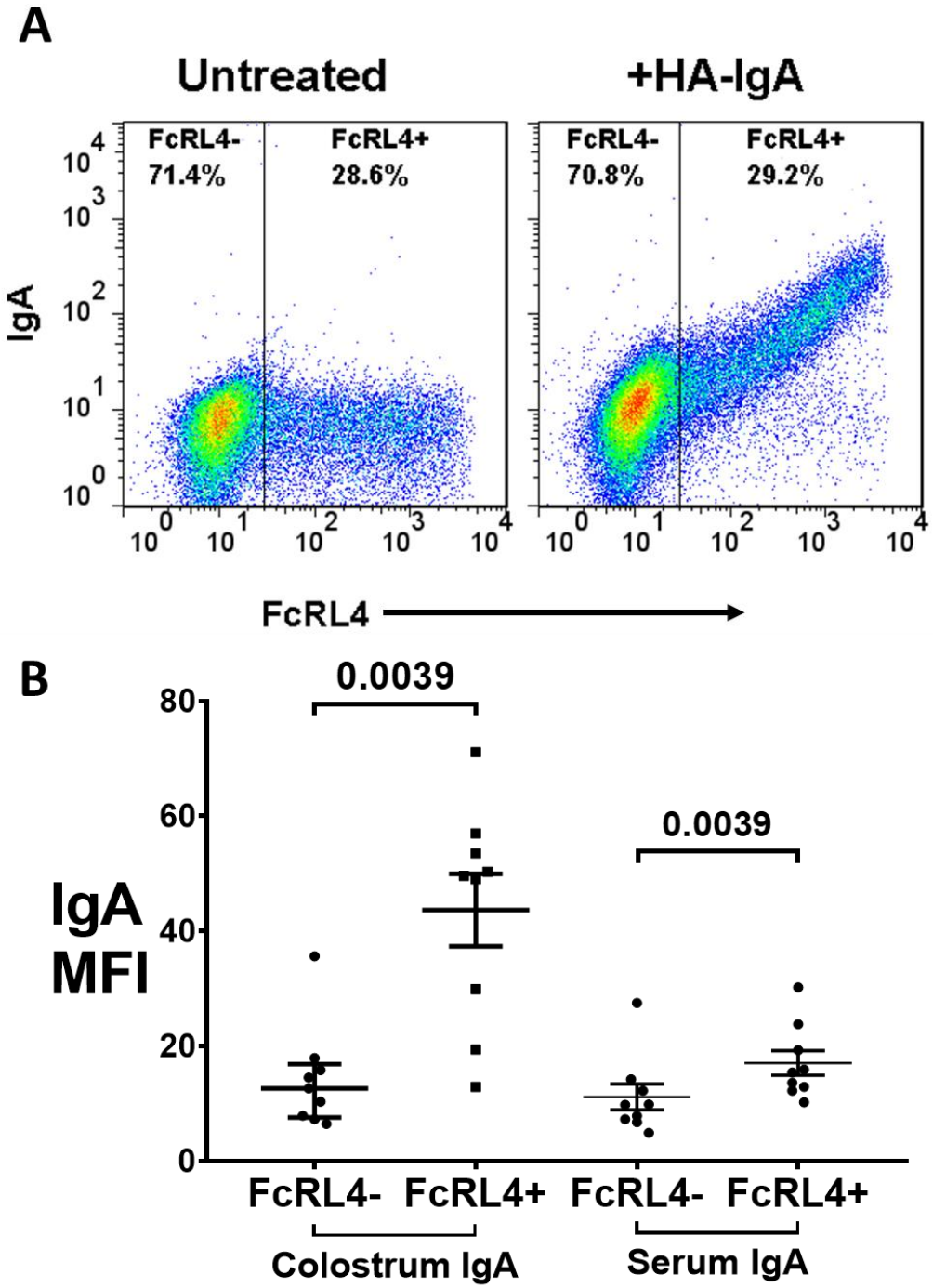

Figure 28 – FcRL4 appears to display a preference for colostrum compared to serum HA-IgA. (A) *Flow cytometry dot plot detailing the gating strategy in (B) showing the FcRL4 vs. IgA MFI, Raji cells were gated on FSC/SSC, Singlets, Zombie$^-$, and either FcRL4$^-$, or FcRL4$^+$.* ***(B)*** *Dot plots showing the IgA MFI for the populations gated upon in (A) following the addition of either colostrum or serum HA-IgA to the cells for 30 minutes. Statistics were carried out using the paired Wilcoxon signed ranked test, (n=9, 9 independent experiments).*

4.2.6 Tonsil FcRL4^{+} B cells demonstrate a preference for aggregated-colostrum IgA over aggregated-serum IgA

In all the previous experiments the IgA used was derived from human colostrum, however, IgA is also present in the serum of individuals and so the ability of both serum and colostrum IgA to bind FcRL4 was tested. These differ in their proportions of IgA1 and IgA2, the two isotypes of IgA present in humans. Serum contains more IgA1, whereas colostrum contains more IgA2. Generally, circulating IgA is present as a monomer in humans, although aggregates are present[538,539]. Isolated TMCs were used and the surface bound proteins removed using the pH3 buffer. Subsequently, the pH3 treated cells were then incubated with HA-IgA derived from either colostrum or serum prior to staining. A significant difference was seen in the ability of FcRL4 to bind the colostrum derived IgA compared to the serum IgA (Figure 29).

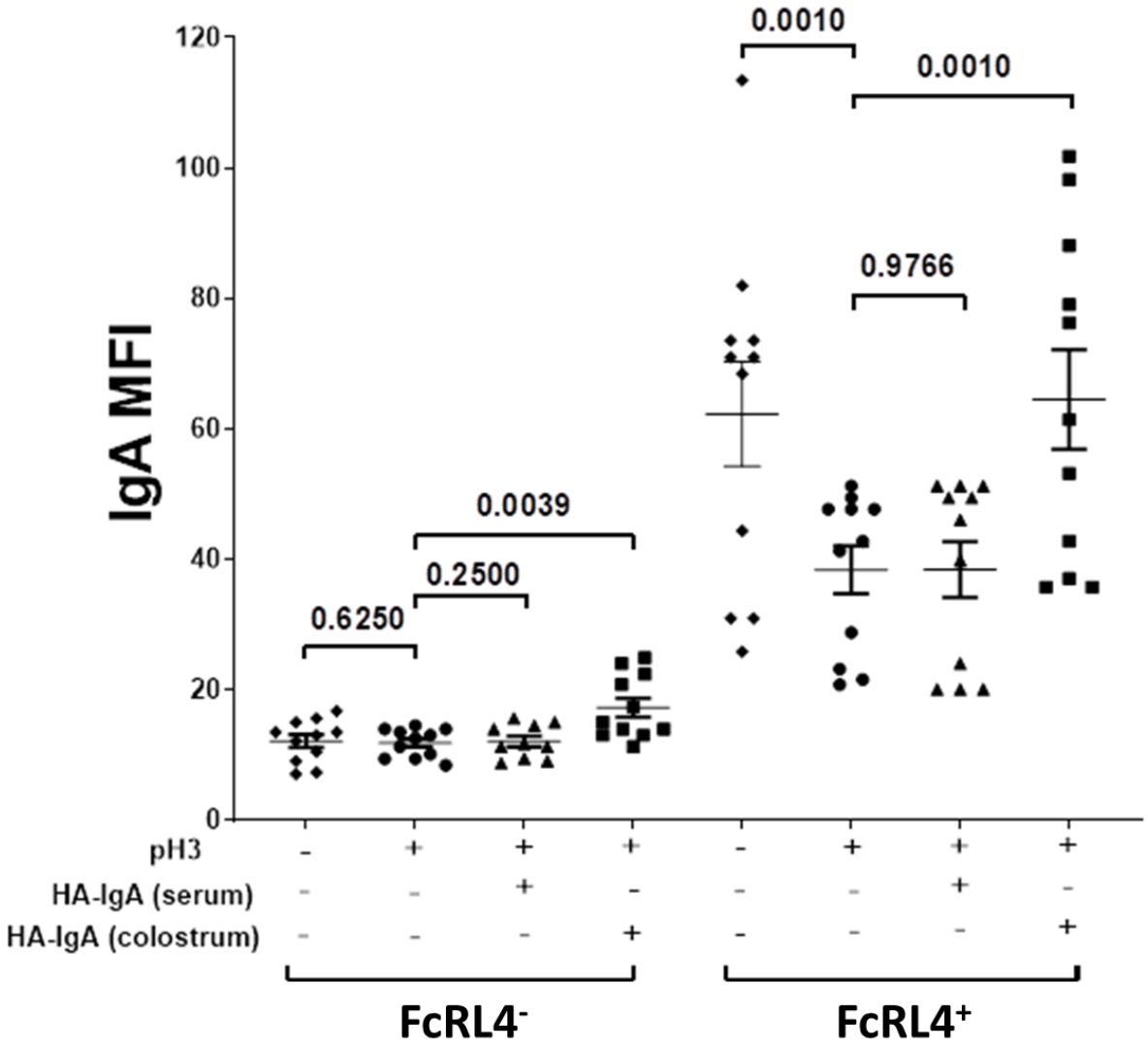

Figure 29 – Tonsil FcRL4⁺ B cells appear to display a preference in their binding of heat-aggregated IgA from colostrum. *Dot plots showing the IgA MFI for the Zombie⁻ CD19⁺ FcRL4⁻ or Zombie⁻ CD19⁺ FcRL4⁺ lymphocyte populations, following the addition of either colostrum or serum HA-IgA to the medium for 30 minutes. Mean and SEM are shown. Statistics were carried out using the paired Wilcoxon signed ranked test, (n=11 separate donors, 11 independent experiments).*

4.2.7 Serum and colostrum IgA differ in their propensity to aggregate

The primary reported difference found in the literature between serum and colostrum IgA is the ratio of the two isotypes present. Although the size of the molecules also differs with the predominantly IgA1 serum molecules existing as monomers and the predominantly IgA2 colostrum molecules existing as dimers and tetramers[281,533]. To investigate whether this resulted in any differences in their ability to aggregate, both untreated and heat-aggregated IgA samples from serum and colostrum were run through size exclusion columns. This was to provide information about the size of the aggregates formed upon heat treatment and any differences in the size of the untreated molecules. This experiment was outsourced to Peakprotein Ltd, Alderley Park.

It was possible to discern differences in their respective aggregation based on elution times though the column as larger molecules were retained longer (Figure 30, Table 5). Molecules of a defined size were run through the columns to provide clearer information on the respective sizes of the IgA samples (Figure 30, Table 5). It appeared as though a higher percentage of the IgA derived from colostrum was aggregated in comparison with the serum derived IgA (Figure 30, Table 4). However, the colostrum IgA also appeared to exist in a larger form than the expected 180kDa, the size of one dimer of IgA, when untreated (Figure 30, Table 5). These data suggested that this colostrum IgA existed as, at least, tetramers. It has been reported in the literature that IgA can exist as dimers, tetramers, or even octamers[533,539]. These differences in ability to aggregate may relate to the glycosylation patterns of the IgA derived from these different sources. These differences also provide a potential explanation for why FcRL4 appears to show a preference for colostrum IgA aggregates over serum IgA aggregates, simply that there is more aggregated IgA or larger IgA aggregates present in the colostrum samples.

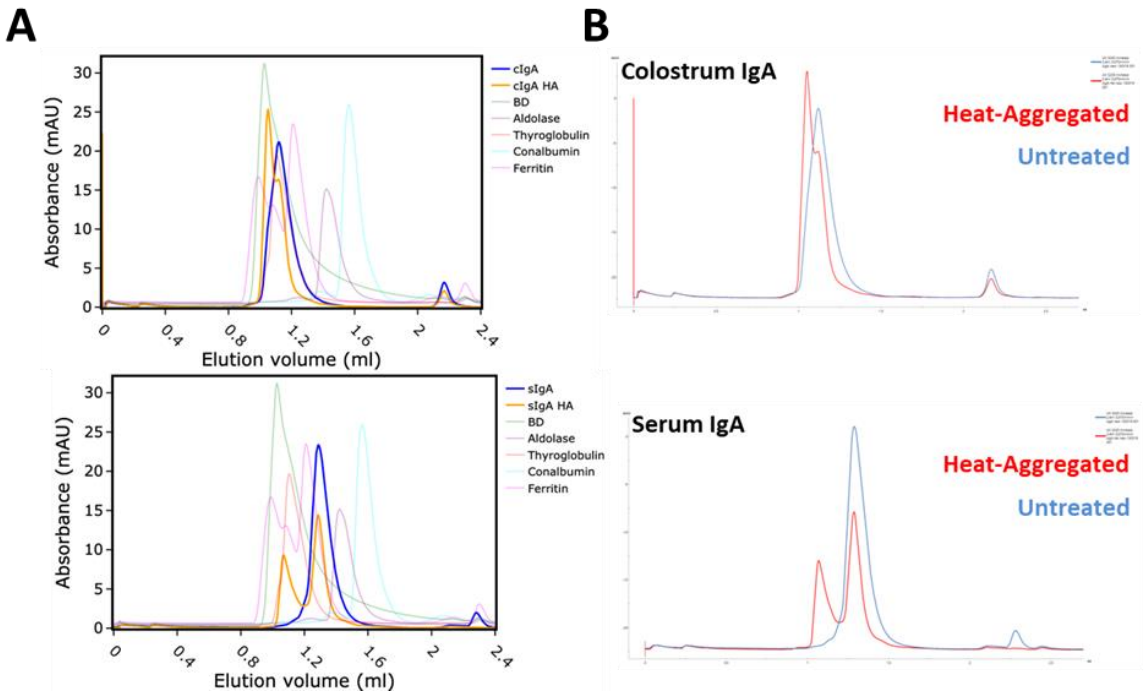

Figure 30 – Colostrum and serum derived IgA aggregate differently under heating. (A) *Histograms showing the Absorbance against Elution volume for untreated and heat-aggregated (63°C) colostrum and serum IgA, overlain with size markers (n=1, 1 experiment).* ***(B)*** *Histograms showing the difference between untreated and heat-aggregated human IgA derived from either colostrum or serum (n=1, 1 experiment).*

Table 5 – Colostrum and serum derived IgA aggregate differently under heating. *Estimated molecular weights calculated from a standard curve of the standards run through a size exclusion column.*

Standard	Molecular weight (Kd)	Retention time (mins)	Estimated Molecular Weight (Kd)
Blue dextran	>2M	1.03	
Thyroglobulin	660	1.1	
Ferritin	440	1.21	
Aldolase	158	1.42	
Conalbumin	76	1.56	
cIgA		1.12	636
cIgA HA		1.05	887
sIgA		1.29	283
sIgA HA		1.07	806

4.2.8 Heating time and temperature alters the level of binding detected for serum IgA

To address the concern raised by the previous experiment about any detected differences in IgA binding preference being solely due to the increased level of aggregation seen in the colostrum IgA sample, several different temperatures and heating times were tested for their ability to affect the binding (and thus aggregation) of serum IgA.

Experiments in which various times and temperatures were used in the IgA aggregation and their ability to affect their binding to transfected cells assessed found that the conditions used for the previous experiments, of 63^{o}C for 30 minutes followed by cooling at room temperature, were not significantly different from the apparent the optimum of 60^{o}C (Figure 31). Above 63^{o}C in temperature there was little significant change and binding appeared weakened above this temperature, likewise for below at 60^{o}C (Figure 31). The time spent at 63^{o}C also failed to improve the level of binding seen and in fact appeared to reduce the binding efficiency (Figure 31).

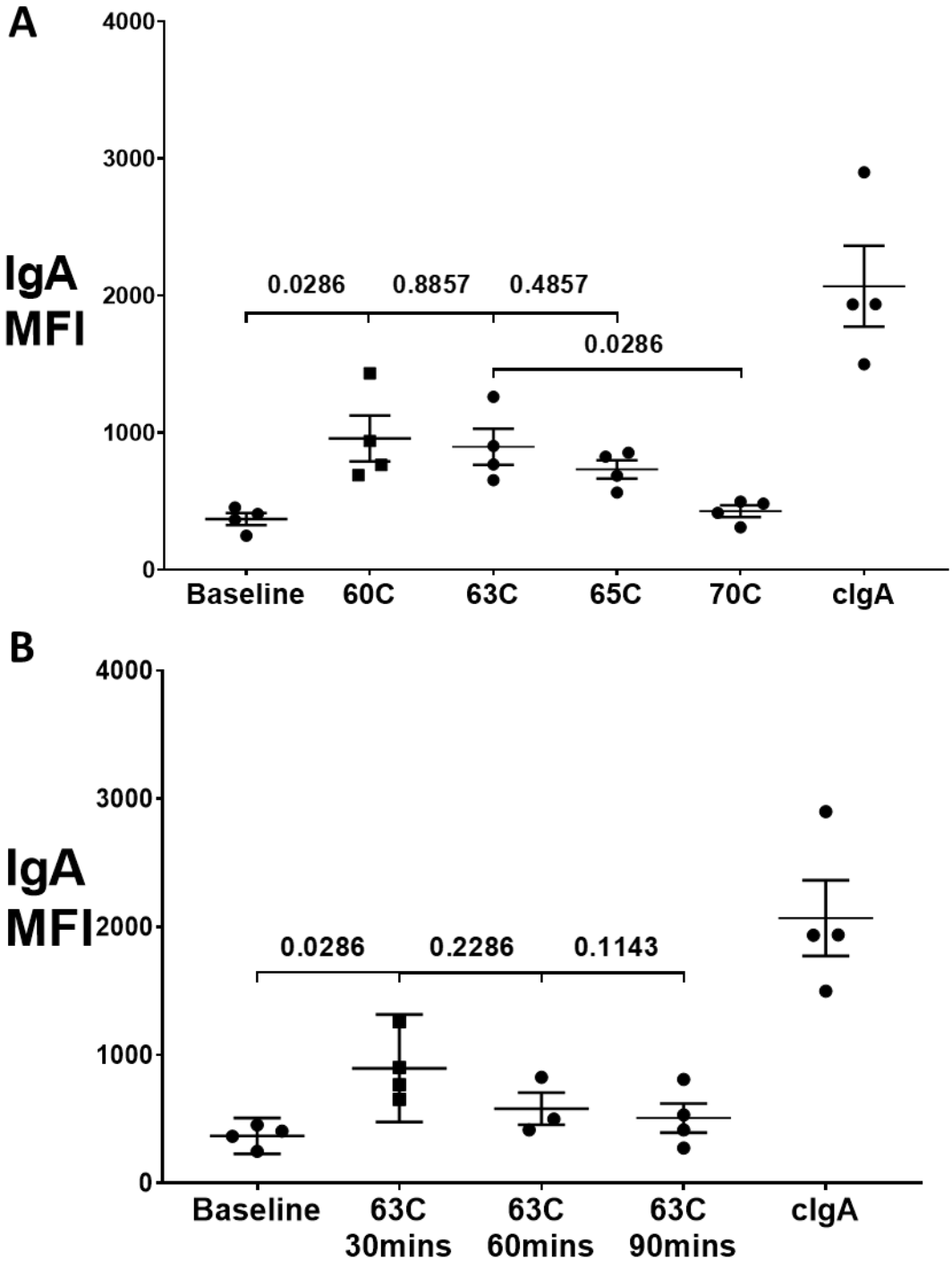

Figure 31 – Serum IgA aggregation is optimal at 60-63°C. (A) *Dot plot showing the effect of temperature on serum-derived IgA as determined by its' binding to transiently transfected Raji cells.* ***(B)*** *Dot plot showing the effect of time heated on serum-derived IgA as determined by its' binding to transiently transfected Raji cells. Raji cells were gated on FSC/SSC, Singlets, Zombie^{-}, and either FcRL4^{-}, or FcRL4^{+}. (n=4, 4 independent experiments). Mean and SEM are shown, statistics were carried out using the paired Wilcoxon signed ranked test.*

4.2.9 Transfected B cells expressing FcRL4 bind heat-aggregated IgA derived from colostrum and non-aggregated IgA1

Other than their apparent differential ability to aggregate, one of the primary differences between colostrum and serum derived IgA is the proportion of the two IgA isotypes present. A greater proportion of IgA1 is expected in the serum and a greater proportion of IgA2, in the mucosa, such as in colostrum. For this reason, the specificity of FcRL4 for IgA1 and IgA2 was tested, alongside comparisons with colostrum IgA and two separate sources of serum IgA.

A significant difference in the ability of FcRL4 to bind the colostrum derived IgA compared to either of the two sources of the serum IgA was seen in these experiments (Figure 32). There was a significant preference for the IgA1 isotype by FcRL4 compared to IgA2 however the IgA2 binding seen was significantly higher compared to one of the sources of serum derived IgA but not the other (Figure 32). There was very little IgA binding seen in the majority of the samples to which monomeric IgA had been added, which was to be expected given FcRL4's reported description as a low affinity IgA receptor, with the exception of IgA1, which showed significantly increased levels of binding as a monomer when compared to any of the other IgA monomers tested (Figure 32).

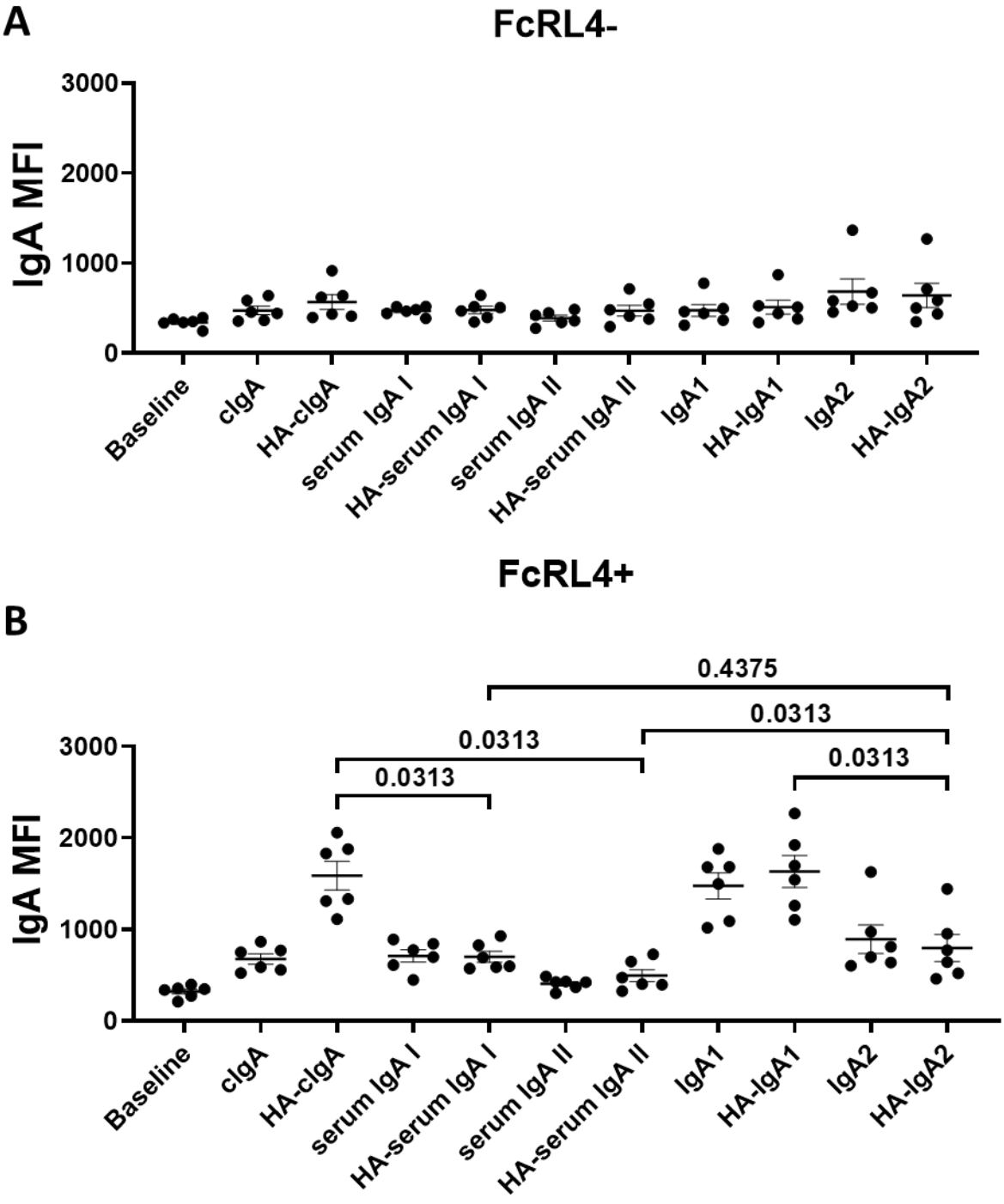

Figure 32 -IgA isotypes differ in the ability to bind to FcRL4. (A) *Scatter dot plots showing IgA MFI of FcRL4⁻ Raji cells following the addition of untreated or heat-aggregated IgA from various sources. Raji cells were gated on FSC/SSC, Singlets, Zombie⁻, and either FcRL4⁻, or FcRL4⁺. (n=4). Mean and SEM are shown.* ***(B)*** *Scatter dot plots showing IgA MFI of FcRL4⁺ Raji cells following the addition of untreated or heat-aggregated IgA from various sources. Raji cells were gated on FSC/SSC, Singlets, Zombie⁻, and either FcRL4⁻, or FcRL4⁺ (n=6, 6 independent experiments). Mean and SEM are shown, statistics were carried out using the paired Wilcoxon signed ranked test.*

4.3 FcRL4 in the Periphery

4.3.1 FcRL4$^+$ B cells are enriched in the ABC population in the periphery of healthy controls and RA patients

As described in the previous chapter, FcRL4$^+$ B cells could be detected in the periphery of healthy blood donors and were found to be enriched in the Age-associated B Cell (ABC) population. In these experiments, direct comparisons were made between healthy controls and RA patients.

Firstly, the percentages of the cells positive for ABC markers and FcRL4 agreed with the previously generated data in the earlier chapter which used healthy controls and a different staining panel (Figure 33). Given the small size of the population a separate panel was required to enable labelling of FcRL4 and IgA before and after the pH3 treatment.

In contrast to what the literature shows, these data suggest that the proportion of ABCs is found in the periphery of RA patients compared against healthy controls is not significantly different (Figure 33). The number of B cells present in the periphery of RA patients was also not significantly in comparison with the healthy controls (Figure 33). The FcRL4+ population in the ABC population was present but not significantly different between healthy controls and RA patients (Figure 33).

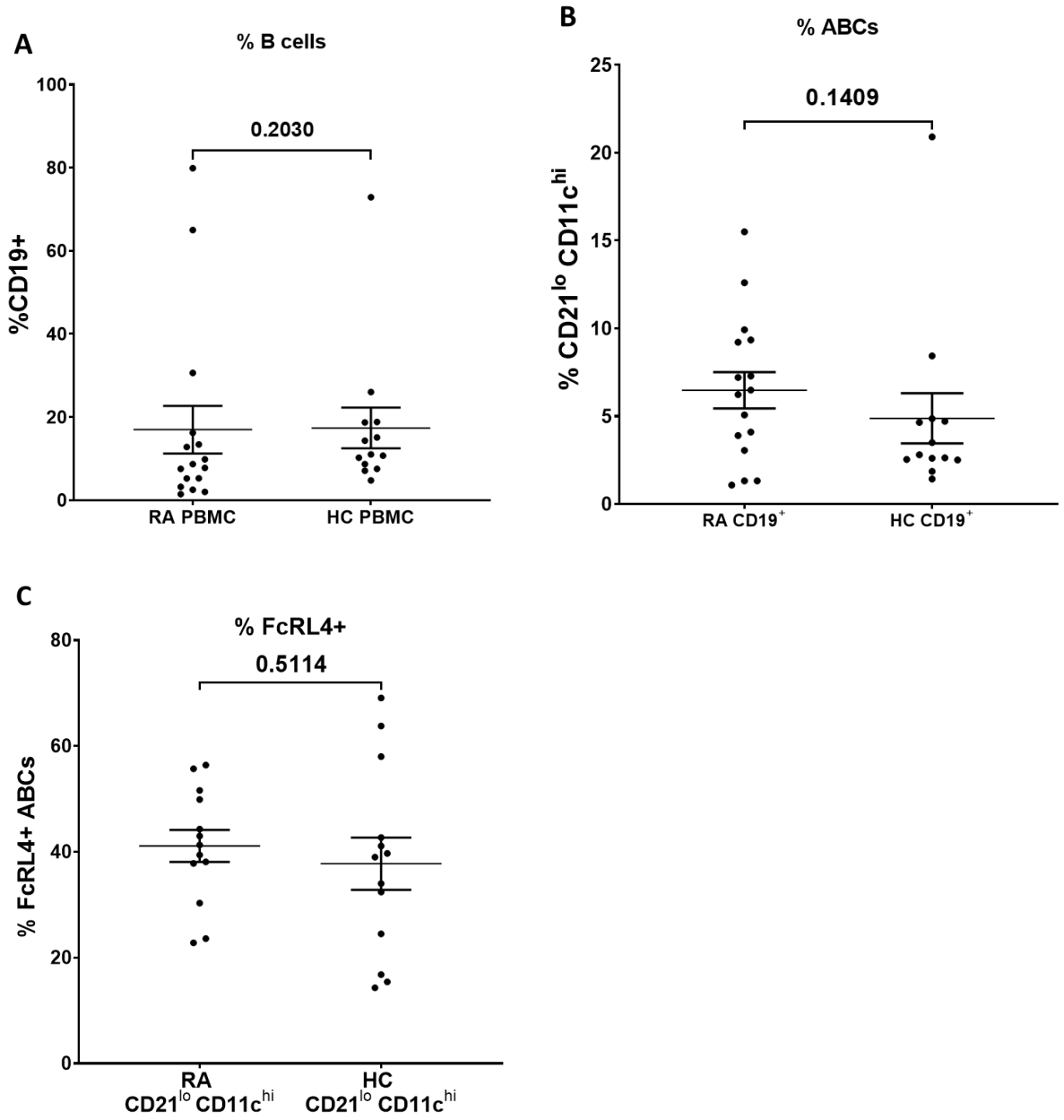

Figure 33 – ABCs are present at a similar percentage in the periphery of RA patients compared to Healthy Controls. (A) *The percentage of CD19⁺ events were quantified as a percentage of total lymphocytes as gated on FSC/SSC, Singlets, Zombie⁻ for RA PBMCs and HC PBMCs.* ***(B)*** *The percentage ABCs quantified were gated on FSC/SSC, Singlets, Zombie⁻, CD19⁺, and CD21^{lo}, CD11c^{hi} events* ***(C)*** *The percentage FcRL4⁺ ABCs quantified were gated on FSC/SSC, Singlets, Zombie⁻, CD19⁺, and CD21^{lo}, CD11c^{hi}, and FcRL4⁺ events. In all plots each dot corresponds to a patient (Healthy Controls, n=10; RA patients, n=10, 10 independent experiments). For statistical comparisons, the Mann-Whitney test was used. Mean and SEM are shown.*

4.3.2 FcRL4 is expressed at a higher level and percentage in the tonsil and SF ABC populations compared to the periphery

The differences between ABC populations between Healthy Control (HCs) and Rheumatoid Arthritis (RA) patients were minimal in terms of their %FcRL4$^+$ and the FcRL4 MFIs detected (Figure 33, 34). Although there appears to be a slightly greater proportion of FcRL4$^+$ ABCs present in the peripheral blood samples of the RA patients, this difference was not statistically significant in the set of patients analysed (Figure 34). In comparison to this there appears to be a significantly higher percentage of FcRL4$^+$ cells in the ABC population present in the synovial fluid of RA patients and in the tonsils, compared to the sites of joint and peripheral blood (Figure 34).

When analysing the differences between the levels of expression as opposed to the relative proportions of FcRL4$^+$ cell in the ABC population, further variation was discernible. There was a significantly greater level of FcRL4 detected on the surface of the synovial fluid derived ABCs and tonsil derived ABCs compared to the periphery (Figure 34). Furthermore, the difference between the synovial fluid and tonsil ABCs was also significantly different, with a higher level of FcRL4 detected on the surface of the tonsil derived ABCs than on those derived from the synovial fluid (Figure 34).

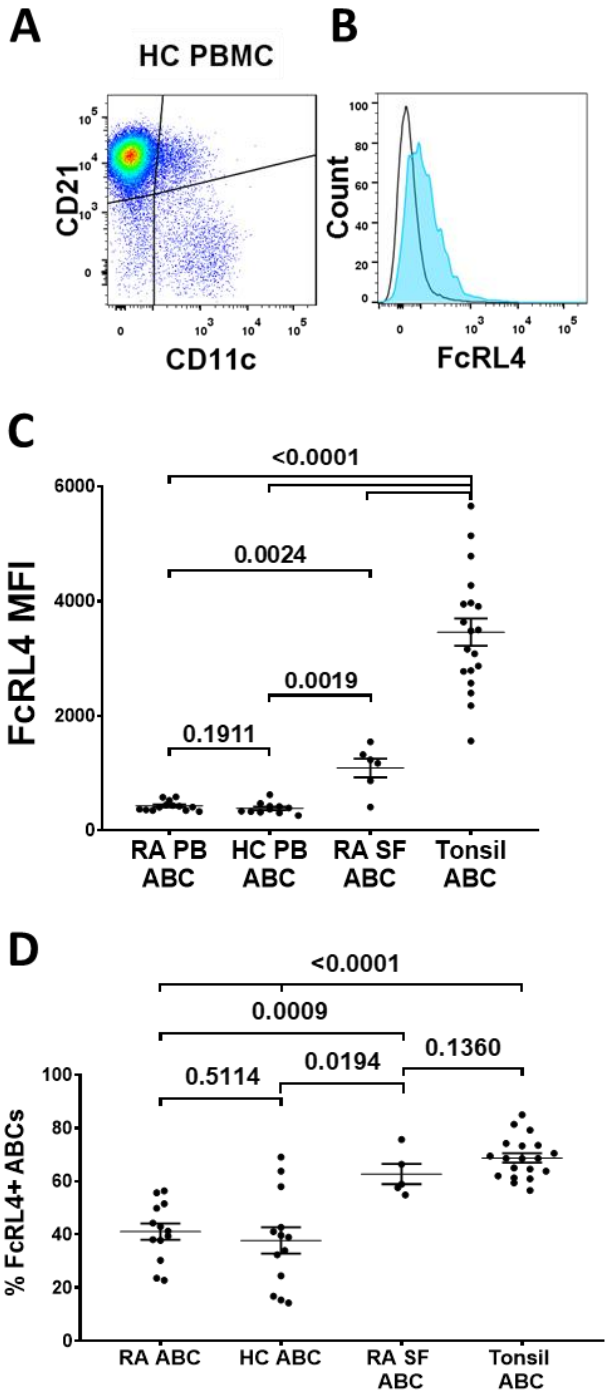

Figure 34 – FcRL4 is expressed on a higher percentage of cells and at a higher level in the Tonsil and RA SF ABC populations than in the RA, and HC peripheral blood ABC populations. (A) *Flow cytometry dot plot detailing the gating strategy in (B), (C), & (D), showing the CD21 vs. CD11c MFI, ABCs were gated on FSC/SSC, Singlets, Zombie^{-}, CD19^{+}, CD21^{lo}, CD11c^{hi}, and FcRL4^{+}.* ***(B)*** *Histogram showing the relative FcRL4 expression on non-ABCs (CD21^{hi}, CD11c^{lo} – black line) and ABCs (CD21^{lo}, CD11c^{hi} – blue filled line).* ***(C)*** *Dot plots showing the %FcRL4^{+} for the population gated upon in (A) for each of the samples.* ***(D)*** *Dot plots showing the FcRL4 MFI for the for the population gated upon in (A), for each of the samples. (RA ABC – Rheumatoid Arthritis Age-associated B Cells, n=10; HC ABC – Healthy Control Age-associated B Cells, n=10; RA SF ABC – Rheumatoid Arthritis Synovial Fluid Age-associated B Cells, n=6; Tonsil ABC – Tonsil Age-associated B Cells, n=15; 10 independent experiments). Statistics were carried out using the paired Wilcoxon signed ranked test, mean and SEM shown.*

4.3.3 ABCs from healthy controls and RA patient's blood don't have surface IgA bound to their surface in contrast to mucosa FcRL4+ B cells

Following from the description of their presence in the periphery of healthy controls it was tested whether these peripheral FcRL4^{+} B cells were carrying surface bound IgA as is the case in the mucosa (tonsil) and the joints (synovial fluid) and whether there were differences between RA patients and healthy controls in this respect. PBMCs from both healthy and RA patients were labelled for CD19, CD21, CD11c, FcRL4, and IgA. These samples were treated with the same pH3 acid buffer used previously to remove surface bound proteins including IgA and stained before and after this procedure. Tonsil mononuclear cells were included as a positive control for the removal of IgA from the surface of the cells, as this activity had been shown in these samples in previous experiments.

No significant changes in the levels of surface IgA on either FcRL4+ or FcRL4- cells in the periphery were detected following the acidic incubation (Figure 35). This suggested that IgA is not bound to the surface of these cells in the peripheral blood, or if it is, then this is a very rare event (Figure 35). Unfortunately, it was not possible to test whether these peripheral FcRL4^{+} B cells were capable of binding IgA as the cell numbers were too limiting in these experiments.

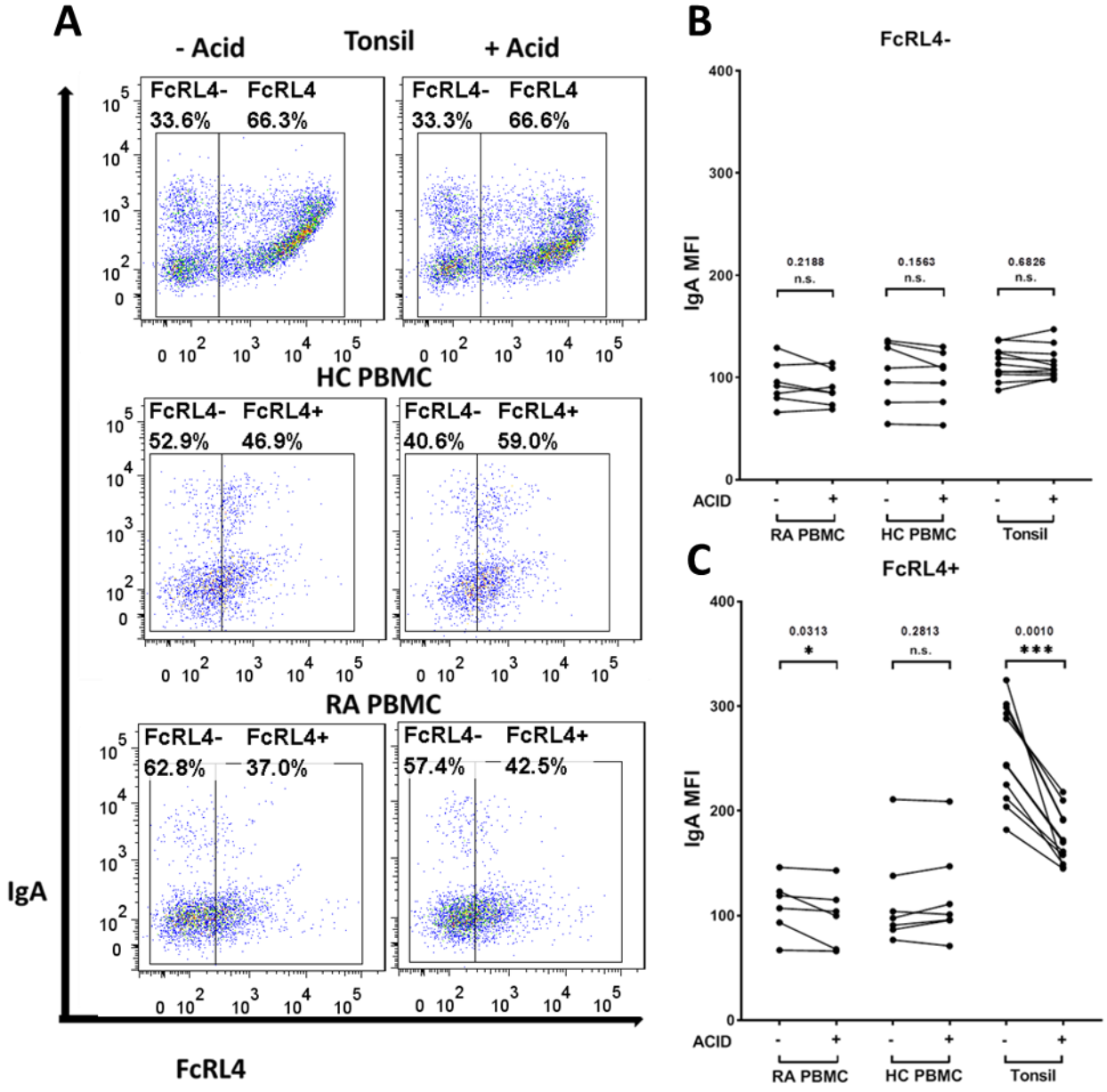

Figure 35 – Peripheral ABCs do not carry a significant level of surface bound IgA. (A) *Flow cytometry dot plot detailing the gating strategy in (B), (C), showing the FcRL4 vs. IgA MFI. PBMCs from RA patients and healthy controls, and Tonsil Mononuclear Cells were gated on FSC/SSC, Singlets, Zombie$^-$, and either FcRL4$^-$ IgA $^+$, or FcRL4$^+$ IgA$^+$.* ***(B)*** *Dot plots showing the IgA MFI for the FcRL4$^-$ populations gated upon in (A) following the addition pH3 buffer for 60 seconds. Statistics were carried out using the paired Wilcoxon signed ranked test, (n=10, 10 experiments).* ***(C)*** *Dot plots showing the IgA MFI for the FcRL4$^+$ populations gated upon in (A) following the addition pH3 buffer for 60 seconds. Statistics were carried out using the paired Wilcoxon signed ranked test, (n=10, 10 independent experiments).*

4.3.4 ABCs from healthy controls and RA patient's blood don't have a significant enrichment of IgA in their BCRs

Following from these experiments and earlier data which demonstrated an enrichment of IgA BCRs at both the gene and protein level, this analysis was repeated using the peripheral blood and tonsil samples from the above experiments. Expression of IgA as a BCR strongly suggests that the B cell has undergone its affinity maturation and somatic hypermutation in the mucosal areas[281,540]. Following the acid wash experiments which remove any surface bound IgA, leaving only the membrane IgA, it was possible to quantify the proportion of B cells expressing IgA BCRs.

Despite appearing as though there is a distinct trend towards $FcRL4^-$ B cells in the periphery of both RA patients and healthy controls having an enrichment for IgA BCRs, the opposite of what was previously seen in the joint, these observations were not statistically significant (Figure 36). However, the opposite is true in the tonsil, with agreement between these data and that reported for the joints (Figure 36).

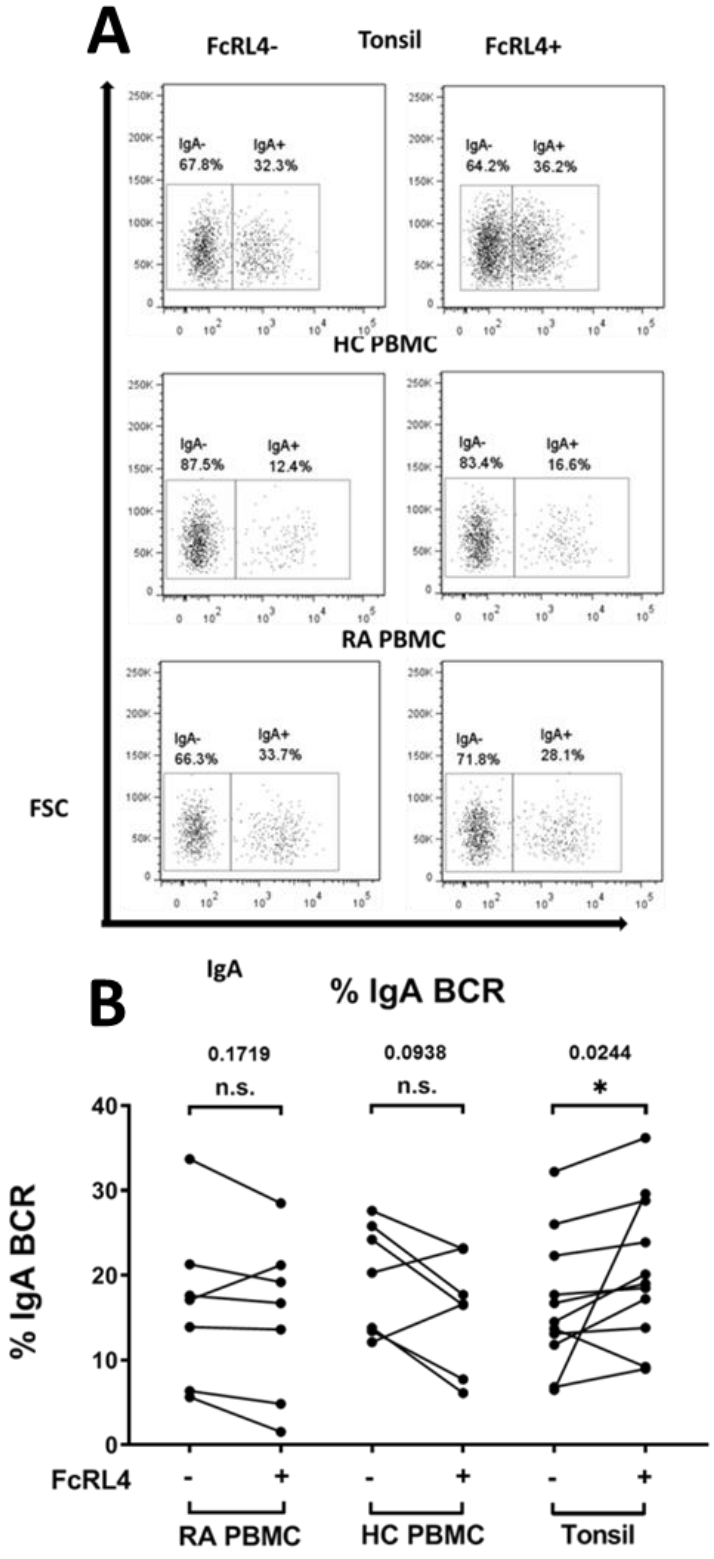

Figure 36 – FcRL4⁺ B cells are enriched for IgA⁺ BCRs in the tonsil but not the periphery. (A) *Flow cytometry dot plot detailing the gating strategy in (B), showing the %IgA⁺ lymphocytes in the FcRL4⁻ and FcRL4⁺. PBMCs from RA patients and healthy controls, and Tonsil Mononuclear Cells were gated on FSC/SSC, Singlets, Zombie⁻, and either FcRL4⁻ IgA ⁺, or FcRL4⁺ IgA⁺.* ***(B)*** *Dot plots showing the IgA MFI for the FcRL4⁻ populations gated upon in (A) following the addition pH3 buffer for 60 seconds. Statistics were carried out using the paired Wilcoxon signed ranked test, (n=10, 10 independent experiments).*

4.4 Summary – FcRL4$^+$ B cells and IgA

These experiments investigate the complex interaction of FcRL4+ B cells with the IgA response. Firstly, I investigated the levels of IgA present on synovial fluid and tonsil FcRL4$^+$ cells, and the ability of these populations to bind IgA. These demonstrated an increased level of IgA present on FcRL4$^+$ B cells and an ability of FcRL4$^+$ B cells to bind IgA, they also demonstrated an overrepresentation of the IgA BCR in the synovial fluid FcRL4$^+$ B cell population. Following this, the specificity of FcRL4 for IgA was investigated using a range of sources of IgA and clinical samples of RA synovial fluid, demonstrating FcRL4's specificity for aggregates of IgA. Further experiments suggested a preference for IgA1 and demonstrated differences in the ability of IgA from different sources to aggregate. Finally, the FcRL4$^+$ B cells in the periphery was further investigated and the presence of IgA on their surface probed. These experiments demonstrated a lack of surface IgA on peripheral FcRL4$^+$ B cells and suggested that the size of the FcRL4$^+$ ABC population and the levels of FcRL4 expression on the peripheral ABC population may explain why they lack surface bound IgA, in contrast to the synovial fluid and tonsil. They furthermore demonstrate a difference to the overrepresentation of the IgA BCR seen in the FcRL4$^+$ populations seen in the tonsil and synovial fluid, with no significant difference between FcRL4$^+$ and FcRL4$^-$ populations in the periphery in this regard.

4.5 Experimental Limitations and Discussion – FcRL4$^+$ B cells and IgA

From the initial experiment in this chapter it is possible to conclude that synovial fluid-derived FcRL4$^+$ B cells have a higher level of IgA present on their surface than their FcRL4$^-$ counterparts. However, in the flow cytometric analysis of these experiments in which the gating strategy is shown, it should be noted that the staining pattern looks similar to a common artefact seen in flow cytometry, where spectral overlap between fluorochromes is not sufficiently accounted for in the instrument compensation settings. However, these experiments were fully compensated using appropriate

controls including fluorescence-minus-ones, isotype controls, and single positive compensation controls. The fluorophores used for the relevant markers were also selected for their distance from each other on the emission spectrum, limiting the likelihood of the fluorescence emitted by these fluorophores leaking into the detection spectra of another, and reducing the possibility of a false positive. This staining pattern was also demonstrated with different combinations of staining panels.

The following experiments show that FcRL4 demarcates a population of B cells which initially carry a higher level of surface IgA than their $FcRL4^-$ counterparts and that following incubation in an acidic buffer at pH3, it is possible to remove these proteins, as indicated by a significant decrease in the IgA MFI detected. This suggests that this IgA is surface-bound protein and not expressed in the membrane of these B cells as a B cell receptor. The use of a pH3 buffer is a common method for the elution of surface bound proteins used for mass spectrometry. Following this, incubation with heat-aggregated IgA (HA-IgA) enabled the demonstration that a significantly higher level of IgA can be bound following incubation in pH3 buffer. Despite a statistically significant level of IgA binding not being limited to $FcRL4^+$ B cells, the increase seen in the $FcRL4^-$ population is proportionally smaller and latter experiments reinforce the specificity of FcRL4 for IgA. However, in this context, background binding is surprising, since prior to the incubation with HA-IgA, the cells are incubated in an Fc-blocking reagent which should minimise any non-specific binding of immunoglobulins to Fc receptors. It is possible that the incubation in the pH3 acid buffer leads to binding sites for IgA being revealed on the $FcRL4^-$ B cells which could explain the background binding seen. Alternatively, it could be that the charge of the cell surface is altered following the addition of H^+ ions to the cells which could then lead to electrostatic attraction between the IgA aggregates and the $FcRL4^-$ cells. A mechanism along these lines was demonstrated in experiments testing the effects of incubating transiently transfected Raji cells with pH3 buffer prior to IgA binding which found an increase in background signal following pH3 treatment. A less likely but potential mechanism for the presence of

IgA on these cells could simply be a lack of washing of the IgA aggregates away from the cells in the subsequent steps, however there are a number of wash steps prior to the staining and fixing.

In those experiments investigating the interaction of tonsil-derived FcRL4$^+$ B cells with IgA, the same pattern of FcRL4 appearing on a population of cells with higher initial levels of surface IgA present is seen. So is the significant decrease following incubation in the pH3 buffer and subsequent increase in IgA MFI signal upon incubation with HA-IgA. Although there appears to be a difference in the initial levels of IgA present on the FcRL4$^+$ cells in the tonsils than those in the synovial fluid, which might be attributable to the higher concentrations of IgA expected in a mucosal location as opposed to the joints and the latter demonstrated higher level of FcRL4, these TMCs experiments were separate to the SFMC experiments, and though the same antibody clones and fluorophores were used, different voltages were applied during the data acquisition, complicating direct comparisons. Thus, these data are only suitable for intra-experimental comparisons and the demonstration of IgA binding to FcRL4$^+$ cells occurring at a site distal to the joint. This implies that it is not a factor of the cell's location but that FcRL4 does appear to demarcate a population of B cells able to bind IgA aggregates.

In both of these SFMC and TMC IgA binding experiments, a high level of variation in the levels of IgA binding detected between samples is seen in the IgA MFI both initially and following the pH3 treatment and subsequent re-addition of HA-IgA. This could potentially be due to variation in the levels of IgA present in the synovial fluid patient samples, but unfortunately this could not be assessed as this is not routinely collected information. Little correlation was seen between the levels of FcRL4 present on these cells and the level of IgA, despite varying levels of FcRL4 found on these cells, this implies that there might be a maximum load these cells can carry. However, this is in contrast to the latter experiments showing *ex vivo* data from the periphery in which a lower level of FcRL4 expression does correlate with lower levels of surface-bound IgA and may explain their lack of

binding. This could be further investigated by using cell lines expressing different levels of FcRL4, or by titrating IgA on a cell line with a defined and stable expression level of FcRL4.

The class-switching to IgA as a BCR receptor has been demonstrated to take place via both T cell dependent and T cell independent routes, generating high- and low- affinity IgA antibodies respectively. T-dependent switching requires signaling via CD40 expressed on B cells and the presence of TGFβ1[534]. T independent IgA switching requires innate signals such as TLR agonism, and signals including B-cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL)[539,541]. IgA class-switching suggests that the immune reaction which generated these B cells took place in the Peyer's Patches (PP) or Isolated Lymphoid Follicles (ILF) of the mucosa associated lymphoid tissue (MALT)[542]. These data demonstrating an over-representation of IgA BCRs in the $FcRL4^+$ population both at the protein and gene level strongly suggest that a higher percentage of $FcRL4^+$ B cells undergo maturation in the MALT than $FcRL4^-$ B cells. However, these data also show that the majority of $FcRL4^+$ B cells are still of the IgG isotype, suggesting that a proportion of these cells were generated in the secondary lymphoid organs and that they are not limited to generation in the mucosa. The significant difference between $FcRL4^+$ and $FcRL4^-$ B cells in the levels of IgM present, showing an almost complete lack of the marker on $FcRL4^+$ cells also appears to indicate that FcRL4 is predominantly found on memory B cells, a conclusion which is also supported by data in the previous chapter.

In those experiments in which transiently transfected cells were used to ascertain the specificity of FcRL4 for IgA, some variation in the level of IgA aggregate bound was seen across experiments despite the number of cells and concentration of IgA being controlled for, which could relate to the variation in the transfection efficiency. Reasons for this variation in transfection efficiency include the cells' passage number as latter passages appeared to transfect less efficiently (personal observation).

There also might be an issue in these experiments might be dead cells providing background signal, although these cells should have been gated out during analysis based on their positivity for the dead cell dye Zombie Aqua. However, the level of dead cells was also assessed across the SFMC, TMC, and Raji experiments prior to, and following incubation in the pH3 buffer and no significant difference in the level of Zombie^{+} lymphocytes seen, and in all samples the percentage of live lymphocytes remained above 95%.

As described in the previous experiment in which heat-aggregated, purified IgA was used, there was some variation in the level of IgA bound. This was seen across experiments despite the number of cells and concentration of IgA being controlled for. In the following experiments using RA synovial fluid this will have been affected by both variation in the transfection efficiency which can vary according to the cells' passage number, and the concentration of IgA ICs in the patients' SF. However, the level of variation seen was comparable to those earlier experiments, which in the case of there being an added variable is unexpected. This either suggests that the transfection efficiency was not the primary cause of the variation, and that merely the presence of enough FcRL4 on the surface sufficed, or that the concentration of IgA ICs present in the SF did not vary significantly between patients. It is also possible that these cells could have internalised the bound IgA over the course of the experiment at differential rates, but this is an unlikely source of variation given that these experiments were carried out on ice until the point of fixation, so any cellular activity, including internalisation will have been minimised. Another deficiency of these experiments is a lack of a positive control for IgG. And although early literature on the Raji cell line does suggest that they were able to pull down IgG from serum this has not been replicated here in experiments using RA synovial fluid[543,544].

The ability of the stably transduced, FcRL4 expressing cell line to pulldown IgA ICs from the synovial fluid of patients with different arthritides is interesting in light of the respective pathologies of these

diseases involving the adaptive immune system and thus (auto-) antibodies to a greater or lesser extent. What is surprising is the lack of significant difference between OA, an inflammatory disease, and RA, a systemic autoimmune disease. These data might indicate that the synovial fluid compartment is capable of sequestering circulating antibodies and immune complexes, irrespective of autoimmune or inflammatory background. It would be useful to obtain synovial fluid from healthy controls to rule this hypothesis out. This again might be related to the number of cells or insufficiently titrated SF, although the IgA IC concentration of these SFs was not measured and so this remains conjecture until they are measured and compared across diseases. However, the intraexperimental variation, i.e. the differences in levels of IgA detected within diseases, suggests that this volume of SF is capable of providing enough of a variation in IgA concentration as to be detected.These experiments rely upon a stably transfected cell line with a less variable level of expression of FcRL4 in contrast to those earlier utilising a transiently transfected cell line (data not shown). Also, of note is the reduction in cells undergoing apoptosis seen in these experiments, this was also seen in the transient transfections and may have been a potential source of error between experiments. A final consideration is unavoidable, that this IgA binding occurs ex vivo, which is the closest that the biological reality of what is occurring in the joints of RA patients and tonsil can be approached experimentally in an ethical fashion. From these experiments it is possible to conclude that FcRL4 is an IgA receptor, capable of pulling IgA ICs down from synovial fluid of various diseases.

Given the demonstrated activity of FcRL4 to specially bind IgA ICs and the link between IgA ACPA and poor disease diagnosis there is potential for the use of FcRL4 as a diagnostic. However, this is perhaps limited by the lack of differences assessed between diseases and suggests that immune complexes (ICs) or aggregates are present in the synovial fluid of all arthritis patients. It is not possible to say that SF ICs are a universal feature of synovial fluid or arthritic diseases, it may be a marker of inflammation, but this data wasn't available for the patients tested. It would of value to

test the SF of healthy patients to determine whether the presence of ICs in the SF is a common feature of SF or if it might indicate pathology. Following up these patients to determine how their disease progresses would also be of use in testing this. Despite this it may still be possible to utilise FcRL4 in a diagnostic capacity. The reactivity of the IgA clones isolated from the synovial fluid is of great interest to researchers, as many hypothesise that the initial breach of immune tolerance which leads to systemic autoimmunity, occurs in the mucosa. The ability to specifically isolate IgA clones from the synovial fluid of RA patients and test their reactivity against mucosal antigens would be useful in testing this hypothesis.

In determining whether or not FcRL4 may display a preference for IgA derived from different sources it was possible to discern a high degree of variation in the level of IgA bound is seen in the tonsil samples. The variation in levels of FcRL4 expression achieved with transient transfection did not directly correlate with the extent of IgA binding, suggesting that at least a portion of the binding seen in these experiments may be background binding enabled by the pH3 acid treatment. This is further reinforced by the experiment comparing the acid treated and subsequently rested cells, although there was a paucity of repeats. It is tempting to conclude from these data that FcRL4 expresses a preference for mucosa-derived IgA. However, there are a number of further points of biological variation that might explain the observations, the IgA from the different sources could itself be different, either in its' glycosylation pattern or its' ability to aggregate. It could also vary in its' composition of the ratio of IgA1 to IgA2 present, which could indicate a preference between IgA isotypes.

The aggregation time did not appear to significantly affect serum IgA and the temperature used (63^{o}C) was not significantly different from the apparent optimum of 60^{o}C. It remains to be seen whether shorter periods of time are capable of improving the binding level detected. An important caveat in these experiments is that they used an increase in IgA MFIas a read-out. This does reveal

functional differences in the aggregates ability to bind but it does not inform as to whether the aggregation has been changed by the different temperatures or time heated. This would require the experiment in 4.2.7 to be repeated, however the time and expense was deemed unreasonable. Thus, these data could still be concealing a preference for IgA isotypes or glycosylation patterns for FcRL4.

The data displaying a preference for the presumably monomeric, myeloma-derived IgA1 over all other IgAs tested are potentially at odds with the primary location of FcRL4 cells described thus far as being in the mucosa as IgA2 is the predominant isotype in this location. The presence of FcRL4 on ABCs or "atypical" B cells described in the earlier chapter would also suggest that if this receptor has a bias for IgA, and is capable of binding IgA1 monomers, that this would be detectable in the periphery. In light of the data demonstrating a lack of IgA present on those $FcRL4^+$ B cells in the periphery, this data is surprising. However, the demonstrated differences in the levels of FcRL4 expression on those cells in the periphery might provide an explanation for why a lack of IgA is detected on their surface despite the increased prevalence of IgA1 in the periphery as the peripheral $FcRL4^+$ cells express significantly lower levels of FcRL4 on their surface. There is also a significantly smaller percentage of $FcRL4^+$ B cells in the periphery. It could also be as a result of the lower level of aggregation detected in the non-heat-treated serum IgA compared to the non-heated treated colostrum IgA tested in the size exclusion experiments. Testing of the IgA1 and IgA2 samples used in the experiments with size exclusion chromatography would clarify this. It would also be possible to isolate *ex vivo* $FcRL4^+$ B cells via FACS and elute any bound IgA, mass spectrometry would then be used to clarify whether IgA1 or IgA2 is the preferred isotype *in vivo*.

A more sensitive staining protocol and gating upon ABCs allowed the for the presence of $FcRL4^+$ B cells in the periphery of both healthy controls and RA patients to be shown. This is in contrast with work previously reported by our group, although an $FcRL4^+$ population has been described in the

periphery of a number of chronic inflammatory conditions[458,462,545]. However, these data demonstrating a comparatively low level of FcRL4$^+$ B cells in the periphery compared to the tonsil and joint could indicate that the signals required for the generation of FcRL4$^+$ B cells are present at higher concentrations in these locations, this is discussed briefly in the previous chapter. Or this could suggest that these represent sites where FcRL4$^+$ B cell migrate to and are then subsequently retained. However, according to transcriptomic data in the literature, little upregulation of typical gut mucosal homing markers such as MAdCAM-1 and $\alpha_4\beta_7$, or lung mucosa markers such as CD62L, CCR7, CCR10, and $\alpha_4\beta_2$ are seen on the FcRL4$^+$ population described in the joint or those in the tonsil[465]. With regard to the joint, it has been shown in the literature that FcRL4$^+$ cells derived from the joints have upregulated CCR1 and CCR5 which are involved in the homing of lymphocytes to points of inflammation, however it is not known if these cells upregulated these receptors in response or prior to entering the inflammatory milieu of the joint[499]. To determine whether or not this is a candidate mechanism, direct comparisons of these homing receptors between these peripheral and tissue-based FcRL4$^+$ B cells is required. Another potential mechanism behind the upregulation of FcRL4 is as a response to inflammation, as both the tonsils from tonsillectomies and the SF from patients with active RA are from inflamed sites and would have contained upregulated levels of inflammatory cytokines compared to the circulation of healthy controls and RA patients. However, results from the previous chapter have demonstrated that few inflammatory cytokines were individually capable of driving FcRL4 expression and this required the presence of both TGFβ and TLR9 agonist.

Reasons for the lack of surface-bound IgA seen on FcRL4$^+$ cells in the periphery could include the comparatively low concentrations of FcRL4$^+$ B cells present in the circulation, it could simply be an uncommon occurrence for these to come into contact. It could also be explained by the significantly lower levels of FcRL4 expressed by these cells in the periphery, where IgA is not found bound to their surface, compared to the tonsil and in the RA joint, where IgA is found bound to their surface

correlating with a higher level of FcRL4 expressed. This hypothesis is more convincing in light of the data suggesting that FcRL4 is capable of binding monomeric IgA1 which is present in the circulation.

Unfortunately, it was not possible to test whether these peripheral FcRL4^{+} B cells were capable of binding IgA as the cell numbers were too limiting. It would be possible to indirectly test this hypothesis that FcRL4 cannot bind IgA at the lower surface concentrations seen in the periphery using either a titration of plasmid in transfected cells or by selecting cells of varying levels of expression from the stably expressing cell line and culturing a titration of surface expression. It would also be possible to obtain much larger volumes of blood from patients and healthy controls in order to test this hypothesis.

Despite these limitations we can conclude that FcRL4^{+} B cells are present in the periphery of both healthy controls and RA patients and that they are over-represented in the ABC or “atypical” population. It can also be concluded that a higher percentage of these ABCs are found in the periphery of RA patients compared to healthy controls and that FcRL4 expression does not vary between health and disease. Furthermore, in the periphery, FcRL4^{+} B cells are most likely not carrying IgA ICs on their surface, which is potentially a function of their low levels of surface expression. However, the possibility that these cells have internalised IgA ICs in the joint or mucosa and migrated into the periphery or vice versa, cannot be ruled out.

The peripheral FcRL4^{+} B cells also differ from their mucosal or synovial fluid counterparts in the percentage of the population expressing an IgA BCR. The reasons for these site-specific inversions are not immediately clear, as being present in the mucosa was no guarantee of IgA expression since both FcRL4^{+} and FcRL4^{-} populations would be equally exposed to that environment, minus any microenvironmental niches which the FcRL4^{+} B cells might inhabit. The literature has reported that

they are preferentially found in the sub-epithelial dome regions and directly next to the epithelium, regions associated with class switching to IgA[514,542]. It is possible that there are differences in the homing capacity of the FcRL4$^+$ B cells or that this marker labels only antigen experienced cells and thus the likelihood that they will express IgA over IgM is higher, leading to a detectable difference. This could be investigated simply through the inclusion of antibodies against IgM, IgD, and IgG in the staining panel, however the previously mentioned issues of limited cell numbers still apply and thus these would require a great deal more patient material. An interesting observation from these data is that, unrelated to FcRL4 expression, a comparable percentage of IgA$^+$ B cells were found in the periphery and in the mucosa. This is a surprising result given the expectation that a greater proportion of tonsil B cells will be IgA$^+$, it could suggest that a large number of the cells present in the tonsil are newly migrated and requires further investigation. To assess whether these populations are linked, for example by migration, the clonality of these from the same patient would have to be assessed.

4.6 Conclusion – FcRL4$^+$ B cells and IgA

The above data illustrate a number of links between FcRL4 and IgA and a complex involvement of these cell in the MALT, illustrated below (Figure 37). For one, in the tonsil and in the synovial fluid of RA patients, FcRL4 distinguishes a population of B cells with an enrichment for IgA BCRs, suggesting that these cells have some level of mucosal experience. The opposite appears to be true in the periphery.

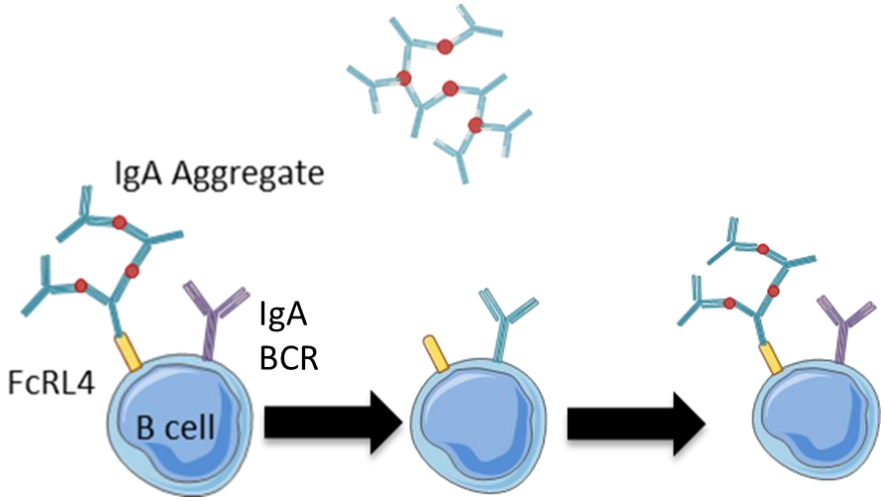

Figure 37 – Schematic of the interaction between FcRL4⁺ B cells and IgA aggregates/complexes. *The schematic details the effect of incubating tonsil or synovial fluid derived FcRL4⁺ B cells in pH3 buffer leading to the removal of surface bound IgA complexes, allowing for the quantification of %IgA BCR, and the subsequent binding of IgA upon addition of HA-IgA to the incubation medium.*

Furthermore, FcRL4 was confirmed to act as a specific IgA receptor. FcRL4's binding of IgA is relatively low affinity, requiring aggregates of IgA, and that this binding is occurs in the joints of RA patients and the tonsils of tonsillectomy patients, as demonstrated both by the initial level of surface IgA enrichment and by their subsequent ability to bind IgA following removal of surface proteins. The level of aggregation or propensity to aggregate for IgA from different locations, the serum or colostrum, appears to be different. And assuming that the purified myeloma-derived IgA1 and IgA2 are equals in their level of aggregation, these data suggest that FcRL4, displays a preference for IgA1.

5 Exploration of reactivity of recombinant antibodies derived from FcRL4+ B cells with the gut microbiome

5.1 Development of sorting protocols for antibody labelled bacteria.

There is increasing evidence for a link between the microbiota and the development of autoimmune diseases, particularly focussing on dysbiosis. This can be as a result of an increased prevalence of inflammatory bacteria in the composition of the commensal microbiota, or as a result of disruption to the mucosa itself leading to infiltration of bacteria into previously untouched spaces such as the lamina propria. One of the primary hypotheses in placing the microbiome at the pathogenesis of autoimmunity suggests that an immune reaction against bacteria leads to immune cells with new specificities, or neo-epitopes, which subsequently cross-react with self-antigens leading to a break in immune tolerance and autoimmunity. As shown in previous chapters, there is an overrepresentation of IgA BCRs in the FcRL4+ population found in the RA SF, which suggests a mucosal origin, potentially implicating these cells in linking joint and mucosal immunity. The literature has also reported an enrichment of commensal-reactive clones within the tonsil $FcRL4^{+}$ population when compared to the $FcRL4^{-}$ population, which merits further investigation utilising joint-derived clones[546].

We have previously reported the reactivity of recombinant monoclonal antibodies generated from RA SF IgG^+ $FcRL4^+$ B cells towards citrullinated antigens common to RA, such as α-enolase, and citrullinated-fibrinogen[521]. However, for the majority of this set of antibodies, no antigen specificity had been determined. A separate selection of monoclonal antibodies were subsequently generated from single sorted RA SF IgA^+ $FcRL4^+$ and IgA^+ $FcRL4^-$ B cells.

In this chapter the possibility of testing the reactivity of monoclonal antibodies (mAbs) towards the microbiota of healthy and diseased donors was assessed. This work is part of a collaborative project within the EU IMI RTCURE with contributions from Birmingham, the Karolinska Institutet in Stockholm, and the German Rheumatology Research Institute in Berlin. I carried out the sorts of IgA+ single B cells. Cloning and molecular characterisation of the variable region sequences was carried out in Vivianne Malmstrom's team at the Karolinska Institute. I travelled to Berlin twice, firstly to establish and secondly to carry out the labelling protocol and the sorting of bacterial from stool samples in the lab of Hyun-Dong Chang. The 16S sequencing was carried out by the team in Berlin.

The mAbs were derived from the cloned variable region sequences of BCRs of single $FcRL4^+$ and $FcRL4^-$ B cells sorted from the joints of patients with active disease. Fluorescent-activated cell sorting (FACS) was utilised to sort bacteria from stool samples which had been stained with directly labelled recombinant mAbs. The V3 and V4 hypervariable regions of these bacteria's 16S rRNA were then amplified using universal primers, further amplified using index primers, and sequenced using the Illumina MiSeq platform, enabling the composition of the labelled bacteria to be assessed.

5.1.1 Initial method development using previously cloned recombinant antibodies

To enable these mAbs to be used in fluorescence activated cell sorting (FACS) they were directly labelled with fluorophores. In these initial experiments the mAbs derived from single IgG^+ $FcRL4^+$ and IgG^+ $FcRL4^-$ cells were directly labelled with APC using the Lightning Link kit. To test them for successful labelling they were incubated with beads which capture antibodies by binding to the Fc portion of human IgG and the APC signal on these beads was determined by flow cytometry. Only 3 of the 8 IgG antibody clones from this previously generated batch appeared to be successfully labelled with APC, as seen by the lack of APC positive peaks (Figure 38).

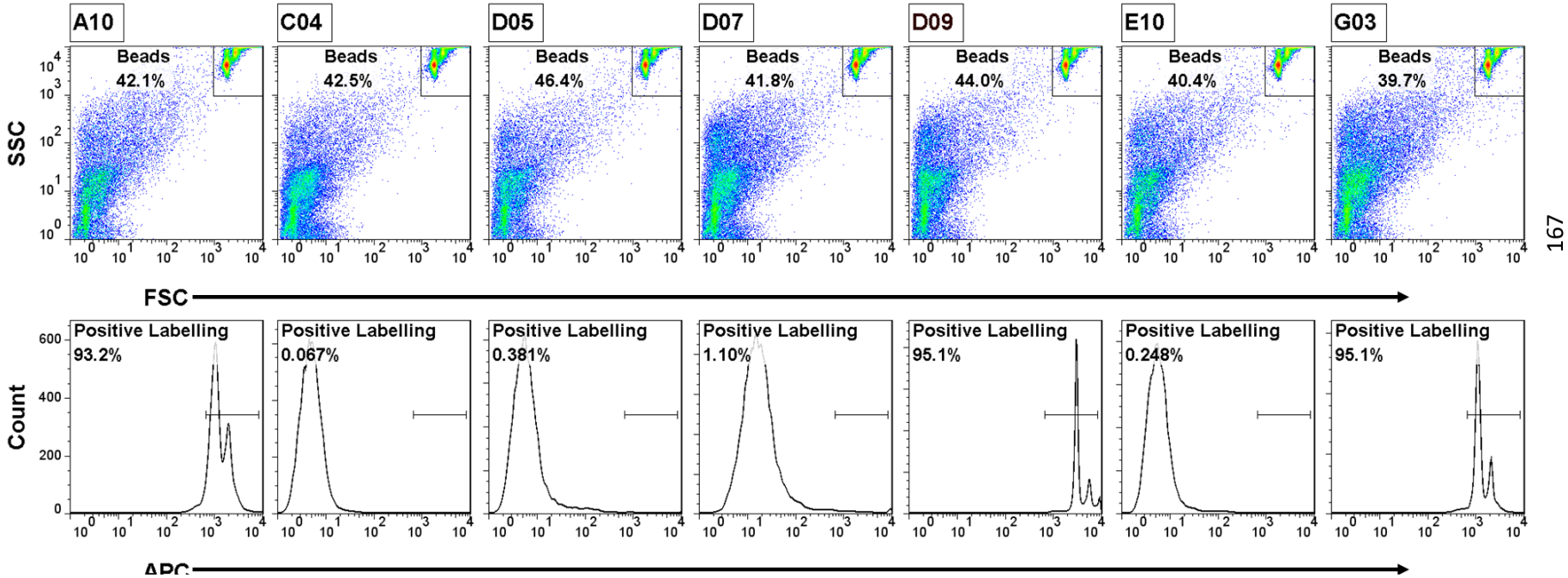

Figure 38 – RA SF joint derived monoclonal antibodies were labelled with APC. *Flow cytometry dot plots showing the gating strategy used in the corresponding histograms beneath each plot. Dot plots are showing size (FSC) against granularity (SSC) and gate used for the labelled bead populations. Histograms show the APC MFI for the mAb labelled beads and gates were established using FMOs.*

Following their labelling, these joint derived mAbs were then tested for their reactivity against bacteria derived from a stool sample from a healthy donor. To provide a guide for the gating on live bacteria, which contain greater levels of DNA than the dead and dying bacteria, this sample was also labelled with SYBR Green, a DNA-intercalating dye. A single positive for SYBR Green was also included as a control for autofluorescence and to enable unbiased gating on live bacteria. Alongside this control, the sample was stained with an anti-IgA antibody in order to provide a positive control. This population of IgA^{hi} bacteria in human stool samples has been reported in the literature as representing a more pathogenic population than the IgA^{lo} in colitis[547].

There was a significant level of background signal seen in the APC channel of the sample labelled only with DNA dye SYBR Green (Figure 39). However, the strong IgA positive signal in the anti-IgA labelled sample demonstrated the possibility of labelling bacteria present in the sample (Figure 39). None of the four mAb clones tested, provided a level of signal comparable with the anti-IgA labelling, making it difficult to discern any specific labelling of bacteria (Figure 39). However, there were minor differences in the percentages of positively labelled bacteria between the clones tested, suggesting that there was a low level of specific binding (Figure 39).

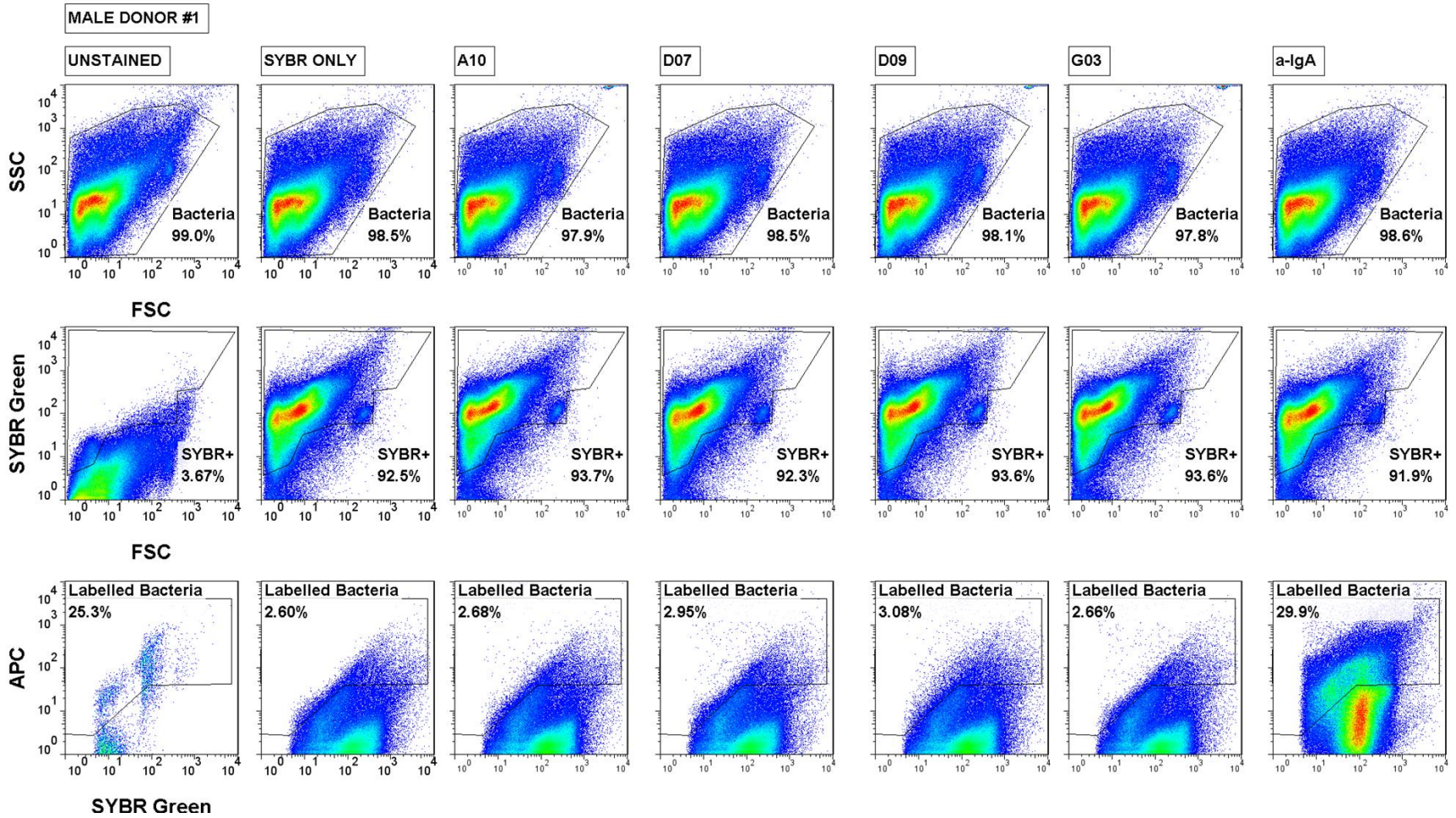

Figure 39 – RA SF joint derived monoclonal antibodies labelled a smaller population of bacteria than the anti-IgA mAbs (A) *Flow cytometry dot plots showing the gating strategy to determine the binding of the mAbs tested based on the FMOs shown, events were gated on FSC/SSC, SYBR⁺, APC⁺ (Clone, IgA).*

Given the small differences seen between the clones the subsequent experiment investigated the possibility of using incubation with an unlabelled mAb of the same clone prior to the A10 and G03 APC labelled clones to demonstrate the specificity of binding. This blocking of potential specific binding would allow for differences to be detected in the percentages of positively labelled bacteria.

Again, the very high background autofluorescence signal from the SYBR Green only sample prevented any significant differences being detected between the specific clones and the blocked samples, although clone D07 did appear to show a higher level of binding than seen previously (Figure 40). Despite the minimal differences in staining pattern, this does not rule out the possibility that the clones are binding specific bacteria or shared bacterial antigens, but it did show that if there is labelling occurring, then it is of a proportionally small population of bacteria, or that the donor lacks clone-reactive species.

When comparing between this stool sample and the previous it demonstrated that there are significant qualitative differences in the microbiota of different individuals. A higher level of IgA positive bacteria was detected in this sample (Figure 40). This is strongly suggestive of a more immunogenic microbiota as these IgA^{hi} bacteria are thought to have stimulated the immune system of the gut more strongly than commensals and become preferentially labelled with greater amounts of IgA^{547}. There are also further qualitative differences apparent in the DNA staining patterns seen with the SYBR staining. In this sample there were fewer bacteria containing large amounts of DNA, as determined by their lower levels of SYBR Green positivity (Figure 40). This may suggest less variation in the bacteria present and clearly show the lack of a population of bacteria seen in the previous sample (Figure 40).

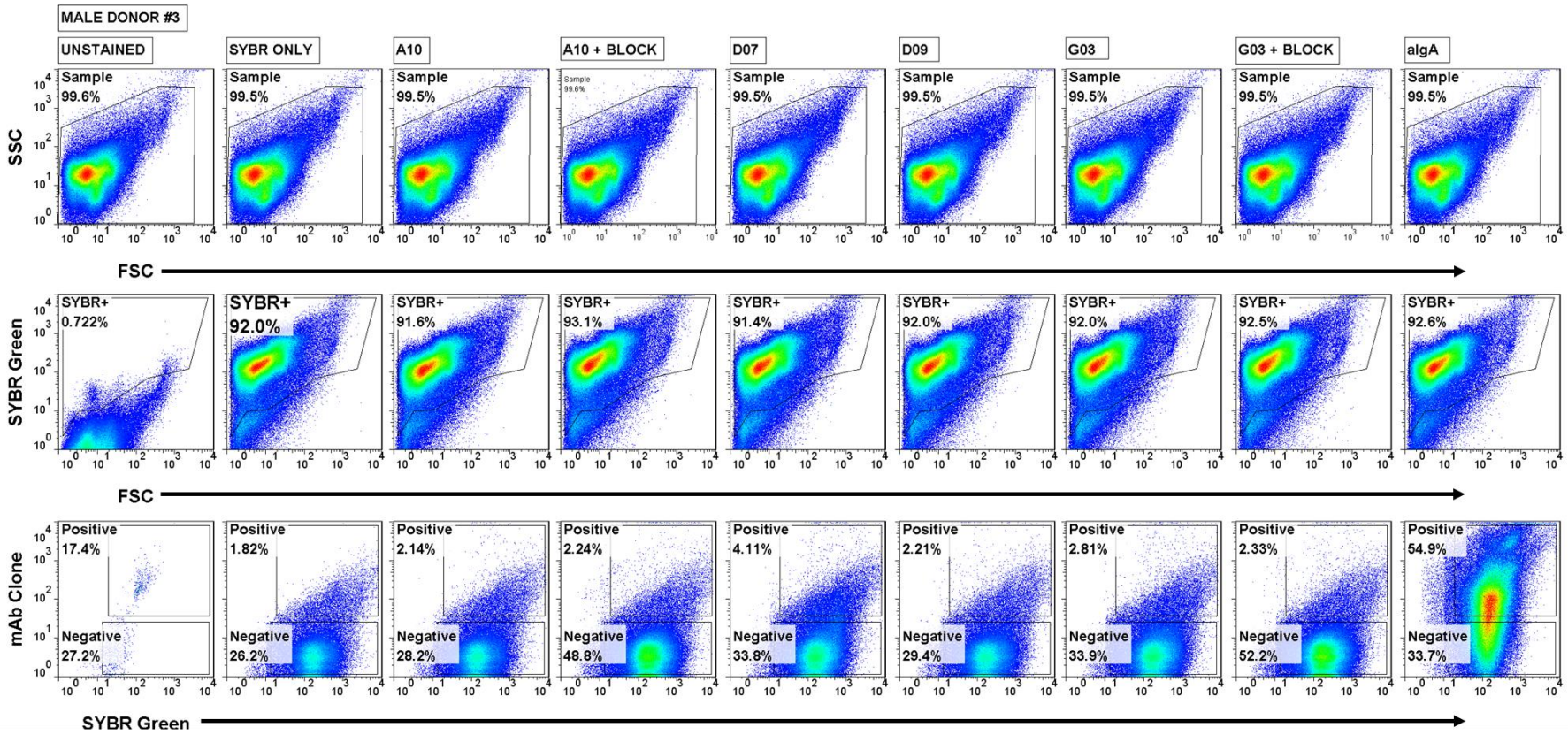

Figure 40 – Specific blocking of RA SF joint derived monoclonal antibodies did not demonstrate specificity *Flow cytometry dot plots showing the gating strategy to determine the binding of the mAbs tested based on the FMOs shown, events were gated on FSC/SSC, SYBR⁺, APC⁺ (Clone, IgA). An apparent failure of unlabelled mAbs to block their labelled counterparts is seen due to a lack of discernible specific binding and high background signal.*

One of the limitations of the previous experiment was a lack of clear binding seen in mAb-labelled samples, this was partially due to the high level of background signal coming from the vastly larger number of unlabelled bacteria present. To attempt to achieve a better resolution, the samples were enriched for the specifically labelled bacteria using magnetic-activated cell sorting (MACS), prior to FACS. This was achieved by labelling the bacterial suspension with the APC-labelled mAb clones or an APC anti-IgA mAb, and then secondarily labelling this with anti-APC beads. These beads enabled for the MACS separation of those bacteria which had been specifically labelled with either the APC-labelled mAb clones or the anti-IgA mAb, thus enriching for the population of interest. The primary reason for this was to improve the resolution available for gating but it also decreased the time taken for the required number of bacteria to be sorted for 16S rRNA sequencing. Separate to the enrichment, blocking was again utilised to demonstrate binding specificity, and a striking decrease in the signal for D07 is seen (Figure 41).

This enrichment proved successful, with clearer staining patterns visible for labelled bacteria apparent (Figure 41). However, it also demonstrated that MACS was not 100% efficient in sorting the labelled population, this could potentially be a result of under-labelling the sample and leaving a large number of unbound bacteria present. However, the enriched sample contained far greater numbers of labelled bacteria and enabled successful sorting for subsequent sequencing (Figure 41). Again, qualitative differences were noticeable between donors with regard to the bacterial DNA staining pattern seen (Figure 41), the importance of this is not known and difficult to quantify without paired sequencing data from the populations.

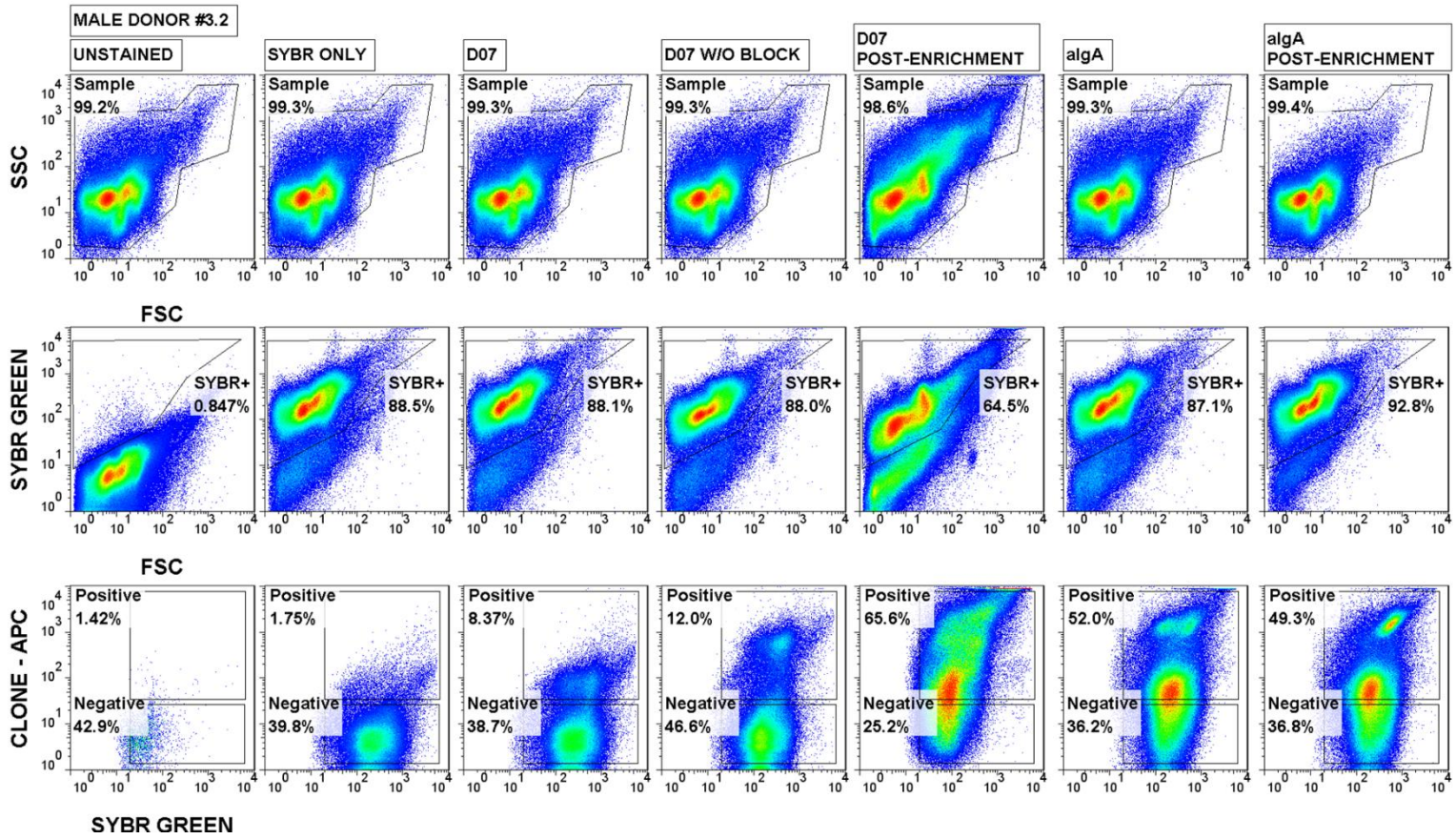

Figure 41 – MACS enrichment enables the determination of labelled bacteria using FcRL4⁺ RA SF joint derived monoclonal antibodies. *Flow cytometry dot plots showing the gating strategy to determine the binding of the mAbs tested based on the FMOs shown, events were gated on FSC/SSC, SYBR⁺, APC⁺ (Clone, IgA). The possibility of sorting labelled bacteria with the mAb clones is demonstrated.*

The method was then applied to another healthy donor's stool sample, although the blocking step was omitted given the lack of specific blocking of mAb binding seen in earlier experiments. In this experiment a single male donor sample was used, and the MACS enrichment step used previously was applied. Following this enrichment for labelled bacteria, a total of 400 000 positive events were sorted from the sample via FACS and subject to 16S rRNA sequencing in order to determine the composition of the bacterial populations present. As can be seen in the flow dot plots from this experiment the enrichment enabled sufficient numbers of bacteria to be sorted and of relative purity (Figure 42).

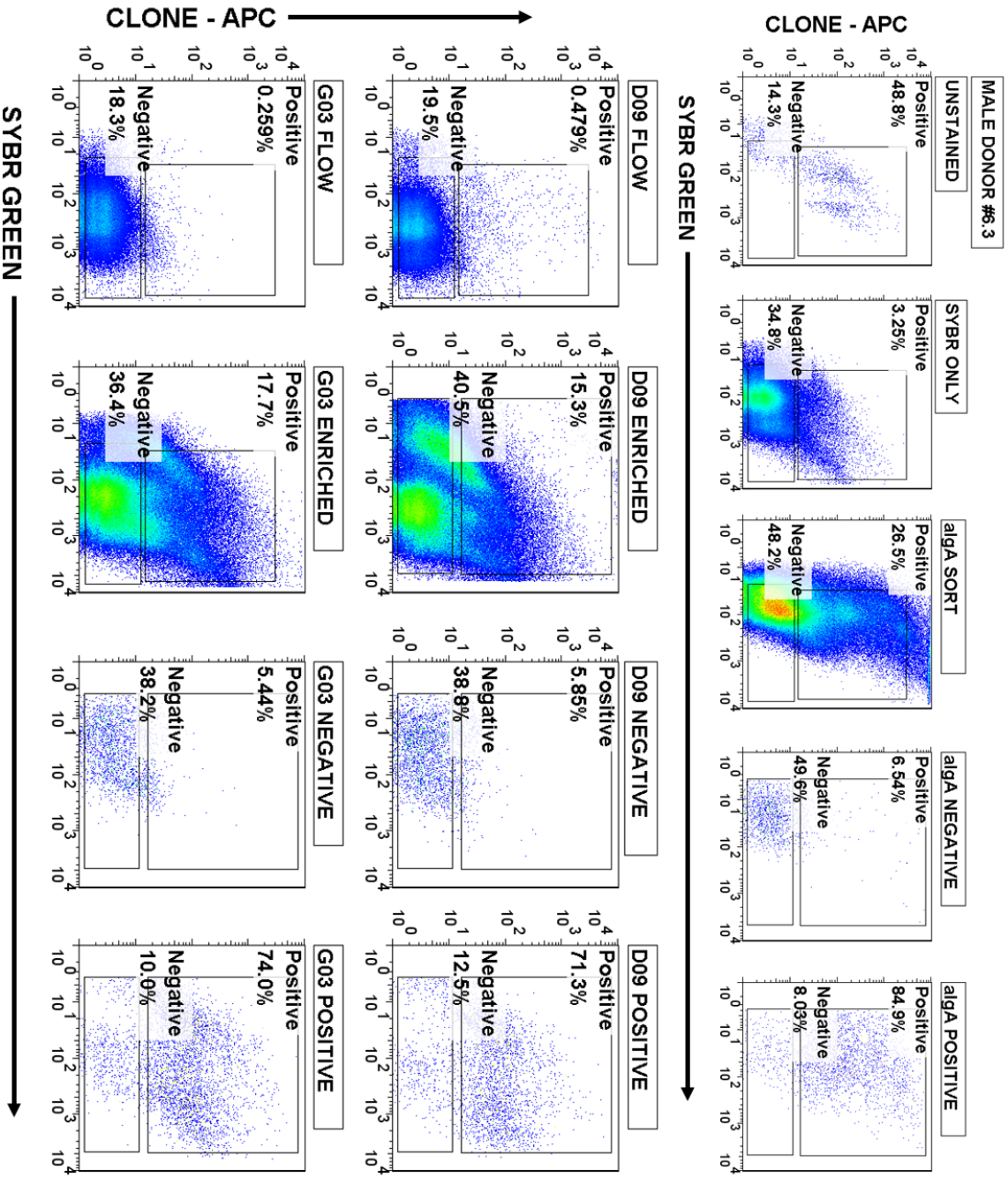

Figure 42 – MACS enrichment enables the sorting of labelled bacteria using FcRL4⁺ RA SF joint derived monoclonal antibodies. *Flow cytometry dot plots showing the gating strategy and purity checks of the sorted populations of stool derived bacterial samples for two clones and anti-IgA antibody, events were gated on FSC/SSC, SYBR⁺, APC⁺ (Clone, IgA).*

5.1.2 16S Sequencing – Identifying the FcRL4⁺ reactive bacteria

16S rRNA sequencing of these bacteria lead to the identification of several over-represented bacterial genera in the labelled fractions including *Prevotella*, *Ruminococcus*, and *Roseburia* (Figure 43). These were enriched 2-, 17-, and 6-fold respectively.

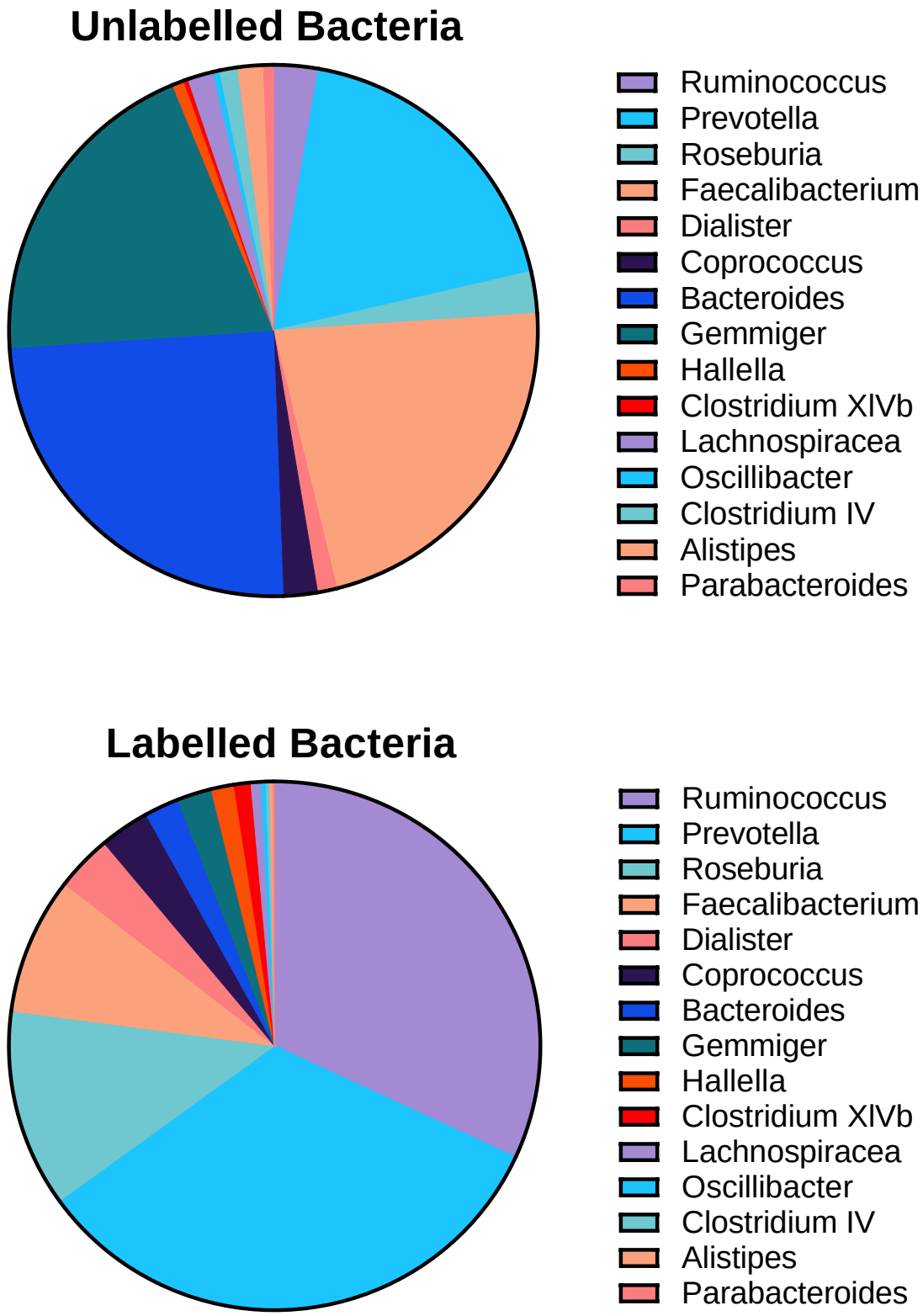

Figure 43 – An RA SF FcRL4⁺ derived mAb clone recognises a unique pattern of bacterial genus present in the healthy human microbiota*. Pie Charts showing the proportion of bacterial species labelled or unlabelled by the clone D09 in the bacterial cell sorting, the top 15 genera represented by relative counts of genus specific variable regions are shown.*

5.2 RA IgA$^+$ Synovial Fluid B cells and their specificity for microbiota

5.2.1 FcRL4$^+$ BCRs are Hypermutated and display differential clonality to FcRL4$^-$ BCRs

Following from the experiments above, which outline the proof of principle for labelling stool samples with mAbs, sorting the labelled bacteria, and sequencing them in single donors; a larger set of experiments was carried out using monoclonal antibodies generated from single cell sorted IgA$^+$ FcRL4$^+$ and IgA$^+$ FcRL4$^-$ B cells, and pooled stool samples from healthy donors and donors with active RA. The cloning work and molecular characterisation of the monoclonal antibody sequences was carried out at the Karolinska Instituet by Khaled Amara and Vivianne Malmstrom.

To investigate the origins of FcRL4$^+$ B cells found in patient's joints, analysis of the IgA repertoire was carried out on single cells isolated from the synovial fluid of RA patients. These data suggest separate generation events for FcRL4$^+$ and FcRL4$^-$ B cells. Single cells with the surface phenotype; CD19$^+$, IgD$^-$, CD27$^{+/-}$, FcRL4$^+$, IgA$^+$& CD19$^+$, IgD$^-$, CD27$^{+/-}$, FcRL4$^-$, IgA$^+$ were sorted and then their Ig regions were amplified (Figure 44). The amplified Ig regions were analysed for mutation rates and locations, the light and heavy chain usages of the clones.

100% of the clones sorted were IgA clones, indicating that the sorting strategy was successful in this regard (Figure 44). The distribution heavily favoured the IgA1 isotype, with >90% of clones representing this isotype and there was no significant difference between FcRL4$^+$ and FcRL4$^-$ cells usage of either the IgA1 or IgA2 isotype (Figure 44). These data suggest that there is little difference in the origins of IgA$^+$ FcRL4$^+$ and IgA$^+$ FcRL4$^-$ B cells in the joints and that the sorting strategy was successful.

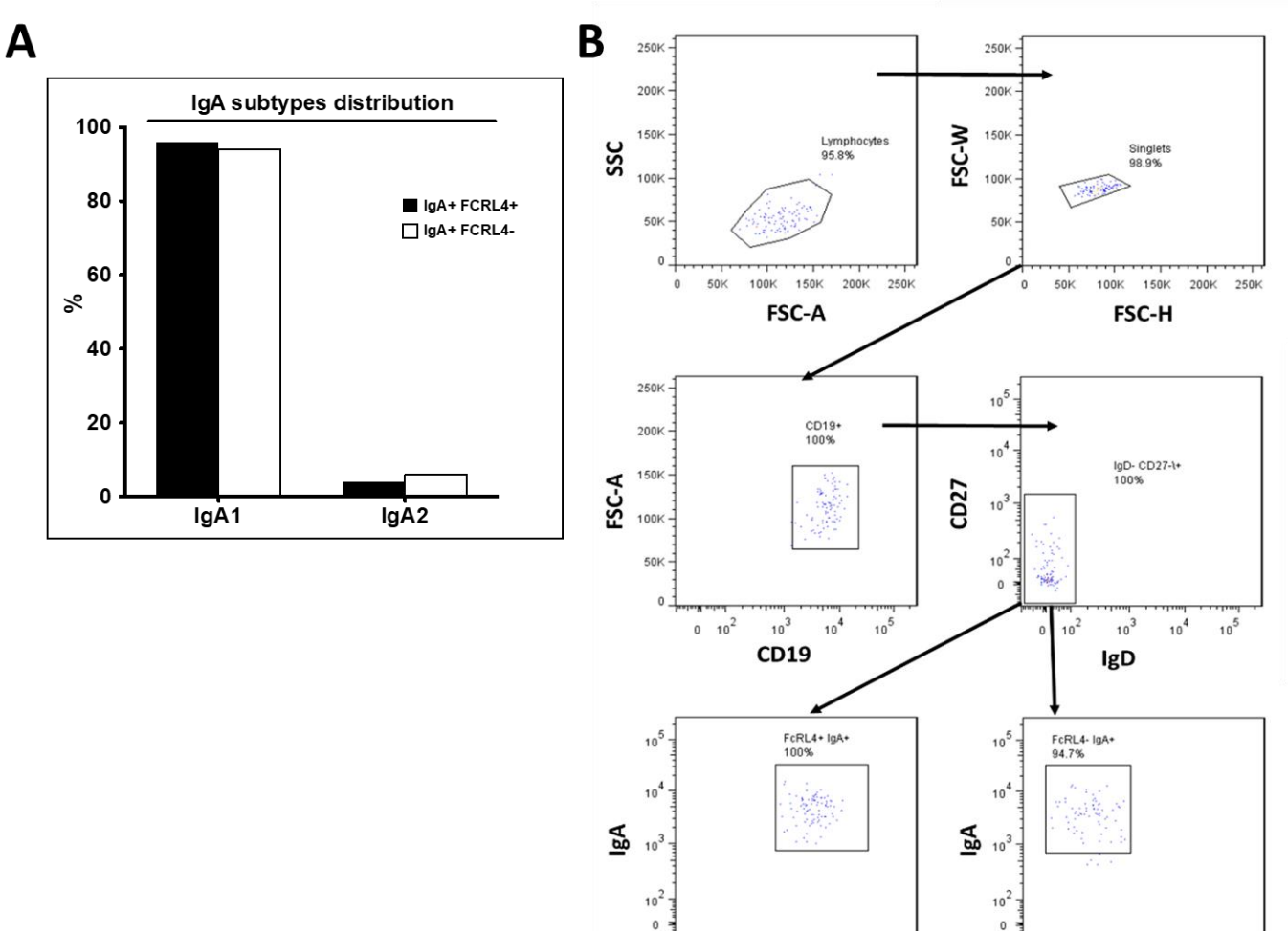

Figure 44- Sorted IgA⁺, FcRL4+ or FcRL4⁻ cells were confirmed as expressing IgA heavy chain genes. (A) *The heavy chain of the immunoglobulin gene in the sorted IgA⁺ FcRL4⁺ and FcRL4⁻ cells was amplified and the subclass and isotype determined. Sorted cells were all of the immunoglobulin A subclass, with the majority of the IgA1 isotype and a small minority representing the IgA2 isotype. 106 FcRL4⁺ IgA⁺ and 122 FcRL4⁻ IgA⁺ clones from 3 RA patient SFs were analysed in total.* ***(B)*** *Flow cytometry dot plots showing the gating strategy used in (A), Events were gated as per the sequential flow cytometry dot plots linked by arrows according to the following markers: CD19⁺, IgD⁻, CD27⁺/⁻, FcRL4⁺, IgA⁺ and CD19⁺, IgD⁻, CD27⁺/⁻, FcRL4⁻, IgA⁺.*

The overall mutation rate was comparable between the two populations (Figure 45) with a marginally higher number of mutations seen in the FcRL4- population, suggesting that both populations are post-SHM memory B cells. Furthermore, the mutations, as determined by replacement/ silent mutation ration, for both populations appeared to be focussed in the CDRs, rather than spread equally across the framework regions (FWR) and complimentarity determining regions (CDRs) of the heavy chain, indicating that the mutations seen are directed and selected

mutations in response to antigenic stimulation, rather than random mutation (Figure 45).

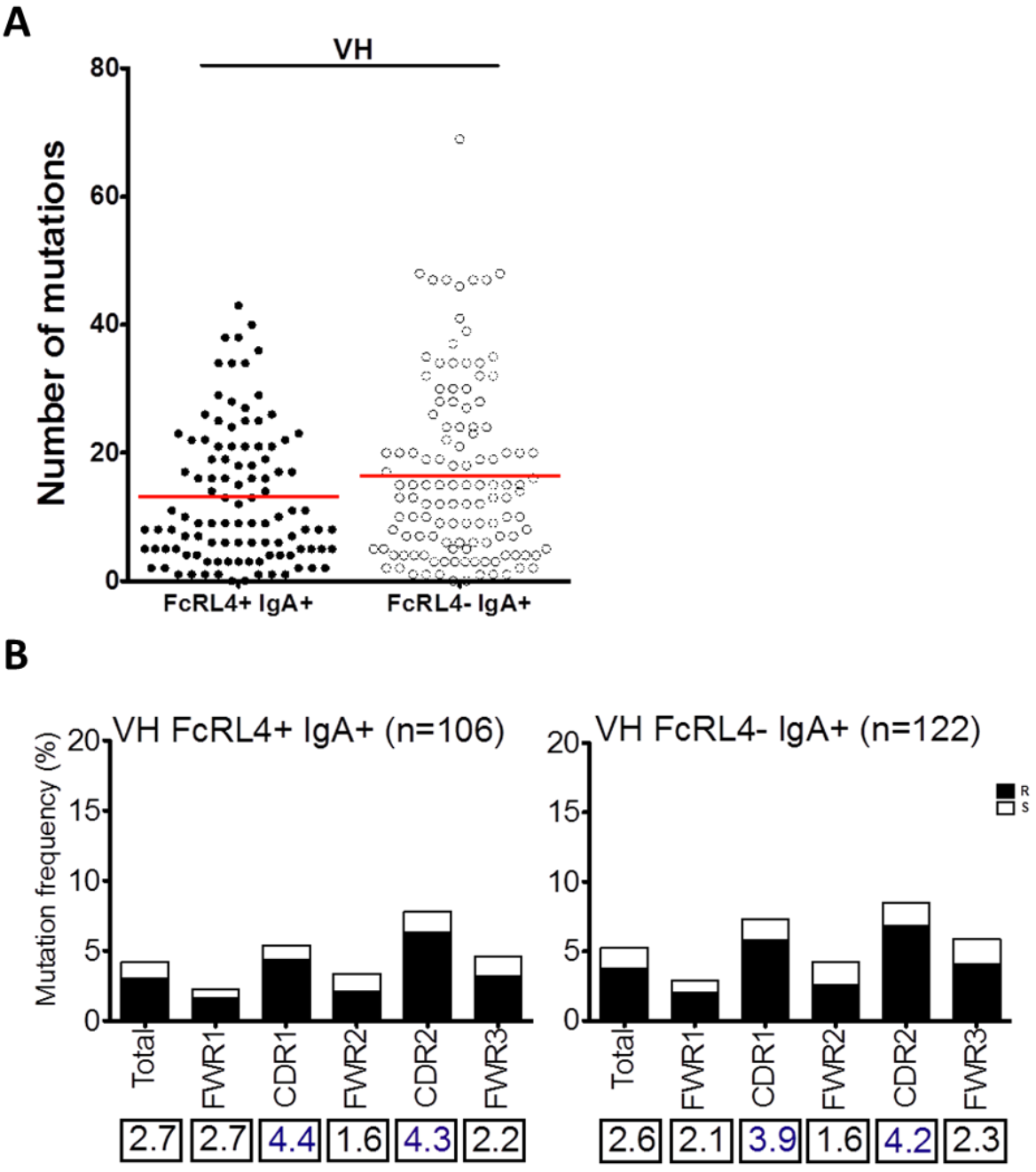

Figure 45- The Complementarity-determining regions (CDR) 1 and 2 of FcRL4+ or FcRL4- clones demonstrate higher levels of mutation than the surrounding areas. (A) *Scatter dot plot showing the number of mutations present in the FcRL4⁺ IgA⁺ and FcRL4⁻ IgA⁺ populations analysed.* ***(B)*** *Stacked column graphs showing the frequency of silent and replacement mutations present in the CDRs of the cloned FcRL4⁺ IgA⁺ and FcRL4⁻ IgA⁺ populations analysed. FcRL4⁺ and FcRL4⁻ clones have comparable levels of mutation in their CDRs, mutations were also lower in the framework regions (FWR) compared to the CDRs suggesting directed selection, in black R – replacement mutation, in white S – silent mutation (n=106 FcRL4⁺ IgA⁺ and n=122 FcRL4⁻ IgA⁺).*

5.2.2 Labelling of mAb clones derived from RA IgA + FcRL4+ B cells

Seven clonally expanded from each, the $FcRL4^{+}$ IgA^{+} and $FcRL4^{-}$ IgA^{+} clones were then selected and generated as recombinant IgG1 mAbs. The mAbs were directly labelled with FITC before being incubated with unlabelled compensation beads which bind the Fc portion of human IgG. This enabled the success of antibody labelling to be tested by flow cytometry. All clones were successfully labelled as demonstrated by the positive signals detected in each (Figure 46).

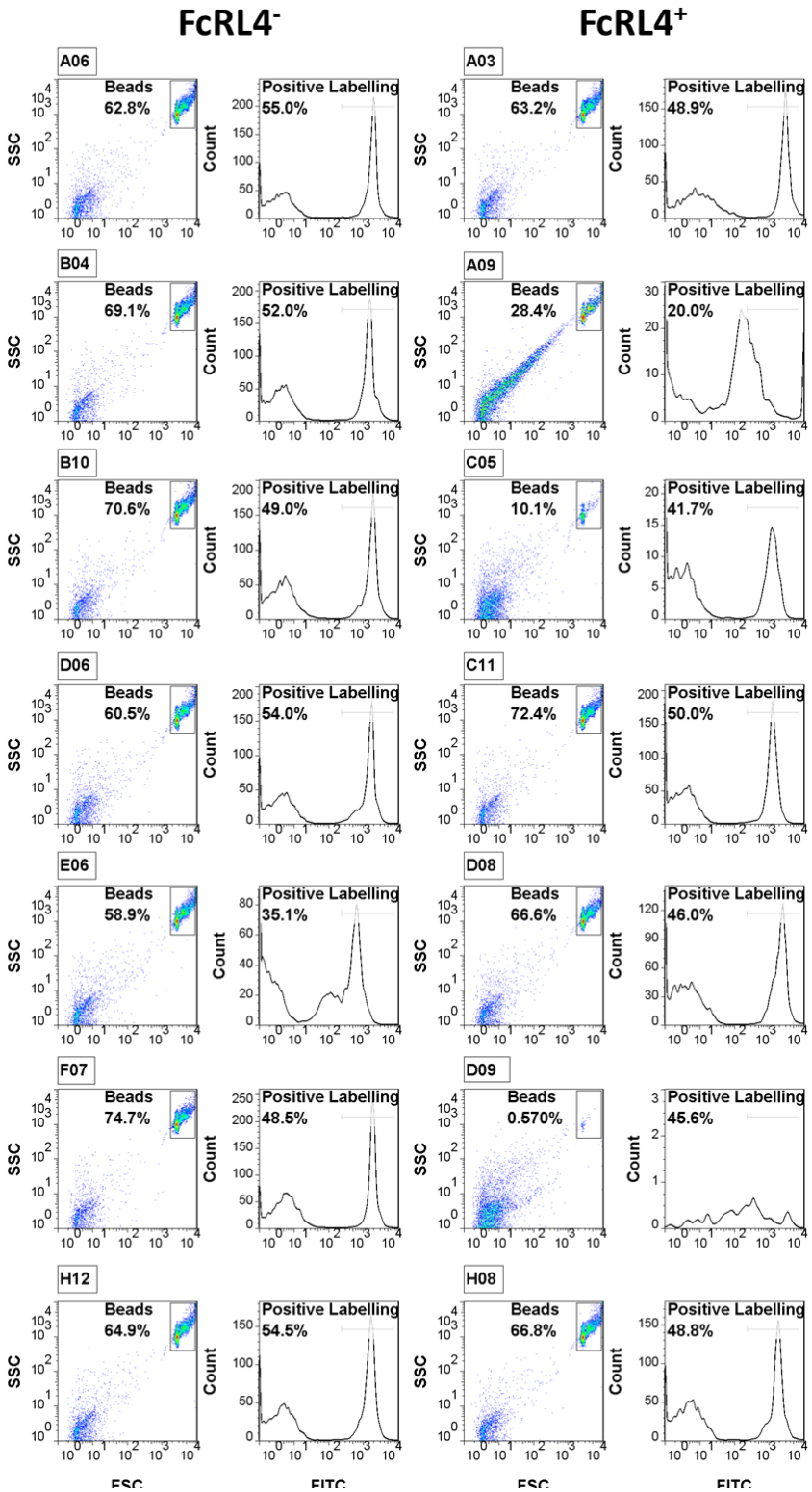

Figure 46 – RA SF joint derived monoclonal antibodies were labelled with FITC. *Flow cytometry dot plots showing the gating strategy used in the corresponding histograms beneath each plot. Dot plots are showing size (FSC) against granularity (SSC) and gating on the labelled bead populations. Histograms show the FITC MFI for the mAb labelled beads and gates were established using FMOs.*

5.2.3 Screening of Pooled Stool Sample Pool from RA patients

Following the successful labelling of all the clones, their reactivity against a pool of stool samples from RA patients was assessed. FMOs were used, as was a labelled blocking antibody of the same isotype. Both $FcRL4^+$ and $FcRL4^-$ clones reacted with the pooled diseased samples, demonstrating that both of these populations were capable of binding bacterial antigens (Figure 48). The percentages of bound bacteria varied between the two groups of clones with a slightly higher degree of binding detected by the $FcRL4^+$ clones, however, this difference cannot be interpreted as directly representing a difference in successful binding as these samples were enriched (Figure 49). This could thus reflect differences in the success of the enrichment process, or the level of fluorophore attached to the clones as opposed to the size of the labelled population.

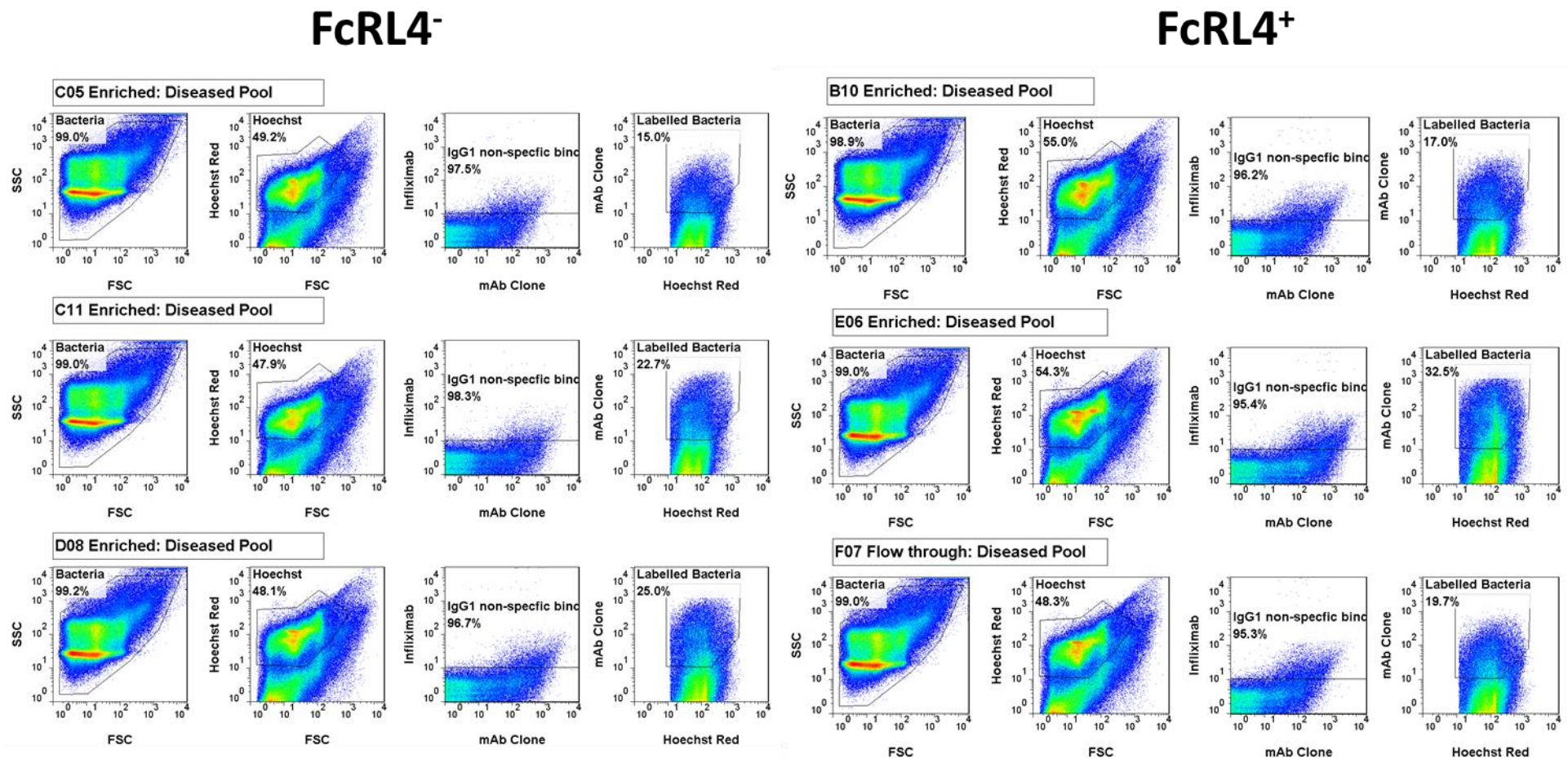

Figure 47 – FcRL4⁺ and FcRL4⁻ B cell-derived clones react with stool derived bacteria. *Flow cytometry dot plots showing the gating strategy used to assess the reactivity with stool derived bacteria of the FcRL4⁺ and FcRL4⁻ clones. Events were gated sequentially on FSC/SSC, Hoechst⁺ and FITC⁺/APC-Cy7⁻ (Clone⁺/Blocking mAb⁻).*

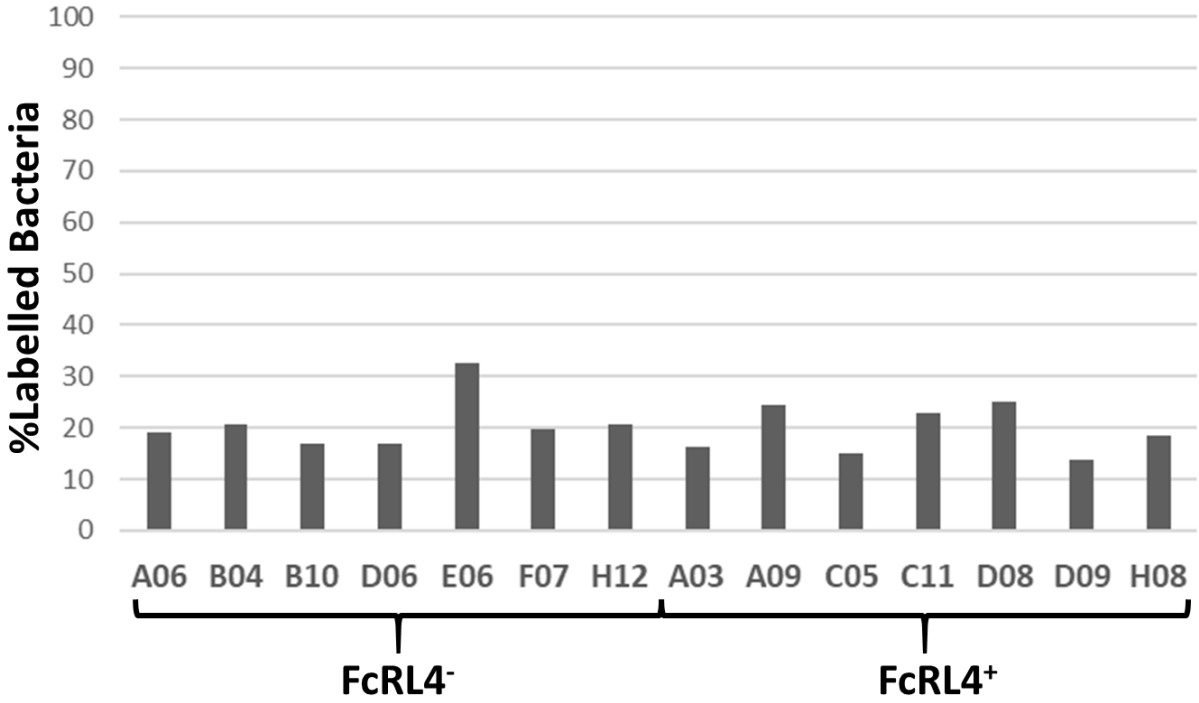

Figure 48 – FcRL4^{+} and FcRL4^{-} derived clones appear to react to differing degrees with RA-stool samples*. A column graph showing the percentage labelled bacteria from the flow cytometry screening of the labelled bacteria for FcRL4^{+} and FcRL4^{-} clones. Events were gated on FSC/SSC, Hoechst^{+} and Clone^{+}/Blocking mAb^{-} and the percentage of Clone^{+}/Blocking mAb^{-} events displayed.*

5.2.4 Bacterial Cell Sorting

Following the screening of the mAb clones against bacteria derived from a pool of stool samples from RA patients, these samples were then sorted. IgA^+ bacteria were also sorted as a positive control and to provide relevant information regarding the immunogenicity of the sorted bacteria (Figure 49).

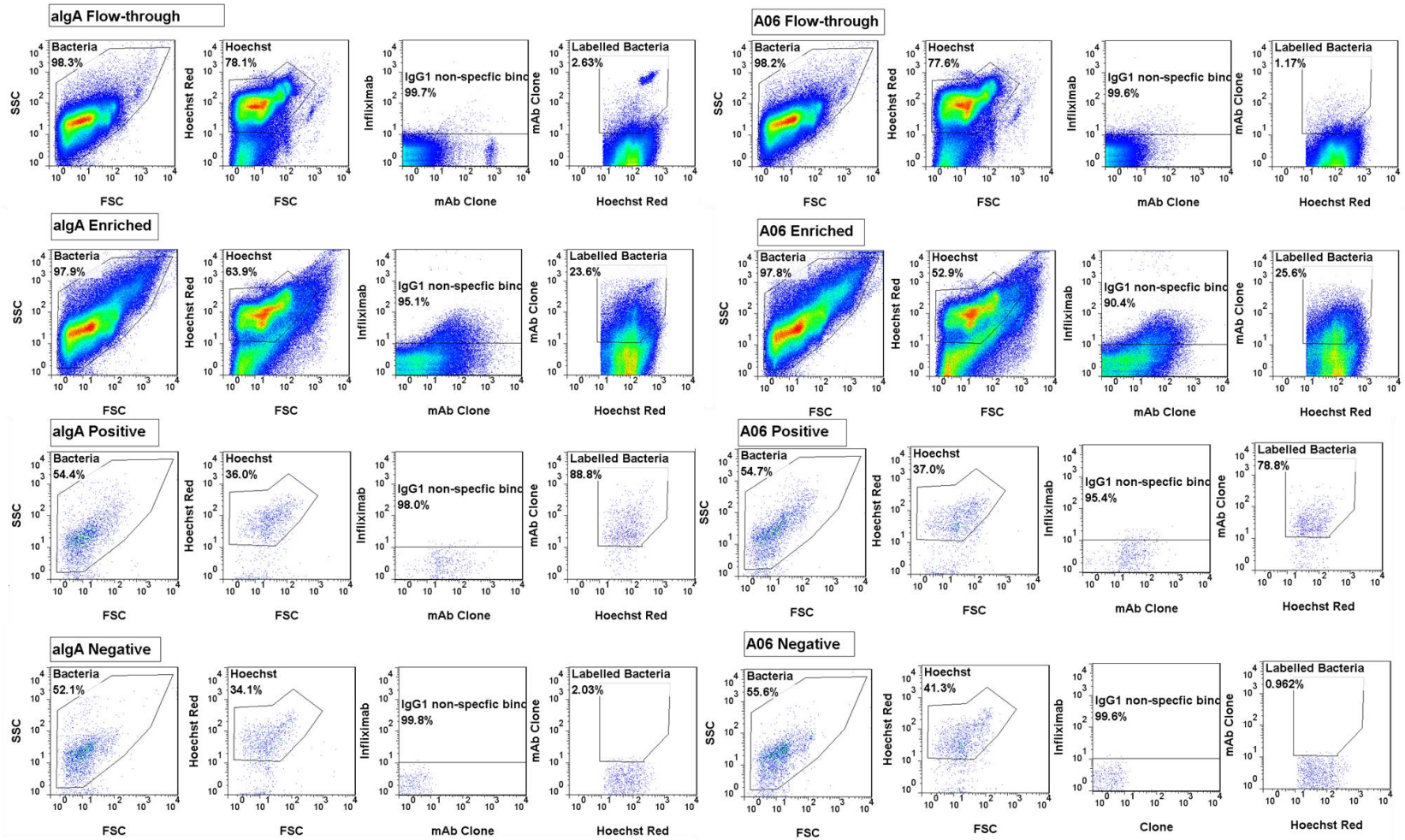

***Figure 49 – Clone⁺ and IgA⁺ bacteria can be sorted via FACS**. Representative flow cytometry plots showing the purity checks and gating strategy to determine the binding of the mAbs tested based on the FMOs, events were gated on FSC/SSC, SYBR⁺, APC⁺ (Clone, IgA). following the sorting of positively and negatively labelled fractions of pooled diseased stool samples.*

5.2.5 16S Sequencing of SF-mAb recognised bacteria

Upon sorting, the labelled bacteria were subject to sequencing of their 16S rRNA in order to provide information on the identity of the recognised bacteria. The genus level analysis demonstrated that there was a core of approximately 5 bacterial genera which both the $FcRL4^+$ and $FcRL4^-$ clones recognised, including *Ruminococcus, Faecalibacterium, Bacteroides, Blautia,* and *Clostridium* (Figure 50). *Prevotella* was recognised by a greater number of the $FcRL4^+$ than $FcRL4^-$ clones, but a greater variety or wider distribution of bacterial genera were seen in the top 8 of the $FcRL4^-$ clones (Figure 50).

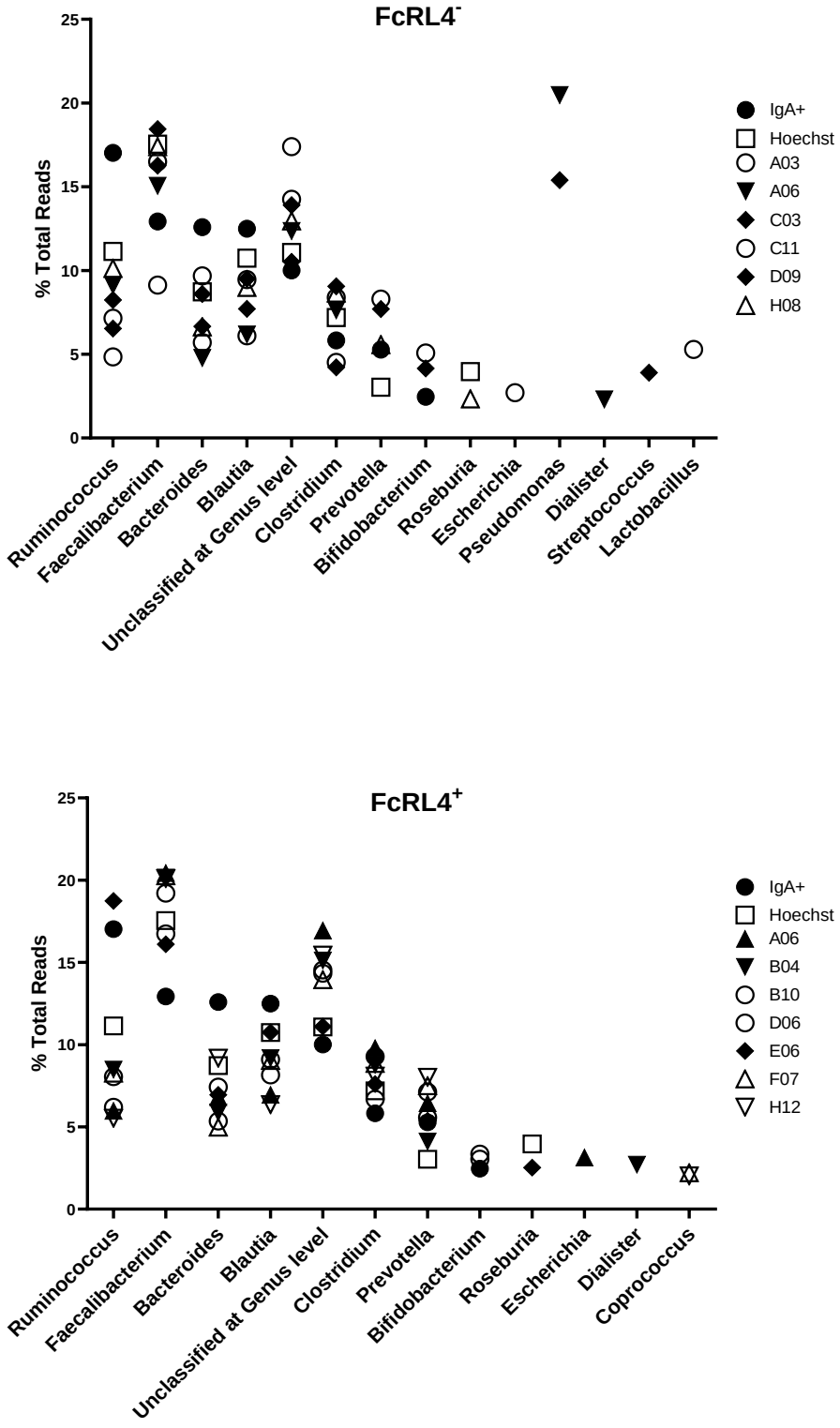

Figure 50 – A greater number of FcRL4⁺ clones recognise Prevotella but FcRL4⁻ clones recognise a wider number of significant bacterial genera. *Dot plots showing the bacteria genera recognised by FcRL4⁻ and FcRL4⁺ clones as sorted in the above experiments. Samples were 16S sequenced and the %Total reads quantified using the Illumina MiSeq platform.*

5.3 Summary – Exploration of reactivity of recombinant antibodies derived from FcRL4+ B cells with the gut microbiome

These experiments outline the possibility of using monoclonal antibodies derived from BCR sequences of synovial fluid derived B cells, to test the hypothesis that an immune response against bacteria present in the gut leads to development of RA, through a mechanism such as epitope spreading. These experiments demonstrated a variety of developments to a method enabling the sorting of specifically labelled bacteria in the stool samples of healthy donors and suggest that Immunoglobulins of B cells derived from the joints of RA patients are reactive against bacteria present in the stool samples of healthy donors.

5.4 Experimental Limitations and Discussion – Exploration of reactivity of recombinant antibodies derived from FcRL4+ B cells with the gut microbiome

In the initial experiments it was possible to distinguish live bacteria by their size and positivity for SYBR Green, a DNA dye. Although this might be considered slightly redundant if we expect bacteria which are dead at the point of staining to be antigenically relevant to the disease process or to the generation of these BCR arrangements. It could be argued that all bacteria were worth including but the ability to subsequently sequence them would have been greatly diminished. It was also possible to discriminate between bacteria which had IgA bound to their surface using an anti-IgA antibody. However, in the early experiments it was more difficult to discern clear differences with the antibodies. Several of the mAbs reacted but the level of background noise (Figure 41) prevented any firm conclusions being drawn as to whether any specific bacteria had been labelled and prevented the sorting of any positively labelled bacteria for sequencing.

These initial experiments all used directly labelled mAbs, another option would be to use a secondary antibody specific for the isotype of these antibodies they were all expressed as human IgG1. This would mean that although it is unlikely that bacteria present in the gut would be labelled with IgG as IgA and IgM are the predominant isotypes seen in the gut, there is still the possibility that some remain coated with IgG. This would lead to background signals and false positive labelling. For this reason, direct labelling was determined to be the most specific method.

Although these experiments from the first half enabled the sorting of specifically labelled bacteria for 16S sequencing, they also demonstrated that none of the mAbs tested recognised a conserved antigen and raised the potential that either they are poly-reactive, or that they react with an antigen which has been shared between species. For this reason the term relative purity was used to describe the labelled populations of bacteria as these will still be comprised of a mixed population of bacteria since the specificity of the mAbs is not known and antigens may be shared or epitopes spread between bacteria.

Interestingly, in the experiment in which the MACS enrichment was attempted for the first time, the same donor was used as in the initial experiment and the uniquely SYBR Green labelled population in that experiment was not present (Figure 39 & 42). Given that these experiments took place on separate days this suggests that certain populations of bacteria in the microbiota are transitory. However, this would require sequencing before and after to determine if a taxonomic shift had occurred. In light of this, it would be useful to collect dietary information to determine whether these transitory bacterial populations detected were linked, for example, to probiotics.

A potential downfall of this MACS enrichment step, is that it may exclude some bacteria which are not labelled with IgA, biasing any results obtained with this method. It also might prevent those

bacteria labelled heavily with IgA, if coated with anti-IgA, from being labelled with the specific antibodies further skewing the bacterial pool away from those uncoated in IgA and away from those coated in very high levels of IgA. However, looking at the results of the anti-IgA it is possible to see that bacteria with lower levels of IgA were still present following MACS enrichment. This suggested that whilst this method could be titrated down to achieve a higher level of purity initially using MACS alone, this "leaky" method might actually enable a less biased sample to be run in the FACS whilst also enabling a suitable staining pattern and high enough efficiency for the FACS.

One source of bias that interpretation of the initial experiments faces is the higher proportion of male donors compared to female donors. This skewing of the sex ratio may affect the species of bacteria present. The initial experiments also utilised very few donors, which as can be appreciated from the varied flow plots for the bacteria between experiments, sequencing from only these could induce biases.

To address these issues, the clones were later tested for their reactivity against pooled stool samples, comprised of RA patients, healthy female, and healthy male donors to enable well-powered sequencing experiments to take place. These were preserved in glycerol in order to best preserve their external structures and maintain epitopes, but which might negatively impact upon their DNA integrity impacting upon the reliability of the generated sequences.

The initial experiments also lacked an isotype control. In the latter experiments a human recombinant IgG1 clone was directly labelled with another fluorophore to allow the discrimination of specific and non-specific binding. To further verify the selectivity of the mAbs, the use of unlabelled mAbs as well as faecal samples deficient in the bacterial species labelled in previous experiments

could be used to assess specificity.

Inferring a difference in the percentage of bacteria bound by the specific clones should only be considered in the first experiment to screen the diseased pool and not the sorted data as these samples are enriched via MACS. Thus, this does not represent an unbiased level of binding but could reflect how well the enrichment worked, or how susceptible certain bacteria were to become non-specifically bound to the column during MACS enrichment. However, these experiments all demonstrate the utility of such an approach in obtain suitable numbers of cells for FACS and subsequent sequencing.

Alongside the clone reactive bacteria sorting, IgASeq was performed, which involved sorting the bacteria stained positive by the anti-human IgA antibody[548]. This provided a positive control and information about the microbial species recognized by the antibodies; such as whether they are more heavily coated with IgA and therefore a commensal or pathogenic bacterium[548]. Sequencing of the Hoechst^{+} bacteria present in the faecal samples provided unbiased information such as their prevalence across microbiota samples, and enrichment in the individual's microbiota. In line with previously published data looking at the reactivity of tonsil derived FcRL4^{+} B cells, the joint derived FcRL4^{+} clones reacted predominantly commensal bacteria genera[546]. This data thus extends the previous work and demonstrates this to be true of both FcRL4^{+} and FcRL4^{-} B cells, with a core group of genera accounting for approximately 80% of the FcRL4^{+} and FcRL4^{-} clones reactivity. This data suggests that this doesn't represent a population of B cells predisposed to react against pathogenic bacteria, but those generated as a product of normal mucosal immune responses. However, at present only the pooled healthy male samples have been analysed, thus biasing the data in terms of gender, especially given the increased prevalence of RA in the female population[549]. The data generated by these future experiments and further analyses of the already generated data will

provide information as to whether joint derived immune cells recognize bacteria derived from the human gut, which would implicate the gut as a site of autoimmunity initiation.

It would also be interesting in light of the bacterial sequencing data which demonstrated the reactivity of joint derived immunoglobulins towards the microbiota, to test wider populations of B cells from the joints in these assays. These assays could include unrelated peripheral IgG isotype BCR expressing B cells, and IgA and IgG B cells derived from the joints of patients with joint disease. These joint diseases could include RA and PsA but would also include diseases primarily with gut symptoms or pathology which also manifest articular symptoms, such as Crohn's, and Ulcerative Colitis[336]. Furthermore, testing the reactivity of a greater number of individuals' joint derived clones against paired stool samples over time would provide information as to whether bacterial populations recognised by joint derived B cells expand or contract prior to disease onset.

5.5 Conclusions – Exploration of reactivity of recombinant antibodies derived from FcRL4+ B cells with the gut microbiome

These data consider the involvement of FcRL4 and the mucosa and outline the reactivity of FcRL4$^+$ B cells to microbial antigens suggesting that joint derived B cells are capable of reacting against antigens found in the microbiota of both healthy controls and patients with active RA. They also suggest that these cells are most likely the product of the normal mucosal immune reaction and not a product of pathogenic infection.

6 Discussion

6.1 A Brief Summary

In this section I will discuss the experiments in this work, the relevance of the results in light of our current understanding of FcRL4$^+$ B cells, and their role in Rheumatoid Arthritis and autoimmune disease in general. It will focus on the three aims, outlined at the beginning of this work. Firstly, investigating the presence of FcRL4$^+$ cells in a variety of tissues and how expression of FcRL4 can be regulated. Secondly, testing the ligand binding properties of FcRL4 and the FcRL4$^+$ B cells found in the different anatomical sites. Thirdly, the reactivity of the RA SF FcRL4$^+$ B cells towards mucosal antigens.

In this work so far, I have introduced the concept of autoimmunity and in particular, Rheumatoid Arthritis, before outlining the various biological roles of B cells in health and in this specific disease context. I have further detailed the relevance of atypical B cells to disease and described a population of B cells with many commonalities to atypical B cells, demarcated by their expression of the cell surface marker FcRL4.

In the results I have demonstrated the presence of the FcRL4$^+$ B cell population in the synovial fluid of arthritis patients, the tonsil, and in the periphery of both RA patients and healthy controls. The regulation of these cells has been detailed. FcRL4's role as a low-affinity IgA receptor has been described and demonstrated in a variety of contexts, and the correlation between FcRL4 and IgA BCR expression shown. Finally, the antigen specificity of FcRL4$^+$ B cells from the joints of patients with active Rheumatoid Arthritis was tested against the microbiome of healthy patients and demonstrated to react with defined bacterial populations.

6.2 FcRL4$^+$ B cells

6.2.1 Background

B cells remain an important population in the context of autoimmunity, responsible not just for the secretion of autoantibodies which are a hallmark of disease, but also for directly pro-inflammatory and regulatory functions through the secretion of cytokines and their interactions with other cells of the immune system[44,469,472,475,476]. They have also been demonstrated to be vital for the induction of disease in mouse models of arthritis in their role as antigen presenting cells (APCs). A job they carry out with a greater specificity than other APCs such as DCs due to the specific nature of BCR recognition [482]. One of the key areas of research demonstrating their importance to the pathology of RA is the clinical success of B cell depletion therapies, most commonly using the anti-CD20 monoclonal antibody Rituximab, which depletes circulating and joint resident CD20$^+$ B cells, leaving the antibody-producing plasma cells intact[227,490]. Despite the varied roles which B cells play in disease, many of these functions have only become more fully appreciated in recent years. The work carried out for this thesis aimed to better characterise a population of B cells found in the synovial fluid of RA patients and investigate the links between this population and the mucosa.

This hypothesis that this population may provide a link between the mucosa and the joint came from several areas of research; firstly, that IgA$^+$ B cells can be found in the joints of RA patients; secondly, that dysbiosis of the mucosa can lead to chronic antigenic stimulation of B cells and the spreading of epitopes leading to autoimmunity. FcRL4 appeared to link these through its expression upon a population of B cells found in both the mucosa and the joints of RA patients, the overrepresentation of IgA BCRs in this population, and the ability of FcRL4 to act as a low affinity IgA receptor[537,550].

FcRL4 is a cell surface marker belonging to a family of 6 receptors, known as “Fc-Like Receptors”, so-

called due to their sequence homology to classical Fc receptors[505,506]. This family of receptors are relatively recent in their description in the literature, and much of their role's as receptors and markers have yet to be elucidated[505,506]. Important to note in the context of this work is that expression of FcRL4 is limited to humans, and no homologue has been found expressed in mice[506,509]. This has implications for understanding the role of this receptor in autoimmune disease as it is not possible to create a mouse with a conditional knock-out of this gene and observe the consequences, nor is it possible to carry out experiments analysing this population *in vivo* in a mouse model. Although humanised mice models exist and could increase the range of possible experiments one can carry out, they currently lack a fully functional B memory compartment and thus cannot be used to further our knowledge of this biology. This complicates the process of understanding these cells, but it also means that any understanding we achieve using human samples is more likely to be representative of human biology as opposed to in mice. For that reason, the majority of the work carried out used human samples, and where appropriate, immortalised human cell lines expressing FcRL4.

6.2.2 FcRL4 Expression and Regulation

This is not the first work to describe the presence of FcRL4$^+$ B cells in the synovial fluid (SF) of RA patients, this was first published by our research group in 2015 by Yeo, et al., following the description of a population of B cells found in the RA SF responsible for the secretion of RANKL[474,551]. These B cells were found to express high levels of the surface receptor FcRL4 in addition to their ability to secrete RANKL[474]. Work on this cell population was later extended by two separate groups, Akashi et al. and Anolik, et al., who both suggested that this population has a pathogenic activity in the context of RA through their ability to secrete RANKL and thus stimulate RANK expressing osteoclast precursors[468,552]. Upon RANKL-RANK activation these precursors differentiate into

osteoclasts (OCs) which are responsible for the degradation of the bone seen in RA[553]. However, the work described in this thesis is the first to demonstrate the presence of FcRL4$^+$ B cells in the SF of patients with other arthritides, suggesting that their activity and development is not disease specific. Although this work did not investigate the OC-stimulating capacity or bone erosive capabilities of these cells, it would be interesting to assess the expression levels of RANKL in the FcRL4$^+$ populations across diseases, given the differential levels of bone erosion seen across the different diagnoses such as Psoriatic Arthritis which see much lower levels of bone involvement than RA. Although those experiments in which the presence of FcRL4$^+$ B cells is demonstrated across different arthritides are suggestive of differences in the size of the population, they used different staining panels and protocols, limiting the utility of further analysis. A consistent staining panel and analysis would make better use of patient material in the future.

Previous transcriptomic data published on the FcRL4$^+$ population in the RA SF have shown significant differences between FcRL4$^+$ and FcRL4$^-$ B cell populations and suggested that the FcRL4$^+$ B cell population have a transcriptomic programme typical of a memory B cell population[465]. In this work, further analysis of the clonality of the IgA$^+$ FcRL4$^+$ B cells suggests that they are an antigenically-experienced, post-somatic hypermutation memory B cell population. As determined by Ki-67 expression FcRL4$^+$ B cells are significantly more proliferative than FcRL4$^-$ B cells, and closer to plasmablasts in levels of proliferation detected, demonstrated by Erhardt et al. and by unpublished data from our group[518]. They also have a significantly lower percentage of IgM$^+$ cells in their population as determined by PCR[521]. These data collectively suggest that FcRL4 is a marker of a memory B cell subset. One important caveat to the RA SF sequencing work carried out by our group previously is that the cells sorted as FcRL4$^-$ B cells are a diverse mixture of cell populations including plasmablasts, naïve B cells and memory B cells and thus differences between the two populations may be biased[521]. However, other published transcriptomic data comparing FcRL4$^+$ and FcRL4$^-$ B cells

in the tonsil described a similar transcriptomic programme, proliferative, and typical of memory B cells[518]. Future work interrogating the transcriptome of this population should account for the above considerations and sort the traditional B cell populations, including naïve, memory, and plasmablast or plasma cells and the FcRL4$^+$ population before making direct comparisons, ideally this work would cover both diseased and healthy patients several different anatomical sites, including the mucosa (tonsil), joint, and peripheral blood in order to generate the most valuable comparisons. However, in planning these experiments it is worth considering the data which demonstrated a significant drop in expression levels of FcRL4 on CD19$^+$ cells following only three hours in culture. This suggests that the marker may not be a stable marker of a memory B cell population and that when making comparisons between FcRL4$^+$ and FcRL4$^-$ populations both could obscure a level of heterogeneity. Furthermore, the transient nature of FcRL4 expression suggests that expression of FcRL4 may identify a transient differentiation stage, rather than a separate stable lineage. These findings also show that while the level of expression was significantly reduced, it does not reach 0% following 24 hours in culture, suggesting there is a stably expressing population, albeit at a lower level of surface expression. This could be further investigated through a transcriptomic approach before and after culture to determine whether there are shared characteristics. Published work on culturing FcRL4$^+$ B cells has focussed on isolated memory B cells from peripheral blood which may have phenotypic differences from those found in the tonsil and joint, although reported phenotypes do appear similar[468,527]. Of particular note is the use of IFN-γ to generate RANKL$^+$ B cells, which in the experiments in this work did not result in the production of FcRL4$^+$ B cells, suggesting that whilst concomitant expression of these markers is a signature of the RA SF FcRL4$^+$ B cells, they do not necessarily rely upon the same expression signals or networks[468,474,499]. The published experiments on generating FcRL4$^+$ B cells utilised a sorted FcRL4$^-$ population and appeared to rely upon greatly more complicated culturing medium and protocol than used in this work[527]. However, TLR9 agonist was present in their culture medium[527]. This suggests that the ability of TLR9 to affect the detected levels

of FcRL4 in the experiments in this work may be due to an upregulating effect. The ability of TGF-β and TLR-9 to affect detected levels of FcRL4 in this work also suggests that simpler culturing conditions may be suitable for the maintenance of FcRL4 expression. Furthermore, from a more biologically relevant perspective, it is possible that the TLR9 agonist mediated upregulation of FcRL4 could play a functional role in allowing B cells to bind IgA ICs, with presumably bacterial antigens, and potentially internalise them and present them to generate an immune response against them or sequester them and dampen the immune reaction.

This heterogeneity of the analysed populations was a consistent issue throughout the culturing experiments that prevented detailed conclusions being made as to the activity of $FcRL4^+$ B cells, for example, clouding the differences between death and down- or up-regulation occurring in culture. Another consideration for these experiments is the anatomical location from which these samples derived, the human tonsil. These samples were initially chosen due to access and quantity of B cells, $CD19^+$ cells comprise approximately 60% of the lymphocyte compartment in human tonsils. This allowed for enough cells to be sorted for the culturing experiments. However, despite being a rich source of B cells, only ~6% of these expressed FcRL4 which prevented sorting of the pure population. Another consideration was avoiding direct interactions of FcRL4 before culturing these cells to prevent unwanted downstream signaling of this receptor, given what little is known about it. This also means that any understanding achieved of these cells in the tonsil may not be directly translatable to their activity in the joints or periphery. Many of the experiment-specific issues are discussed in the limitation's sections of the respective results chapter.

A future avenue of work to determine specific signals capable of upregulating FcRL4 expression in B cell populations could involve isolating several classical defined populations of B cells from peripheral blood or tonsil; enriching for $CD19^+$ using MACS; subsequently staining and sorting using FACS,

removing any FcRL4$^+$ cells; before culturing using a checkerboard style titration of multiple signals, particularly focusing on those suggested by the work in this thesis. These included IgA, the TLR9 agonist ODN 2006, and TGFβ. Furthermore, it would be interesting to carry out concordant ELISAs in order to determine whether any of these signals lead to the secretion of cytokines or factors. Following from this, analysis of the transcriptomics and metabolomics of the cells before, during, and after culture could provide information as to the signaling networks implicated in the regulation of FcRL4$^+$ and RANKL$^+$ B cells.

The implications for RA based solely on the presence of FcRL4$^+$ B cells depend further upon their functional role. Although the potential for these cells to act as stimulators of bone erosion has been demonstrated, RANKL also is responsible for important interactions in the formation of tertiary lymphoid organs[554]. The functions of FcRL4 as an IgA receptor also require further analysis, although currently the literature suggests that it functions as an inhibitory receptor, dampening the ability of FcRL4$^+$ B cells to differentiate towards plasma cells[501,513].

6.3 FcRL4 and its' interaction with IgA

This work demonstrates FcRL4's specificity for IgA in a disease relevant context, namely binding IgA immune complexes in RA SF. It furthermore demonstrates that both joint and tonsil derived FcRL4$^+$ B cells are capable of binding IgA aggregates and have significantly higher levels of IgA on their surface than FcRL4$^-$ B cells. This suggests that this activity is occurring *in vivo*. However, this work did not assess the consequences of this binding in these contexts. FcRL4 has been reported as an inhibitory receptor, through phosphorylation of SHP-1 leading to inhibition of BCR signaling, although that work did not use FcRL4's natural ligand, instead relying upon an FcRL4 specific mAb[512]. However, further work by the same group used IgA aggregates, and demonstrated that FcRL4's immunomodulatory

activity is highly dependent upon the co-expression of src-family kinases HCK and FGR[513]. The authors reported that FcRL4 had inhibitory activity in cells co-expressing FGR but an activating function in cells coexpressing HCK p59[513]. Although this paper did not show the data, they suggest that binding of IgA to FcRL4 alone was not sufficient to detect any increase in tyrosine phosphorylation, and that this depended upon subsequent BCR cross-linking[513]. In light of this It might be interesting to titrate the IgA aggregates used, or to use an IgA immune complex containing the antigen for which the B cells are specific in order to achieve cross-linking. One potential criticism of those published results is that they were carried out in a B cell lymphoma cell line and transiently transfected PBMCs. It would be again be interesting to see how well these results translate into primary FcRL4$^+$ expressing B cell samples from joints and tonsils. One indirect method for interrogating whether the presumed outcome would be activating, or inhibiting would be to investigate the transcriptomic data of both tonsil-derived and RA SF-derived FcRL4$^+$ B cells and determine whether they had higher expression levels of FGR or HCK. However, in the previously published transcriptomic data from our group, both HCK and FGR are more highly expressed in the FcRL4$^+$ B cells than in the FcRL4$^-$ B cells[521,522]. This could have many implications or suggesting that there is a heterogeneity in the population, which would require single cell sorting; that expression of HCK or FGR behave differently in the joint derived population; but ultimately that further validation work at the protein or functional level would be required.

In assessing the ability of IgA from different sources to bind FcRL4 the data suggested that a large proportion of the FcRL4's apparent preference for colostrum derived IgA, was as a result of its propensity to form larger aggregates more easily. This was indicated by the consistent demonstration of higher IgA MFI throughout the binding experiments, and larger aggregates as determined by the size exclusion chromatography. Serum derived IgA appeared to be bound optimally after aggregation at 60-63^{o}C with an incubation time of 30 minutes, but never reached the

levels of binding seen with the colostrum IgA, which the chromatography experiments suggest was due to smaller aggregates being formed. These aggregation conditions were used throughout the binding experiments for all the different IgAs, unless otherwise indicated. The reasons for this difference in aggregation were not answered in this work but can be speculated as to include differences in glycosylation. However, reported work investigating the FcαR1, another IgA receptor suggested that glycosylation had little to no impact on its' binding affinity for IgA[555]. Furthermore, it has been reported that there is no detectable difference in the glycosylation of IgA1 in RA compared to healthy controls, suggesting that alterations in glycosylation, even if they have a functional effect on FcRL4 binding, are unlikely to be present in the diseased state[556]. This could be investigated using a mass spectrometric analysis of the different sources of IgA. Throughout the experiments artificially generated ICs analogues were used, real ICs may be of different sizes or structures which may lead to greater or lesser binding.

However, markedly less clear are the reasons behind FcRL4's apparent preference for the IgA1 isotype over IgA2 in a monomeric and aggregated form. This fact is complicated further by the lack of detectable IgA binding in the periphery, where a greater proportion of IgA1 is present, compared to the mucosa where the opposite is true. The IgA1 and IgA2 monomers used in this work were derived from purified monoclonal myeloma cell lines, and it is possible that either the source, or differences in processing from these sources as compared to the serum and colostrum derived IgA, led to differences in their aggregation. However, this would not explain the significant difference seen between these isotypes seen when these were left untreated. Unfortunately, these IgA isotypes were not compared by size exclusion chromatography due to the expense of the reagents and assay, this could have illuminated differences in their sizes which could potentially explain this preference.

The implications for RA in having a population of B cells capable of binding IgA ICs in the synovial

fluid (SF) are numerous. IgA has been demonstrated to prognose a greater degree of joint damage in RA[195,557,558]. As such, there is a potential for a link between IgA and the RANKL$^+$, FcRL4$^+$ B cells found in the RA joint[474]. There is the potential for these cells to present IgA ICs to other cells which could worsen inflammation, such as the activation of neutrophils via FcαR leading to the secretion of neutrophil extracellular traps (NETs)[559]. However, it is also possible that FcRL4$^+$ cells could act to sequester IgA away from such interactions dampening the immune reaction, or internalise these IgA ICs, and if they are internalised it could result in processing and presentation of IgA IC-bound antigens to T cells present in the joint. Furthermore, if these IgA IC-bound antigens are self-antigens this could result in further perpetuation of the ongoing autoimmune reactions of the RA joint. One method for analysing this could be to use FcRL4 to specifically pulldown IgA ICs from RS SF and subject the eluted proteins to mass spectrometric analysis. If autoantigens are detected, they could be tested for their ability to activate FcRL4$^+$ B cells and T cells from the same SF and depending on the antigen this could indicate that these cells are reacting against antigen that had been internalised via FcRL4. Alternatively, FcRL4$^+$ B cells derived from the RA SF could be tested for their ability to internalise a defined antigen bound to an IgA IC and present them to T cells specific for this antigen. This would provide evidence for the potential of FcRL4 to act as a novel route of internalisation, with implications in linking the mucosa and RA joint.

6.4 FcRL4 in the periphery

Previous work has suggested that a population of FcRL4$^+$ cells are present in the periphery of patients with malaria, as a result of chronic antigenic stimulation, and it has been demonstrated that FcRL4 is upregulated in response to the HIV envelope protein gp120[501,560]. However, later work demonstrated that the antibody used to demarcate the FcRL4$^+$ population in malaria was in fact cross-reactive with FcRL5[500]. More robust findings have been demonstrated in the periphery of hepatitis virus C (HCV) patients and in HIV[561]. However, little to no work has demonstrated the presence of FcRL4$^+$ cells in

the periphery of healthy controls and RA patients, although one paper reported the presence of FcRL4 transcript in the periphery of ~30% of RA patients[520]. In this work, phenotypic and functional comparisons were made between the two.

The presence of FcRL4^{+} cells was demonstrated to be overrepresented in the age-associated B cell (ABC) population of both healthy controls and RA patients. First discovered in aged female mice, and later in humans, ABCs have been shown to have unique phenotypic and functional properties and comprise a population of peripheral B cells which increase in proportion with increasing age[523,562]. This population of cells are generally typified by the surface phenotype CD11c^{hi}, CD21^{lo}, and their high expression of the transcription factor T-bet although a unified description has yet to be elucidated[562–564]. These cells are found in many situations of chronic antigenic exposure such as HCV and HIV, and correlate with autoimmunity, however, their precise function is not yet fully understood[458,459,563,564]. In this work, further comparisons where then made between FcRL4^{+} B cells in the periphery and those found in the synovial fluid of RA patients and in tonsils. These demonstrated significant differences in the percentage of ABCs which expressed FcRL4, with far fewer detected in the periphery than in the tonsil or RA synovial fluid; there were also significant differences in the levels of FcRL4 expressed in the ABCs detected in the periphery compared to those in the tonsil or RA synovial fluid with much lower levels of expression detected on peripheral ABCs. Furthermore, these data seem to follow the reported pattern in the literature of FcRL4^{+} B cells being linked to sites of chronic inflammation, and populations associated with chronic antigenic inflammation. These data also demonstrate a potential phenotypic reason why the peripheral FcRL4^{+} B cells do not carry IgA immune complexes/aggregates on their surface, in contrast to those in the mucosa and synovial fluid. Namely that there are far fewer of them in the periphery, and that they express far lower levels of FcRL4, meaning that they are unlikely to come into contact with IgA immune complexes and when they do, they are far less likely to be able to bind them.

The implications of FcRL4$^+$ B cells being found in the periphery of both healthy controls and RA patients are, firstly, that their presence is not disease-specific, despite commonalities with atypical FcRL4$^+$ B cells found in HCV and HIV. Thus, suggesting that FcRL4$^+$ B cells do not require the chronic antigenic stimulation associated with those diseases for their generation. If we were to hypothesise that the FcRL4$^+$ B cells were migrating from the mucosa to the joint, this evidence suggests that these cells are found in transit between the two, indicating that this migration could occur. Phenotyping studies could further investigate the homing molecules expressed on the surface of these peripheral FcRL4$^+$ B cells, MAdCAm, and $\alpha_4\beta_7$ or CD62L, CCR7, CCR10, and $\alpha_4\beta_1$ would suggest gut or lung homing respectively and upregulated chemokine receptors that these cells were homing to the inflamed joint. Previously published transcriptomic data by numerous groups, including ours, has demonstrated upregulated CCR1 and CCR5 in the RA SF and in the tonsil FcRL4$^+$ B cells, suggesting that they have migrated towards inflammatory mediators[474,518,565].

6.5 FcRL4 and the mucosa

The interactions between the mucosa and autoimmune disease are under a great deal of scrutiny in light of the growing recognition of the importance of the microbiome upon all aspects of human biology, particularly metabolism and immunity[232]. This work sought to investigate further the links between the lymphocytes of the joint and their reactivity to antigens derived from the gut. To achieve this a method was developed to sort bacteria from human stool samples using monoclonal antibodies generated from isolated single B cells from the joints of patients with active RA.

The BCR variable regions of these cells' genomes were then amplified, sequenced, and cloned into vectors to be expressed as monoclonal IgG antibodies. These antibodies were then directly labelled

and their reactivity against stool samples from healthy donors was tested. This work managed to demonstrate the feasibility of such an approach and demonstrate that mAbs generated from BCR sequences from B cells in the joints of RA patients can bind to bacteria present in the stool of healthy controls and RA patients.

This work was further developed in the various controls required and in the numbers of samples from stratified groups which need to be sequenced, to allow more robust conclusions to be made about differences in specificity between $FcRL4^+$ and $FcRL4^-$ B cells. Controls included isotype controls of an unrelated specificity such as infliximab, a chimeric IgG monoclonal, and sequencing of samples prior to sorting and, following sorting, sequencing of those bacteria labelled solely with anti-IgA, i.e. those bacteria which are already labelled with host antibody. This enabled the analysis of the composition of the total microbiome and the distinguishing of commensals or more pathogenic strains and allow for comparisons to be made between those bacteria labelled with the specific mAbs. Further controls not included in this work but of interest in future studies could also include IgA^+ and IgG^+ clones of unrelated sequences derived from the joints of RA patients to determine whether there is cross-reactivity between non-mucosa experienced RA joint-derived B cells and those expressing IgA, a majority of which will have presumably undergone affinity maturation at a mucosal site. In terms of patient populations stratifying patient stool samples, by sex, diet, and disease would provide a great deal of information about potential risks for RA or potential prophylactics. For example, whether adherents of a particular diet appear to have reduced incidences of RA. In terms of patient samples used in these experiments it might also be of interest to test bronchial lavage (BAL) samples, as one of the prime candidates of the initiation of the immune reaction which develops into RA is the lung, further reinforced by the epidemiological evidence implicating smoking[175,566]. This method would provide a powerful tool to interrogate the reactivity of RA joint-derived B cells against antigens from a variety of mucosal sites. It would also be possible to

undertake a computational approach based on the antibody structure, using the BCR sequences generated from the single cell sorting and avoiding the costly generation of monoclonal antibodies and staining of patient samples. However, positive results would require further validation to confirm their reactivity. If specific bacterial genera or species are flagged, then introducing these bacteria into mouse models with a genetic predisposition to develop RA.

6.6 Final Conclusions and Future Work

The experiments described in this thesis were complicated by the small size of the cell population investigated. For this reason, many compromises were made in population heterogeneity, breadth of staining panels, and level of functional experiments carried out.

The peripheral blood-, synovial fluid-, and tonsil- mononuclear cell staining experiments and the culturing experiments suggest that FcRL4 expression is limited to $CD19^+$ B cells. The culturing experiments further suggest that FcRL4 expression exists at a low level on a small stable population but may be transiently expressed by B cells and is affected by TLR9 agonism in conjunction with TGFβ. Understanding this process in finer detail will require pure sorted populations of $FcRL4^+$ and $FcRL4^-$ B cells from the various sites to be analysed for their proneness for apoptosis and dynamics of FcRL4 expression under the most promising conditions suggested by this work. Furthermore, in light of published results suggesting that the effect of TLR9 agonism in a mouse model of SLE is to diminish the autoantibody response, this could suggest that the $FcRL4^+$ population has a regulatory function *in vivo*[567].

The IgA interaction experiments demonstrate that FcRL4 is a receptor specific for IgA and that this activity occurs *ex vivo* in both the tonsil and the RA SF and hint that FcRL4 may display a preference

for IgA1. Further analysis of this is necessary, including binding tests using titrations of IgA1 and IgA2 aggregates by size, and investigation of the degree of aggregation of the myeloma derived IgA. Furthermore, methods of removing surface bound IgA which don't lead to high levels of background binding are also required. Using labelled IgA aggregates and incubating them in culture with the cells could potentially lead to an FcRL4$^+$ B cell specific uptake of labelled IgA aggregates. However, given the specificity for IgA demonstrated in the other experiments this might not be needed. Other than investigations into the downstream signaling of FcRL4, analysis of whether FcRL4$^+$ cells can internalise IgA ICs via FcRL4 would be of great interest given that B cells are highly efficient APCs. The potential of BCR cross-linking would also be interesting to assess at this point using IgA bound to BCR-specific antigen.

The over-representation of IgA BCRs at the protein and gene level in SFMCs FcRL4$^+$ cells and at the protein level in tonsil FcRL4$^+$ suggests that these cells may have a mucosal origin in common, this is in contrast to the peripheral FcRL4$^+$ cells which demonstrate no significant over-representation of IgA BCRs. This could potentially implicate different origins for these populations. However, this difference in ratio is difficult to interpret as there is little data available on the dynamics of this population's regulation or migration. To determine if it is possible for FcRL4$^+$ B cells to migrate from the mucosa to the joints and reveal the links between these populations, FcRL4$^+$ B cells could be sorted from paired samples from peripheral blood, gut, and joint biopsies and their BCR genes sequenced. Commonality would indicate a shared origin, until this information is available, the origin of RA SF FcRL4$^+$ B cells remains speculative.

In the bacterial sorting and sequencing experiments, more clones need to be tested against a variety of stratified patient samples using more controls. However, it is clear that there is a degree of reactivity from mAbs derived from RA SF and that certain bacteria are bound specifically.

Furthermore, once specific bacteria have been identified, transcriptomic approaches could allow for the identification of commonly expressed proteins between bacterial strains, potentially allowing for the identification of antigens. Once these antigens are identified further experiments could investigate structural homology to common disease-associated antigens. Using mouse models these antigens could then be assessed for their ability to induce disease via gavage or injection into the gut, investigating whether an immune response can spread from the MALT to the joints. Remaining at the species-level it would be interesting to see whether the presence of absence of specific bacteria could lead to disease, the generated antibodies could be utilised as treatments to deplete specific bacteria to investigate this.

7 Appendix

Figure	Patient Identifier	Sample Date	Diagnosis	Sex	Age at sample (years)	Age at onset of RA (years)	Disease duration (years)	Rheumatoid Factor	anti-CCP	CRP	ESR	DAS 28 ESR	DAS 28 CRP	TJC (28)	SJC (28)	VAS general health	Steroids or DMARDs	Other medication
12, 20, 23	T15.068	12/05/2015	RA	Male	54	54	0.25	<14	1	88	58	7.52	7.25	23	12	73	N/A	co-codamol, paracetamol
	T15.069	13/05/2015	RA + OA	Female	63	62	1	pos	pos	30	60	4.13	3.46	1	2	22	methotrexate, prednisolone	metformin , gliclazide, sitagliptin
	T15.074	04/06/2015	RA	Female	60	29	31	neg	pos	57	28	6.75	6.84	16	11	89	prednisolone	celecoxib, cocodamol, omeprazole
	T15.078	09/06/2015	RA	Female	60	57	3	pos	pos	122	104	5.88	5.32	2	4	91	sulfasalazine	Nil
	T15.083	14/07/2015	RA	Female	70	68	2	pos	neg	142	21	4.8	5.42	5	2	73	prednisolone	paracetamol
	T15.088	27/07/2015	RA	Female	77	68	9	NA	NA	71	62	NA	NA	NA		NA	Prednisolone 5 mg, Methotrexate 10 mg, Amlodipine, Etanercept 50 mg,	Folic Acid 5 mg, Calcium & vitamin D, Omeprazole, Ventolin inhaler, Doxazosin, Alendronic Acid 70 mg, Amitriptyline
	T15.051	30/03/2015	RA (1987 &2010)	Fish Oil, Vitamin D	72	63	9	24.7	>340	20	53	7.26	6.54	24	2	96	Methotrexate 15mg, Abatacept infusions	Folic Acid, Adcal D3
	T16.025	24/03/2016	RA	Female	60	51	9	NA	Pos	NA	45	13	5	6	NA	NA	Methotrexate	Ibuprofen, atenolol, tramadol , oxytetracycline
	T16.028	18/04/2016	RA	Male	68	56	12	19	19	15	NA	NA	NA	NA	NA	NA	Methotrexate	Folic Acid
	T16.053	29/06/2016	RA	Female	60	57	3	Pos	Pos	20	55	7.05	6.3	15	10	85		Naproxen, codeine 30mg co-codamol 8/500 2 tablets QDS
	T16.095	19/10/2016	RA	Male	58	57	1	71	>340	53	70	NA	NA	6	1	NA	Methotrexate, depomedrone 120mg on 02/09/2016	Allopurinol, Folic Acid
	T16.105	21/12/2016	RA	Male	62	48	14	46	8	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Methotrexate 17.5 mg weekly	

26	T16.089	27/07/2015	RA	Female	68	79	9	No results	No results	71	62	NA	NA	NA	NA	NA	Prednisolone 5 mg, Methotrexate 10 mg, Etanercept 50 mg,	Folic Acid 5 mg, Calcium & vitamin D, Omeprazole, Amlodipine, Ventolin inhaler, Doxazosin, Alendronic Acid 70 mg, Amitriptyline
	T17.005	27/01/2017	RA	Male	45	45	0	26	>340	13	37	NA	NA	NA	NA	NA		Paracetamol
	T17.084	17/10/2017	KR to check	Female	78	61	17	80	57	41	69	4.48	3.82	1	1	48	Methotrexate Injections, Enbrel injections	Folic acid, Co-codamol
	T17.085	20/10/2017	KR to check	Female	71	69	2	189.8	>340	16	54	6.09	5.27	5	7	93	Prednisolone	Paracetamol
	T17.088	31.10.2017	Unclassified Inflammatory Arthritis	Male	51	44	6 years	<11.0	1.6	16	5	1.46	2.32	0	0	24	None	Ramipril, Amlodipine, Citolopran, Co-dydromol
	T18.009	01/02/2018	RA	Female	74	72	2	122	NA	65	12	NA	NA	NA	NA			Codeine, Paracetamol, Colecalciferol, Carbonate, Alendronic Acid
	T13.152	27/06/2013	RA	Female	59	42	17	476	272	NA	NA	NA	NA	NA	NA		Leflunomide 20 mg,	OxyContin 90 mg, Thyroxine 75 mcg, Frusemide, Metoclopramide, Loratadine
	T13.164	16/07/2013	KR to check	Female	70	63	7	24.7	>340	56	NA	NA	7.09	19	12	90	Methotrexate	Folic acid, Co-codamol, alendronate, Calcichew
	T13.190	01/08/2013	RA	Male	56	41	15	661	188	7	17	NA	NA	NA	NA		Etanercept 50 mg, Methotrexate 15 mg, Hydroxychloroquine 200 mg,	Folic Acid 5 mg, Paracetamol, Dihydrocodeine
	T13.142	18/06/2013	RA	Female	37	27	10	140	91	17	34	NA	NA	NA	NA		Leflunomide 20 mg	

T13.233	13/11/2013	Sero pos RA	Male	54	54	0.75	583	>340	0	NA	NA	3.74	7	5	48	Methotrexate, Diclofanac, Prednisolone	
T13.235	18/11/2013	PsA	Female	48	46	2	417	>340	22	70	6.45	5.57	12	7	57	Methotrexate, Hydroxychloroquine, Ibuprofen	Folic Acid, Co-Codamol
T13.252	11/12/2013	RA	Male	60	59	1	61	61	21	30	NA	NA	NA	NA		Prednisolone 7.5 mg, Leflunomide 20 mg	Ramipril 10 mg, Amlodipine 5 mg, Ventolin Evohaler, Symbicort 20/6, Simvastatin 40, NovoMix 30, Tramadol 50 mg, Lansoprazole, Metformin 500, Codeine 30 /Paracetamol, Calcium and Vitamin D2,
T14.041	28/02/2014	RA	Female	49	48	1	48	>340	64	49	NA	NA	NA	NA		Hydroxychloroquine 200 mg, Methotrexate	Vitamin D, Ibuprofen, Omeprazole,
T18.004	24/01/2018	RA	Female	39	33	6	379	>340	4	7	NA	NA	NA	NA		Methotrexate 20 mg, Rituximab infusions,	Folic Acid 5 mg, Paracetamol,

	T17-033 RA	28/04/2017	RA	Female	81	74	7	<14	96	10	28	NA	NA	NA	NA			Calcium and Vitamin D, Gaviscon, Fybogel Orangem, Salbutamol inhaler, Symbicort inhaler, Carmellose eye drops, Paracetamol, Amlodipine
	T17-034 RA	05/05/2017	RA	Female	48	41	7	NA	261	12	12	NA	NA	NA	NA			Calcichew D3 Forte, Piroxicam, Paracetamol, Solpadol, Fluoxetine, Folic Acid, Ferrous Sulphate, Lansoprazole, Methotrexate, Folic acid
	T17-088 RA	31.10.2017	Unclassified Inflammatory Arthritis	Male	51	44	6 years	<11.0	1.6	16	5	1.46	2.32	0	0	24	None	Ramipril, Amlodipine, Citolopran, Co-dydromol
21	T17-095 RA	12/12/2017	RA	Female	62	56	6	<14	1	13	26	NA		NA	NA	NA	Methotrexate	Folic acid
	T17-098 RA	22/12/2017	RA	Female	62	61	1.5	127.3	28	114	107	6.04	5.44	4	7	65	Methotrexate, Naproxen	Folic Acid
	T17-093 RA	07/12/2017	JIA	Male	58	9	11	142.2	30	14	25	3.5	3.18	0	6	40	Methotrexate, Hydroxychloroquine, Prednisolone	Folic Acid
	T18-009 RA	01/02/2018	RA	Female	74	72	2	122	NA	65	12	NA	NA	NA	NA			Codeine, Paracetamol, Colecalciferol, Carbonate, Alendronic Acid
	T18-033 RA	26/04/2018	RA	Male	78	73	5	605	>340	258	35	NA	NA	NA	NA	NA	Etanercept, Methotrexate, Hydroxychloroquine	
	T18-092 RA	27/07/2018	Unclassified Inflammatory Arthritis	Female	47	44	4	<14	0.5	17	NA	NA	NA	NA	NA	NA	Methotrexate	Progestogen, Ibuprofen, Paracetamol, Oxybutynin,

33, 34, 35, 36,	T18-113 (RA SFMC)	06/09/2018	Psoriatic Arthritis	Male	43	40	3	<14	0.4	143	24	3.6	NA	1	1	38	Sulfasalazine	Omeprazole, Eucerin emollient, Ibuprofen
	T18-044 (RA) SFMC	21/05/2018	Seropositive RA	Male	74	57	17	24.6	>340	66	95	5.12	4.4	2	2	53	Etoricoxib 60mg, Leflunomide 10mg	Bisoprolol, Metformin, Gliclazide, Nicorandril, Pigolitazone
	T19-002 (RA) SFMC Female	09/01/2019	RA	Female	43	27	17	NA	>340	NA	NA	NA	NA	NA	NA	NA	Prednisolone	Iron supplements, Multivitamins
	T18-120 (RA) SFMCs	27/09/2018	RA	Female	86	74	11	NA	NA	NA	NA	NA	NA	NA	NA	NA	Methotrexate, Etanercept, Prednisolone,	Aspirin, Simvastatin, , Folic Acid, Ferrous Sulphate
	T19-005 (RA SFMCs) Female	15/01/2019	RA (1987 & 2010)	Female	75	63	12	247	>340	24	28	7.16	6.94	28	4	93	Methotrexate, Cimzia,	lansoperazole, Atorvastatin, Alendronate, Calcichew
	T18.100 (RA)	14/08/2018	Seropositive RA	Female	27	15	12	15.3	>340	2	12	3.53	3.15	6	0	30	Methotrexate 10mg	Folic Acid, Adcal D3, Paracetamol
	T18.090 (RA)	17/07/2018	Seropositive RA	Male	75	61	14	31.3	>340	1	NA	NA	1.81	0	0	43	Methotrexate 15 mg OW	Folic Acid, Adcal D3, Asprin, Atorvastatin, Quinine, Lactulose
	T17.025 (RA)																	
	T17.094 (JIA)	12/12/2017	JIA	Female	20	9	11	<11.0	0.7	6	16	3.17	2.89	1	1	28	None	Ibuprofen
	T18.107 (RA)	21/08/2018	RA (1987 & 2010)	Female	61	49	12	194	172	5	9	2.93	3	3	2	2	Methotrexate 25mg, Sulfasalazine	Folic Acid, Paracetamol, Vitamin D
	T18.044 (RA)**	21/05/2018	Seropositive RA	Male	74	57	17	24.6	>340	66	95	5.12	4.4	2	2	53	Etoricoxib 60mg, Leflunomide 10mg	Bisoprolol, Metformin, Gliclazide, Nicorandril, Pigolitazone
	T18-115 (RA)	11/09/2018	RA (1987 & 2010)	Male	59	49	10	456	>340	2	2	0.49	1.36	0	0	0	Methotrexate, Sulfasalazine Humira,	Folic Acid, Thyroxine

T18.112 (RA)	04/09/2018	RA (1987 & 2010)	Male	55	61	6	50.6	208	1	8	1.9	1.66	0	0	32	MTX, Hydroxychloriquine,	Folic Acid, Adcal D3
T18-138 (RA) Female	30/10/2018	RA (1987 & 2010)	Female	33	31	2	45.1	153	1	2	0.53	1.25	0	0	3	Methotrexate	Folic Acid, Loratidine, Mometasone, Calcium/ Vit D
T18-154 (RA) Male	18/12/2018	RA (1987 & 2010)	Male	60	NA	NA	503	>340.0	6	23	4.92	4.39	3	11	59	Sulfasalazine, prednosolone	Adcal D3, Lansoperazole, Indomethacin
T19-005 (RA) PBMC Female	15/01/2019	RA (1987 & 2010)	Female	75	63	12	247	>340	24	28	7.16	6.94	28	4	93	Methotrexate, Cimzia,	lansoperazole, Atorvastatin, Alendronate, Calcichew
T18-150 (RA) PBMC	27/11/2018	RA (1987 & 2010)	Female	41	36	5	127	158	8	36	2.51	1.75	0	0	0	Sulfasalzine	Folic Acid, Ferrous Fumarate
T18.124 (RA)	09/10/2018	RA (1987 & 2010)	Female	71	NA	10	<11.0	1.3	1	2	3.6	4.33	12	0	84	Methotrexate, tocilizumab	Apixaben, Bisoprolol, Atorvastin, Omeprazole, Adcal D3, Folic Acid, Co-codamol
T18.129 (HC)(	16/10/2018	RA (1987 & 2010)	Female	30	28	2	30.9	12	23	NA	NA	5.29	7	4	82	Methotrexate, Prednisolone	Folic Acid, Co-Codamol
T18-143 (HC) PBMC	13/11/2018	RA (1987 & 2010)	Female	57	51	6	827	108	1	5	2.01	2.09	1	1	3	Hydroxychlolroquin e	

T18.039 (HC/male		None	Male	32
T18.098 (HC)		None	Female	n/a
T18.116 (HC)		None	Female	54
T18.133 (HC)		None	Female	62
T18.139 (HC)		None	Female	54
T18.147 (HC)		None	Female	54
T18.097 (HC)		T1D	Female	61
T19-006 (HC) PBMC		None	Male	62
T19-007 (HC) PBMC Male		None	Male	62
T18-151 (HC) PBMC		None	Female	44
T18-125 HC PBMC		None	Female	54
S315341 30-08-18		Recurrent tonsilitis	Male	20
S317663 24-08-18		Recurrent tonsilitis	Male	43
S341674 04-09-18		Recurrent tonsilitis	Male	34
S304233 09-09-18		Recurrent tonsilitis	Female	19
S345942 10-09-18		Recurrent tonsilitis	Female	32
S304234 10-09-18		Recurrent tonsilitis	Female	24
S340673 10-10-18		Recurrent tonsilitis	Female	35
S315341 13-09-18		Recurrent tonsilitis	Male	20
S346989 02-10-18		Quinsy/peritonsillar abscess infection	Male	19
S321308 24-10-18		Recurrent tonsilitis	Female	25
S331250 15-11-18		Recurrent tonsilitis	Female	42
S340673 25-11-18		Recurrent tonsilitis	Female	41
S326465 10-01-19		Recurrent sore throats	Male	19
S321769 14-01-18		Recurrent tonsilitis	Female	20

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