Regulation of alpha 2,6-sialylation in B-cells and the role of sialylation in the pathogenesis of Rheumatoid Arthritis

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Sialylation is a common post-translational modification involving the addition of sialic acid to glycoprotein chains. Sialic acid within the Fc fragment of IgG molecules can influence binding to Fc receptors. In rheumatoid arthritis (RA) and other autoimmune conditions, in disease specific auto-antibodies, Fc fragment sialylation is reduced compared to total IgG. Furthermore, plasmablasts from patients with RA display reduced cell surface sialylation compared to cells from healthy donors. Factors which determine B-cell surface sialylation and consequences of altered sialylation are not well understood. α2,6-sialylation was measured in B-cells isolated from healthy donors (HD), patients with pre-RA (PRA) or early RA (ERA) using SNA lectin flow cytometry. Sialylation and markers of activation were measured at baseline or following stimulation with TLR ligands or anti-IgM/G ± CD40L; treatment with neuraminidase (Neu) to digest sialic acid; or culture with serum from HD or patients with ERA. B-cells were differentiated to plasma cells *in vitro* and sialylation measured at each stage of differentiation. Furthermore, Neu activity in serum was measured by fluorescent assay. Sialylation was confirmed to be decreased in patients with ERA and PRA at baseline compared with HD B-cells. Upon stimulation with TLR ligands, sialylation was increased in HD cells but not cells from patients with ERA or PRA. Differentiated cells showed an initial increase in sialylation before decreasing in terminally differentiated cells. Exposure to serum in culture led to reduced B-cell sialylation and Neu activity was highest in serum from patients with ERA. Exposure to serum in culture as well as direct treatment with Neu led to reduced B-cell activation potential. These results suggest that B-cell sialylation influences activation and function, and control of surface sialylation may be disrupted in RA.

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Abbreviations

ACPA	Anti-citrullinated Peptide Antibodies
AHR	Aryl Hydrocarbon Receptor
AID	Activation Induced Deaminase
Anti-CarP	Anti-Carbamylated Protein Antibodies
APCs	Antigen Presenting Cells
AREG	Amphiregulin
ASCs	Antibody Secreting Cells
BCL-6	B-cell Lymphoma 6 Protein
BCR	B-cell Receptor
bDMARDs	Biological Disease Modifying Anti-Rheumatic Drugs
bMAA	Biotinylated Maackia Amurensis
BSA	Bovine Serum Albumin
bSNA	Biotinylated SNA
CDR3	Complimetarity-determining Region 3
CMP	Cytposine 5-monophosphate
CpG	Cytidine-phosphate-guanosine
CSR	Class Switch Recombination
CTLA-4	Cytotoxic T-lymphocyte-associated Protein 4
DAS28	Disease Activity Score
DCs	Dendritic Cells
dsDNA	Double-stranded DNA

ELS	Ectopic Lymphoid Structures
ERA	Early Rheumatoid Arthritis
ESR	Erythrocyte Sedimentation Rate
F(ab')2	F(ab')2 Anti-IgM/IgG Antibodies
Fab	Fragment of Antibody Binding
FBS	Foetal Bovine Serum
Fc	Fragment Crystallisable
FcγRs	Fc gamma Receptors
fDCs	Follicular Dendritic Cells
FLS	Fibroblast-like Synoviocytes
fSNA	SNA-fluorescein
GC	Germinal Centre
GM-CSF	Granulocyte-monocyte Colony-stimulating Factor
HD	Healthy Donors
HEV	High Endothelial Venules
HLA	Human Leukocyte Antigen
ICOS	Inducible Co-stimulator
ICOSL	Inducible Co-stimulator Ligand
ICs	Immune Complexes
IFN	Interferon
Ig	Immunoglobulin
IgH	Immunoglobulin Heavy Chain

IgL	Immunoglobulin Light Chain
IL	Interleukin
INFγ	Interferon-gamma
ITIM	Immunoreceptor Tyrosine Based Inhibitory Motif
IVIG	Intravenous Immunoglobulin
L-ERA	Later Early Rheumatoid Arthritis
LPS	Lipopolysaccharide
MAA	Maackia Amurensis
ManNAc	N-Acetyl-D-mannosamine
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MMPs	Matrix Metalloproteinases
N-ERA	New Early Rheumatoid Arthritis
NET	Neutrophil Extracellular Trap
Neu	Neuraminidase
NEU1	Neuraminidase 1
Neu5Ac	N-acetyl Neuraminic Acid
NRA	Newly Diagnosed Rheumatoid Arthritis
ODN	Oligodeoxynucleotides
PAD	Peptidylarginine Deiminase
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline

PRA	Pre-Rheumatoid Arthritis
RA	Rheumatoid Arthritis
RAG	Recombination Activation Gene
RANKL	Receptor activator of nuclear factor kappa-B ligand
RF	Rheumatoid Factor
ROS	Reactive Oxygen Species
RT	Reverse Transcription
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
SA	Sialic Acid
SE	Shared Epitope
SHM	Somatic Hypermutation
Siglecs	Sialic Acid Binding Immunoglobulin-like Lectins
SLE	Systemic Lupus Erythematosus
SNA	Sambucus Nigra
SOCS	Suppresors of Cytokine Signalling
ST6Gal1	Beta-galactoside Alpha 2 6 sialyltransferase 1
TD	T-cell Dependent
TdT	Terminal Deoxynucleotidyl Transferase
Tfh	T-follicular Helper Cells
TGF-β	Transforming Growth Factor Beta
Th	T-helper
TI	T-cell Independent

TLR	Toll-like Recptor
TNF	Tumour Necrosis Factor
Treg	Regulatory T-cells

Chapter 1
General Introduction

1.1 Rheumatoid Arthritis Pathogenesis

1.1.1 General Background

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease which comprises chronic, usually symmetrical inflammation in the joints, and leads to destruction of synovial tissue and underlying bone erosion. It has a prevalence of around 0.5 - 1% in the majority of North American and European populations studied[1], with women affected around three times more often than men[2]. If joint inflammation is not effectively controlled, it can result in pain, loss of function in affected joints and associated severe disability[3]. The global burden of RA - measured by disability-adjusted life years - has increased over the last two decades despite earlier diagnosis and more effective treatment strategies. This is likely attributable to an increase in ageing populations[4,5]. Nevertheless, this highlights the continued importance of preventing disease progression. Biologic disease modifying anti-rheumatic drugs (bDMARDs), introduced over 20 years ago, are highly effective at suppressing inflammation. These treatments have revolutionised outcomes in RA[6]. However, response is not universal, and a trial and error approach to choice of therapy means a clinically meaningful proportion of patients require cycling through several drugs and develop poorer outcomes[7]. Although drug free remission may be achievable in a proportion, there are no clear therapeutic approaches which permanently halt disease progression, nor are there any effective primary preventative measures, emphasising the need for continued research into events during the onset of disease, with a view to informing future drug targets.

1.1.2 Clinical Features of Rheumatoid Arthritis

Clinical features of RA include pain and swelling in the joints, as well as extra-articular features which can affect the skin, eyes, lungs, gastrointestinal tract and vascular system[8]. Importantly, patients with RA have around a 1.5 - 2 fold

increased risk of developing cardiovascular pathology, increasing mortality in patients with RA compared with the general population[9]. The increased risk of cardiovascular pathology is thought to be linked to chronic, systemic inflammation increasing the risk of atherosclerosis[10].

The clinical presentation of RA can be widely variable between patients. Patients typically present with pain and swelling in the joints which is usually symmetrical. Inflammation commonly occurs in the proximal joints of the hands and feet, as well as the wrists and knees. Some patients may also present with other symptoms such as palindromic onset inflammation or mono/oligo large joint articular inflammation. In addition, more constitutional symptoms such as malaise and fatigue are common[11].

A combination of factors, including laboratory parameters and clinical observations as well as the overall health of the patient, aid in the diagnosis of RA and in judging disease severity[12]. Disease activity is often measured using the Disease Activity Score 28 (DAS28) which takes into account swelling across 28 joints, measures of systemic inflammation including C-reactive protein (CRP), Erythrocyte Sedimentation Rate (ESR) and the patient's general health, which is reported via a questionnaire completed by the patient[12]. Response to treatment may be measured using the European League Against Rheumatism (EULAR) response criteria which takes into account the change in DAS28 following treatment and overall disease activity state, to determine if a patient is a good, moderate or non-responder to a particular therapy[13]. In treating RA, the aim is to achieve a period of sustained remission or at least low diseases activity, where the patient is relatively asymptomatic and measures of disease activity such as inflammatory markers are low. Clinicians adopt a "treat to target" approach where a target disease activity state (remission or low disease activity) is set for each patient and drug treatment is escalated, and patients are switched to new therapies if a sufficient decrease in disease activity (and the target disease activity) is achieved[14].

1.1.3 Environmental Factors Which Contribute to the Development of Rheumatoid Arthritis

The aetiology of RA is complex and multifactorial. It is widely thought that several environmental factors may contribute to disease initiation in combination with

genetic factors. The most widely studied environmental factors thought to contribute to RA aetiology are smoking and periodontal disease. Smoking is a well-established risk factor for the development of RA, with the risk increasing by 2 fold in male smokers vs non-smokers, and by 1.3 in female smokers[15].

There are a number of mechanisms by which smoking may influence RA pathogenesis, including the induction of oxidative stress, apoptosis, inflammation, production of autoantibodies and epigenetic changes[16].

Studies have shown that smoking can increase expression of citrullinated peptides in the lungs, which is associated with an increase in expression of peptidylarginine deiminase (PAD) 2, which catalyses the conversion of arginine to citrulline[17]. Anti-citrullinated peptide antibodies (ACPA) can be detected in around 60-80% of patients with RA[18] and are thought to play an important role in the onset and prognosis of the disease. Smoking has been associated with autoantibody positivity in RA, however a recent study suggested that smoking is not specifically associated with the development of ACPA, but rather with the development of multiple autoantibodies, including an antibody to the Fc fragment of IgG – rheumatoid factor (RF)[19].

It has also been shown that exposure to smoke during the development of disease worsens inflammation by promoting T-helper (Th) 17 cell differentiation via the transcription factor aryl hydrocarbon receptor (AHR). The ligand for AHR, polycyclic aromatic hydrocarbons, are common environmental pollutants which can also be found in cigarette smoke[20]. Despite this, smoking was not found to be associated with the development of RA in RF^- $ACPA^-$ patients[21], and no association has been found between ACPA and smoking in RF^- patients[22].

Periodontal disease has also been shown to have a strong link to RA. The diseases have a similar pathogenesis, which includes inflammation in response to citrullinated peptides, driven by the production of inflammatory cytokines including tumour necrosis factor (TNF) and Interleukin – (IL) 6. The majority of periodontal disease is caused by a combination of 3 bacteria, which multiply in healthy tissue and outcompete healthy oral bacteria. One of the bacterial species which leads to disease, *porphorymonas gingivalis* has been found to express porphorymonas gingivalis peptidylarginine deiminase (PPAD). This bacterial

PAD leads to citrullination of peptides, which is thought to lead to the production of autoantibodies and destruction of tissues within the oral cavity[23]. The prevalence of RA in patients with periodontitis is higher than in healthy individuals, and vice versa. It has been suggested that generation of a response to citrullinated peptides in oral tissues may lead to the production of ACPA which contributes to joint destruction in RA[24].

There is also evidence to suggest that in the gut of some patients with RA there is an increase in citrullinated peptides which may trigger the generation of ACPAs[25].This, in combination with the strong body of evidence which supports the link between RA and periodontitis, as well as the increase in citrullination in the lung tissue induced by damage caused by smoking, suggest that increased citrullination at one or more mucosal site may lead to the initial development of autoimmunity and the presence of APCAs, long before the onset of symptomatic joint inflammation and clinical presentation. It is thought that the development of ACPA due to increased citrullination at mucosal sites may represent the first hit of the “two-hit” hypothesis for the aetiology of RA, however, the second hit which leads to the development of joint inflammation after the appearance of ACPA is not yet well understood[24]. Intestinal dysbiosis has also been suggested as a potential mechanism for triggering the onset of RA, however the exact mechanism of this is unclear[26].

1.1.4 Genetic Factors Which Contribute to the Development of Rheumatoid Arthritis

There are several genetic factors which may contribute to the development of RA. Heritability is estimated to be around 60% based on twin studies[27,28]. However, a more recent study found that although the risk of developing disease was higher in those with an affected first degree relative, environmental triggers and/or epigenetic events may play a more significant role[29]. Association with class II human leukocyte antigen (HLA) is thought to account for around 11% of overall genetic susceptibility[30]. Certain HLA-DRB1 alleles which contain the shared epitope (SE), a common 5 amino acid sequence at positions 70-74, are thought to convey predisposition to ACPA positive RA[31]. The SE has been linked to the development of ACPA, as HLA-DRB1 alleles with the shared epitope have a higher affinity for citrullinated peptides[32]. This leads to the strong presentation

of citrullinated peptides to T-cells, which can then stimulate production of ACPAs via co-stimulation of autoreactive B-cells. ACPA can be detected in the blood long before the onset of disease, and can be present in many individuals who never go on to develop RA. It is thought that the presence of the shared epitope may contribute to the development of RA in $ACPA^+$ individuals. There is also a strong risk of developing RA associated with smoking and presence of the SE[21].

There have been a number of single nucleotide polymorphisms which have been reported to be associated with RA. Of these, PTPN22 R620W - a gain of function missense variant - is the most widely reported[33], and it has been shown to be associated with RA risk in Caucasian but not Asian populations[34]. PTPN22 encodes a protein tyrosine phosphatase which is a negative regulator of T and B-cell receptor signalling. The variant allele associated with RA leads to decreased responsiveness to antigen stimulation[35], and impaired induction of PTPN22 leads to increased PAD activity and hyper-citrullination of PBMCs[36]. PTPN22 R620W has also been associated with impaired regulatory functions of Treg cells[37], and decreased signalling via toll-like receptor (TLR) 7 in plasmacytoid dendritic cells[38]. Taken together this evidence suggests that the PTPN22 R620W variant may contribute to several stages of the RA disease pathogenesis. Genome wide association studies have identified several other gene loci which may also be associated with RA pathogenesis, including IL-23R, PADI4, TRAF1, CTLA-4, IRF5 and numerous others[30].

1.1.5 Contribution of innate immune cells to pathology in Rheumatoid Arthritis

The precise causes of joint-specific inflammation in RA, are not yet well understood, however the process of inflammation and joint destruction has been well described. Once inflammation is triggered in the joint, cells of both the innate and adaptive immune system begin to infiltrate the joint tissues, driven by chemokine gradients produced by cells in inflamed tissues. Innate immune cells such as monocytes/macrophages, neutrophils and dendritic cells play a key role in orchestrating and propagating chronic autoimmune inflammation within the joints, by producing factors which promote inflammation and activate cells of the adaptive immune system to drive autoimmunity.

1.1.5.1 Fibroblast-like Synoviocytes

The intimal lining layer of a healthy synovium is made up of 2-3 layers of specialised fibroblast-like synoviocytes (FLS) and synovial macrophage-like synovial cells[39]. During inflammation, the sublining layer is thickened and is mainly populated by invading T and B-lymphocytes. The intimal lining layer increases in thickness to around 10-20 cells. The macrophage-like cells become highly activated and produce numerous inflammatory mediators, which can in turn activate FLS. Activated FLS produce their own combination of inflammatory mediators – mainly IL-6 and matrix metalloproteinases (MMPs), perpetuating synovial inflammation and recruiting more inflammatory cells to the joint[40]. A section of invasive synovial tissue called the pannus is formed, made up of FLS, macrophages and neutrophils. At the pannus/cartilage interface, osteoclasts become activated and begin to resorb bone, while FLS produce MMPs which begin to break down cartilage[41] (Figure 1.1).

1.1.5.2 Neutrophils

As well as having potent cytotoxic activity, neutrophils are thought to play an important role in orchestrating synovial inflammation in RA. When activated they can release an array of molecules which lead to damage in synovial tissues including reactive oxygen species (ROS), cytokines and chemokines as well as granule proteins, which can activate other immune cells[41]. They are also able to act as antigen presenting cells, leading to the activation of T-cells in the joints[42]. Neutrophils isolated from the blood of patients with RA have a more active phenotype than cells from healthy donors, and they are primed to produce ROS[43]. In the RA synovium, neutrophils can be activated by immune complexes (ICs) formed of autoantibodies, which bind Fc receptors on the neutrophil surface and trigger degranulation, releasing granule proteins and promoting cartilage destruction[41].

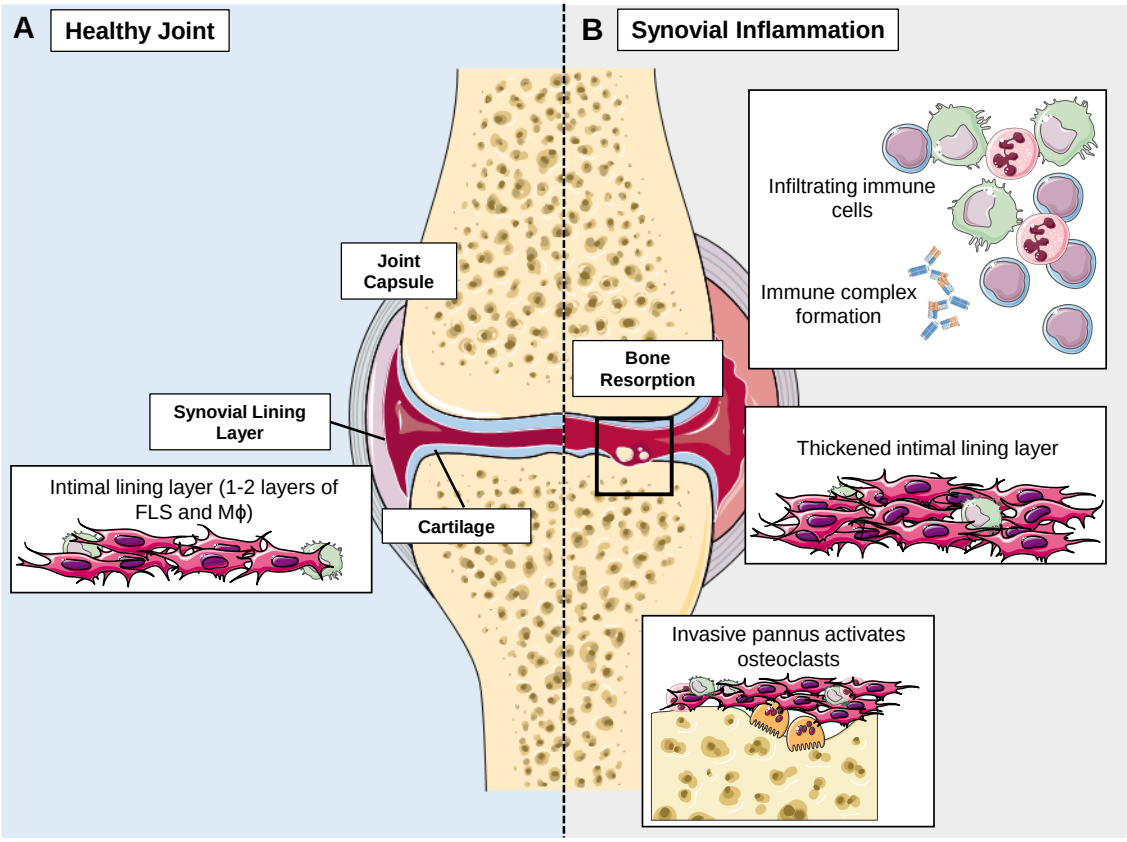


Figure 1.1 Synovial inflammation in Rheumatoid Arthritis

(A) In a healthy, non-inflamed joint, the joint capsule is protected by the synovial lining layer. The intimal lining layer is made up of 1-2 rows of FLS and macrophage-like synoviocytes, which produce synovial fluid to lubricate the joint. The surface of the bone is protected by articular cartilage and there is a balance between bone resorption and bone building to maintain homeostasis. **(B)** In the inflamed joint, the intimal lining layer becomes thickened, and activated immune cells infiltrate the joint space. These can produce inflammatory mediators which active FLS and macrophage-like synoviocytes and lead to the formation of invasive pannus tissue. Cells in the pannus stimulate osteoclast differentiation and activation, leading to destruction of the cartilage and bone. This figure was created using templates from Servier Medical Art which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

1.1.5.3 Dendritic cells

One of the main functions of dendritic cells (DCs) is to act as antigen presenting cells (APCs), therefore, in RA they may be involved in presenting self-peptides to adaptive immune cells such as T and B-cells in the lymph nodes and promoting autoimmunity[44]. Additionally, DCs produce cytokines, and in RA they have been shown to produce increased levels of IL-6 and IL-23, which can promote inflammation and promote T-cell differentiation to highly inflammatory Th17 cells respectively[45]. DCs in RA have also been shown to produce more CXCL8, which may promote leukocyte migration into the inflamed synovium, propagating inflammation[46].

1.1.5.4 Monocytes and macrophages

Circulating monocytes in RA may be recruited to the synovium by chemokines produced by FLS[47]. Monocytes in RA are skewed towards the intermediate phenotype, with upregulated expression of CD14 and CD16. These intermediate monocytes produce inflammatory cytokines such as TNF, IL-1 and IL-6 within the synovium in RA, and are primed to differentiate to highly inflammatory M1 macrophages. M1 macrophages also produce high levels of TNF, IL-1, IL-6, IL-23 and ROS, which can drive the inflammatory response within the synovium[48]. Although both infiltrating monocytes and tissue resident macrophages are thought to be able to play a role in promoting synovial inflammation, treatment with anti-TNF therapy has been shown to rapidly reduce infiltrating monocytes, suggesting that they may play a more critical role in driving synovitis[49]. Circulating monocytes and tissue resident macrophages are also the precursors to osteoclasts. IL-17 produced by Th17 cells can promote upregulation of receptor activator of nuclear factor kappa-B ligand (RANKL) and differentiation to osteoclasts, which promote bone resorption in the inflamed joint[50]. Osteoclasts can bind to the surface of the bone and release factors which dissolve calcium and break down the bone matrix. Bone erosions are a common radiographic feature of RA, in which sections of cortical and adjacent trabecular bone are lost in joints affected by synovitis. The presence of bone erosions in RA indicates the severity of disease and is a predictor of poor functional prognosis[51].

1.1.6 Role of T-helper cells in the pathogenesis of rheumatoid arthritis

$CD4^+$ Th cells are thought to play an important role in driving inflammation in RA. The association between RA and HLA-DRB1 is such that HLA-DRB1 major histocompatibility complex (MHC) class II molecules with the SE have a higher affinity for citrullinated peptides[32], leading to the presentation of these peptides and activation of autoreactive T-cells. Activated T-cells then provide co-stimulation for autoreactive B-cells with a cognate receptor, thereby leading to the production of autoantibodies to citrullinated peptides. Th cells in the RA synovium have been found to express characteristics of both Th1 and Th2 phenotypes, producing Interferon-gamma (INFγ), and IL-4, IL-5 and IL-13 respectively[52]. However a large proportion of T-cells in the inflamed synovium are though to adopt a Th17 cell phenotype[53]. Large infiltrates of T and B-cells as well as formation of tertiary lymphoid structures are a common feature of the inflamed synovial joint in RA. Establishing tertiary lymphoid structures induces local production of autoantibodies, propagating inflammation in surrounding tissues[54]. A role for $CD8^+$ cytotoxic T-cells in promoting inflammation in RA has been suggested, but is not yet well understood[55].

Regulatory T-cells (Treg) generally promote tolerance to autoantigens, by suppressing activation of Th cells by production of IL-10 and expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), an inhibitory co-receptor for Th cells[56]. However, in RA this mechanism of tolerance may be disrupted. It is thought that high levels of TNF in the RA synovium can block the suppressive action of Tregs[57]. Further to this, differentiation to Tregs is initiated in the periphery by transforming growth factor beta (TGF-β), however, if IL-1β and IL-6 are also present cells differentiate to inflammatory Th17 cells. It has been suggested that the balance between Treg and Th17 may be shifted in favour of Th17 cells in RA, due to higher production of IL-1β and IL-6[56]. It has also been reported that effector cells in RA may be less susceptible to inhibition mediated by Tregs[58].

1.1.7 IL-17 and Th17 cells in RA

In recent times, Th17 cells have been reported to play an important role in a number of autoimmune diseases. Th17 cells are potent inducers of inflammation, and in autoimmune disease, they drive the recruitment of inflammatory cells to diseased tissues and promote tissue inflammation[59]. In RA, Th17 cells have been found in the blood of patients with RA[60], and cytokines associated with Th17 cells have been found to be increased in the serum of patients with RA[61]. Production of Th17-associated cytokines in RA has been associated with inducing the production of inflammatory cytokines in synovial fibroblasts[62], stimulating production of MMPs[60] and the activation of osteoclasts[61]. All of these features can promote inflammation and destruction of tissues within affected joints in RA.

1.1.8 Role of Cytokines in RA

The important role played cytokine networks in driving the pathogenesis in RA is demonstrated by the clinical successes of targeted anti-cytokine therapies – in particular, those targeting TNF and IL-6. Anti-TNF therapy likely proves to be particularly effective at limiting inflammation in RA due to the wide-ranging effects of TNF. It is involved in numerous inflammatory processes, including leukocyte activation, adhesion and migration, chemokine expression, stromal cell activation and osteoclast function (via RANKL)[63]. Anti-IL-6 therapy has also shown great clinical benefits. IL-6 has similar functions to TNF in the synovium, as well as driving the acute phase response.

Several other cytokines have been shown to play a role in driving the pathogenesis of RA, but have had less success clinically, these include IL-1, interferon (IFN) and granulocyte-monocyte colony-stimulating factor (GM-CSF). IL-1 promotes inflammation in the joints by upregulating production of other cytokines, including TNF. Considering the lack of clinical efficacy of anti-IL-1 therapeutics, it is possible that the pro-inflammatory roles of IL-1 may be secondary to the effects of TNF[64,65].

IFN is widely known for its antiviral effects, however it has also been shown to be involved in various other immune pathways including activation of T-cells and dendritic cells; and in the upregulation of MHC[66]. In RA, patients have been shown to have upregulated expression of genes induced by Type I IFN[67] – known

as the IFN signature. However, IFN signature has not been found to be correlated with clinical parameters in RA[68]. Since a large proportion of the effects of type I IFNs are anti-inflammatory, IFNβ was trialled as a therapeutic agent in RA, but this trial was largely unsuccessful – showing no clinical benefit[69].

GM-CSF is able to mediate effector function in macrophages, neutrophils and DCs by promoting differentiation to inflammatory phenotypes of these cells, driving cytokine production and activation[63].The potential roles of GM-CSF in RA and its potential as a therapeutic target, are currently being explored[70,71], with initial results suggesting that GM-CSF inhibition may be of therapeutic benefit in RA[71].

1.2 B-cell Development and Immunoglobulin Production

1.2.1 General B-cell Development

B-cells play an important role in both the onset and dissemination of inflammation in RA. As well as producing inflammatory cytokines and potentially activating autoreactive T-cells, they can produce autoantibodies which can be detected long before the onset of symptomatic inflammation, and contribute to inflammation in the joints. B-cells originate in the bone marrow, where they undergo several stages of development before entering the circulation as transitional B-cells. Naïve transitional B-cells circulate between tissues and lymph nodes via the blood, and upon encountering their cognate antigen in the lymph nodes they can undergo differentiation to either long-lived memory B-cells or plasmablasts. Development of B-cells and their expansive repertoire of antibody specificity is a complex process which will be discussed in brief below (summarised in Figure 1.2).

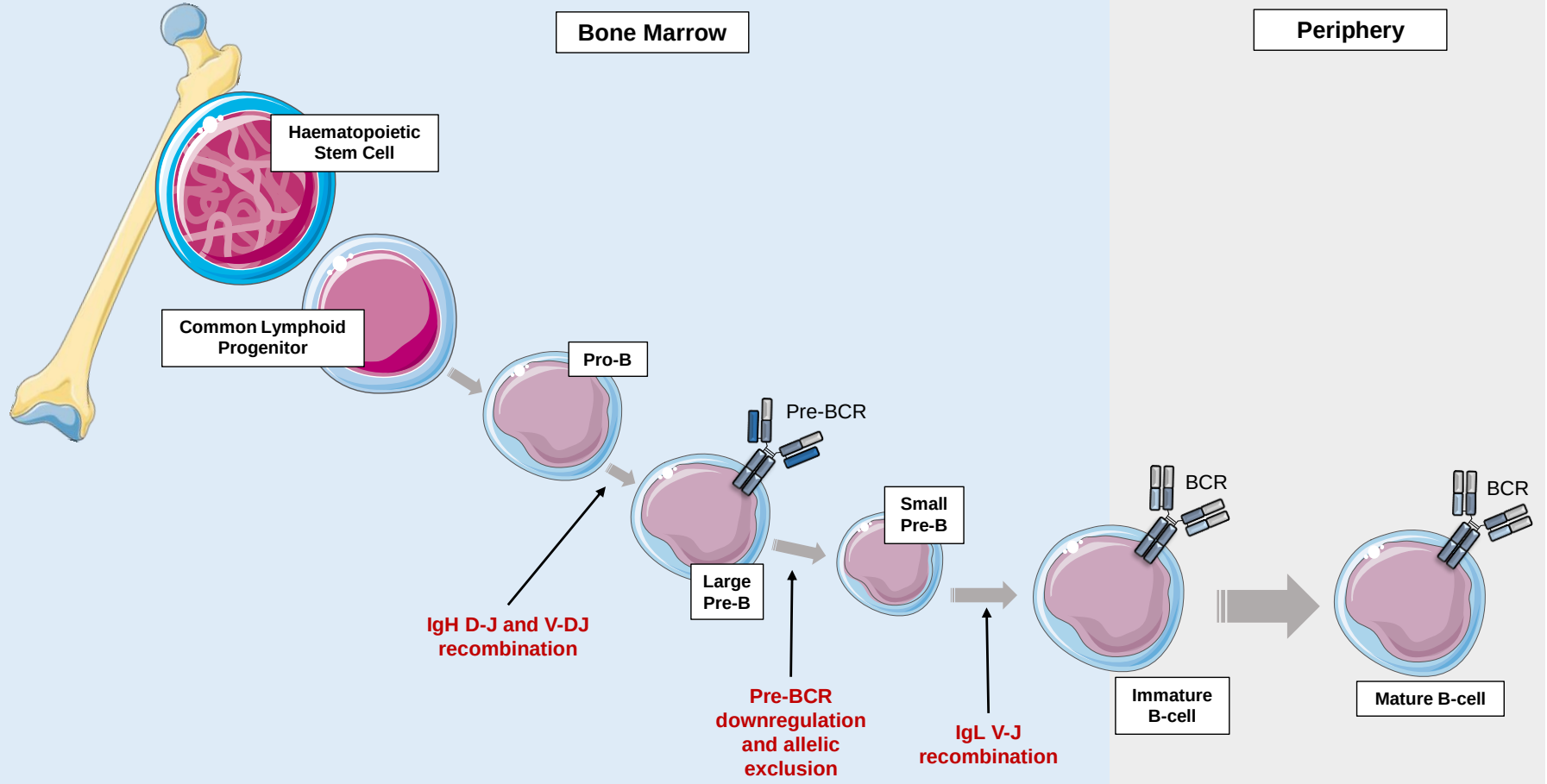


Figure 1.2 B-cell development pathway

B-cells are differentiated from haematopoietic stem cells in the bone marrow which become common lymphoid progenitor cells then are committed to B-cell lineage in the pro-B stage where cells express a number of B-cell lineage markers. IgH chains undergo recombination of V,D and J regions and successful rearrangements are displayed on the cell surface during the large pre-B-cell stage, associated with surrogate light chains as a pre-BCR. Pre-BCRs are downregulated, allelic exclusion occurs and during the small pre-B stage, IgL chains undergo recombination of V and J regions. Successful recombination leads to transcription and translation of complete IgM protein and display on the cell surface as the BCR on immature B-cells. This figure was created using templates from Servier Medical Art which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

1.2.2 Development of Pro-B-cells

B-cells begin life as multipotent stem cells in the bone marrow, in which the immunoglobulin (Ig) gene loci is in a germline configuration. Rearrangement of the gene loci, or recombination, is initiated by recombination activation gene (RAG) 1 and RAG2. These enzymes create breaks in the DNA strand within segments then the cleaved ends are joined by another set of proteins which repair the breaks in the strand – creating a new sequence[72]. In the common lymphoid progenitor stage, gene rearrangement is initiated in the immunoglobulin heavy chain locus (IgH) by RAG proteins.

First, one distal (D) and one joining (J) segment are brought together, with intervening DNA deleted. Following D to J recombination, one of the variable (V) gene segments is joined with the DJ unit, with remaining V and D segments which lie between being deleted. This gives rise to a rearranged VDJ exon. The constant (C) region of the IgH locus is separated from the VDJ region by distal J segments and a J-C intron. Following VDJ recombination, the IgH gene is transcribed, producing a primary transcript of the rearranged VDJ segment and Cμ exons. Through splicing, joining DNA and subsequent regions of Cμ are removed. If the mRNA produced shows a productive rearrangement of VDJ, the μ protein is translated and synthesised[73,74].

1.2.3 Pre-BCR expression and signalling

Cells with a productive Igμ gene rearrangement have differentiated from the pro-B stage to the pre-B stage and the Igμ protein is expressed on the cell surface in association with surrogate light chain proteins, and signalling subunits Igα and Igβ to form the pre-B-cell receptor (pre-BCR)[73]. Unlike the light chains of complete BCRs, surrogate light chains are germline encoded invariant proteins expressed in all pre-B-cells. Expression of the pre-BCR is an important checkpoint for B-cell development[75].

Signalling via the pre-BCR leads to downregulation of surrogate light chain expression and then termination of pre-BCR expression and initiation of immunoglobulin light chain (IgL) gene rearrangement. Pre-BCR signalling also

prevents VDJ recombination in the second IgH allele – to ensure only BCRs of the same specificity are expressed on each B-cell, a process called allelic exclusion[76].

1.2.4 Development of Immature B-cells

Following the pre-B-cell stage, IgL chains are rearranged (involving only V and J recombination), starting with the κ chain. If the κ chain is productively rearranged, the κ light chain protein is produced and associates with the Igμ chain to form a complete IgM protein. If the κ rearrangement is unproductive, then the λ chain is rearranged and this may also associate with the Igμ protein[77]. The completed IgM protein can then be expressed on the cell surface of immature B-cells. The immature B-cell stage is the first checkpoint for B-cell tolerance, as cells which express IgM which strongly bind self-peptides within the bone marrow undergo apoptosis or receptor editing[78]. Cells which do not respond to self-peptides are released from the bone marrow as naïve transitional B-cells[77].

1.2.5 B-cell maturation in the spleen

Immature cells which exit the bone marrow are first trafficked to the spleen where they encounter self-antigens. Cells which respond to self-antigens may undergo a further round of receptor editing, distinct from initial stages of BCR development[79]; they may also undergo apoptosis in some cases or be rendered anergic to prevent autoreactive cells from circulating. The process of selection and receptor editing is thought to be dependent on BCR signalling within the spleen[80]. Cells which exit the spleen following selection are now classed as mature naïve B-cells which are able to respond to antigen stimulation, and these cells circulate between tissues and lymph nodes.

1.2.6 Antibody Structure and Function

As discussed, mature B-cells express Ig protein on the cell surface in the form of the BCR. Mature B-cells can also produce a soluble form of the Ig molecule, for opsonisation of the target antigen. The most abundant class of antibody in the serum is immunoglobulin G (IgG) which is a glycoprotein molecule comprised of two identical paired heavy and light chains held together by disulphide bonds[81] (Figure 1.3). Each chain contains a variable domain and at least one constant domain. The light chains of IgG contain one constant domain, whereas the heavy

chains have 3 constant domains. As well as IgG, there are four other classes of Ig, IgM, IgA, IgE and IgG, which share the same basic structure with variations in the heavy chain constant region which determines their differing effector functions[82] (Ig isotype structure and function summarised in Figure 1.3).

The antigen binding regions of the IgG molecule are formed by the variable domains of each heavy and light chain. A high degree of variability in the antigen binding region can be achieved through VDJ rearrangement which occurs during B-cell development. Further mutations which increase specificity and affinity occur during germinal centre reactions following B-cell activation. The variable regions and one constant region of each chain form the fragment of antigen binding (Fab) and the remaining constant domains of the heavy chain form the fragment crystallisable (Fc)[82]. The function of Ig in serum is to bind to its cognate antigen. Antibody binding can lead to the formation of ICs or opsonisation of the surface of a particular cell or organism the antigen is expressed on. Immune complex formation on a cell or pathogen surface can lead to clearance by phagocytes, activation of the complement cascade or activation of an inflammatory response. The Fc region of the antibody molecule can bind a group of receptors called Fc gamma receptors (FcγRs) which are present on the surface of immune cells and can either promote or inhibit inflammatory activity[83].

There are several structural determinants of IgG which have been shown to be associated with autoimmunity, including enrichment of Ig heavy chain gene segment V4-34 (IGH4-34)[84], increased length of the complementarity-determining region 3 (CDR3) region in the heavy chain[85] and N-linked glycan glycosylation patterns, which will be discussed later in this chapter. Enriched IGH4-34 and lengthened CDR3 regions have also been associated with dominant B-cell clones found in synovial tissues in patients with RA[86].

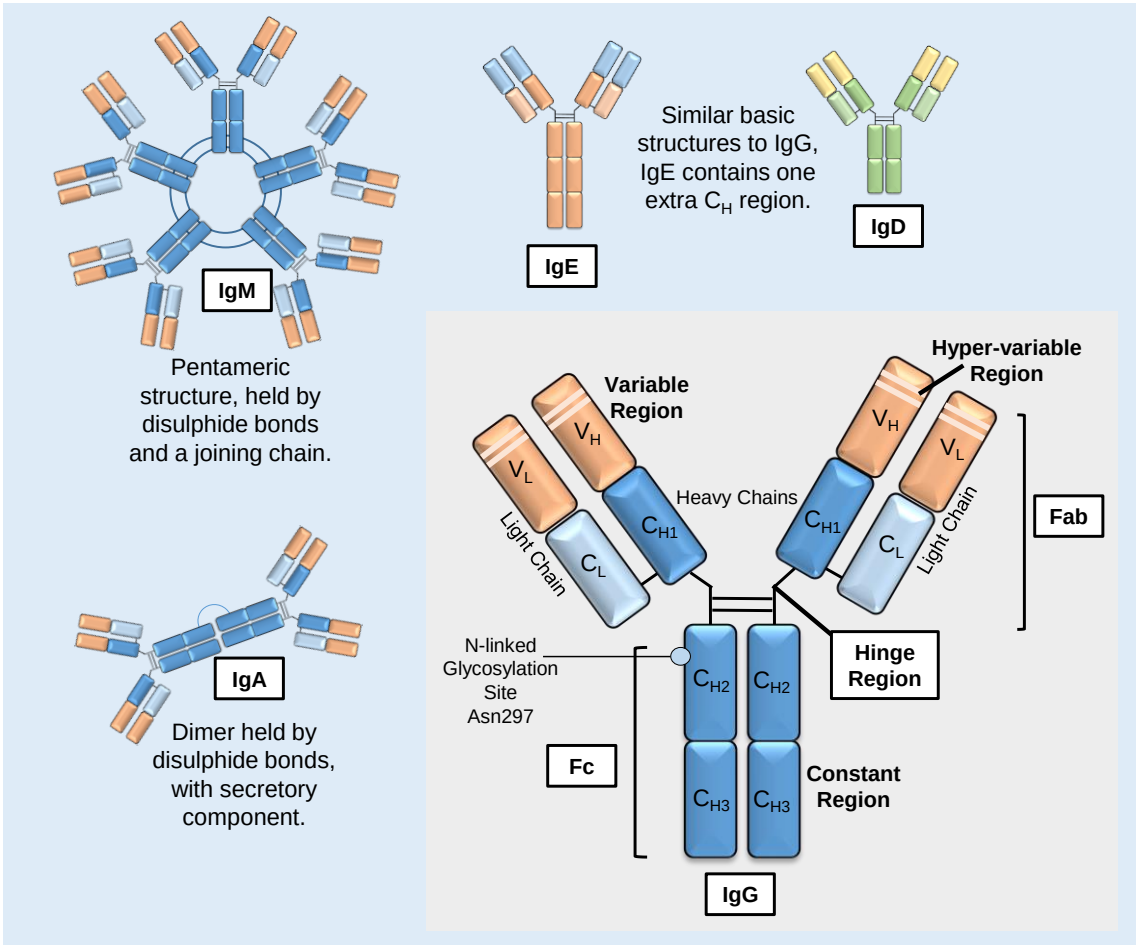


Figure 1.3 Structure of immunoglobulins

The basic structure of IgG, IgM, IgA, IgE and IgD is shown, with key features of IgG structure highlighted. The IgG molecule is a biantennary structure which is arranged in a “Y” formation and consists of four chains, two identical heavy and two light chains held together by disulphide bonds. The variable region forms the antigen binding domain and variability is achieved through rounds of somatic mutation. The Fc region binds IgG Fc receptors and determines its effector function. The glycan at Asn297 is an important determinant of Fc receptor binding.

1.3 B-cell activation and the Germinal Centre Reaction

1.3.1 B-cell Circulation

Mature-naïve B-cells which circulate between tissues and lymph nodes, driven by chemokine gradients, can be activated when they meet their cognate antigen in the lymph nodes. B-cells enter the lymph node via high endothelial venules (HEV) – highly specialised endothelium which allows the cells to pass into the lymph node via the circulatory system[87]. HEVs of the lymph nodes express CXCL13, which is essential for B-cell transport into the lymph nodes via its ligand CXCR5 which is expressed on the B-cell surface[88].

1.3.2 B-cell Activation in the Lymph Nodes

Whilst in the lymph nodes, B-cells may be activated by soluble antigen, or by antigen presented by dendritic cells. Lymph nodes are organised into B-cell follicles and T-cell zones. B-cell antigen capture occurs within the follicles, where antigen is displayed by dendritic cells. BCR engagement induces antigen uptake, processing and presentation via MHC II[89]. Activated B-cells upregulate CCR7 and downregulate CXCR5, which enables migration to the border of the T-cell zone, drawn by an increasing gradient of CCR7 ligands CCL19 and CCL21[90]. Within the T-cell zone, naïve T-cells are activated by antigen presented by dendritic cells via T-cell receptor engagement. Activated T-cells then upregulate CXCR5 and down-regulate CCR7, allowing them to migrate towards the follicle. Activated T-cells also upregulate expression of CD40L – a co-stimulatory molecule which binds CD40 expressed on B-cells[91].

In the T-cell zone a small amount of activated T cells differentiate to follicular helper T-cells (Tfh). In the early stages of Tfh differentiation, IL-6 produced by dendritic cells is key in upregulating the transcription factor B-cell lymphoma 6 protein (BCL-6), a major regulator of germinal centre formation and maintenance in B-cells. BCL-6 drives the upregulation of CXCR5 which allows Tfh cell migration into the follicle[92]. Inducible co-stimulator (ICOS) is also important for the polarisation of Tfh from naïve Th cells, and is induced by ICOS-ligand (ICOSL), expressed by B-cells at the border of the T-cell zone and follicle[93].

At the border between the follicle and the T-cell zone, activated T and B-cells meet. T-cells are able to recognise antigen presented by B-cell MHC II and the cells form an immunological synapse. During the T and B-cell interaction, CD40L expressed by T-cells binds CD40 on B-cells, stimulating B-cell activation and contributing to induction of B-cell proliferation, a small degree of isotype switching and differentiation to short-lived plasmablasts. A small number of B-cells which are activated by T-cells at the border of the follicle will also migrate back into the follicle to form a germinal centre (GC) reaction[94].

1.3.3 Germinal centre independent B-cell activation

B-cells which undergo a degree of isotype switching and differentiation to plasmablasts within the T-cell zone are thought to be important for the early stages of the humoral immune response. It is likely that these cells are short-lived and are not able to migrate to sites such as the bone marrow in order to become long-lived plasma cells[95]. There is also evidence that some B-cells may be able to directly differentiate into memory B-cells, foregoing the germinal centre reaction. In mouse models, these cells have been shown to be induced by strong CD40 stimuli, and express memory B-cell markers CD38 and GL7, however they do not express CD73 – a marker thought to be induced by upregulation of AID during somatic hypermutation[96]. In humans there is no clear marker which may delineate GC and non-GC memory B-cells, but they are thought to be within the $CD27^-$ memory cell population[97].

1.3.4 Germinal centre formation and maintenance

After the initial interaction with T-cells at the border of the follicle, a select few activated B-cells move towards the centre of the follicle and begin to rapidly divide. These cells form the basis of the densely packed dark zone of the germinal centre where cells undergo several rounds of mutations and division to improve antigen specificity. Surrounding the dark zone is the light zone, which comprises of blasts which have migrated from the dark zone, a network of follicular dendritic cells (fDCs), macrophages and Tfh cells[98].

The transcriptional repressor BCL-6 is essential for the induction of the germinal centre reaction and is expressed by both GC B-cells and Tfh cells[99]. MEF2B, and IRF4 are also upregulated in GC B-cells, and they are thought to play a role in

the induction of BCL-6[98]. BCL-6 is able to repress BLIMP-1, which is key for differentiation to plasmablasts, thus preventing GC B-cells from differentiating during proliferation in the dark zone[100].

1.3.5 Class switch recombination

During the germinal centre reaction, B-cells undergo rounds of somatic hypermutation (SHM) to increase the affinity of the BCR for antigen, and class switch recombination (CSR) to promote the production of the most appropriate class of Ig, dependent on the antigen. CSR is in part determined by T-cell cytokines in the lymph node, in response to a particular antigen. CD40 ligation in combination with IL-4 leads to the induction of activation induced deaminase (AID)[101]. Each Ig isotype is encoded for by a different exon cluster in the Ig H constant region locus C_H[102]. The sequence of the Ig C gene contains a switch region upstream of the isotype determining exon clusters. AID induces deamination of deoxycytosines to form deoxyuracils, forming U:G mispairings[103] in both the region of the current Ig isotype and the downstream desired Ig isotype. The processing of deoxyuracils leads to double stranded DNA breaks in both of these regions. The intervening DNA of the switch region is then deleted during transcription, bringing the downstream C_H isotype region adjacent to the VDJ region, resulting in the production of a new Ig isotype with the same V region as the original molecule[104].

1.3.6 Somatic hypermutation

Somatic hypermutation is the process of diversifying antibody variable regions. Single nucleotide point mutations are introduced into the Ig V regions in a stepwise manner, primarily driven by AID[105]. AID induces deamination of deoxycytodine to form deoxyuridine, forming U:G mispairings[103]. The mispairing can result in C to T or G to A transition mutations during DNA replication or the removal of uracil to create an abasic site - leading to further mutations during replication. Furthermore, the mispairing may be recognised by mismatch repair machinery Msh2/Msh6, triggering excision and re-synthesis of a section of DNA. This is a highly error prone process which therefore spreads the mutations to surrounding base pairs[106]. Following somatic hypermutation within the dark zone, cells will migrate to the light zone, where B-cells with the most favourable

mutations – i.e. increased antibody specificity - will be selected, either for further rounds of somatic mutation, or differentiation to plasmablasts or memory cells.

1.3.7 Selection following class switch recombination and somatic hypermutation.

When B-cells enter the light zone they encounter antigen displayed by fDCs. Antigen can then be taken up via the BCR and presented on the surface via MHC II. Efficiency of antigen presentation is dependent on the affinity of the BCR for the antigen. Efficient antigen presentation is crucial for strengthening and prolonging the interaction between B-cells and Tfh cells in the light zone, therefore those with the strongest BCR affinity, and most efficient antigen presentation will provoke the strongest interaction[98]. Signals provided via Tfh cells induce B-cell re-circulation into the dark zone, so they may undergo further rounds of mutation, and eventually commitment to plasma cell precursors or memory B-cells. B-cell interaction with Tfh cells promotes upregulation of CD40L, ICOSL, IL-4 and IL-21 in Tfh cells, which stimulate B-cells to migrate back into the dark zone, or differentiate depending on the nature and strength of the signal[93]. B-cells which bind antigen insufficiently, and therefore do not receive signals from Tfh cells undergo apoptosis. Cells which are selected positively may also undergo CSR before recirculating into the dark zone for further rounds of SHM, or they may directly differentiate into plasma cell precursors or memory B-cells[98].

1.3.8 B-cell differentiation to plasmablasts and memory cells

The fate of germinal centre B-cells is determined following selection of clones with the highest affinity for antigen after several rounds of mutation. Cells either differentiate to plasmablasts - the precursors to plasma cells, or to memory B-cells[94]. Long lived plasma cells survive in bone marrow niches and are able to produce large quantities of antibodies for several years following activation. Memory cells circulate between tissues and the blood and are primed to initiate a rapid recall antigen response, upon stimulation with their cognate antigen. In order for cells to become plasmablasts, they must downregulate expression of BCL-6 and Pax-5. Downregulation of BCL-6 allows for BLIMP-1 upregulation, which is key in plasmablasts differentiation[100]. IRF4, a transcriptional activator is also induced. Together IRF4 and BLIMP-1 induce expression of XBP1, which is

required in plasmablasts to account for their secretory ability[107]. It is thought that perhaps cells which express lower levels of IRF4 become long-lived memory B-cells and those with higher expression are able to differentiate to plasmablasts. Signals which may determine expression of IRF4 and control the fate of GC B-cells are not currently well understood[108].

1.3.9 Ectopic Lymphoid Structures

Although the majority of germinal centre reactions are confined to the lymph nodes, it has also been described that ectopic lymphoid structures (ELS) can form in the target tissues of a number of autoimmune diseases. These structures provide *in situ* class switching and affinity maturation, contributing to the production of autoreactive B-cells in the target tissues[54]. Within the lymph node Tfh cells drive formation of isotype switched, antigen specific B-cells, however a recent study has shown that this process is not directly dependent on Tfh cells specifically, and is most likely driven solely by CD40/CD154 (CD40L) signalling between B-cells and Th cells. This provides support for the notion that isotype switched B-cells may be generated in the periphery, outwith the Tfh-rich environment of the lymph node[109]. It was found that in RA, B-cells isolated from synovial tissues with ELS present contained IgV genes which targeted citrullinated neutrophil extracellular trap (NET) proteins. This suggests a preference for autoantigens in B-cells derived from ELS[110], which may be a contributing factor for the maintenance of autoimmune inflammation in the joints.

Studies of influenza infections in murine lungs have revealed that following infection, a subset of B-cells persist in the lungs – dubbed resident memory B-cells, which are thought to be generated by early antigen exposure in the lungs and these cells do not recirculate[111]. Further to this it has been described that persistence of germinal centres for influenza viruses in the lungs contributes to cross reactive immunity[112]. This may support the theory of antigen cross-reactivity caused by infection or damage due to smoking in the lung leading to the development and persistence of autoreactive B-cells.

1.4 Breakdown of B-cell Tolerance and Development of Autoimmunity in Rheumatoid Arthritis

1.4.1 B-cell Tolerance Checkpoints

There are several points at which B-cell tolerance is monitored, and where cells which react to self-peptides are deleted or undergo receptor editing, both centrally and in the periphery. A breakdown in these key tolerance mechanisms is an important factor in the development of autoimmunity, and is likely to play a role in the production of autoantibodies in RA.

Since the process of B-cell antibody repertoire generation is a result of random gene rearrangements, antibody specificities for “self” peptides are generated as a result. Therefore, to maintain tolerance and prevent autoimmunity, there has to be several “checkpoints”, during which self-reactive cells are filtered out, preventing them from initiating an autoimmune response.

The first checkpoint occurs centrally within the bone marrow after rearrangement of the V-D and J genes of Igμ and expression of the pre-BCR. Cells which have an antigen receptor specific for self-peptides either undergo apoptosis or receptor editing, depending on the strength of the signal. High affinity binding to self-antigen leads to receptor editing where RAG genes are reactivated and V and J regions are rearranged. If receptor editing fails and the cell still receives a signal from binding self-peptides then the cell undergoes apoptosis[77]. Lower affinity reactions lead to a weaker signal via the antigen receptor and induces anergy, by lowering expression of antigen receptor and blocking signalling. Cells which have been negatively selected through this process exit the bone marrow and traffic to the spleen, where they undergo a further tolerance checkpoint, where binding strongly to self-antigens can induce anergy or apoptosis[113]. Negatively selected mature naïve B-cells can then enter the blood and begin to circulate between tissues and lymph nodes. In the periphery, B-cell tolerance is thought to be dependent on CD40/CD40L interactions. It is also thought that Tregs play a role in maintaining peripheral tolerance, by downregulating T-cell activation in the periphery via co-inhibitory molecules and production of anti-inflammatory cytokines[114].

1.4.2 Breakdown of B-cell Tolerance in Rheumatoid Arthritis

There is some evidence that in patients with RA, there is a failure in tolerance checkpoints which allows autoreactive cells to escape detection and deletion, even prior to the induction of disease specific autoimmune responses[115]. It was found that in RA, autoreactive cells were not removed by tolerance checkpoints and that between 30 and 52% of mature B-cells displayed autoreactivity – compared with 20% in healthy individuals[116].

It is likely that the breakdown in RA of B-cell tolerance is linked to an inherent genetic defect rather than a consequence of inflammation, as it was shown that numbers of autoreactive B-cells did not change despite decreased inflammation following treatment with methotrexate and anti-TNF therapies[117].

Additionally, B-cells from patients with RA appear to have extended Igκ chains containing 11 or more amino acids in the CDR3 regions, which is thought to be related to the production of autoantibodies[116]. This is likely mediated by addition of non-template nucleotides by terminal deoxynucleotidyl transferase (TdT). TdT is downregulated by pre-BCR expression, so it is hypothesised that defective signalling in the pre-BCR in RA may lead to continued expression of TdT and the generation of longer amino acid sequences in CDR3[115,118].

1.4.3 Rheumatoid Factor

Several antibodies have been identified which are thought to play a role in RA pathogenesis. The first to be identified was rheumatoid factor (RF)[119], which is an autoantibody directed against the Fc portion of IgG[120] and is produced by B-cells in lymphoid follicles and GCs. Physiologically, IgM RF is transiently produced in response to B-cell activation by invading microbes. RF binds to IgG on the microbe surface, forming ICs and thereby increasing the rate of clearance by phagocytes[121]. There are several infectious agents which have been shown to be associated with RA, and it is possible that during infection, formation of ICs may trigger the production of RF[64]. The presence of RF in the serum has also been found to be strongly associated with smoking[19]. Unlike in healthy individuals, RF produced in RA has shown evidence of affinity maturation and class switching to IgG RF, potentially increasing the affinity for IgG[122]. In patients with RA, it is likely that formation of ICs in the synovial fluid and synovial tissue, when high affinity

RF binds to ACPA[120], worsens inflammation. IgM-RF bound to ACPA was shown to enhance the ability of ACPA to stimulate cytokine production in macrophages[123]. RF^+ B-cells also activate ACPA-specific T-cells by presenting peptides derived from antigen-Ig ICs[124].

1.4.4 Anti-Citrullinated Peptide Antibodies

ACPAs are another important autoantibody in RA, and they bind antigens containing the amino acid citrulline. Citrullination is a post-translational modification which converts arginine to citrulline, mediated by peptidyl-arginine deiminase enzymes[125]. Citrullination is a cellular response to stress and is induced in the lung through smoking and in the oral cavity by bacteria that cause periodontitis – key environmental factors associated with RA[125].

HLA-DRB1 alleles containing the SE convey particular susceptibility to $ACPA^+$ RA[126]. Major Histocompatibility Complex (MHC) class II molecules containing the SE bind citrullinated peptide antigens with a higher affinity than control HLA alleles[126]. Citrullinated antigens presented by APCs lead to the activation of autoreactive B and T-cells – and the production of ACPAs by plasma cells[127]. Citrullinated proteins have been found to be increased in the inflamed RA synovium and B-cells isolated from synovial tissue can produce ACPAs *in vitro*[128]. It has been hypothesised that HLA-SE may present joint-specific peptides - leading to an inflammatory response in the synovium[129].

Although there is an increase in the citrullination of peptides within the joints in RA, this is not exclusive to RA and there is also an increase in citrullination within non-RA inflamed joints. ACPA is highly specific for RA however, so it suggests an aberrant response to the presence of citrullinated peptides in RA[130]. ACPA can often be detected in the serum of patients with RA a median of around 5 years before the onset of clinically relevant joint inflammation[131]. A subset of $ACPA^+$ individuals may remain asymptomatic indefinitely. However, a proportion will develop general musculoskeletal symptoms or arthralgia – so-called Pre-RA, which may then progress to meet the criteria for diagnosis of RA. Around 30% of $ACPA^+$ individuals with Pre-RA go on to be diagnosed with RA within 12 months[132]. Factors which determine and drive the development of RA in $ACPA^+$ individuals are not well understood. Nor is it fully understood whether ACPAs

contribute to the onset of disease or if they are present as a consequence of early disease processes.

In established disease, ACPA antibodies may contribute to pathology in RA via a number of different pathways – via Fc receptor binding, complement activation or stimulation of NETosis within the joint [129]. ACPA deposition within the joints likely promotes synovial inflammation by activating immune cells to produce cytokines, which in turn activate and recruit further immune cells, propagating synovial inflammation[133]. ACPA undergoes limited avidity maturation which mostly takes place before the onset of disease, producing low avidity antibodies[134]. Further to this, patients with ACPAs of lower avidity have increased risk of joint destruction[129], suggesting this may also contribute to pathology.

1.4.5 Contribution of ACPA to bone loss in RA

Bone loss, and changes to bone metabolism can also be detected in otherwise asymptomatic individuals who are $ACPA^+$ without the presence of synovial inflammation, suggesting that ACPA-mediated bone loss is not a direct consequence of inflammation, but rather a direct consequence of $ACPA^-$ stimulated osteoclastogenesis[135,136].

It has been found that antibodies against citrullinated proteins are able to bind directly to osteoclasts, promoting bone resorptive activity[125]. Citrullination is required for the differentiation of osteoclasts, and specific PAD enzymes are upregulated at different stages during differentiation from monocytes, possibly facilitating ACPA binding[137]. It has also been shown recently that ACPAs are able to induce dendritic cell trans-differentiation to osteoclasts, mediated by IL-8, further driving bone destruction in $ACPA^+$ individuals[138].

Results published in 2016 suggested that ACPA molecules can also enhance osteoclast differentiation via IL-8-mediated upregulation of PADs[137]. It was also reported that ACPA may contribute to the induction of pain in the joints in RA independent of inflammation, via osteoclast production of CXCL1 and IL-8 which may target nociceptors in the joint[139]. These results must be interpreted with caution however, as the authors later reported their antibodies were not specific for citrullinated peptides[140,141]. Despite this, there may yet be a further mechanism of antibody mediated osteoclastogenesis, independent to recognition of

citrullinated targets. Independent to Fc receptor binding, Fab fragments of the antibodies used experimentally were also able to induce osteoclast activation[137].

Further to this, one study which examined specific characteristics of autoreactive B-cells in RA found that amphiregulin (AREG) signalling was upregulated, which is thought to stimulate osteoclastogenesis in combination with ACPAs[142]. It was also found that expression of IL-15RA was enriched in $ACPA^+$ cells compared to $ACPA^-$ B-cells from patients with RA[142]. Genetic variations in IL-15 have also been associated with increased osteoclastogenesis and joint destruction in RA[143]. This evidence suggests that $ACPA^+$ B-cells undergo specific phenotypic changes, enabling them to promote autoimmune inflammation and joint destruction in RA.

1.4.6 Seronegative disease and seropositive disease pathotypes

Although up to two thirds of patients with RA test positive for ACPA and/or RF[144], there is a subset of RA patients in which neither antibody can be detected – so-called seronegative RA. It is possible that some of these patients may have autoantibody specificities which are not picked up by widely used tests. However, there is also some evidence to suggest a distinct disease pathogenesis in seronegative RA, potentially due to differing roles for T-cells in pathogenesis. Studies have shown that there was less incidence of ectopic lymphoid structures in seronegative patient synovial tissues, however T-cell numbers were similar, suggesting less of a role for autoantigens in driving synovial inflammation[145]. Further to this, links have been drawn between seronegative disease and IL-6 signalling via STAT3 pathways, which suggest a stronger response to IL-6 may occur in seronegative patients, potentially driving inflammation[146]. It is also known that heritability is reduced in cases of seronegative RA, however there is still a link to the SE and seronegative disease[147]. Smoking appears to be a less important risk factor for seronegative disease, despite its strong links to seropositive disease[148], possibly suggesting an alternative pathway for the induction of inflammation. It has also been suggested that cases of seronegative RA may be better classified using mechanistic characteristics, to include auto-inflammatory diseases which share certain similarities with RA[149].

RA is known to be an extremely heterogeneous disease, and even within the seropositive disease group, it has been suggested that distinct phenotypes may

exist, based on patterns of synovial inflammation. One study used expression of serum biomarkers - CXCL13 and ICAM-1 - to classify patients into groups based on the dominant cells in synovial infiltrates. Patients with a high baseline CXCL13, classed as lymphoid phenotype, with a stronger presence of lymphoid cells in infiltrates, showed a better response to anti-IL-6 therapy than ICAM-1 high "myeloid" patients whose infiltrates were dominated by myeloid cells, and vice versa[150]. Further investigations on the classification of patients into distinct groups based on synovial infiltrate and mechanisms of inflammation may help to achieve more informed targeted treatment regimens in future.

1.4.7 Further autoantibodies to post-translational modifications

As mentioned previously, it is thought that a proportion of $ACPA^-RF^-$ patients with RA may have disease driven by other antibodies which are not picked up by conventional testing. Several other classes of autoantibodies have been identified in such patients' serum, which are thought to play a role in driving pathogenesis. One of the most widely reported classifications is anti-carbamylated protein antibodies (Anti-CarP). Carbamylation is a spontaneous reaction between a primary amine or free sulfhydryl group with cyanate, converting lysine residues to homocitrulline. Cyanate exists in equilibrium with urea in the body, therefore under normal conditions the levels of cyanate are inadequate for extensive carbamylation. However, during inflammation, the enzyme myeloperoxidase is released by neutrophils which converts thiocyanate to cyanate, increasing its availability to react with lysine residues[151]. Carbamylated peptides were shown to be able to induce arthritis and the production of autoantibodies in mice[152]. Anti-CarP antibodies were also detected in the serum of a number of patients with RA, which appeared to predict a more aggressive diseases phenotype in $ACPA^-$ individuals[153].

Antibodies against PAD4 have also been detected in RA patients[154]. It is thought that PAD4 can undergo auto-citrullination, leading to the development of anti-PAD4 antibodies. These antibodies have been shown to be able to stimulate PAD4 activity[155], potentially increasing citrullination of other peptides. Some studies have also shown that antibodies to anti-acetylated vimentin can be detected in patients with RA and may also contribute to the disease process[156]. Several other types of autoantibodies have also been identified as potential

contributors to pathology in RA, these include Anti-BRAF, Anti-RA-33, Anti-oxidised protein, Anti-malondialdehyde and anti-malondialdehyde acetaldehyde antibodies[154].

1.4.8 Further roles of B-cells in RA pathogenesis

As well as contributing to disease via the production of autoantibodies, autoreactive B-cells may also have further roles in perpetuating synovial inflammation. This may occur via inflammatory cytokine production or via antigen presentation and the activation of autoreactive T-cells[124]. One study which profiled the transcriptome of $ACPA^+$ B-cells in RA found that beyond production of autoantibodies, they also produced AREG, which could activate FLS[142]. B-cells in RA have also been shown to produce RANKL, which can also stimulate osteoclast differentiation[157]. Production of autoantibodies as well as their roles in promoting inflammation in the joints makes B-cells key players in driving RA pathogenesis. Further understanding of the development of auto-reactivity in B-cells in RA is likely to reveal potential targets for therapy, and further understanding their role could help guide use of current therapeutics, with a more targeted approach.

1.5 Sialic Acids

1.5.1 Sialic Acid Structure

Sialic acids are a group of around 40 derivatives of a 9-carbon sugar – neuraminic acid. A common structural feature is an amino group at carbon 5 and a carboxyl group at carbon 1 which gives the molecule a negative charge. The amino group on the molecule is usually acetylated which gives rise to N-acetylneuraminic acid (Neu5Ac) – the most common form of sialic acid (SA), and the molecule which is being referred to in the remainder of this report where SA is referenced[158] (Figure 1.4).

1.5.2 Sialic Acid Synthesis and Transport

SAs are synthesised in the cytosol from UDP-GlcNAc which is first converted to ManNAc, then phosphorylated to form ManNAc-6-P by the enzyme GNE. Neu5Ac is produced by condensation and dephosphorylation reactions catalysed

by Neu5Ac 9-phosphate synthase and Neu5Ac-9-phosphate phosphatase respectively[159].

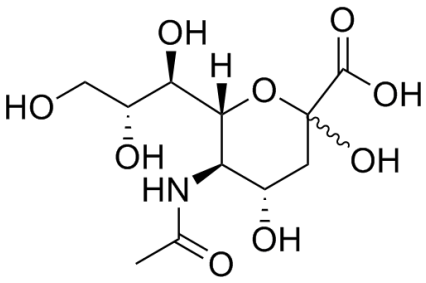
SAs are then transported to the nucleus and activated by cytosine 5'-monophosphate N-acetylneuraminic acid synthetase to form cytosine 5'-monophosphate (CMP) Neu5Ac, which is transported to the Golgi apparatus where glycoconjugates are synthesised[160] (Figure 1.5).

1.5.3 Sialyltransferase Enzymes

One measure of SA diversity comes from different α-linkages which can be formed between the C-2 of SA and the underlying glycan structures, most commonly with the C-3 or C-6 on galactose or C-6 on N-acetylgalactosamine residues[159]. These distinct linkages are formed by specific sialyltransferase enzymes, which catalyse the addition of SA to galactose residues. The enzyme which catalyses the addition of SA to C-6 on galactose is beta-galactoside alpha 2,6 sialyltransferase 1 (ST6Gal1). α2,6-SA is the most widely studied SA linkage, and is thought to have the most relevance in pathologies.

ST6Gal1 is a type II membrane glycoprotein found mainly within the membrane of the Golgi apparatus. It consists of a single short NH_2 terminal transmembrane domain which tethers the enzyme within the membrane, a COOH-terminal catalytic domain facing the Golgi lumen and a stem domain which connects the two[161-163]. ST6Gal1 activity involves SA residues being transferred from the substrate, CMP-SA, to type 2 galactose residues, which are found as free disaccharides or as a terminal N-acteyllactosamine units of N- or O-linked glycan structures[163].

Although predominantly a Golgi membrane bound protein, free ST6Gal1 can also be found in plasma. ST6Gal1 is expressed abundantly in the liver by hepatocytes, where it can be cleaved by β-site amyloid precursor protein-cleaving enzyme-1 and secreted by the cell[164]. ST6Gal1 is thought to be upregulated in hepatocytes as part of the acute phase response, and this leads to an increase in secreted ST6Gal1[165]. The function of upregulated ST6Gal1 is likely to increase sialylation of acute phase proteins to protect them from clearance by liver asialoglycoprotein receptors[166].



Neu5Ac – Sialic Acid (SA)

Figure 1.4 Sialic acid structure

Chemical structure of Neu5Ac sialic acid. 9 carbon molecule with an amino group at carbon 5 and a carboxyl group at carbon 1 which gives the molecules its negative charge, playing a part in SA masking/inhibitory functions.

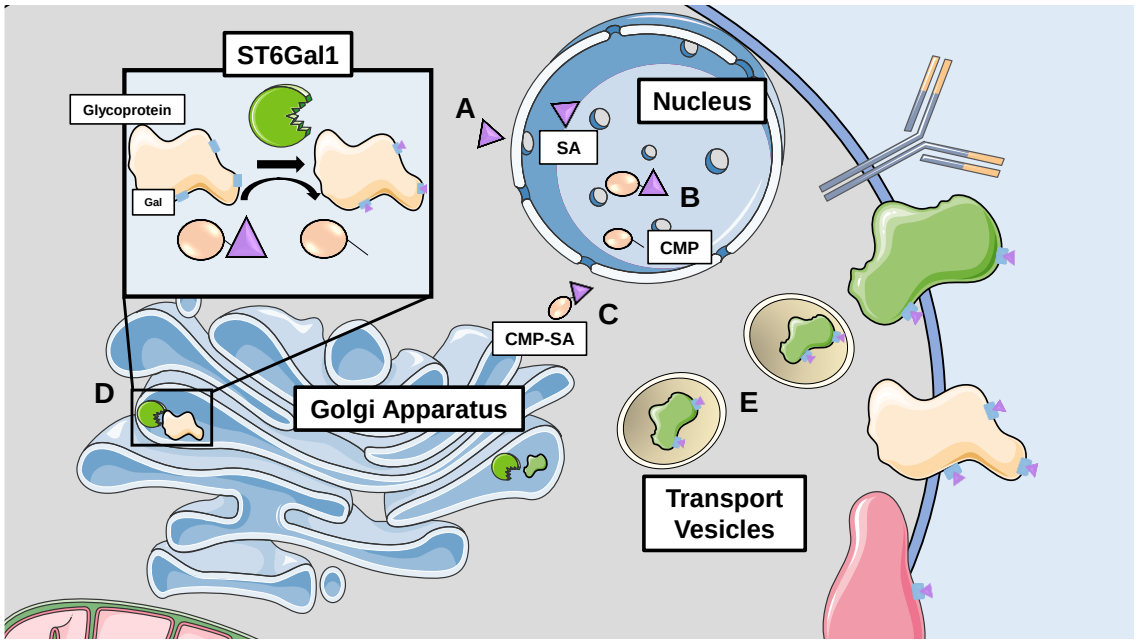


Figure 1.5 Sialic acid transport and sialyltransferase activity

SA is synthesised in the cytoplasm from UDP-GlcNAc **(A)**. SA is then translocates to the nucleus where it is picked up by carrier molecule CMP **(B)**. CMP-SA exits the nucleus and is transported to the Golgi apparatus **(C)** where ST6Gal1 catalyses the addition of SA to a Gal moiety on a glycoprotein chain **(D)**. Sialylated glycans are then packaged for transport and may be secreted from the cell or delivered to the cell surface **(E)**. This figure was created using templates from Servier Medical Art which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

Serum concentrations of plasma glycosyltransferases which have been released from cells, can vary according to age, and are found to be increased in children and those over 80 compared to individuals aged 18-80[167]. Some studies have shown that an increase in soluble ST6Gal1 has an effect on the α2,6-sialylation of secreted glycoproteins, but not of cell surface glycoproteins[166]. However, a study of haematopoietic stem cell differentiation suggested that ST6Gal1 secreted by the liver was a negative regulator of haematopoiesis using a mouse model with liver-specific ST6Gal1 knockdown. This regulation is thought to be driven by soluble ST6Gal1 sialylation of haematopoietic stem cell surface glycans[168].

1.5.4 Neuraminidases

Neuraminidases (also called sialidases) are a group of enzymes which carry out cleavage of SA residues from glycoprotein chains. Neuraminidases are glycoside hydrolase enzymes and they cleave glycosidic bonds within neuraminic acids. They are expressed by a wide range of organisms, playing a notable role in virus entry into cells – making them an attractive target for anti-viral drugs such as oseltamivir[169]. There are four mammalian neuraminidases which have been identified – neuraminidase-1-4. Neuraminidase-1 (NEU1) is universally expressed by all tissues, and is found within lysosomes. However it has also been shown to associate with the plasma membrane, and can exist free in the serum[170].

1.5.5 Functions of Sialic Acid

Terminal SA residues on glycan chains may contribute to glycoprotein biological function either by forming or masking glycan recognition sites[162]. SAs are also able to inhibit cell-cell interactions in a nonspecific manner due to their negative charge[171]. Sialylated glycoproteins have an increased half-life in the serum as they are protected from clearance by the liver via asialoglycoprotein receptors[166]. Sialylated glycoproteins on the cell surface can also protect cells from being removed from circulation, as SA masks ligands from detection by liver macrophages. In particular, erythrocyte surface proteins are heavily sialylated when the cell is first generated, then sialylation is lost as the cell ages or is damaged, thus allowing them to be removed from circulation[172].

Sialylated cell-surface glycoproteins also have an important role in mediating cellular interactions, including cell-cell adhesion and migration of cells into tissues. In the vascular endothelium, a key step in the initiation of inflammation is monocyte adhesion and transmigration, which is driven by glycoprotein receptors selectins and integrins[173]. Integrin function is heavily dependent on α2,6-sialylation and hyposialylation of monocyte integrins can lead to increased adhesion to vessel walls and in increase in endothelial transmigration[174]. Therefore, during vessel wall inflammation, monocyte surface sialylation is downregulated.

1.5.6 Sialic Acid Receptors

α2,6 sialylation also plays an important role in the regulation of immune cell activity. Sialic acid binding immunoglobulin-like lectins (Siglecs) are immune receptors which recognise sialylated glycans, and are present mostly on innate immune cells. There have been 15 distinct Siglecs identified in humans, the majority of which contain an immunoreceptor tyrosine based inhibitory motif (ITIM)[175], which function to negatively regulate immune cell activation[176]. An exception to this is Siglec-1 or sialoadhesin, which lacks ITIMs but contains 17 Ig-like domains[177]. It is expressed on macrophages and is thought to be involved in the uptake of sialylated antigens. Most Siglecs are “masked” on the cell surface, meaning they are ligated by SA molecules expressed on neighbouring receptors, and are therefore prevented from interacting in a trans manner with sialylated proteins in the extracellular environment and with other cells[177,178]. Sialoadhesin however is “unmasked” which is thought to allow its interaction with sialylated antigens and promote their uptake. Siglecs mainly function to recognise self-associated ligands, playing a role in maintaining self-tolerance[179].

1.5.7 Regulation of B-cell activation by CD22

α2,6-SA is the ligand for CD22, a Siglec which is expressed exclusively in B-cells. It is thought to act as a negative regulator of B-cell signalling, by recognising self-antigens and preventing B-cell over-activation and autoimmune reactivity[180]. Following BCR crosslinking, CD22 is rapidly phosphorylated, mediated by SHP-1[176], which has an attenuating effect on calcium signalling[181,182]. On the surface of resting B-cells, CD22 molecules are thought to exist as multimers, as neighbouring CD22 molecules carry CD22 ligands, meaning the molecules are

ligated in a cis manner[183]. CD22 ligands are abundant on the surface of B-cells and it is thought that this may limit the capacity of CD22 for trans signalling, however in certain situations, CD22 may be ligated by sialylated proteins of other cells during interactions or by free sialylated glycoproteins[176].

It is thought that following B-cell stimulation with anti-IgM to cross-link the BCR and CD40 ligand to provide a co-stimulatory signal, CD22 becomes unmasked and therefore becomes more available to receive trans signals[184]. However, simultaneous stimulation of the BCR and CD22 by a sialylated antigen may lead to suppression of BCR signalling[180], and interactions with cells which display sialylated proteins may also inhibit B-cell activation[182].

ST6Gal1 is responsible for the production of Sia6LacNAc – which is the predominant ligand for CD22. Mice which lack ST6Gal1 are viable but are severely immunodeficient, have decreased levels of circulating IgM and decreased expression of CD22. Mice also showed a deficient response to BCR stimulation, however in the presence of IL-4, response was close to wild type cells[185]. ST6Gal1 and CD22 double knockout mice also showed a reduced response to BCR stimulation and had a lack of circulating B-cells and IgM[186]. However, early B-cell development in the bone marrow was not found to be affected. B-cell homing to bone marrow was deficient in ST6Gal1 knockouts and double-knockout mice, suggesting that homing may be determined by CD22-sialylation glycoprotein interactions[186].

It is also thought that CD22 may help to distinguish between self and non-self – as ligands for CD22 are generally only displayed on mammalian cells, and stimulation of B-cells with antigens displaying ligands for CD22 can lead to induction of anergy[187]. It is also thought that signalling via TLRs regulates the development of autoimmunity in combination with BCR signalling[188]. CD22 is also thought to play a role in TLR signalling, since B-cells from CD22-deficient mice exhibit hyperproliferation in response to TLR stimulation[189]. Therefore CD22 may help to downregulate B-cell responses to antigens which trigger both BCR and TLR signalling.

Currently, no convincing links have been found between CD22 polymorphisms in humans and autoimmune disease[176]. Despite this, there has been some

evidence which suggests that CD22 may be downregulated in B-cells in patients with rheumatoid arthritis, in B-cells which express CD5 – a marker associated with the production of autoantibodies[190]. Further study of CD22 and ST6Gal1 expression in human B-cells is required to further understand the link between dysregulated B-cell activation and the development of autoimmunity.

1.5.8 Sialylation in Pathology

As discussed previously, α2,6 sialylation has a wide range of functions which vary between cell and tissue type. Sialylated glycans are involved in various processes related to cell-cell interactions and cell activation, therefore, aberrant ST6Gal1 activity and a change in regulation of α2,6 sialylation can contribute a number of pathologic states, particularly inflammatory states and carcinogenesis.

1.5.8.1 ST6Gal1 in cardiovascular pathology

ST6Gal1 is known to play a role in several aspects of cardiovascular pathology. Recently, ST6Gal1 has been shown to have a role in driving atherosclerotic plaque formation. It was found that ST6Gal1 was downregulated in endothelial tissues during lesion development, leading to decreased sialylation of integrins. This facilitated monocyte transendothelial migration into the vessel wall, propagating vessel inflammation[191]. Patients with acute coronary syndrome also showed reduced endothelial cell expression of ST6Gal1 mRNA compared to controls, supporting its role in promoting vessel inflammation[173]. Some studies also suggest that a reduction in serum SA could predict the onset of cardiovascular pathology, however natural variations due to age, race, gender etc. complicate its use as a biomarker[192].

Hyposialylation of low-density lipoproteins has also been associated with its increased rate of accumulation in patients with atherosclerosis[193], however this may be counteracted by an increased rate of clearance of hyposialylated proteins[192]. It has also been suggested that hyposialylation of erythrocytes and platelets may contribute to thrombotic plaque formation, but the power of this over the effect of increased clearance from circulation is yet to be confirmed[192]. There is also evidence for a susceptibility loci in ST6Gal1 for coronary artery disease[194].

1.5.8.2 ST6Gal1 in cancer

Sialylation and ST6Gal1 expression has been shown to be altered in a number of different cancers, and changes to the glycocalyx have been reported to have varying effects on tumour cell behaviours, dependent on the tissue type. There a number of mechanisms by which altering ST6Gal1 expression may contribute to tumour progression, invasion, chemoresistance and immune evasion[195]. These include a decrease in complement binding by increasing sialylation, the introduction of xenosialylation with non-human sialic acid from food sources triggering antibody production and a low-grade tumorigenic inflammation, and the increase of sialyloglycans to engage inhibitory receptors and avoid immune cell activation[179].

In numerous tumour types, increased sialylation has been associated with invasiveness, survival, and cell proliferation. In cervical cancer cells, for example, ST6Gal1 knockdown not only decreased cell proliferation and invasiveness, but also increased sensitivity of the cells to chemotherapeutics[196]. Manipulation of epidermal growth factor receptor has also been shown to promote cell survival and resistance to therapy[197,198].

A link between ST6Gal1 expression and activation of the PI3K/Akt pathway has also been suggested as a possible mechanism of promoting cell survival and inhibiting apoptosis[199]. ST6Gal1 has been found to be over-expressed in prostate cancer tissues, and it promotes proliferation, migration and invasiveness, mediated by the PI3K/Akt/GSK-3β/β-catenin signalling pathway[200]. Overexpression of ST6Gal1 has also been shown to be associated with the development of multi-drug resistance in leukemic cells, mediated by PI3K/Akt signalling[201]. Furthermore, ST6Gal1 has been found to be a target for MicroRNA-199a, which is a negative regulator of the PI3K/Akt signalling pathway[202].

ST6Gal1 has also been shown to play a role in cell survival in growth factor deprived conditions. Cells overexpressing ST6Gal1 upregulated pAkt in response to serum starvation and were resistant to apoptosis. Cyclin D2 was also upregulated by ST6Gal1 in serum starved conditions, which prevented cells from entering cell cycle arrest. These results suggested ST6Gal1 has a role in protecting cell proliferative activity, to promote cell survival in cytotoxic conditions[203].

The role of ST6Gal1 in tumour cells is diverse however, and it has been shown that in bladder cancers, downregulation of ST6Gal1 is associated with a more invasive phenotype[204]. A study of ST6Gal1 expression in colorectal carcinomas also found it to be increased in non-metastatic tumours compared to metastatic tumours[205].

Regarding control of ST6Gal1 expression in tumour cells, one study linked ST6Gal1 expression in prostate cancer to androgen signalling. Transcription of ST6Gal1 mRNA was found to be increased when prostate cells were stimulated by androgens. However, this was not coupled with an increase in surface SA expression[206]. In pancreatic ductal adenocarcinoma, which is known to be associated with high fructose intake, it has been shown that *in vitro*, exposure of pancreatic ductal adenocarcinoma cells to fructose increases their metastatic potential, in part by upregulating ST6Gal1[207]. It was also found that exposure to IL-1β in culture increased the level of α2,6-sialylation in pancreatic ductal adenocarcinoma cells[208].The important and diverse role of ST6Gal1 in cancers makes it a popular target for research and evidence is constantly evolving. Potential for targeting sialylation with therapies is also currently being investigated.

1.6 Sialylation in B-cells and Autoimmunity

1.6.1 Roles for B-cell Surface Sialylation

As well as playing an important role in many aspects of inflammation, dysregulated sialylation is also known to play a part in the development and propagation of autoimmunity. Sialylation of the B-cell surface plays a role in determining B-cell activation as described previously, and may be linked to the development of autoreactive cells. Antibody sialylation has been widely studied in a number of autoimmune diseases and is widely thought to be a crucial part of promoting autoimmune inflammation once self-tolerance is broken down.

It has been described that treating resting B-cells with neuraminidase leads to an increase in their capacity to present antigen to T-cells. This observation was dubbed the neuraminidase effect[209]. However, it is not clear if the mechanism of increased antigen presentation capability is directly linked to expression of surface SA, since blocking the activity of LFA-1 inhibited T-B cell interactions in

neuraminidase treated but not lipopolysaccharide (LPS) treated cells[210], suggesting instead that SA on the cell surface may have an impact on the interaction of T and B cells only. These experiments did not confirm that SA expression was reduced in cells stimulated with LPS, nor in cells activated via the BCR. In murine splenic B-cells, it was found that activating B-cells with anti-IgM leads to a downregulation of SA, whereas stimulation with anti-CD40 did not. It was also found that SA on the cell surface blocked access to co-stimulatory ligands, offering a possible explanation for the neuraminidase effect[211]. The same group also described an increase in B-cell activation induced by culture in media alone for 24 hrs[211]. Taken together, this data shows that B-cell surface sialylation likely plays a role in determining cell function, however further study is required to further understand the consequences of altered sialylation in pathology.

1.6.2 Antibody Sialylation

All five classes of antibody molecule contain several sites for glycosylation within the Fab and Fc fragments, and dependent on the structure and placement of the glycan, they may play a role in antibody function[212]. The functions of glycans in IgG molecules have been the most widely studied, and there is a large body of evidence which suggests sialylation of glycans within the Fc fragment of the molecule help determine its effector function.

The N-linked glycan at position Asn-297 on the heavy chain near the hinge region in the Fc fragment has a bi-antennary structure consisting of N-acetylglucosamine and mannose residues with varying amounts of terminal galactose and core fucose moieties[213]. The most common glycoforms of IgG can be categorised according to number of terminal galactose moieties - IgG-G2 has two (16% of total in healthy subjects), IgG-G1 only one (35%) and IgG-G0 has no galactose moieties (20-35%)[214]. Since α2,6 sialylation is dependent on the presence of galactose, absence of galactose equates to absence of SA residues[163] (Figure 1.6). Sialylation of Fc glycans, as well as the presence or lack of core fucose molecules contributes to determining the affinity of IgG for FcγRs[215-218] and the ability to bind complement[219-221].

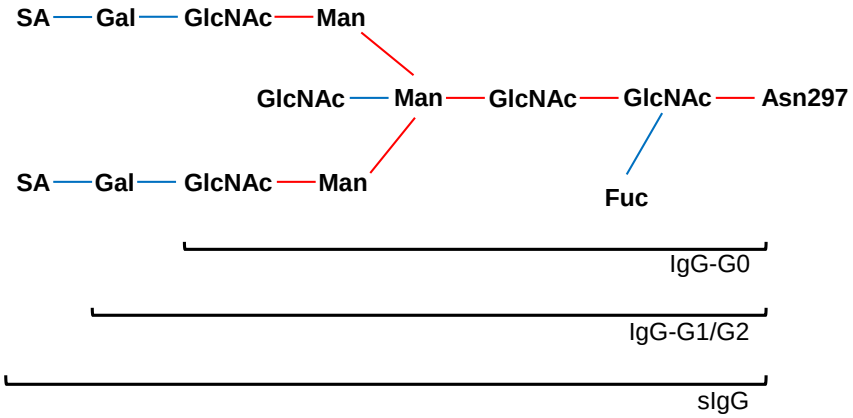


Figure 1.6 Structure of IgG N-linked glycans

Basic structure of biantennary glycan found at Asn297 in the Fc fragment of IgG near the hinge region. Basic structure contains mannose (Man) and N-acetylglucosamine (GlcNAc) residues with variable addition (shown in blue) of Fucose (Fuc), bisecting GlcNAc, galactose (Gal) and terminal sialic acid (SA) residues. IgG-G0 contain no glactose, IgG-G1 and G2 contain one or two Gal and sIgG contans two Gal and at least one SA residue.

Asialylated glycoforms of IgG bind to activating FcγRs – FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA, and FcγRIIIB, which are expressed on innate immune cells, with varying affinity[83] and promote inflammatory activity by increasing phagocytosis, cytokine production and antibody dependent cellular cytotoxicity (ADCC)[222] However, the presence of SA leads to an increase in affinity for inhibitory FcγRs-FcγRIIB, CD23 and DC-SIGN. The presence of SA results in a more closed conformation – exposing more binding sites for the inhibitory receptors [217].

Sialylated IgG binding to DC-SIGN on regulatory myeloid cells induces production of IL-33 - expanding basophils, which produce IL-4. This promotes the upregulation of inhibitory receptor FcγRIIB – increasing the activation threshold of effector Mϕ to ICs [218]. Sialylated IgG in ICs also bind to CD23 expressed on B cells, upregulating FcγRIIB expression and elevating the threshold for BCR signalling[216] (summarised in Figure 1.7). The absence of fucose moieties in IgG leads to higher affinity for FcγRIIIA, which enhances ADCC and monocyte/Mϕ activation [215].

Intravenous immunoglobulin (IVIG) is a treatment sometimes used in patients with autoimmune disease, which can decrease inflammation[223]. The positive effects of IVIG treatment on autoimmunity have long been thought to be linked to the isoforms of IgG with higher Fc sialylation[224]. It has been suggested that CD22 binding sialylated isoforms of IgG during IVIG treatment may contribute to B-cell suppression and induction of apoptosis[225]. Increased Fc glycan sialylation can also be manipulated to increase serum half-life of biologic drugs[226].

1.6.3 Disruptions in Antibody Sialylation in autoimmunity

It was first described in 1985 that patients with RA had a shift in glycosylation and that their IgG contained lower amounts of galactose than healthy control IgG. It was then confirmed that ACPA IgG Fc glycans contained less SA than non-ACPA IgG from the same patient[227]. Reduced sialylation of disease specific autoantibodies can also be seen in systemic lupus erythematosus, inflammatory bowel disease, multiple sclerosis, myasthenia gravis, Sjogren's syndrome and a number of other autoimmune conditions[228].

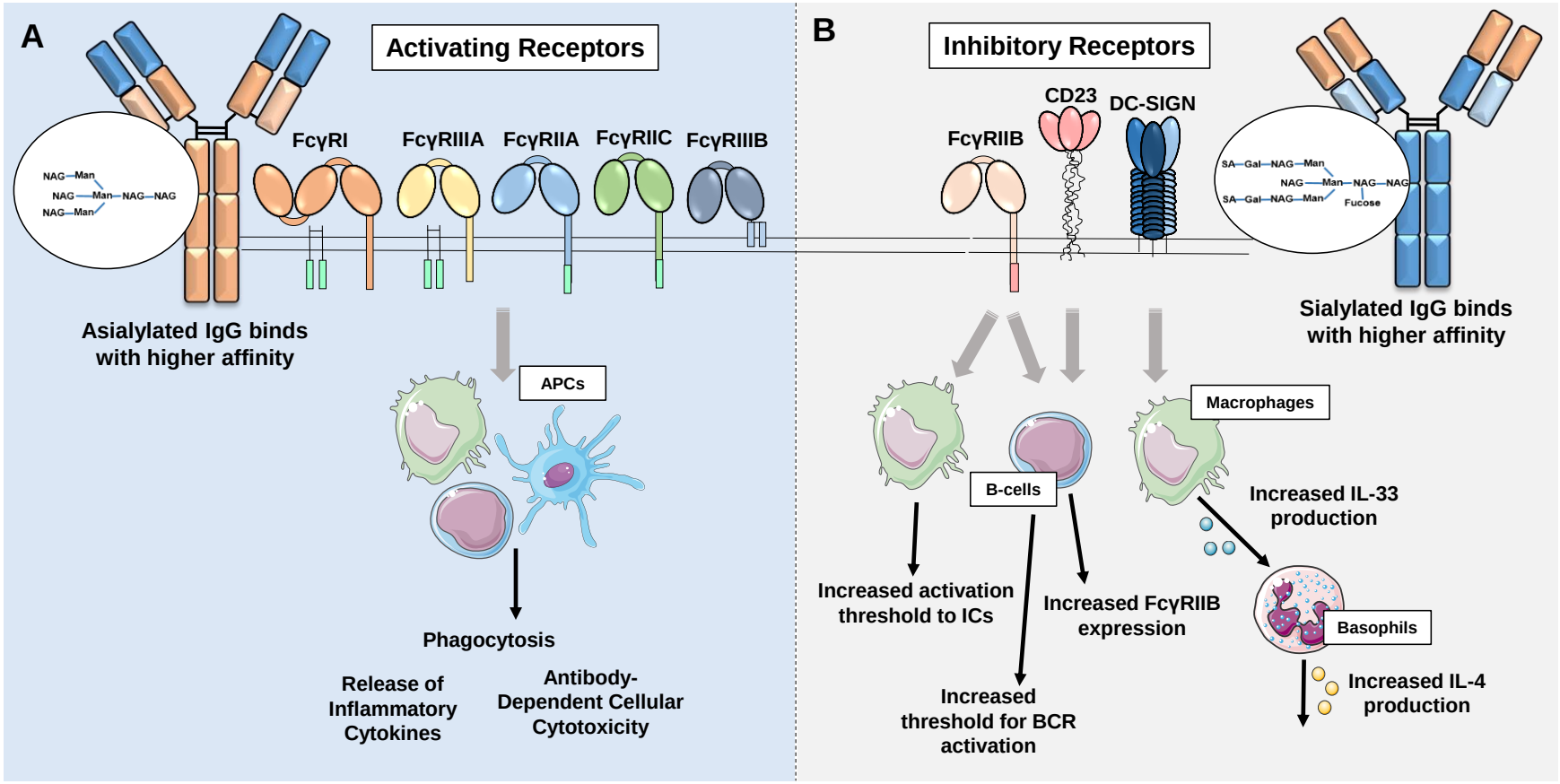


Figure 1.7 IgG Fc receptors and the impact of IgG sialylation

(A) IgG which lacks sialylation of Fc glycans has a higher affinity for activating FcγRs FcγRI, FcγRIIIA, FcγRIIA, FcγRIIC and FcγRIIIB. Binding these receptors on APCs and effector cells leads to upregulated phagocytosis, cytokine release and increased ADCC. **(B)** IgG with sialylated Fc glycans has a higher affinity for inhibitory FcγRs FcγRIIB, CD23 and DC-SIGN. Binding FcγRIIB inhibits ITAM-initiated activation increasing the activation threshold of innate immune cells to ICs, and inducing apoptosis in B-cells with low affinity BCRs. DC-SIGN binding induces production of IL-33 - expanding basophils, which produce IL-4. This promotes the upregulation of inhibitory type I receptor FcγRIIB – increasing the activation threshold of effector cells to ICs. CD23 binding in B-cells also upregulates FcγRIIB expression. This figure was created using templates from Servier Medical Art which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

As discussed previously, asialylated IgG molecules bind with higher affinity to activating receptors than inhibitory receptors, giving them a more inflammatory phenotype. Further to this, asialylated ACPA was found to be able to enhance osteoclastogenesis, whereas sialylated ACPA did not, suggesting desialylation may be responsible for the direct role of ACPA in promoting bone loss[125]. Additionally, mice with collagen-induced arthritis treated with an SA precursor to induce ACPA sialylation showed a less severe disease phenotype than controls[229]

There is also some emerging evidence that not only is ACPA Fc sialylation disturbed, Fab fragment sialylation is also altered. Fab sialylation has been found to be increased in ACPA IgG, in glycans located near the antigen binding region[230]. It is thought that Fab sialylation occurs during the many rounds of somatic hypermutation that ACPA B-cells undergo during germinal centre reactions[231,232].

The exact mechanisms which control sialylation of Fab or Fc glycans in RA are not fully understood, there is however some evidence of factors which may influence IgG sialylation in autoimmunity. It was described that IgG sialylation was reduced when B-cells were activated via the TLR9 ligand cytidine-phosphate-guanosine (CpG) and stimulated with IL-21 and IFN-γ[233]. More recently it has also been shown that sialylation of Fc glycans was increased, as was expression of ST6Gal1 when antibody producing cells were treated with oestrogen. Treatment of post-menopausal women with oestrogen was also able to increase Fc glycan sialylation, suggesting a protective role for oestrogen in preventing autoimmunity[234].

Recently it has also been described that the IL-17/IL-23 axis may influence the Fc sialylation of ACPA antibodies, since IL-21/IL-22 produced by Th17 cells was shown to downregulate B-cell expression of ST6Gal1 and reduced IgG Fc sialylation in mice. It was also found that when measuring expression of sialylated proteins of the B-cell surface, a decrease in sialylation can be seen in patients with RA and $ACPA^+$ individuals in the at-risk group for developing RA[235]. Detection of reduced sialylation on the cell surface was also correlated with

progression to RA from an asymptomatic autoimmune inflammatory state[235,236], suggesting that changes to sialylation may correlate with the onset of autoimmune pathology in $ACPA^+$ individuals. It is thought that measuring the surface sialylation may give an indication of overall sialyltransferase activity, however the decrease observed does not account for increased sialylation of Fab glycans. It is possible therefore that there may be changes to B-cell surface sialylated proteins in RA, independent of changes to antibody sialylation. Although studies have shown how certain cytokines can affect IgG sialylation[233,235], little is known about the regulation of SA on cell surface proteins.

1.7 Current Strategies for the Treatment of Rheumatoid Arthritis

1.7.1 Treat to Target Approach

The introduction of biologic drugs has revolutionised the treatment of RA. Several drugs which target multiple mechanisms of inflammation have been successful in reducing disease activity for many patients. However, there is still a number of patients for whom these drugs are not effective, and the disease progresses despite multiple drug interventions. The usual first line of treatment in RA is with conventional synthetic diseases modifying anti-rheumatic drugs which include methotrexate, leflunomide and sulfasalazine, which provide broad immunosuppression through various mechanisms, in combination with glucocorticoids. If this treatment strategy is unsuccessful, the next line of defence is biological DMARDS (bDMARDs).

1.7.2 Biological DMARDs

Several therapies targeting cytokines have been trialled in the treatment of RA, and several show promising results. In particular, therapeutic agents targeting TNF have proven to be effective in reducing inflammation in RA. There are several drugs on the market which block the action of TNF. Anti-TNF agents adalimumab, certolizumab and etanercept bind to TNF in the serum and prevent it from binding its receptor[237].

Another strategy which has delivered success in the treatment of RA is anti-IL-6 therapies. Antibodies to the IL-6 receptor in both its soluble and surface-bound forms such as sarilumab and tocilizumab prevent IL-6 from binding the receptor. Sirukumab binds IL-6 directly, blocking its interaction with soluble and surface bound IL-6R[238].

Other anti-cytokine therapies which have shown success in treating other autoimmune conditions have also been trialled in RA, including anti-IL-1β and anti-IL-17. Anti-IL-1β agent anakinra has proven only moderately effective in treating RA[239], with one study finding it no more effective than methotrexate monotherapy in early stage RA[240]. The lack of efficacious response possibly indicates a less dominant role for IL-1β in RA pathogenesis. Similarly, anti-IL-17 therapy such as secukinumab targets IL-17A directly, and has been successful in treating patients with psoriasis and psoriatic arthritis, but is less effective in treating patients with RA[7]. This may also indicate that the impact of IL-17 cells may be secondary to other inflammatory processes or that the role of IL-17 in RA is more diverse.

Other biologic drugs which target T-cell co-stimulation have also been used to treat RA. Abatacept is a fusion protein which combines the Fc region of IgG1 and CTLA-4[241]. The drug binds to B7, which is the ligand for CD28, a T-cell co-stimulatory molecule, and prevents T-cell co-activation via APCs[242]. In RA, abatacept has been shown to be effective in reducing synovitis, with a relatively low rate of adverse events, and is indicated for the treatment of patients who are bDMARD naïve, or who have failed treatment with anti-TNF therapy[241]. Finally, the recent introduction of small molecule inhibitors such as toficitinib and barcitinib that target the JAK/STAT signalling pathway, which is involved in several cytokine signalling pathways, is proving to be effective in treating RA in patients with RA across the disease stages, including those that are refractory to treatment with other bDMARDs[243].

1.7.3 B-cell Depletion Therapy

B-cell depletion therapy has shown particular success in treating patients with RA and as with other therapeutic approaches, has demonstrated benefit in a proportion of patients refractory to treatment with other DMARDs, including anti-TNF therapies[244]. B-cells are depleted via rituximab – a monoclonal chimeric

antibody to CD20, a B-cell surface marker. Rituximab binds to CD20 on the B-cell surface and can induce B-cell death via activation of the complement cascade, or via antibody dependent cellular cytotoxicity[245]. Despite its success there are a number of patients who, following B-cell repopulation, have a relapse in disease activity and inflammation. After the first infusion of rituximab, B-cells are found to be variably depleted, and those with incomplete depletion are less likely to respond well to treatment than those who achieve complete depletion after the first infusion[246].

B-cell repopulation of the blood tends to occur at around 8 months following treatment. The first subset of B-cells to return are thought to be immature B-cells, followed by naïve mature cells, then memory cells, however memory cells have been found to be reduced in the blood of patients with RA for up to two years following rituximab treatment[247]. Disease relapse was associated with increased numbers of returning memory B-cells in some patients[248].

Treatment with rituximab leads to a reduction in the levels of serum immunoglobulins, however one study showed that levels did not decrease below the normal range[249], this suggests that its therapeutic benefits may be related to B-cell functions other than antibody production. Rituximab treatment has also been shown to decrease the population of Th17 cells in the synovial tissue[250], suggesting B-cells promote Th17 differentiation in the synovium, possibly through production of IL-6 and the presentation of autoreactive antigens. Depletion of B-cells with rituximab also led to improved endothelial function and reduced systemic inflammation – which may reduce the cardiovascular risk[251].

Despite successes of treatment, the rate of relapse is high, and it has been shown that B-cell depletion is not enough to “reset” B-cell tolerance. In patients with type I diabetes, relative frequency of autoreactive B-cells was unaltered following treatment, suggesting a possible mechanism of relapse following complete depletion in this and other autoimmune diseases[252].

One possible contributing factor to the return of inflammation following B-cell depletion may include the survival of $ACPA^+$ B-cells which reside within synovial tissues[253]. Synovial tissue in RA also harbours dominant B-cell clones which are

not found in the blood[86], providing a potential survival niche for dominant clones that may propagate autoimmunity following treatment with rituximab.

1.7.4 Sialylation and Response to Therapy

The changes in IgG sialylation which occur in RA have been shown to be reversible, as treatment with tumour necrosis factor inhibitors, methotrexate or both lead to an increase in galactosylation, associated with clinical response[254,255]. Sialylation may also have an influence on how well patients respond to treatment. In many cancers chemosensitivity is largely dependent on surface sialylation, and the same may be true in the treatment of RA. Research of the glycosylation profile of immune cells in RA and how this may affect the efficacy of treatment is lacking. However, it is hypothesised that sialylation may play a role in the efficacy of rituximab treatment. A study has shown that the sialylation of the Fc receptor on innate immune cells which recognises the rituximab antibody on the B-cell surface determines the affinity of the rituximab-Fc receptor interaction, and found that large sialylated glycan chains interfered with binding[256]. Since the efficacy of rituximab depends on its binding to the B-cell surface, it is hypothesised that glycoproteins on the B-cell surface may also interfere with rituximab binding and affect response to therapy.

1.8 Hypotheses

- Decreased sialylation is a feature of autoimmunity that contributes to the onset of inflammation in RA.
- Sialylation of surface proteins is reduced in peripheral B-cells in patients with RA and pre-RA.
- Decreased surface sialylation in RA influences B-cell function.
- Changes to B-cell sialylation are induced by B-cell intrinsic and extrinsic factors which may be altered in RA.
- B-cell sialylation may play a role in determining response to depletion by rituximab in RA.

1.9 Aims and Objectives

1.9.1 Aims

- To investigate factors which influence B-cell surface molecule sialylation
- To determine the functional consequences of reduced B-cell sialylation in patients with RA

1.9.2 Objectives

- To confirm that sialylation is reduced in resting B-cells in patients with RA and pre-RA compared to cells from healthy donors
- To measure the impact of B-cell activation, via mitogens and cytokines, on B-cell sialylation
- To compare response to mitogens and cytokines in cells from healthy donors and patients with RA
- To investigate factors which may alter B-cell sialylation in patients with RA
- To investigate the consequences of reduced sialylation on B-cell activation and antibody production activation *in vitro*
- To investigate the impact of B-cell sialylation on efficacy of *in vitro* B-cell depletion by rituximab.

Chapter 2
Methods

2.1 Healthy Donors and Patient Cohorts

2.1.1 Patient and healthy donor samples

Samples of up to 50ml of peripheral blood were collected by venepuncture from a pool of healthy donors (n=27). Samples of up to 30ml of peripheral blood were collected by venepuncture from the cohort of patients attending Chapel Allerton Hospital for treatment (Leeds Teaching Hospitals Trust). The patient cohort was divided between two main categories. The first, Pre-RA, consists of patients whose serum is $ACPA^+$ and who have musculoskeletal symptoms but do not meet the criteria for diagnosis with RA (based on 2010 EULAR/ACR criteria[257] (PRA, n=12). The second were those with biologic-naïve early RA (ERA, n=41), with a symptom duration of less than 12 months. For certain experiments, the ERA group was also sub-divided into two groups - newly diagnosed (samples taken upon first clinic attendance), treatment naïve patients – new early RA (N-ERA, n=18) and biologic-naïve (± csDMARD treatment according to local protocol) patients attending clinic following initial diagnosis, but with a symptom duration of less than 12 months – later early RA (L-ERA, n=23). Unless stated otherwise in the text, patients with RA in the ERA group were a mixture of individuals with newly diagnosed or later early RA. Patient and healthy donor characteristics are summarised in (Table 2.1).

2.1.2 Ethics

All healthy volunteers and patients gave informed consent, with ethical approval for healthy donors and patients with early RA (RADAR) and pre-RA (NHS-CCP) obtained from West Yorkshire Research Ethics Committee REC references: [09/H1307/98] and [17/YH/0177] respectively.

Table 2.1 Summary of patient and healthy donor characteristics

	HD	Pre-RA	ERA	ERA	
				N-ERA	L-ERA
n	27	12	41	18	23
Age (years)	42.4 ± 10.9	53.3 ± 13.4	56.5 ± 11.6	54.9 ± 12.0	57.8 ± 11.0
% Male	44.0	9.1	19.5	16.7	21.7
DAS28-CRP	NA	1.79 ± 0.85 (n=11)	3.93 ± 1.40 (n=8)	4.66 ± 0.66 (n=6)	1.74 ± 0.35 (n=2)
% ACPA+	NA	100.0 (n=12)	83.3 (n=24)	92.3 (n=11)	72.7 (n=13)
% RF+	NA	27.3 (n=11)	60.9 (n=23)	54.5 (n=13)	66.7 (n=10)

Samples were collected from healthy donors and from ACPA+ individuals with generalised rheumatic symptoms (Pre-RA) or patients with bDMARD-naïve early RA (symptom duration <12 months) (ERA). ERA patients were further divided into two categories – newly diagnosed patients (sample collected at first clinic visit) (N-ERA) and later bDMARD-naïve early RA (± csDMARD treatment, sample taken within 12 months of symptom onset) (L-ERA). DAS28 was calculated using the DAS28-CRP calculator found at https://www.das-score.nl/das28/DAScalculators/dasculators.html. Data displayed shows mean with standard deviation. N numbers in brackets indicate the number of patients for which clinical data was available for each parameter.

2.2 Sample Processing

2.2.1 Isolation of Peripheral Blood Mononuclear Cells from Blood

Peripheral blood was collected in 9 ml EDTA tubes from healthy donors and patients. In the majority of cases samples were processed shortly after collection (within 1 hr), though some cases necessitated storage at room temperature for up to 24 hrs following collection.

To isolate peripheral blood mononuclear cells (PBMCs), blood was diluted 1:1 in sterile phosphate buffered saline (PBS), then layered on top of 14 ml Lymphoprep™ density gradient medium in SepMate™ tubes (both Stemcell Technologies Inc.). Tubes were centrifuged for 10 mins (20 mins for samples more than 12 hrs old) at 1200 x g at room temperature. After centrifugation, cell fraction containing PBMCs was poured into a fresh tube, then washed once with PBS by centrifugation at 500 x g for 8 mins. Supernatant was then removed and cell pellet re-suspended in 10 ml red cell lysis buffer (155mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM ETDA) and incubated protected from light for at least 10 mins to lyse contaminating erythrocytes. Cells were then washed once in 10 ml cold PBS then counted using trypan blue exclusion.

2.2.2 Isolation of B-cells from Peripheral Blood Mononuclear Cells

B-cells were isolated from PBMCs using the Pan B-cell Enrichment Kit (Stemcell Technologies Inc.). Cells were isolated largely following the manufacturer's protocol. Briefly, PBMCs were suspended in autoMACS Running Buffer + 5% bovine serum albumin (BSA) Stock Solution (cell isolation buffer, both Miltenyi Biotech) at $5x10^7$ cells/ml. Cells were then incubated for 10 mins at room temperature with B-cell enrichment antibody cocktail at 50 µl/ml cell suspension. 75 µl/ml cell suspension of magnetic beads were then added and incubated with cells for 5 mins at room temperature. Following incubation tubes were place into an EasySep magnet (Stemcell Technologies Inc.), and incubated for 5 mins, then supernatants poured into a fresh tube. Enriched B-cells were washed once in PBS then counted using trypan blue exclusion. Two further methods of B-cell isolation were tested – Pan B-cell Isolation kit (Miltenyi Biotech) and RosetteSep™ Human B-cell Isolation Kit (Stemcell Technologies Inc.), however the method using the Stemcell Technologies Pan B-cell Enrichment kit proved to

be most suitable and delivered the highest B-cell purity for smaller starting cell numbers (results not shown).

2.2.3 Isolation of T-cells from Peripheral Blood Mononuclear Cells

T-cells were isolated from PBMCs via positive selection by CD4 microbeads (Miltenyi Biotech). Following counting, PBMCs were washed once in cell isolation buffer. Supernatant was then completely removed and cells were re-suspended in buffer at $12.5x10^7$ cells/ml. 20 µl of CD4 microbeads was then added and the suspension incubated in the fridge for 15-20 mins. 1 ml buffer per $1x10^7$ cells was then added and the cells centrifuged at 300 x g for 10 mins. Supernatant was then removed and cells re-suspended in 500 µl buffer.

MS columns (Miltenyi Biotech) were used to isolate positively labelled T-cells, and to prepare for cell separation, columns were placed within a magnetic field (OctoMACS magnet, Miltenyi Biotech), then washed with 500 µl buffer, by allowing it to drip completely through the column into a waste tube. Labelled cell suspension was then added directly into the column well, and flow through containing unlabelled cells collected in a new tube. Column was then washed 3 times with 500 µl buffer, each time allowing liquid to completely flow through before the next wash was added.

After the third wash column was removed from magnet and placed into a fresh tube. 1ml buffer was added and the plunger depressed to release labelled $CD4^+$ T-cells. T-cells were then counted using trypan blue exclusion, then $CD4^+$ T cells and unlabelled cells washed by centrifugation at 500 x g for 5 mins. In some cases unlabelled fraction was then used for negative isolation of B-cells in cases where both T-cells and B-cells were required from the same donor.

2.3 Cell Culture

2.3.1 Cell Culture conditions

PBMCs or isolated T-cells and B-cells were cultured in RPMI (Gibco) containing 10% foetal bovine serum (FBS) (Gibco), 2% L-glutamine (Fisher Scientific Ltd.) and 2% penicillin + streptomycin (Gibco) in suspension unless stated otherwise. Mixed PBMC populations were cultured at $2.5\text{-}5x10^6$ cells/ml and B-cells at $1x10^6$

cells/ml unless stated otherwise. Cells were cultured in 96 well plates (200 µl/well) or 24 well plates (1 ml/well).

2.3.2 B-cell Activation Experiments

Isolated B-cells from healthy donor or patients with RA were cultured at $1x10^6$ cells/ml in RPMI supplemented with either 1 µg/ml Type B CpG oligodeoxynucleotides (ODN) (Alpha Diagnostics), 5 µg/ml $F(ab')_2$ Anti-IgM/IgG ($F(ab')_2$) (Jackson ImmunoResearch Europe Ltd.) 1 µg/ml R848 (InvivoGen), 2 µg/ml soluble CD40L (BioLegend, Inc.) or a combination of more than one stimuli. Type B CpG ODN was used since these have been shown to produce a strong activation signal in B-cells[258]. $F(ab')_2$ were used for stimulation of B-cells since the antibodies are modified to contain only Fab fragments – to prevent inhibitory stimulation of B-cells via FcγRIIB. For dose response experiments, three concentrations of each stimuli were tested. For CpG and R848 1 µg/ml, 2 µg/ml or 5 µg/ml. For $F(ab')_2$ 5 µg/ml, 10 µg/ml or 15 µg/ml and for CD40L, 2 µg/ml, 5 µg/ml or 10 µg/ml. Cells were incubated for up to 72 hrs at 37°C, 5% CO_2 in round-bottom 96 well plates.

2.3.3 B-cell Cytokine Stimulations

Isolated B-cells from healthy donors or patients with RA were cultured at $1x10^6$ cells/ml in RPMI supplemented with IL-4, IL-6, TNF, IL-17, IL-23 at 5, 20 or 50 ng/ml (Peprotech, Inc.). In some cases media was also supplemented with 5 µg/ml $F(ab')_2$ and 2 µg/ml CD40L. Cells were incubated for up to 48 hrs at 37°C, 5% CO_2 in round-bottom 96 well plates.

2.3.4 Neuraminidase Treatment of B-cells

To digest SA from the B-cell surface, cells were incubated with 100 mU of the enzyme Neuraminidase (Sigma-Aldrich Co.) for 4 or 18 hrs at 37°C in RPMI ($1x10^6$ cells/ml). Cells were then washed in fresh RPMI before being re-suspended at $1x10^6$ cell/ml. Following this either 1 µg/ml CpG or 5 µg/ml $F(ab')_2$ + 2 µg/ml CD40L was added and cells were cultured for 24 hrs at 37°C, 5% CO_2 in round-bottom 96 well plates.

2.3.5 T and B-cell Co-stimulation Assays

PBMCs isolated from healthy donor blood were suspended in RPMI at $5x10^5$ cells/ml. T-cells were activated using Dynabeads™ Human T Activator CD3/CD28 (Invitrogen). Beads were used at a 2:1 cells to beads ratio, and were suspended in a stock solution at $4x10^7$ beads/ml. Therefore, 6 µl beads were required per well of PBMCs. Required volume of beads was added to 1 ml PBS in a 1.5 ml Eppendorf, mixed well and placed into a DynaMag™-2 (Invitrogen) for 1 min then buffer removed. Tube was then removed from the magnet and beads re-suspended in the same volume of media as original volume of beads. 6 µl washed beads were then added to each well containing $5x10^5$ PBMCs in 1 ml RPMI. To activate B-cells concurrently, 5 µg/ml $F(ab')_2$ and 2 µg/ml CD40L or 1 µg/ml CpG was also added to certain wells. Cells were then incubated for 24 hrs at 37°C, 5% CO_2.

To remove beads from cell suspensions before staining for flow cytometry, cells + beads were harvested from culture plates and transferred to Eppendorf tubes then placed in magnet for 1 min. The cell suspension was then transferred to FACS tubes for staining.

2.3.6 CD40L blocking experiment

T-cells and B-cells isolated from peripheral blood were re-suspended at $2x10^6$ cells/ml and $1x10^6$ cells/ml in RPMI respectively.100 µl of each cell suspension was then combined in 4 wells of 96 well plate. CD3/CD28 beads were washed and 5 µl beads added to 2 wells (1:1 beads:T-cell ratio). To block CD40/CD40L mediated T-B-cell activation anti-CD40L (CD154, monoclonal, mouse anti-human, 24-31, eBioscience, Invitrogen) was added at 2 µg/ml. T and B-cell co-cultures were incubated for 24 hrs at 37°C, 5% CO_2. Following 24 hrs of incubation, cell suspensions were placed within magnetic field to remove beads as described above.

2.3.7 *In vitro* differentiation of B-cells to plasma cells

To generate a pure population of plasma cells from B-cells *in vitro,* various cytokines and mitogens were added to drive B-cell activation and differentiation over a period of 13 days (described previously[259], and summarised in Figure 2.1).

B-cells were first isolated from 50 ml peripheral blood from healthy donors and patients with RA as described earlier (Section 2.2.2).

Prior to isolation of B-cells from PBMCs, an aliquot of gamma irradiated CD40L expressing L-cells was defrosted in a water bath, then washed twice in IMDM media (Gibco), being careful to completely remove supernatant between washes. Cells were then suspended at ~$2x10^4$ cells/ml and added to 1-2 24-well plates in 1 ml volume and allowed to attach for 6-18 hrs prior to use.

Day 0: On day 0, B-cells were either isolated from freshly collected peripheral blood, or from PBMCs isolated from peripheral blood the day before and stored at 4°C in media overnight. Isolated B-cells were then suspended at $5x10^5$ cells/ml in IMDM supplemented with Glutamax (Gibco) + 10% heat inactivated FBS (Gibco). Supernatant was removed from wells containing CD40L L-cells and replaced with 500 µl IMDM supplemented with 40u/ml IL-2, 100ng/ml IL-21 (Peprotech) and 4µg/ml $F(ab')_2$. 500 µl cell suspension was then added to wells to give a final cell suspension of $2.5x10^5$ cells/ml and cytokine/mitogen concentrations of 20 u/ml IL-2, 50 ng/ml IL-21, and 2 µg/ml $F(ab')_2$. Plates were then incubated for 3 days at 37°C, 5% CO_2. Cytokines and mitogens which are added to B-cell cultures to induce differentiation are summarised in Table 2.2.

Day 3: After 3 days in culture, B-cells were aspirated from CD40L L-cell-containing wells and counted by trypan blue exclusion. Cells were then washed and re-suspended in media supplemented with Lipid Mixture 1 and MEM Amino Acids Solution (Sigma-Aldrich Co., both at a final concentration of 1X) plus 20 u/ml IL-2 and 50 ng/ml IL-21 at $1x10^5$ cells/ml. Up to 40 ml cell suspension was added to T75 culture flasks or added to 24 well plates at 1 ml/well.

Day 6: At day 6, cells were washed and re-suspend at $5x10^5$cells/ml in media supplemented with Lipid Mixture 1 and MEM Amino Acids Solution plus 50 ng/ml IL-21, 10 ng/ml IL-6 and 100 u/ml IFNα (Peprotech) at $1x10^6$ cells/ml and cultured in 24 well plates. Every 3.5 days 50% of the media from each well was removed and replaced with fresh media + cytokines.

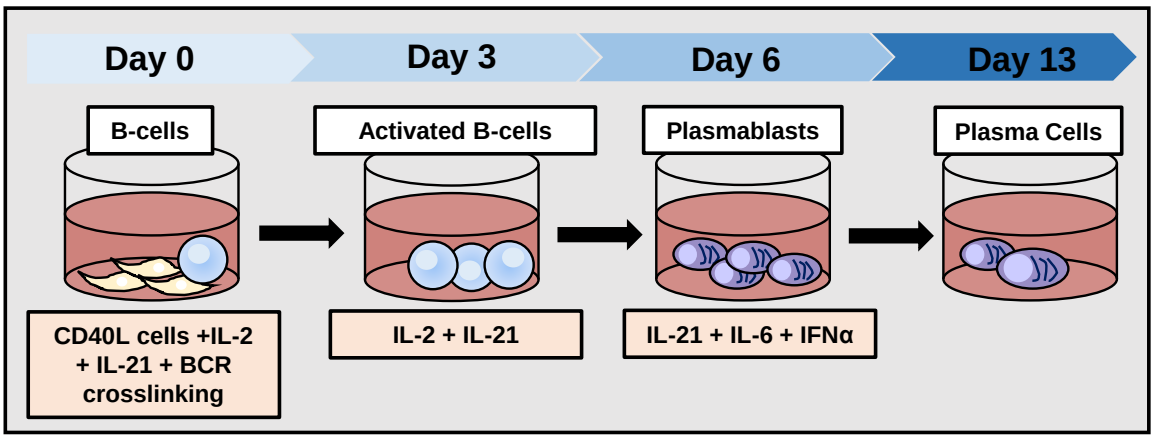


Figure 2.1 *In vitro* plasma cell differentiation workflow

B-cells isolated from peripheral blood are first cultured with IL-2, IL-21, F(ab')$_2$ and CD40L expressing fibroblasts to activate cells. At day 3 activated B-cells are cultured with IL-2 and IL-21 and CD40L stimuli is removed to induce differentiation to plasmablasts. At day 6, plasmablasts are then cultured with IL-21, IL-6 and IFNα to induce plasma cell differentiation. At Day 13 a pure population of plasma cells is achieved.

Table 2.2 Summary of cytokines and mitogens used for *in vitro* B-cell differentiation

Time-Point	Media Supplements
Day 0	20 u/ml IL-2 500 ng/ml IL-21 2 µg/ml F(ab')$_2$
Day 3	20 u/ml IL-2 50 ng/ml IL-21 5 µl/ml Lipids 20 µl/ml Amino Acids
Day 6	50 ng/ml IL-21 10 ng/ml IL-6 100 u/ml IFNα 5 µl/ml Lipids 20 µl/ml Amino Acids
Day 13 Onwards	10 ng/ml IL-6 100 u/ml IFNα 5 µl/ml Lipids 20 µl/ml Amino Acids

Day 13 onwards: at day 13 50% of media was removed and replaced with fresh media plus 10 ng/ml IL-6 and 100 u/ml IFNα. Following this, every 3.5 days 50% of the media from each well was removed and replaced with fresh cytokine supplemented media.

On days 0, 3, 6 and 13 an aliquot of cells were reserved and stained for flow cytometry ($1x10^5$ cells/test) and another prepared for RNA extraction and gene expression analysis by RT-qPCR ($2x10^5$/sample).

Under certain conditions, media was also supplemented with 20 ng/ml TNF or IL-6 between days 0 and 3. Where relevant, IMDM media was also prepared with only 5% FBS and supplemented with 5% serum isolated from a healthy donor or a patient with RA, between days 0 and 3. For certain conditions, $F(ab')_2$ stimulation was substituted with 1µg/ml TLR ligands CpG and R848. For each sample, and each treatment condition, 1 ml supernatant was collected at days 6, 10 and 13, then stored at -20°C for later analysis by IgM quantification ELISA (Section 2.8).

2.4 Flow Cytometry

2.4.1 Viability Stain

Cells were prepared for staining by washing in DPBS (Gibco), either from culture medium or PBS directly following isolation from blood. To allow for distinction between viable and non-viable cells during analysis, cells were first stained with FV780 a fixable viability dye which exhibits 10-20 fold higher staining in non-viable permeable cells (BD Bioscience). Cells ($2x10^5$-$2x10^6$ depending on application) were suspended in 200 µl DPBS containing 1:2000 FV780 and incubated for 10-15 mins at room temperature or 15-30 mins at 4°C. Cells were then washed twice (for each wash cells were centrifuged at 500 x g for 5 mins at 4°C unless otherwise stated).

2.4.2 SNA Lectin Staining

Following viability dye staining, samples of PBMCs collected from patients and healthy donors for surface sialylation study were stained with biotinylated sambucus nigra lectin (SNA) (bSNA, Vector Laboratories). Cells from B-cell activation studies and plasma cell differentiations were stained with FITC

conjugated SNA (fSNA, Vector Laboratories). Cells were suspended in 100 µl DPBS then SNA added at 1:400 (5 µg/ml). Cells were incubated at 4°C for 15 mins then washed twice in autoMACS^{TM} Running Buffer (MACS buffer, Miltenyi Biotech).

2.4.3 Antibody stain for resting B-cell surface sialylation study

Samples of PBMCs collected from patients and healthy donors for surface sialylation study were then suspended in 100 µl staining buffer 1 (50% Brilliant Stain Buffer (BD Bioscience) and 50% Blocking buffer (50 µl human IgG (Invitrogen), 50 µl Mouse IgG (Sigma) + 900 µl MACS buffer).

Cells were then stained with CD19 BV421, CD27 PE-Cy7, CD38 BV605, CD3 APC-Cy7, CD14 APC-Cy7 and CD45 FITC, for 20 mins at 4°C (See Table 2.3 for details of antibodies used for flow cytometry). Cells were then washed twice in MACS buffer then re-suspended in 100µl. PE-Streptavidin (BioLegend) was added at 0.2µg/ml and incubated for 15mins at 4°C. Cells were then washed twice in MACS buffer then re-suspended in 100µl 3% Formaldehyde (CytoFix, BD Biosciences) diluted 1:1 in PBS and incubated for 15mins to fix cells. After fixing cells were washed once then re-suspended in 400µl MACS buffer and stored at 4°C for up to 7 days before analysis.

2.4.4 B-cell Activation Assays

Cells from B-cell activation assays were stained with FV 780, SNA FITC then re-suspended in 100µl staining buffer (50% MACS buffer + 50% blocking buffer) and stained with CD19 BV421, and either CD69 PE + CD80 PE/PE-Cy7 + CD86 APC for 20mins. Cells were then washed twice and fixed if not being analysed same day as described previously, or re-suspended in 150 µl MACS buffer for immediate analysis.

2.4.5 B-cell Differentiation Assays

Cells from B-cell differentiation assays were stained with FV 780, SNA FITC then re-suspended in 100µl staining buffer 1 and stained with CD19 BV421, CD20 PE, CD27 PE-Cy7 CD38 BV605 and CD138 APC for 20mins. Cells were then washed twice and fixed if not being analysed same day as described previously, or re-suspended in 200µl MACS buffer for immediate analysis.

Table 2.3 Antibodies used for flow cytometry

Target	Fluorochrome	Clone	Manufacturer	Concentration
CD19	BV421	HIB19	BioLegend	1 µg/ml
CD20	eFlour 450	2H7	eBioscience	0.02 µg/ml
CD20	PE	2H7	Thermo Fisher	0.0024 µg/ml
CD27	PE-Cy7	M-T271	BD Bioscience	20 µl/ml
CD38	BV605	HB7	BD Bioscience	20 µl/ml
CD45	FITC	HI30	BD Bioscience	100 µl/ml
CD69	PE	FN50	BioLegend	1 µg/ml
CD69	BUV395	FN50	BD Bioscience	20 µl/ml
CD80	PE/PE-Cy7	2D10	BioLegend	4 µg/ml
CD86	APC	BU63	BioLegend	2 µg/ml
CD138	APC	44F9	Miltenyi Biotech	20 µl/ml
HLA-DR	FITC	G46-6	BD Bioscience	100 µl/ml

2.4.6 Analysis on CytoFlex S / CytoFlex LX

All flow cytometry experiments were analysed using either the CytoFlex S (Beckman Coulter, Inc.) with a 4 laser configuration – 375 nm near UV, 405 nm Violet, 488 nm Blue and 63 3nm Red, or the Cytoflex LX (Beckman Coulter, Inc.) with a 6 laser configuration – 355 nm UV, 405 nm Violet, 488 nm Blue, 560nm Yellow-Green, 633nm Red and 800 nm Infrared. Thresholds were set to exclude small particulate debris, and up to 500000 events were collected during analysis depending on application. Gates were set using fluorescence minus one (FMO) controls to determine positive staining populations.

In order to calculate compensation required to account for bleeding of one fluorescent channel into another, prior to analysis of samples, comp beads (BD Bioscience) were suspended in 200µl MACS buffer (one drop of positive and one drop of negative control) and stained with antibodies used in each experiment. Beads were then incubated on ice for 20mins then washed once in MACS buffer. For analysis, 10000 events were captured for each set of beads and automatic compensation applied (calculated by CytExpert 2.3 (Beckman Coulter, Inc.)). Compensation matrices were calculated separately for each machine due to differences in laser and filter configurations.

2.4.7 Flow Cytometry Data Analysis

All flow cytometry data collected by either the CytoFlex S or CytoFlex LX was analysed using CytExpert 2.3 software. FlowJo (BD Bioscience) was also used in some cases to analyse data and produce some histograms and overlays for figures. Compensation was applied in CytExpert using the compensation matrix feature. In all cases, single cells were selected by excluding doublets by plotting FSC-A with FSC-H. Cells were then further gated based on size and granularity using FSC-A with SSC-A to identify lymphocyte populations, or based on staining with FV780 and SSC-A to identify live lymphocytes. Depending on application cells were then gated on expression of B-cell lineage markers, then mean fluorescence intensity (MFI) of fSNA, bSNA + streptavidin-PE or activation markers CD69 PE, CD80 PE-Cy7 or CD86 APC measured using statistics function (Figure 2.2).

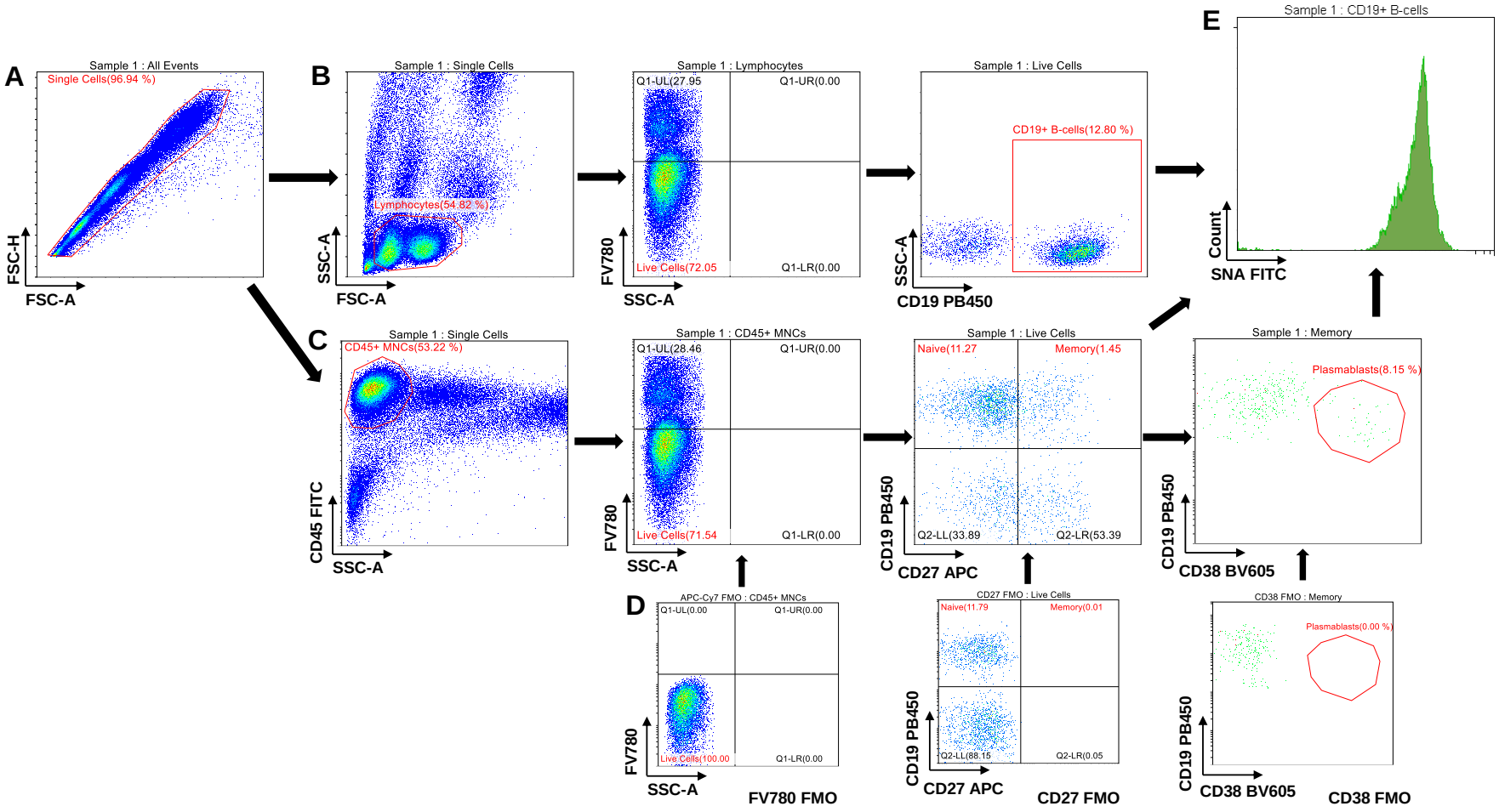


Figure 2.2 Gating strategy for selection of B-cells and B-cell subsets

Single cells are first selected based on size **(A)**, cells were then gated based on forward/side scatter **(B)** or on expression of CD45 and side scatter to select lymphocytes **(C)**. Dead cells were then excluded based on expression of FV780 vs side scatter, then lineage markers were used to select for total B-cells **(B)** or B-cell subsets **(C)**. $CD19^{+}CD27^{-}$ naïve B-cells, $CD19^{+}CD27^{+}$ memory B-cells and $CD19^{+}CD27^{+}CD38^{++}$ plasmablasts were selected based on FMO controls **(D)**. SNA binding was then measured in subsets using MFI of SNA-FITC or PE when using biotinylated SNA with PE streptavidin **(E)**.

2.5 Gene Expression Studies

2.5.1 RNA Extraction

RNA was extracted from cells using the Direct-zol™ RNA Microprep kit (Zymo Research). Cells were harvested from cultures and washed twice in 1 ml PBS. Cells were then re-suspended in 300µl (>1x10^5 cells) or 100µl (≤1x10^5 cells) TriZol (Thermo Fisher). For extended storage, samples were stored at -80°C or kept on ice for immediate extraction.

RNA was then extracted according to the manufacturer protocol. Briefly, an equal volume of 100% ethanol was added to each sample and passed through a column, flow through was discarded then the column washed with RNA wash buffer (Zymo Research). Columns were then treated with DNase I to remove contaminating genomic DNA and then the column washed and RNA eluted in 10 µl Nuclease-free water. RNA concentrations were measured using the NanoDrop Spectrophotometer (Thermo Fisher) and stored at -80°C for extended storage, or kept on ice for further application.

2.5.2 cDNA Synthesis

cDNA was synthesised from RNA by Reverse Transcription (RT). The RT reaction was prepared with reagents from High Capacity cDNA Reverse Transcription Kit with RNase (Applied Biosystems). RNA was first normalised to the lowest RNA concentration value being used in the experiment by dilution in Nuclease-free water. A master mix was then made, containing all reagents from the RT kit (Table 2.4) 10 µl master mix was then added to 10 µl diluted RNA in 0.2 ml PCR tubes, reaction mixtures mixed well then centrifuged briefly. Reactions were then placed in thermal cycler (TC-512, Techne) and RT reaction run as specified by the kit manufacturer (Table 2.5). Following RT reaction cDNA was stored at 4°C for up to 3 days or at -20°C for extended storage.

2.5.3 RT-qPCR

To examine B-cell expression of genes related to sialylation at baseline, or in response to stimuli – quantitative reverse transcription polymerase chain reaction

(RT-qPCR) was used to determine expression relative to a reference gene. cDNA samples from B-cells were analysed for expression of NEU1 and ST6Gal relative to expression of HPRT1 or PPP6C. 96 or 384 well PCR plates containing 10 or 5 µl reactions respectively, were used with all samples run in duplicate for each target and housekeeping gene. Housekeeping genes HPRT1 and PPP6C were selected due to their relatively stable and ubiquitous expression across B-cell subsets during activation[260,261]. Before loading plates, master mixes were made up containing a Taqman assay mixture (Thermo Fisher Scientific) – either HPRT1 (Hs99999909_m1), PPP6C (Hs00254827_m1) ST6Gal1 (Hs00949382_m1) or NEU1 (Hs00166421_m1), Luna Universal qPCR Probe Master Mix (New England Biolabs) and nuclease-free water (Table 2.6). 8 or 4 µl master mix was then added to each well, followed by 2 or 1 µl cDNA. Plates were run and Ct values recorded on QuantStudio 5 or QuantStudio 7 Real Time PCR System (Applied Biosystems), for 96 and 384 well plates respectively.

2.5.4 qPCR Data Analysis

Ct values obtained from RT-qPCR were used to calculate ΔCt values for each sample in each target gene by first calculating the mean Ct of repeats, then by applying the following equation ΔCt = 2^(Ct Reference – Ct Target).

Table 2.4 RT Reaction Mixture

Reagent	Volume/reaction (µl)
RT Buffer	2
dNTPs	0.8
RT Random Primers	2
Multiscribe Reverse Transcriptase	1
RNase Inhibitor	1
Nuclease-Free Water	3.2

Table 2.5 RT Reaction Protocol

	Phase 1	Phase 2	Phase 3	Phase 4
Time (mins)	10	120	5	∞
Temp (°C)	25	37	85	4

Table 2.6 RT-qPCR Reaction Mixture

Reagent	Volume/ 10 µl Reaction (µl)	Volume/ 5 µl Reaction (µl)
Master Mix	5	2.5
Probe	0.5	0.25
Water	2.5	1.25

2.6 Rituximab B-cell Killing Assay

To determine the effects of neuraminidase (Neu) treatment on B-cell susceptibility to killing by rituximab, and *in vitro* rituximab B-cell killing assay was performed based on a previously described assay[262]. $1x10^6$ B-cells isolated from healthy donors were first treated for 1 hr ± 100 mU Neu. B-cells were then washed and stained in 500 µl CFSE (Biolegend) diluted 1:1000 in PBS (5 µM). Cells were incubated with CFSE for 15 mins at 37°C, then an equal volume of FBS was added and reaction was incubated for a further 5 mins at room temperature protected from light. Cells were then washed twice in media at re-suspended at $1x10^6$ cells/ml then 50 µl cell suspension added to a 96 well plate. Wells were then topped up to 200 µl volume with either media alone, 50% media + 50% healthy donor serum, media containing $9x10^6$ cells/ml PBMCs isolated from the same donor ± 50% healthy donor serum. Rituximab (Truxima, Celltrion Healthcare) was then added to half of the wells at 10 µg/ml and the reaction was incubated at 37°C for 4 hrs. Cells were then washed twice in PBS and stained with FV780 as described previously then fixed prior to analysis on the CytoFlex S. Percentage of viable CFSE-stained B-cells following rituximab was calculated, using Precision Count Beads™ (Biolegend) to produce an accurate count of B-cells within each population.

2.7 Serum Neuraminidase Assay

Activity of neuraminidase in serum was determined using the Amplex™ Red Neuraminidase (Sialidase) Assay Kit (Thermo Fisher Scientific). Serum was collected from healthy donors and patients with RA in red-top serum collection tubes which were allowed to sit at room temperature for at least 30 mins to allow blood to coagulate, then centrifuged at 1200 x g for 10 mins and serum supernatant aspirated away from clot. Samples were stored at -80°C prior to analysis. Serum neuraminidase activity was measured using the kit, following the manufacturer's protocol. Briefly, serum was diluted to 30% in reaction buffer (from kit) and added to wells of 96 well flat-bottom plate. A 2X solution of Amplex red was then added to wells in equal volumes to serum (1X final concentration). The reaction was then incubated at 37°C, and absorbance was measured at 560 nm using the Cytation™ 5 (Biotek Instruments, Inc.) plate reader after 30, 60, 90 and 120 mins. Absorbance, which indicates increasing neuraminidase activity, for

each sample was calculated by subtracting the absorbance measurement from blank wells, removing background absorbance levels.

2.8 IgM quantification ELISA

IgM produced by B-cells during differentiation was measured using the Human IgM ELISA Quantitation Set (Bethyl Laboratories, Inc.). IgM in the culture supernatants were measured following the kit protocol. Briefly, 96 well plates (Nunc MaxiSorp C bottom well Modules & Frame, Bethyl Laboratories, Inc.) were coated with anti-IgM antibody diluted 1:100 in ELISA coating buffer (Bethyl Laboratories, Inc.) by incubating at room temperature for 1 hr. Plates were then washed and blocked with blocking solution (Bethyl Laboratories, Inc.) for 30 mins at room temperature or overnight at 4°C. Samples of supernatant were then diluted 1 in 50 or 1 in 500 in sample diluent (Bethyl Laboratories, Inc.) then added to the plate and incubated for 1 hr at room temperature. Samples were analysed in duplicate, as were standard dilutions containing 0 – 1000 ng/ml IgM from human reference serum (Bethyl Laboratories, Inc.). The plate was then washed and then incubated for 1 hr with the HRP detection antibody, before the last wash then the addition of TMB substrate (Bethyl Laboratories, Inc.) which was incubated for up to 15 mins protected from light. After 15 mins the stop solution was added then the fluorescence measured within 30 mins. Fluorescence was measured using the Cytation^{TM} 5 (Biotek Instruments, Inc.) plate reader at 450 nm. For each plate, a standard curve was calculated using GraphPad Prism v7 from the standard dilutions. Concentrations in the supernatants were then interpolated based on the standard curves.

2.9 Statistics

All statistical tests were carried out using features of GraphPad Prism v7. ANOVA with additional tests for multiple comparisons were used to compare samples in stimulation experiments. When multiple conditions were compared with an unstimulated sample Dunnett's multiple comparisons test was selected. When comparisons were relevant between treatment conditions as well as in relation to unstimulated cells, Tukey's multiple comparisons test was selected. When unpaired samples were compared across patient or healthy donor cohorts, Sidak's multiple comparisons tests were used. Paired t tests were used to analyse data with only one variable. Where sialylation – measured as PE or FITC

MFI - was the desired output of experiments, data is presented as fold change in relation to unstimulated or healthy donor samples. Data is presented in this way to avoid bias introduced by MFI measurements taken across several different biological repeats which may have taken place over a number days. MFI measurements in human primary cells were found to be highly variable, and fold change allowed for easier visualisation of trends in the data.

Chapter 3
Investigating B-cell sialylation, and the impact of B-cell activation in rheumatoid arthritis.

3.1 Introduction

Sialic acids are involved in a range of biological processes, and can perform multiple functions in glycan chains, including forming or masking receptors and binding sites[162]. The arrangement of SA in glycoproteins can also determine the outcome of receptor-ligand binding. As discussed in Chapter 1, in relation to humoral immunity, isoforms of IgG with reduced or absent sialylation of N-linked Fc glycans have a higher affinity for activating Fc receptors, which promote immune cell activation[222]. Isoforms with sialylated Fc glycans have a higher affinity for inhibitory receptors – which increase cell activation thresholds[217]. Reduced Fc glycan sialylation is a common feature of autoimmune diseases, including in ACPA IgG in RA, and is thought to contribute to increased autoimmune inflammatory activity[228]. Further to this well-established feature of ACPA IgG, it has been recently described that ACPA IgG also contains increased quantities of sialylated Fab glycans[230]. The sialylated Fab glycans are added during rounds of somatic hypermutation, suggesting they add a selection advantage for ACPA antibodies, however the exact consequences of increased ACPA Fab sialylation are not yet fully understood[231,232].

Additionally, recent research has shown that expression of α2,6 SA on the surface of plasmablasts – the precursors to plasma cells – is reduced in patients with RA and in patients with asymptomatic autoimmunity in the pre-clinical stage of disease[235]. Surface sialylation was measured as a substitute for directly measuring IgG sialylation, to indicate overall sialyltransferase activity in plasmablasts. The observation that sialylation is reduced on the cell surface is intriguing, considering the discord between Fc and Fab sialylation activity. It was therefore hypothesised that in RA there is downregulated expression of SA on the B-cell surface, independent to changes to Ig sialylation, and that this downregulation may be important for the progression of asymptomatic autoimmunity to chronic inflammation.

Expression of surface SA is likely to have an important impact on B-cell function, due to its wide ranging effects, and due to the presence of numerous receptors for sialylated ligands. However, it is currently unclear how sialyltransferase activity, and surface sialylation are regulated in B-cells. To understand how B-cell sialylation may be dysregulated in disease, it was first important to understand how sialylation is regulated in cells from healthy individuals. Firstly, the impact of B-cell activation status on sialylation was considered. The two mechanisms of B-cell activation considered were via BCR crosslinking and via TLR ligation. Since the fate of the cell following activation is dependent on the type of stimulus it receives, it was hypothesised that mode of activation may lead to variances in surface sialylation. The focus of the work in this chapter was to evaluate the impact of signalling via TLRs and the BCR plus co-stimulatory molecules on B-cell sialylation.

When B-cells encounter their cognate antigen *in vivo*, BCR engagement triggers endocytosis of the receptor and the antigen, which is then processed and displayed on MHC class II molecules on the B-cell surface and is presented to activated T-cells in the lymph node[263]. An immune synapse is formed between B and T-cells and a co-stimulatory signal from CD40L on the T-cell surface is delivered to the B-cell – a requirement for full B-cell activation. Activation of B-cells by these T-cell dependent (TD) antigens leads to proliferation and differentiation of some B-cells to short lived antibody secreting cells (ASCs), with some cells migrating towards the B-cell follicle to undergo CSR and SHM to produce long lived memory B-cells and plasma cells[264]. To emulate this interaction *in vivo*, cells will be stimulated with antibodies to surface bound IgM/G molecules along with soluble CD40L to simulate BCR activation T-cell co-stimulation.

When B-cells encounter antigens which activate the BCR and are also able to activate TLRs, they begin to proliferate and differentiate to short-lived ASCs, foregoing the need for a second signal from T-cells – these are so-called T-cell independent (TI) antigens[264]. B-cells express a number of TLRs, including 1, 6, 7, 9 and 10[265]. TLR7 recognises single-stranded RNA and can be activated *in vitro* using the compound resiquimod or R848[266]. TLR9 recognises unmethylated CpG dinucleotides in bacterial or viral DNA[267]. Both TLR7 and TLR9 are endosomal receptors and they signal via MyD88 to activate B-cells, inducing

cytokine and antibody production, and promoting cell survival[268]. R848 and CpG were added to cultures to stimulate B-cells via TLR pathways, to examine the changes to sialylation induced by different pathways of activation. BCR and TLR signalling pathways are summarised in Figure 3.1.

In order to measure B-cell surface expression of sialic acid, plant lectins were utilised in flow cytometry analyses. Lectins can be detected by flow cytometers, either by direct fluorochrome conjugation or by using biotin and streptavidin secondary stains. Lectins produced by SNA preferentially bind to α2,6 SA[269], which is the most widely studied form of SA linkage, and the one which appears to have the most clinical relevance in autoimmunity. As a result, α2,6 sialylation was the main focus of this chapter. However, the impact of B-cell activation on α2,3 SA expression was also briefly explored. Lectins from Maackia amurensis (MAA) were used to study α2,3 sialylation[270].As well as directly measuring the expression of SA on the B-cell surface, mRNA expression of ST6Gal1 - the sialyltransferase which adds α2,6 linked SA to galactose molecules, and NEU1 - the sialidase which preferentially cleaves α2,6 SA from glycan chains, was measured by RT-qPCR.

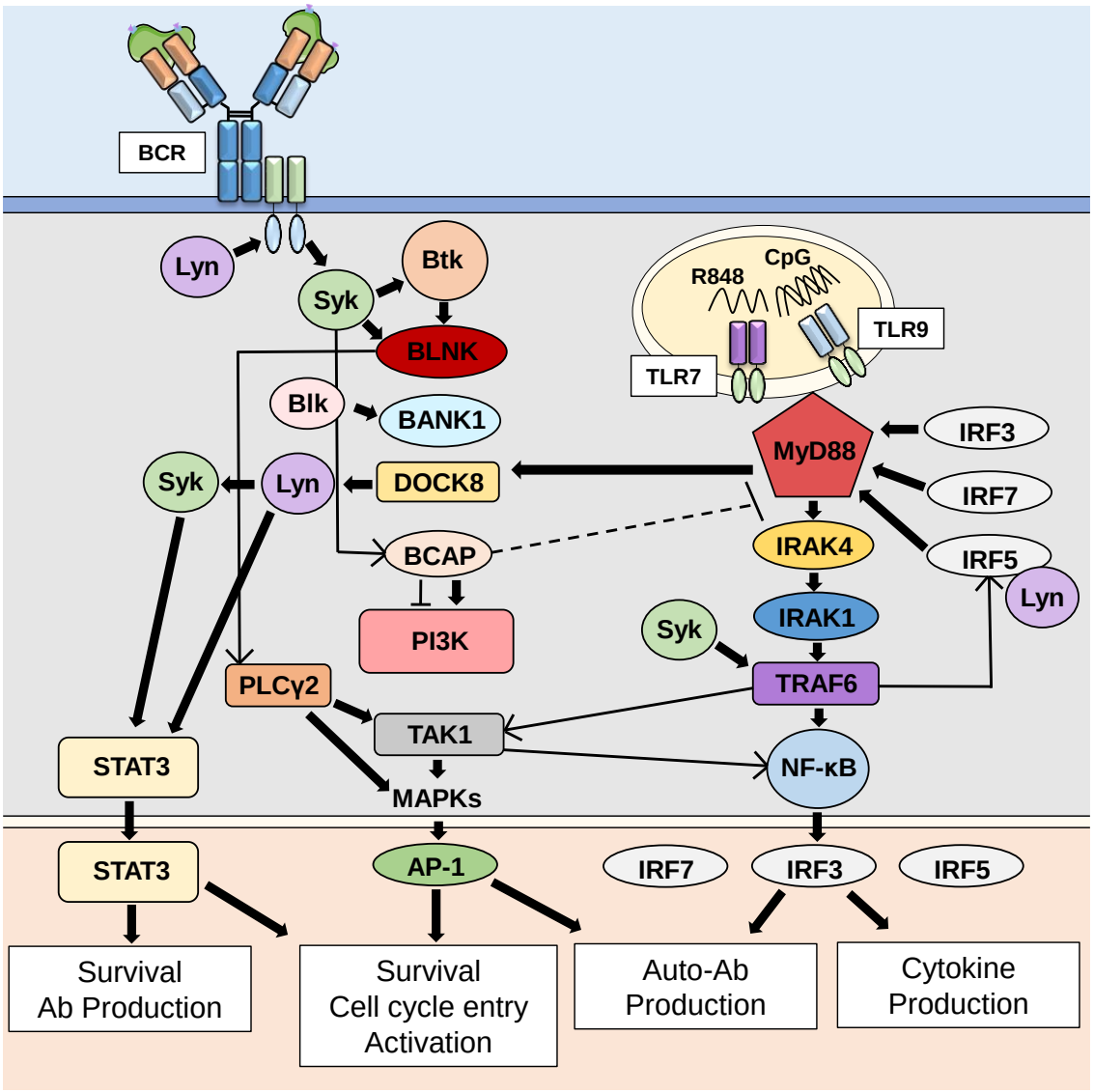


Figure 3.1 Summary of signalling pathways utilised by the BCR and TLRs 7/9

BCR crosslinking by antigen recognition triggers the recruitment of Lck/Yes-related novel protein tyrosine kinase (Lyn) to the CD79 intracellular ITAM domains. Phosphorylated ITAMs recruit and phosphorylate spleen tyrosine kinase (Syk), Bruton's tyrosine kinase (Btk) or B-lymphoid tyrosine kinase (Blk), which can in turn phosphorylate adaptor molecules B-cell linker protein (BLNK), B-cell adaptor for phosphoinositide 3-kinase (BCAP) and B-cell scaffold protein with Ankyrin repeats (BANK1), which can facilitate activation of several divergent pathways including phosphoinositide-specific C phospholipase gamma 2 (PLCγ2), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), mitogen-activated kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB). Activation of NFκB and MAPK pathways leads to further activation of transcription factors which determine functional outcomes, triggering antibody production, cytokine production, cell cycle entry and promoting cell survival. Engagement of TLR7/9 by single stranded RNA or unmethylated CpG dinucleotides respectively, leads to recruitment of myeloid differentiation primary response gene 88 (MyD88). MyD88 recruitment leads to the formation of the Myddsome complex, which is comprised of MyD88, interleukin-1 receptor-associated kinase (IRAK) 4 and 1, and then recruitment of TNF receptor-associated kinase 6 (TRAF6) occurs. TRAF6 associates with TGFβ-activated kinase 1 (TAK1) which autophosphorylates and activated the MAPK or NFκB signalling pathways. MyD88 recruitment can also activate dedicator of cytokinesis 8 (DOCK8), which can lead to initiation of signal transducer and activator of transcription 3 (STAT3) via kinases Lyn and Syk. Figure adapted from Suthers and Sarantopolous, 2017[268].

3.2 Results

3.2.1 Measuring B-cell surface α2,6-sialylation by flow cytometry

Lectins produced by the SNA plant preferentially bind α2,6-SA. SNA lectin can therefore be used to detect α2,6 SA in a mix of glycoproteins or on the surface of cells[269]. The use of fluorophore conjugated SNA lectin, or biotinylated lectin in combination with streptavidin, allows SA on the cell surface to be measured by flow cytometry. Both SNA-fluorescein (fSNA) and biotinylated SNA (bSNA) were optimised for use in further flow cytometry applications. bSNA was used in combination with streptavidin-PE for detection.

3.2.1.1 Optimisation of SNA lectin staining for flow cytometry by titration and neuraminidase digestion of sialic acid

Using lectins for flow cytometry presents a unique set of challenges for optimisation, including the titration of staining concentrations and establishing a valid negative control. A conventional titration to optimise staining, where staining index is calculated using MFI of negative and positive populations, cannot be performed since SA is expressed on the surface of all cells, making it difficult to establish a negative population. Therefore, in order to achieve the best signal to allow identification of positive and negative populations, four concentrations of SNA lectin were tested and the lowest concentration with an acceptable signal relative to unstained cells was selected for further experiments (Figure 3.2). A concentration of 5 µg/ml was selected as the optimal value for staining with both bSNA and fSNA for subsequent experiments.

Isotype controls can be used in conventional flow cytometry to confirm the binding specificity of an antibody and indicate the level of off-target binding. For SNA lectin staining, to confirm the SNA lectin was binding specifically to SA on the cell surface, cells were treated - prior to staining - with Neuraminidase (Neu) to digest SA. PBMCs from healthy donors (HD) were treated for 18 hrs with 100 mU Neu at 37°C then washed and stained with 5 µg/ml bSNA and streptavidin-PE. Treatment with Neu produced a clear decrease in PE signal – confirming the SA binding specificity and sensitivity (Figure 3.2C). Since streptavidin-PE was used as a secondary stain to determine surface sialylation, it was important to determine the degree of non-specific binding of streptavidin-PE. Cells were

stained with streptavidin-PE with/without prior staining with bSNA or left unstained. A small amount of background staining was detected in the sample stained with streptavidin-PE only, leading to the implementation of a further wash step prior to analysis and following staining with streptavidin-PE (Figure 3.2D).

3.2.1.2 Off target lectin binding can impact strength of signal in flow cytometry

Since SNA lectin can bind SA on any protein, using SNA lectin to stain cells as well as using conventional flow antibodies to determine B-cell surface markers, introduced a potential source of off-target binding. Furthermore, a blocking buffer containing 5% human and 5% mouse serum was used to saturate B-cell Fc receptors and avoid non-specific binding of antibodies, introducing further sites for SNA off-target binding. Therefore, the order in which SNA lectin and antibodies - as well as blocking buffer - should be added to cells during staining also had to be taken into consideration. To determine the impact of SNA binding to antibody molecules on the readout, five staining protocols were tested: 1. Blocking buffer first, then a mixed cocktail of antibodies and fSNA second; 2. Blocking buffer first, then fSNA second and antibody cocktail third; 3. Blocking buffer first, then antibody cocktail second, fSNA third; 4. fSNA first, then blocking buffer second and antibody cocktail third; 5. fSNA first, then antibody cocktail mixed with blocking buffer second (Figure 3.3C). It was found that the blocking buffer interfered with fSNA binding and reduced the fSNA signal (Figure 3.3). It was also found that staining cells with antibodies prior to fSNA led to an increased fSNA signal – suggesting fSNA was binding to SA on the antibody molecules. It was therefore determined that SNA lectin should be added prior to the addition of blocking buffer and staining with conventional flow antibodies.

3.2.2 Expression of surface α2,6-sialic acid in resting B-cells from patients with rheumatoid arthritis and healthy donors

Since a previous report showed that plasmablasts from patients with RA have reduced expression of SA on the cell surface, as measured by flow cytometry[235], it was important to first validate these results in patients from the Leeds cohort. Reduction in surface sialylation was also previously described in patients with “pre-RA” (PRA) who test positive for ACPA ± RF but have no specific clinical evidence of RA, suggesting that a reduction in plasmablast or B-cell sialylation

may be established during the early stages of autoimmunity before joint inflammation is apparent, and therefore may be an important mechanism which promotes B-cell autoimmunity.

3.2.2.1 Expression of α2,6-sialic acid is lower in B-cells from patients with RA than in cells from healthy donors

Samples of peripheral blood were collected from patients with biologic-naïve early RA (ERA) (n=10) with a symptom duration of less than 12 months, and from healthy donors (n=5).Since bSNA and streptavidin-PE were used to assess surface SA expression, the output for the measurement of surface SA expression used in this case is PE MFI. Initial experiments on fresh samples of PBMCs isolated from the blood of healthy donors and patients with ERA showed a large degree of variation in PE MFI of samples analysed on different days (results not shown). This was thought to be due to possible differences in machine calibration and/or experimental variation. It was therefore decided that samples of PBMCs should be collected and cryopreserved, to be analysed later in one batch experiment - minimising the chances of experimental and instrument variation. Frozen samples were thawed then stained with a panel of antibodies which allowed sialylation to be measured in $CD27^{-}CD19^{+}$ naïve B-cells, $CD27^{+}CD19^{+}$ memory B-cells and $CD27^{+}CD19^{+}CD38^{++}$ plasmablasts. Samples were also stained with bSNA and streptavidin-PE and PE MFI was measured (Figure 3.4). Results are described as fold change in PE MFI relative to the mean PE MFI of healthy donor samples.

It was found that naïve B-cells, memory B-cells and plasmablasts from patients with RA showed a trend of reduced sialylation compared to healthy donor cells (naïve: 0.658 ± 0.127 vs 1.000 ± 0.391, p=0.1292; memory: 0.722 ± 0.185 vs 1.000 ± 0.406, p=0.2065; plasmablasts: 0.719 ± 0.298 vs 1.000 ± 0.589, p=0.4396) (Figure 3.4). Although subtle changes were observed which were not statistically significant due to small numbers, this result was consistent with the previous reports that plasmablasts from patients with ERA show reduced sialylation[235], as well as suggesting that expression of SA may also be reduced in naïve and memory B-cells from patients with ERA.

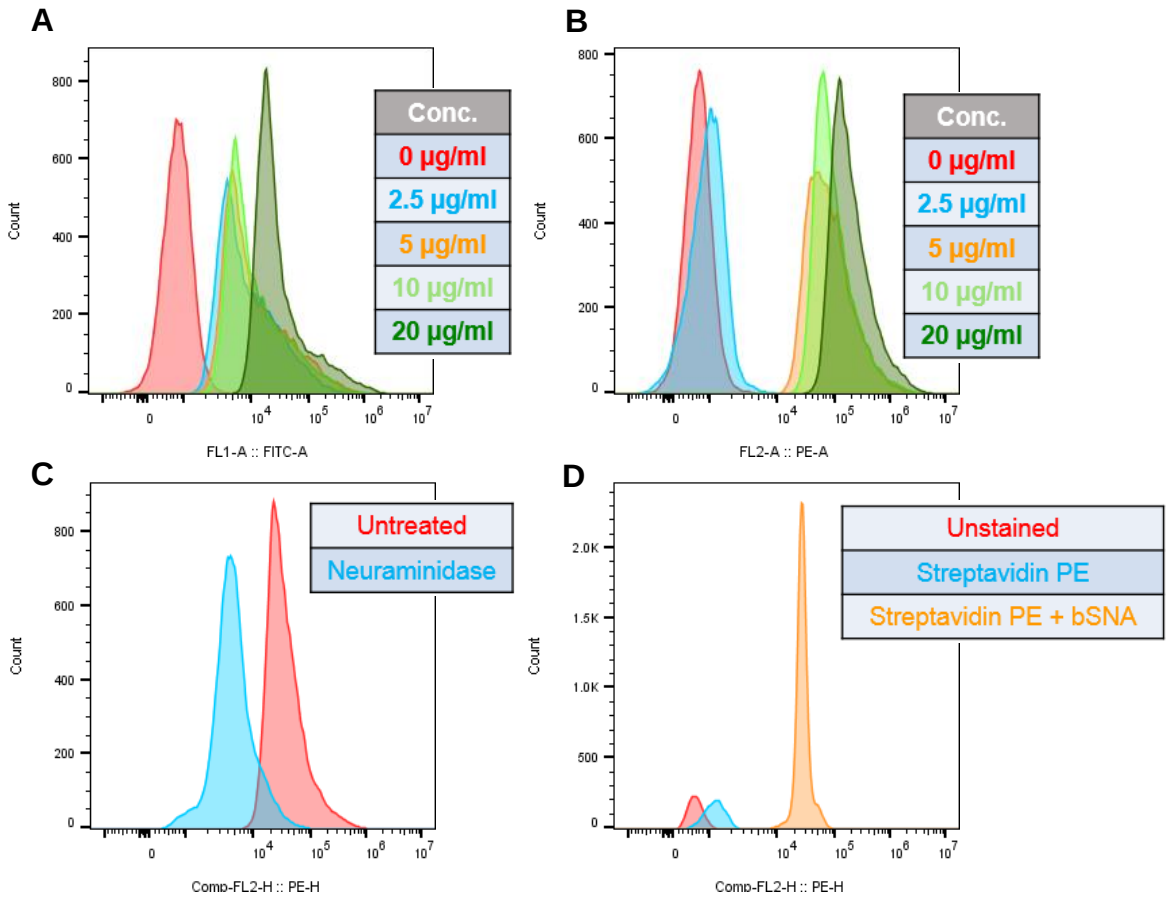


Figure 3.2 Optimisation of SNA lectin staining for flow cytometry

PBMCs from a healthy donor were stained with increasing doses (2.5 - 20 µg/ml) of fluorescein conjugated **(A)** or biotinylated SNA (bSNA) **(B)** or left unstained. Cells stained with bSNA were then stained with streptavidin-PE then fluorescence measured. **(C)** PBMCs from a healthy donor were treated with 100 mU neuraminidase for 18 hrs to digest surface sialic acid then stained with bSNA and streptavidin-PE and fluorescence compared. **(D)** To elucidate streptavidin non-specific binding, PBMCs were stained with 5 µg/ml bSNA and streptavidin-PE, streptavidin-PE only or left unstained and fluorescence measured. Histograms show PE or FITC MFI.

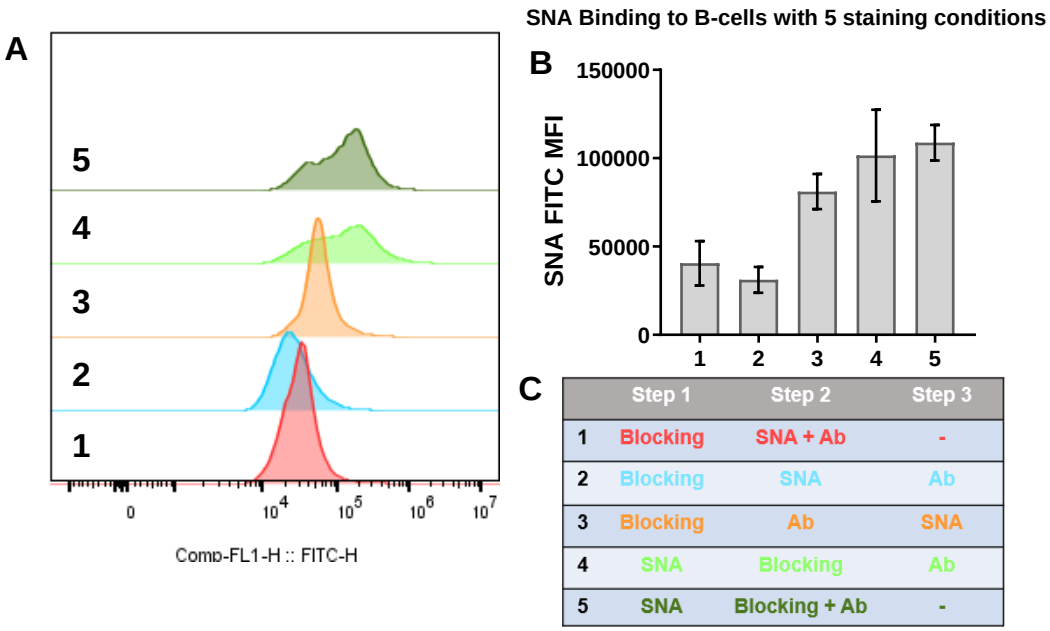


Figure 3.3 Optimising SNA lectin staining in combination with conventional flow antibodies

PBMCs from healthy donors (n=3) were stained with SNA-fluorescein (fSNA), B-cell lineage marker antibodies (CD45 PE-Cy7, CD19 BV421, CD27 APC and CD38 BV605) with or without a blocking step/inclusion of blocking buffer in the staining cocktail. The order of staining/blocking was changed in each of 5 conditions **(C)**. **(A)** Histogram overlay shows FITC MFI of merged samples. **(B)** Bar chart shows mean with SD of FITC MFI.

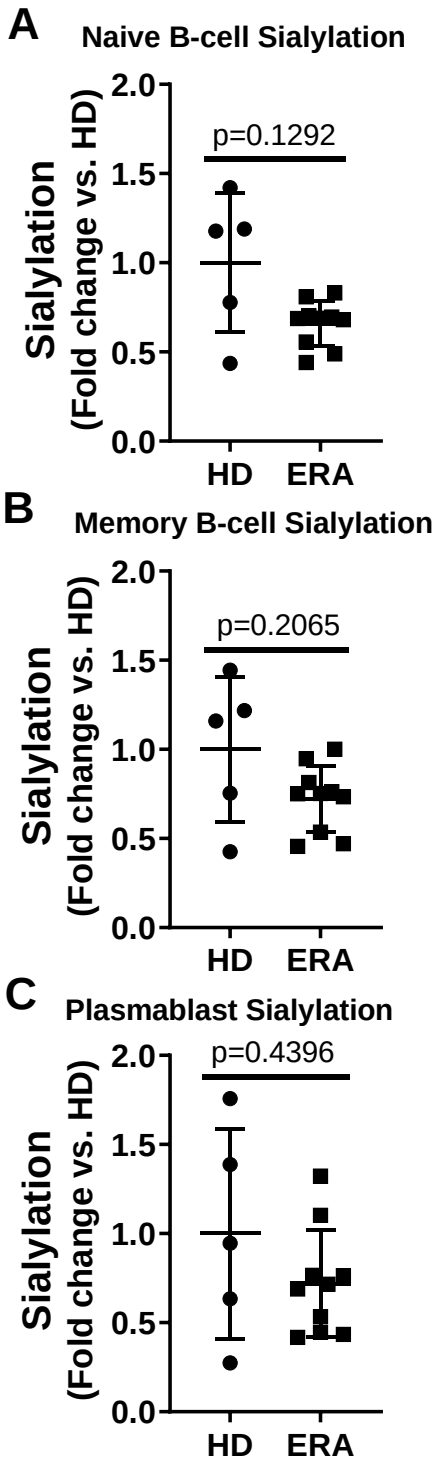


Figure 3.4 Baseline sialylation in B-cell subsets from healthy donors and patients with ERA

PBMCs from healthy donors (HD) (n=5) and patients with early RA (ERA) (n=10) were stained with B-cell lineage markers and bSNA with streptavidin PE. Cells were analysed by flow cytometry and gated based on expression of surface markers – $CD19^{+}CD27^{-}$ naïve B-cells **(A)**, $CD19^{+}CD27^{+}$ memory B-cells **(B)** and $CD19^{+}CD27^{+}CD38^{++}$ plasmablasts **(C)**. Graphs show mean PE MFI with SD, expressed as a ratio based on the mean MFI of samples from healthy donors. Mann Whitney tests were used to calculate p values.

3.2.2.2 Expression of α2,6-sialic acid is reduced in patients with RA prior to the onset of symptomatic inflammation

Samples were collected from patients with PRA (n=5) and healthy donors (n=5) to validate previous results which showed reduced surface SA in plasmablasts from patients with PRA[235]. As in section 3.2.2.1, cells were stained with B-cell surface marker antibodies and bSNA with streptavidin-PE. Results suggested a trend for reduced sialylation in cells from patients with PRA compared to healthy donors in plasmablasts (0.541 ± 0.279 vs 1.000 ± 0.589, p=0.2222- fold change in PE MFI relative to healthy donor samples) (Figure 3.5C), as well as in naïve (0.592 ± 0.289 vs 1.000 ± 0.391, p=0.1508) (Figure 3.5A) and memory B-cells (0.670 ± 0.333 vs 1.000 ± 0.406, p=0.1508) (Figure 3.5B). This agrees with previously reported findings[235] and suggests that there is a trend for reduces sialylation in naïve and memory B-cells even in the pre-clinical stage of disease and thus may play a role in the onset of symptomatic autoimmunity.

3.2.2.3 Expression of α2,6-sialic acid decreases in B-cells during the pre-clinical stages of RA and remains lower upon progression to symptomatic inflammation

To examine changes in B-cell sialylation in patients at different stages of disease, samples of B-cells from healthy donors (n=5), patients with PRA (n=5) and patients with early RA, which were divided into two categories based on whether sample was collected at the time of diagnosis – new early RA (N-ERA, n=5) or if the sample was taken at a later clinic visit, still within 12 months of symptom onset/diagnosis – later early RA (L-ERA, n=5). All patients were bDMARD naïve but L-ERA patients were treated with csDMARDs according to local protocol.

Differences in sialylation between cell types were subtle, however sialylation tended to be lowest in naïve and memory B-cells in patients in the PRA group (Figure 3.6A, 3.6B), however this group also had the highest degree of variation. In plasmablasts it was also found that expression of SA was slightly higher in the early RA groups than the PRA group, however these changes were not statistically significant (Figure 3.6C). The results suggest that the decrease in sialylation which occurs in the pre-clinical phase of disease is maintained at the onset of symptomatic inflammation, indicating that this may be an important feature which drives the progression of autoimmune synovial inflammation.

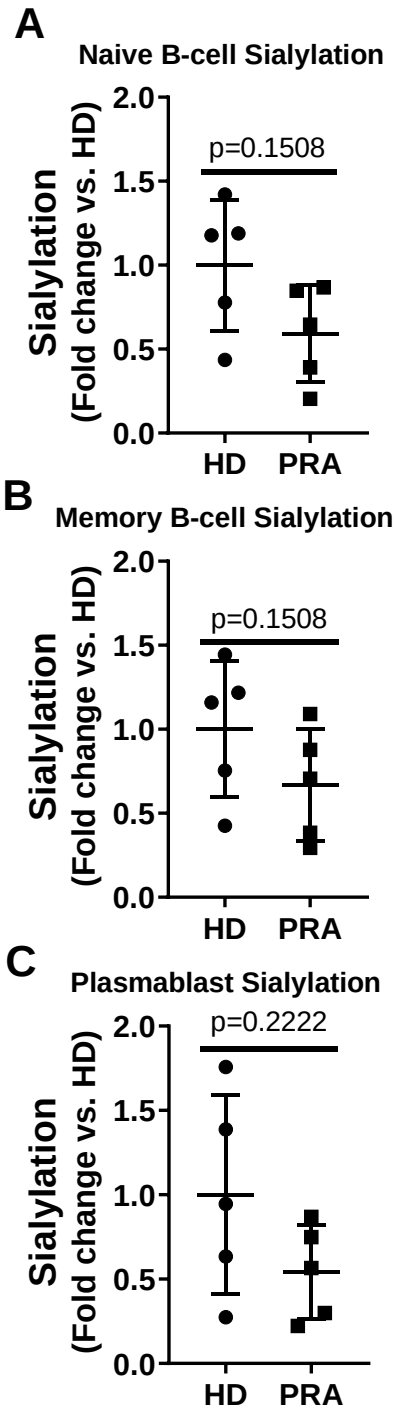


Figure 3.5 Baseline sialylation in B-cell subsets from healthy donors and patients with pre-RA

PBMCs from healthy donors (HD) (n=5) and patients with a pre-RA diagnosis (PRA) (n=5) were stained with B-cell lineage markers and bSNA with streptavidin PE. Cells were analysed by flow cytometry and gated based on expression of surface markers – $CD19^{+}CD27^{-}$ naïve B-cells **(A)**, $CD19^{+}CD27^{+}$ memory B-cells **(B)** and $CD19^{+}CD27^{+}CD38^{++}$ plasmablasts **(C)**. Graphs show mean PE MFI with SD, expressed as a ratio based on the mean MFI of samples from healthy donors. Mann Whitney tests were used to calculate p values.

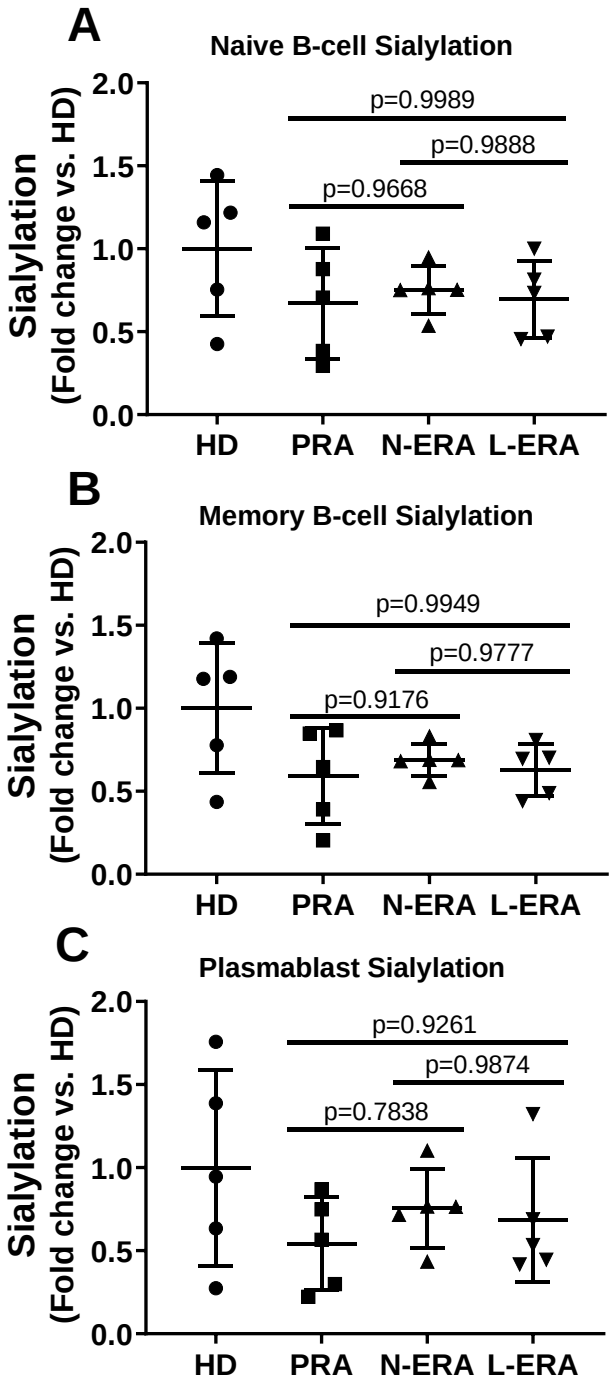


Figure 3.6 Baseline sialylation in B-cell subsets from healthy donors and patients with RA or pre-RA

PBMCs from healthy donors (n=5) and patients with a pre-RA diagnosis (PRA) (n=5), newly diagnosed RA (NRA) or established early RA (ERA) were stained with B-cell lineage markers and bSNA with streptavidin PE. Cells were analysed by flow cytometry and gated based on expression of surface markers – $CD19^{+}CD27^{-}$ naïve B-cells **(A)**, $CD19^{+}CD27^{+}$ memory B-cells **(B)** and $CD19^{+}CD27^{+}CD38^{++}$ plasmablasts **(C)**. Graphs show mean PE MFI with SD, expressed as a ratio based on the mean MFI of samples from healthy donors. One-way ANOVA followed by Sidak's multiple comparisons tests were then used to analyse statistical significance and generate p values.

3.2.3 Impact of activation via the BCR and TLRs on B-cell surface sialylation in cells from healthy donors and patients with RA

Since B-cell expression of SA has been shown to be reduced in cells from patients with ERA and PRA, it was hypothesised that this may be a feature of autoimmune inflammation which contributes to the onset of disease in RA. However, the regulation of sialylation in B-cells during homeostasis is not well understood. One of the key events in B-cell autoimmunity is the activation of autoreactive B-cells, which leads to proliferation and differentiation, and the production of autoantibodies. Therefore the impact of B-cell activation on expression of surface SA was investigated.

To determine the influence of B-cell activation on expression of surface SA, several B-cell activating factors were tested *in vitro*. B-cells may be activated via several pathways, including BCR activation, TLR activation, T-cell co-stimulation and cytokine receptor activation. In this section, induction of B-cell activation via CpG and R848, ligands for TLR9 and TLR7 respectively, as well as crosslinking the BCR with antibodies to IgM and IgG were tested. Activation of B-cells leads to upregulation of several surface markers, including CD69 and HLA-DR[271], which were measured alongside sialylation in stimulated cells.

3.2.3.1 B-cells are activated *in vitro* with TLR ligands and BCR crosslinking

Initially, to optimise *in vitro* activation of B-cells, CpG and anti-IgM/G F(ab') fragments ($F(ab')_2$) were added in increasing concentrations (1, 2.5 and 5 µg/ml and 5, 10 and 20 µg/ml respectively) to B-cell cultures with 20 ng/ml IL-4 (included to promote B-cell survival in cultures). Expression of CD69 and HLA-DR were measured by flow cytometry at baseline and after 24, 48 and 72 hrs. Both stimuli induced a robust increase in expression of CD69 at 24 hrs, which was decreased slightly at 48 and 72 hrs (Figure 3.7A, 3.7B). In contrast, HLA-DR expression was highest after 72 hrs of stimulation with CpG, and stimulation with $F(ab')_2$ did not lead to a significant increase in HLA-DR over 72 hrs (Figure 3.7C, 3.7D). It was also found that in both cases, the lowest tested doses of CpG and $F(ab')_2$ (1 µg/ml and 5 µg/ml respectively) were sufficient to induce a robust response, in terms of CD69 upregulation. Similar to CpG, activation of B-cells with TLR7 agonist R848 was also optimal at 1 µg/ml (data not shown). Expression of HLA-DR was also

influenced by IL-4, which was excluded from further experiments in this section and was studied separately in Chapter 4.

3.2.3.2 Expression of sialylated surface proteins is increased in B-cells stimulated with TLR ligands and F(ab')$_2$ from healthy donors but not in cells from patients with RA

To determine the influence of B-cell activation by BCR crosslinking and TLR stimulation on expression of surface SA, sialylation was measured in B-cells from healthy donors (n=5) stimulated for 48 hrs with either 5 µg/ml F(ab')$_2$ or 1 µg/ml CpG or R848. It was found that stimulation with CpG or R848 led to an increase in B-cell sialylation after 48 hrs (1.953 ± 0.273, p=0.0014, and 1.466 ± 0.421, p=0.0684 respectively - expressed as fold change relative to unstimulated samples), however this effect was more modest in F(ab')$_2$ stimulated cells, which led to a small increase in sialylation (1.171 ± 0.200, p=0.0103) (Figure 3.8A). Cells stimulated with R848 showed the highest percentage of CD69$^+$ cells, suggesting the highest degree of activation (Figure 3.8B), however the greatest increase in levels of SA expression were detected in cells stimulated with CpG, suggesting that increased expression of SA may not directly correlate with increased B-cell activation at least in terms of CD69 upregulation, and that changes to B-cell sialylation may be dependent on the type of stimulus.

B-cells from patients with ERA (n=5) were also stimulated for 48 hrs with either 5 µg/ml F(ab')$_2$ or 1 µg/ml CpG or R848. Strikingly, sialylation in cells these cells did not significantly increase in response to either F(ab')$_2$ (0.992 ± 0.109, p=0.9990), CpG (1.004 ± 0.118, p=0.990) or R848 (1.036 ± 0.127, p=0.9309) stimulation (Figure 3.8C). It did not appear that this was due to lack of cell activation, as there were similar percentages of CD69$^+$ cells following stimulation to cells from healthy donors (Figure 3.8D). Despite this, the percentage of CD69$^+$ cells was lower in cells stimulated with CpG compared with F(ab')$_2$-stimulated cells (54.35 ± 6.13 vs. 71.62 ± 5.87, p=0.0020) whereas, in cells from healthy donors, differences in CD69+ cells in CpG and F(ab')$_2$ stimulated conditions were not statistically significant (63.22 ± 19.43 vs. 51.48 ± 29.07, p=0.9999). This suggests there is a decreased response to TLR9 in cells from patients with ERA. Overall these results suggest that mechanisms which regulate sialylation in B-

cells in response to activation may be dysregulated in patients with ERA, and there may be a decreased response to TLR9 activation in patients with RA.

To determine if dysregulated sialylation in response to stimulation is present from the onset of B-cell autoimmunity, B-cells from patients with PRA were also stimulated with F(ab')$_2$, CpG or R848. Results showed a slight increase in sialylation in response to stimulation with F(ab')$_2$ (1.327 ± 0.118, p=0.1708)(Figure 3.8E). However, similarly to cells from patients with ERA, there was no change in sialylation in response to stimulation with CpG (1.094 ± 0.094, p=0.9999) and there was only a slight increase in response to R848 (1.224 ± 0.153, p=0.1708). Interestingly, in the cells from patients with PRA, the percentage of CD69$^+$ cells was much lower in TLR ligand-stimulated cells compared to F(ab')$_2$-stimulated cells (F(ab')$_2$: 81.15 ± 9.91 vs CpG: 35.38 ± 8.50 p=0.0078 and R848: 48.58 ± 13.11 p=0.0312) (Figure 3.8F), suggesting that there may be dysregulated response to TLR stimuli at this stage of disease. Taken together, the results from patients with ERA and PRA suggest that mechanisms which control expression of SA in response to stimuli may be dysregulated, and response to TLR ligands may be dampened even in the asymptomatic autoimmunity phase of disease.

3.2.3.3 Expression of sialylated surface proteins is not increased in B-cells stimulated with TLR ligands and F(ab')$_2$ from patients with RA in response to increasing doses of stimuli

It was hypothesised that B-cells from patients with ERA and PRA may require a higher dose of stimuli for activation to be achieved, since B-cells from patients with ERA and PRA showed less upregulation of CD69 in response to TLR stimulation. To determine if higher doses of TLR stimuli or induction of BCR cross-linking could induced an increase in sialylation, B-cells from patients with ERA were stimulated with increasing doses of CpG (1, 2 and 5 µg/ml – previous dose 1 µg/ml) (n=4) and F(ab')$_2$ (5, 10 and 15 µg/ml – previous dose 5 µg/ml) (n=2). B-cell sialylation did not increase in response to higher doses of CpG (Figure 3.9A), despite percentage of CD69$^+$ cells increasing between 1 and 2 µg/ml (64.18 ± 12.65 vs. 72.01 ± 6.621, p=0.4970) (Figure 3.9B). In these experiments, sialylation was slightly increased in response to 5 µg/ml of F(ab')$_2$ similar to the changes seen previously in cells from healthy donors (1.244 ± 0.244,

p=0.5867 and 1.171 ± 0.200, p=0.3543 respectively), and was also slightly increased in cells stimulated with 10 and 15 µg/ml F(ab')$_2$ (Figure 3.9C). Expression of CD69 increased in response to F(ab')$_2$ in a dose dependent manner (Figure 3.9D). These results suggest that mechanisms which lead to the upregulation of sialylation in response to TLR9 stimulation may be impaired in cells from patients with ERA, however the cells appear to respond in a similar manner to healthy donor cells to stimulation with F(ab')$_2$.

3.2.4 Impact of co-stimulation via CD40/CD40L on B-cell surface sialylation in cells from healthy donors and patients with RA

Previous results have shown that B-cells from healthy donors upregulate sialylation in response to TLR stimulation and, to a lesser extent, in response to BCR cross-linking via F(ab')$_2$ (Figure 3.8A). *In vivo*, when B-cells are activated via the BCR with TD antigens, they require a second signal via a co-stimulation from activated T-cells to become fully activated. One such co-stimulatory molecule expressed on the T-cell surface is CD40L, the ligand for CD40, which is expressed on the B-cell surface. To determine the impact of co-stimulation on B-cell activation and regulation of sialylation, cells were stimulated *in vitro* with soluble CD40L ± F(ab')$_2$.

3.2.4.1 Expression of sialylated surface proteins is increased in B-cells stimulated with F(ab')$_2$ and CD40L from healthy donors patients with pre-RA but not ERA

To determine the impact of co-stimulatory molecule CD40L on B-cell sialylation, B-cells from healthy donors (n=4) were stimulated with 2 µg/ml CD40L in isolation or in combination with 5 µg/ml F(ab')$_2$. It was found that sialylation was increased slightly in cells stimulated with CD40L alone, but was increased to a greater extent in cells stimulated with both CD40L and F(ab')$_2$ (1.231 ± 0.284, p=0.3820 and 1.377 ± 0.498, p=0.4308 respectively - expressed as fold change relative to unstimulated samples) (Figure 3.10A), however none of these changes reached statistical significance. Stimulation with a combination of CD40L and F(ab')$_2$ also produced the highest percentage of CD69$^+$ cells, indicating better activation was achieved with the dual stimuli, as anticipated (Figure 3.10B). Despite the high level of activation induced by F(ab')$_2$ + CD40L co-stimulation compared to CpG (86.74 ± 7.62 vs. 63.22 ± 19.43 – percentage CD69$^+$ cells), the corresponding

increase in sialylation was less than in cells stimulated by CpG (Figure 3.8B) (1.377 ± 0.498 and 1.953 ± 0.273 respectively), once more suggesting that changes to sialylation are dependent on the mode of activation rather than the strength of the signal.

The response of B-cells from patients with ERA to CD40L co-stimulation was also tested. As previous, cells from patients with ERA (n=5) were stimulated with CD40L and $F(ab')_2$ and sialylation along with activation measured by flow cytometry. Interestingly, cells from patients with RA showed no change in sialylation in response to CD40L in isolation or in combination with $F(ab')_2$ (1.043 ± 0.127, p=0.3569 and 0.946 ± 0.176, p=0.4338 respectively) (Figure 3.10C). Percentage $CD69^+$ cells was comparable to cells from healthy donors following stimulation, though there were fewer $CD69^+$ cells from patients with ERA than from healthy donors stimulated with $F(ab')_2$ + CD40L (69.18 ± 12.98 vs. 86.74 ± 7.62) (Figure 3.10D).

In addition, B-cells from patients with PRA (n=4) were also stimulated with CD40L ± $F(ab')_2$. B-cells from patients with PRA showed similar responses to the cells from healthy donors – with upregulated sialylation in $F(ab')_2$ stimulated, CD40L stimulated and dual stimulated cells (1.327 ± 0.172 p=0.2132, 1.321 ± 0.123 p=0.1243 and 1.401 ± 0.038 p=0.0274 respectively) (Figure 3.10E). Percentages of $CD69^+$ cells were also comparable to healthy donor cells in the CD40L stimulated and dual stimulated cells, however the response to isolated $F(ab')_2$ was increased compared to cells from healthy donors (81.15 ± 9.91 vs. 58.27 ± 24.63) (Figure 3.10F, 3.10A). Taken together, these results suggest that B-cells from patients with ERA and PRA can be activated by CD40L co-stimulation to a similar extent as in cells from healthy donors. The effect on sialylation – a mild increase induced by $F(ab')_2$, CD40L and dual stimulation – is also similar in cells from healthy donors and in cells from patients with PRA, however this response is dampened in cells from ERA.

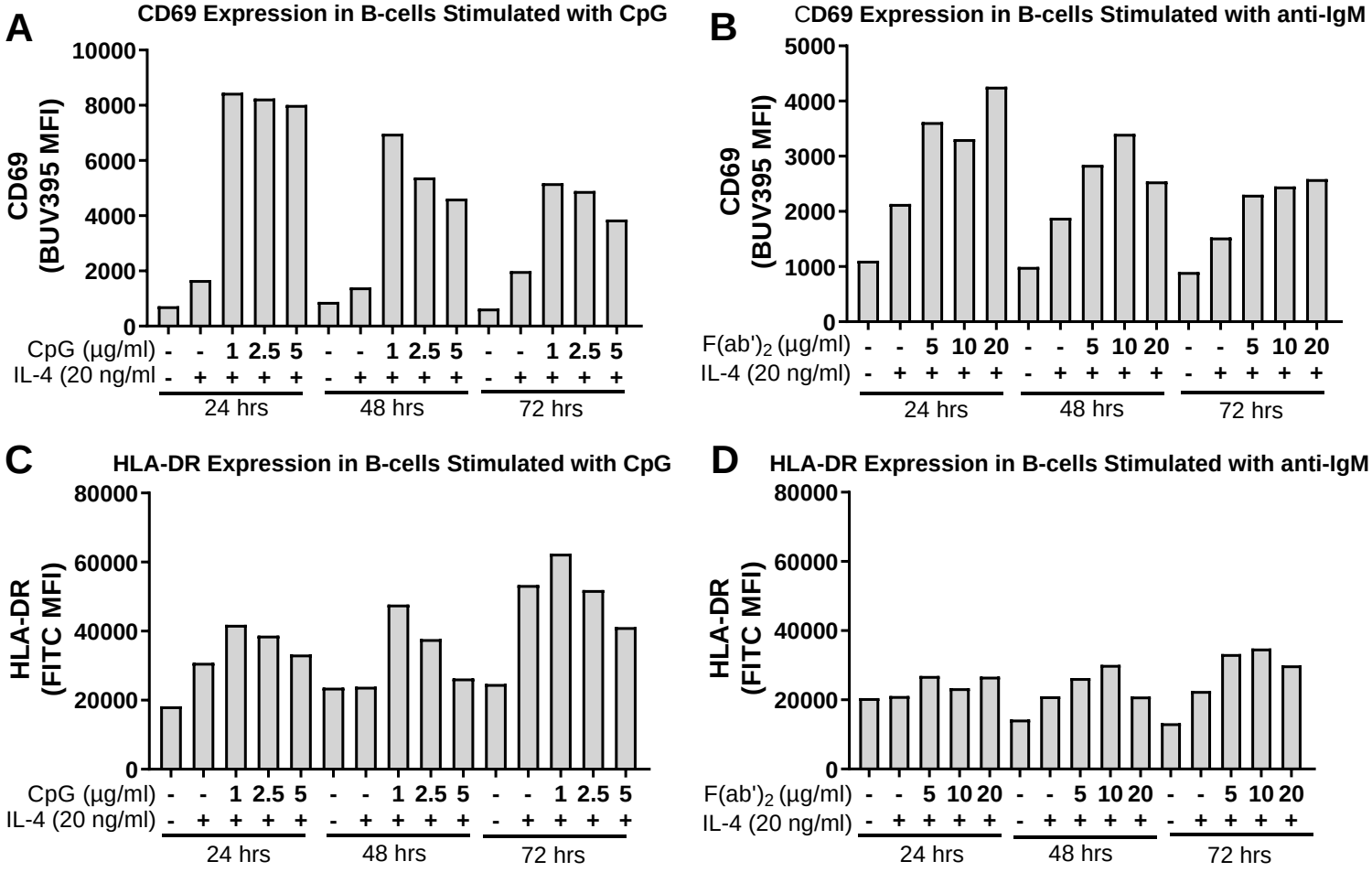


Figure 3.7 Optimising *in vitro* B-cell activation via TLR stimulation and BCR crosslinking

B-cells isolated from healthy donors (n=2) were stimulated for 72 hrs with 20 ng/ml IL-4 and increasing doses of CpG **(A,C)** or F(ab')$_2$ **(B,D).** Expression of activation markers CD69 **(A-B)** and HLA-DR **(C-D)** were recorded at 24, 48 and 72 hrs. Graphs show one representative experiment and either CD69 BUV395 MFI or HLA-DR FITC MFI.

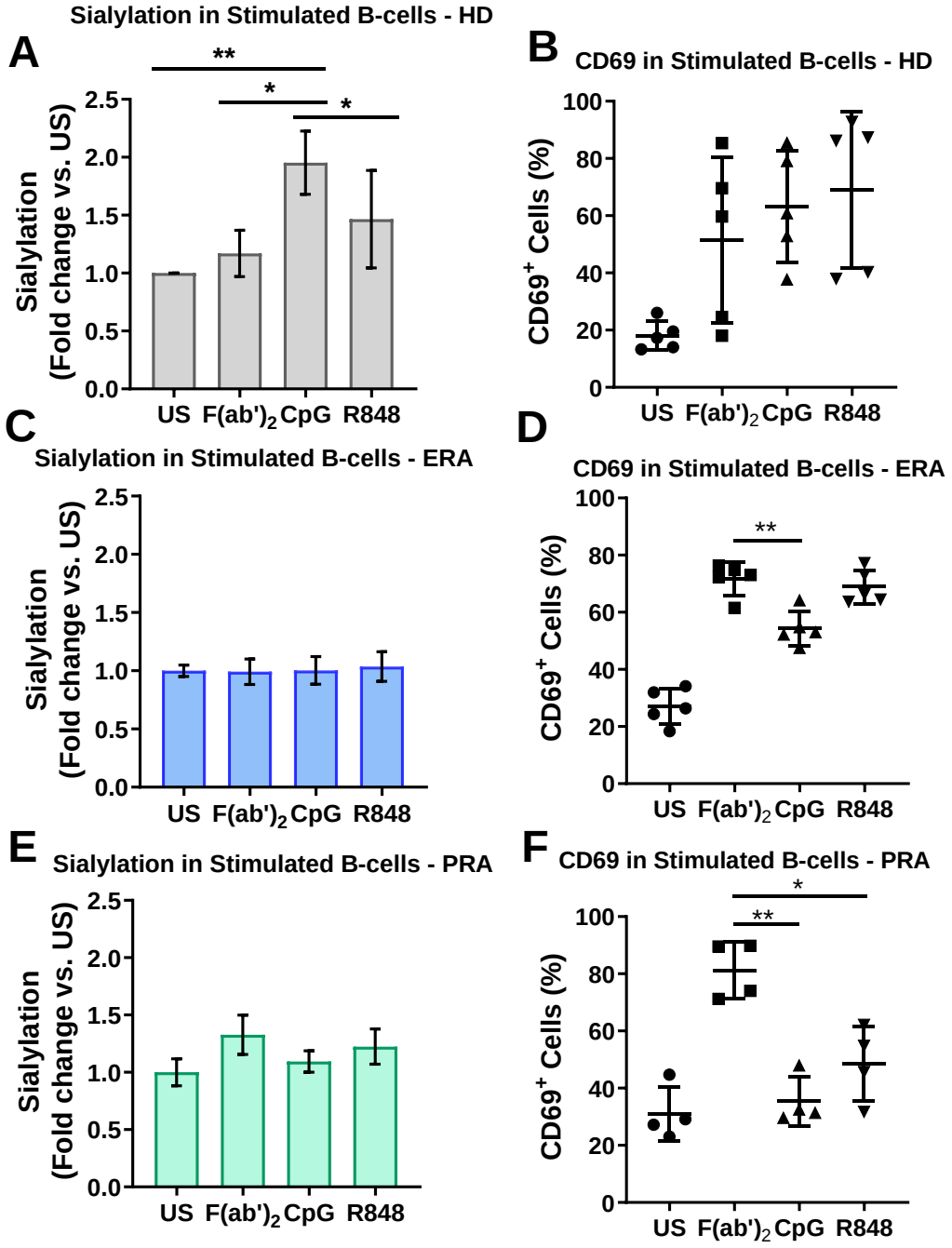


Figure 3.8 Changes to sialylation elicited by *in vitro* B-cell activation via TLR stimulation and BCR crosslinking

B-cells isolated from healthy donors (n=5) **(A-B)**, patients with early RA (ERA) (n=5) **(C-D)** or patients in the pre-RA group (PRA) (n=4) **(E-F)**, were stimulated with 5 µg/ml F(ab')$_2$ or 1µg/ml CpG or R848 for 48 hrs. After 48 hrs sialylation was measured by flow cytometry along with expression of CD69. **(A,C,E)** Graphs show mean with SD of SNA FITC MFI, expressed as a ratio relative to SNA FITC MFI of unstimulated samples. **(B,D,F)** Graphs show mean with SD of the percentage of CD69$^+$ cells. One-way ANOVA followed by Tukey's multiple comparisons tests were used to analyse statistical significance, with relevant statistically significant comparisons highlighted on graphs (* $p<0.05$, ** $p<0.005$).

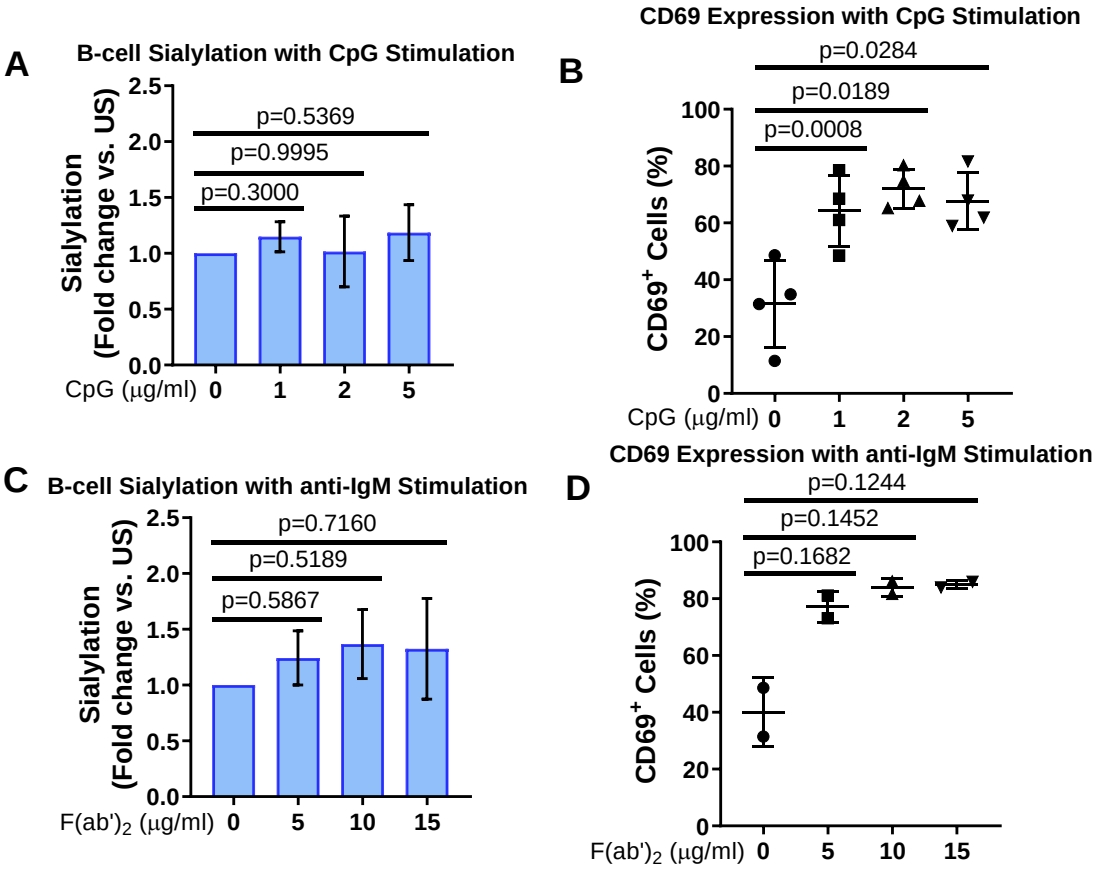


Figure 3.9 Dose dependent response of B-cells from patients with ERA to TLR stimulation and BCR crosslinking

B-cells from patients with early RA (ERA) were stimulated for 48 hrs with increasing doses (1 – 5 μg/ml) of CpG (n=4) **(A-B)** or F(ab')$_2$ (5 – 15 μg/ml) (n=2) **(C-D)**. After 48 hrs sialylation was measured by flow cytometry along with expression of CD69. **(A,C)** Graphs show mean with SEM of SNA FITC MFI, expressed as a ratio relative to SNA FITC MFI of unstimulated samples. **(B,D)** Graphs show mean with SD of the percentage of CD69$^+$ cells. ANOVA and Tukey's multiple comparisons tests were used to generate p values, differences were statistically significant where $p<0.05$.

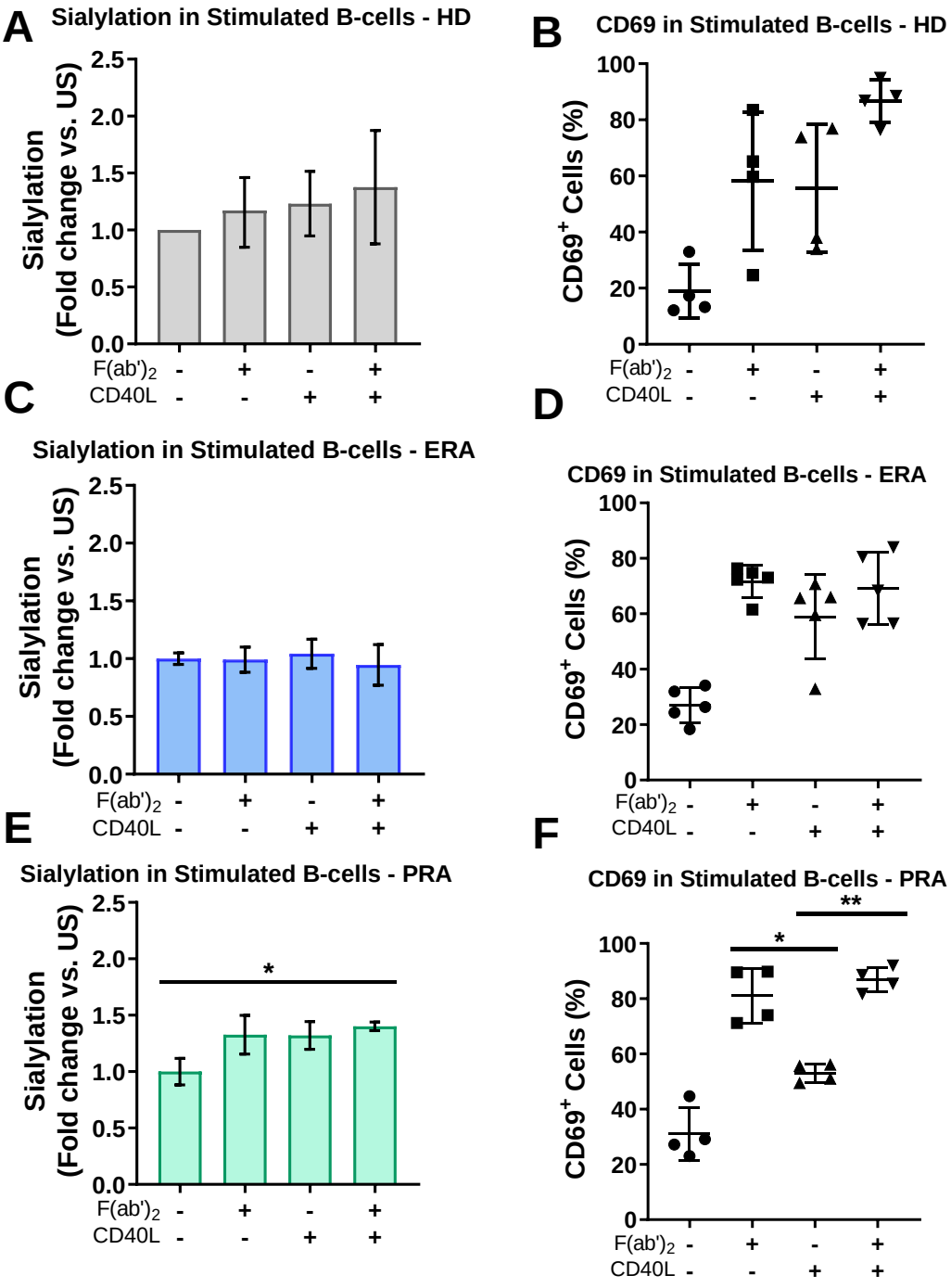


Figure 3.10 Changes to sialylation elicited by *in vitro* B-cell activation via BCR crosslinking and CD40L co-stimulation

B-cells isolated from healthy donors (n=5) **(A-B)**, patients with early RA (n=5) **(C-D)** or patients in the pre-RA group (n=4) **(E-F)**, were stimulated with 5 µg/ml $F(ab')_2$, 2 µg/ml soluble CD40L or both for 48 hrs. After 48 hrs sialylation was measured by flow cytometry along with expression of CD69. **(A,C,E)** Graphs show mean with SEM of SNA FITC MFI, expressed as a ratio relative to SNA FITC MFI of unstimulated (US) samples. **(B,D,F)** Graphs show mean with SD of the percentage of $CD69^+$ cells. One-way ANOVA followed by Tukey's multiple comparisons tests were used to analyse statistical significance, with relevant statistically significant comparisons highlighted on graphs (* $p<0.05$, ** $p<0.005$).

3.2.4.2 Expression of sialylated surface proteins is not increased in B-cells stimulated with F(ab')$_2$ and CD40L from patients with RA in response to increasing doses of stimuli

As in section 3.2.3.3, to determine if cells from patients with ERA required a higher dose of CD40L in order to upregulate sialylation, B-cells were treated with increasing doses of CD40L (2, 5 or 10 µg/ml) alone (n=2) or in combination with increasing doses of F(ab')$_2$ (5, 10 or 15 µg/ml) (n=4). Results showed very little change to sialylation at all tested concentrations of CD40L (Figure 3.11A) and CD40L + F(ab')$_2$ (Figure 3.11C). Activation in response to CD40L appeared to increase in a dose-dependent manner (Figure 3.11B), and response to CD40L + F(ab')$_2$ was generally high, and comparable to cells from healthy donors (Figure 3.11D, 3.11B). These results suggest that, even at higher doses of stimuli, despite B-cells from patients with RA being activated by CD40L and F(ab')$_2$ to a similar level as cells from healthy donors, there is no corresponding upregulation of sialylation.

3.2.5 Changes to B-cell sialylation following activation are dependent on mode of stimuli received and the mechanisms which induce such changes may be disrupted in ERA and PRA

The results of the previous sections (3.2.3 and 3.2.4) described changes to B-cell sialylation dependent on the mode of cell activation, which may be altered in patients with PRA and ERA. The changes to B-cell sialylation following activation were further studied by comparing data from each treatment condition between groups of patients and healthy donors. It was found that differences between expression of SA in healthy donor cells and cells in both patient groups following activation with CpG were highly statistically significant (increased expression in cells from healthy donors: HD 1.953 ± 0.273, PRA 1.094 ± 0.094 $p<0.0001$ and ERA 1.004 ± 0.118 $p<0.0001$ – fold change relative to unstimulated samples) (Figure 3.12A). There was also found to be a difference in sialylation of cells stimulated with R848, which was significantly higher in cells from healthy donors that in patients with ERA (HD 1.466 ± 0.421 vs. PRA 1.224 ± 0.153, $p=0.4063$ and vs. ERA 0.971 ± 0.173, $p=0.0211$). Expression of SA in cells stimulated with F(ab')$_2$ was also significantly higher in cells from patients with PRA than in patients with ERA (PRA 1.327 ± 0.172 vs. ERA 0.992 ± 0.110, $p=0.0235$).

Expression of SA following activation with CD40L and F(ab')$_2$ + CD40L, was higher in cells from patients with PRA compared to those with ERA (CD40L: PRA 1.321 ± 0.123 p=0.024 and ERA 1.043 ± 0.127, p=0.1368; F(ab')$_2$ + CD40L: PRA 1.401 ± 0.038 p=0.011 and ERA 0.946 ± 0.151, p=0.1851). Expression was also lower in cells from patients with ERA stimulated with CD40L and F(ab')$_2$ + CD40L than in cells from healthy donors, though not statistically significant (CD40L: HD 1.231 ± 0.284, p=0.3261 and ERA 1.043 ± 0.127; F(ab')$_2$ + CD40L: HD 1.377 ± 0.498 p=0.145 and ERA 0.946 ± 0.151, p=0.1869)(Figure 3.12A).

Differences in percentage of CD69$^+$ cells were also studied between groups. It was found that the percentage of CD69$^+$ cells in patients with PRA stimulated with CpG was significantly reduced compared to cells from healthy donors (35.38 ± 8.50 vs. 63.22 ± 19.43 p=0.0221) and was reduced compared to cells from patients with ERA (35.38 ± 8.50 vs. 54.35 ± 6.13 p=0.5494)(Figure 3.12B). Similarly, the percentage of CD69$^+$ cells was reduced in cells stimulated with R848 from patients with PRA compared to cells from healthy donors (though not statistically significant, 48.58 ± 13.11 vs. 68.92 ± 27.33, p=0.2606) and compared to cells from patients with ERA (48.58 ± 13.11 vs. 68.93 ± 6.90, p=0.9999). The percentage of CD69$^+$ cells was also reduced in patients with ERA stimulated with F(ab')$_2$ + CD40L compared to cells from healthy donors and cells from patients with PRA (ERA 69.18 ± 12.98, HD 86.74 ± 7.62, p=0.0492, PRA 86.97 ± 4.39, p=0.0465). In contrast, the percentage of CD69$^+$ cells was reduced in cells stimulated with F(ab')$_2$ from healthy donors compared with cells from patients with PRA and ERA (HD 58.27 ± 24.63 vs. PRA 81.15 ± 9.91, p=0.0008, and vs. ERA 71.62 ± 5.87, p=0.4427)(Figure 3.12B). CD69$^+$ cells were also significantly increased in cells from patients with PRA versus cells from patients with ERA (PRA 81.15 ± 9.91 vs. ERA 71.62 ± 5.87, p=0.0002).

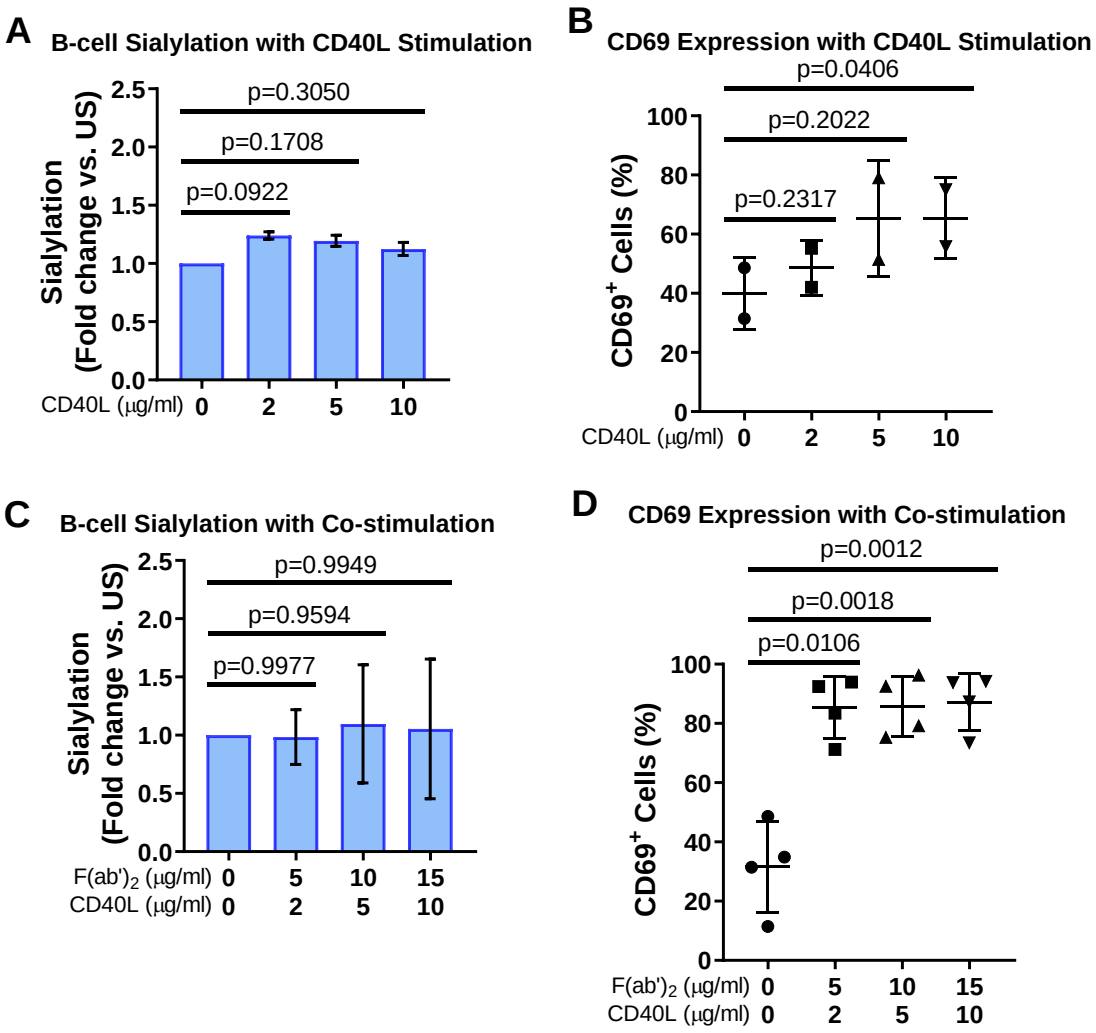


Figure 3.11 Dose dependent response of B-cells from patients with ERA to CD40L co-stimulation and BCR crosslinking

B-cells from patients with early RA (ERA) were stimulated for 48 hrs with increasing doses of CD40L (2 – 10 μg/ml) (n=2) **(A-B)** or F(ab')$_2$ (5 – 15 μg/ml) + CD40L (n=4) **(C-D)**. After 48 hrs sialylation was measured by flow cytometry along with expression of CD69. **(A,C)** Graphs show mean with SEM of SNA FITC MFI, expressed as a ratio relative to SNA FITC MFI of unstimulated (US) samples. **(B,D)** Graphs show mean with SEM of the percentage of CD69$^+$ cells. ANOVA and Tukey's multiple comparisons tests were used to generate p values, differences were statistically significant where $p<0.05$.

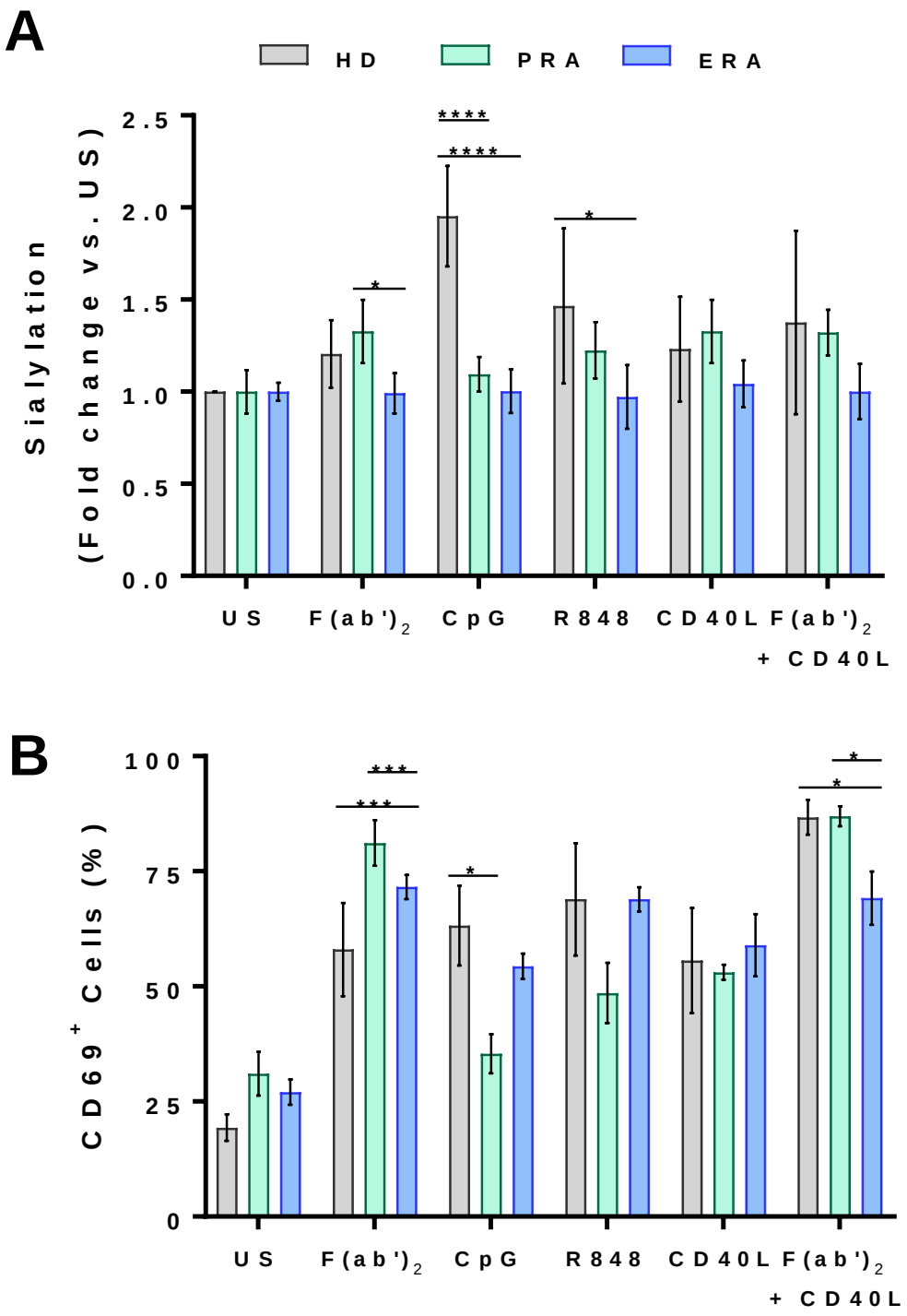


Figure 3.12 Influence of B-cell activation on sialylation and CD69 expression in cells from healthy donors, patients with ERA and patients with PRA

B-cells isolated from healthy donors (HD) (n=5), patients with early RA (ERA) (n=5) or patients with pre-RA (PRA) (n=4), were stimulated with CpG, R848, F(ab')$_2$, CD40L or F(ab')$_2$ + CD40L for 48 hrs. Sialylation **(A)** and % of CD69$^+$ cells **(B)** was measured after 48 hrs. Graphs show mean with SEM of SNA FITC MFI, expressed as a ratio relative to SNA FITC MFI of unstimulated (US) samples or mean with SEM of the percentage of CD69$^+$ cells. ANOVA and Tukey's multiple comparisons tests were used to assess statistical significance with relevant statistically significant comparisons highlighted on graphs (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$ and **** $p<0.0001$).

In summary, these results show the mechanism of upregulated sialylation induced by activation via TLR stimulation is depressed in patients with ERA and PRA. Activation via TLRs is also reduced in patients with PRA but not ERA suggesting the mechanism of response to TLR stimulation may be disrupted during the development of B-cell autoimmunity. However, activation response appears to be recovered upon progression to active disease without recovery of SA upregulation.

Sialylation is also upregulated in response to co-stimulation via the BCR and CD40 in healthy donor cells, and this response is observed in cells from patients with PRA but not ERA, suggesting this mechanism may be dysregulated upon progression to active auto-inflammation, but unaffected in the asymptomatic autoimmune phase.

3.2.6 Impact of activated T-cells on activated B-cell sialylation

As described previously, in section 3.2.4.1, expression of sialic acid on the B-cell surface was increased in B-cells from healthy donors in response to BCR crosslinking with $F(ab')_2$ and co-stimulation with CD40L (Figure 3.10A). Therefore, it was hypothesised that activated T-cells would also be able to activate B-cells in an *in vitro* co-culture system and induce an upregulation of B-cell sialylation.

3.2.6.1 Activated T-cells stimulate B-cells to increase expression of surface sialylated proteins

To determine if T-cells activated *in vitro* would have an impact on B-cell sialylation, PBMCs and B-cells isolated from the same healthy donors (n=3) were co-cultured with CD3/CD28 T-cell activation beads, with or without $F(ab')_2$. It was found that even in the absence of direct B-cell activation, T-cells activated with CD3/CD28 beads led to an upregulation in B-cell sialylation (1.502 ± 0.109, p=0.0378 – fold change relative to unstimulated samples) (Figure 3.13A). Sialylation was also upregulated in cells stimulated with $F(ab')_2$ + activated T-cells (1.616 ± 0.286, p=0.1588). Thus, suggesting that T-cell co-stimulation provides a strong signal for B-cell activation, and leads to upregulation of SA in B-cells from healthy donors.

To determine if sialylation in B-cells from patients with ERA could also be influenced by activated T-cells, PBMCs and B-cells isolated from the same patients with ERA (n=5) were also co-cultured with CD3/CD28 T-cell activation beads, with or without $F(ab')_2$. In line with previous results, which showed very little change in B-cell sialylation in response to stimulation with CD40L (Figure 3.10C), the B-cells from patients with ERA showed no change in sialylation in response to activated T-cells (1.097 ± 0.101, p=0.2137) or activated T-cells in combination with $F(ab')_2$ (1.022 ± 0.184, p=0.9826) (Figure 3.13B). These results confirm previous findings, that B-cells from patients with ERA, have dysregulated control of SA expression, in response to activation via CD40L co-stimulation and BCR crosslinking via $F(ab')_2$.

3.2.6.2 The influence of activated T-cells on B-cell sialylation is in part mediated by CD40L co-stimulation

To confirm if the effects of activated T-cells on B-cell sialylation in cells from healthy donors were, at least in part, driven by CD40L signalling, a blocking antibody to CD40L was used to inhibit this interaction. It was found that, as described previously (Figure 3.13A), cells stimulated with activated T-cells showed an increase in sialylation (1.281 ± 0.212, p=0.1695 – fold change relative to unstimulated samples). Addition of the blocking antibody led to a decrease in B-cell sialylation (1.124 ± 0.098 with blocking vs. 1.281 ± 0.212 without, p=0.2623), however sialylation did not decrease to the level of unstimulated cells (1.000 ± 0.018) (Figure 3.13C). These results suggest that the effects of activated T-cells on B-cell sialylation may be partially driven by CD40L signalling, however there are likely other factors which could impact B-cell sialylation such as T-cell produced cytokines.

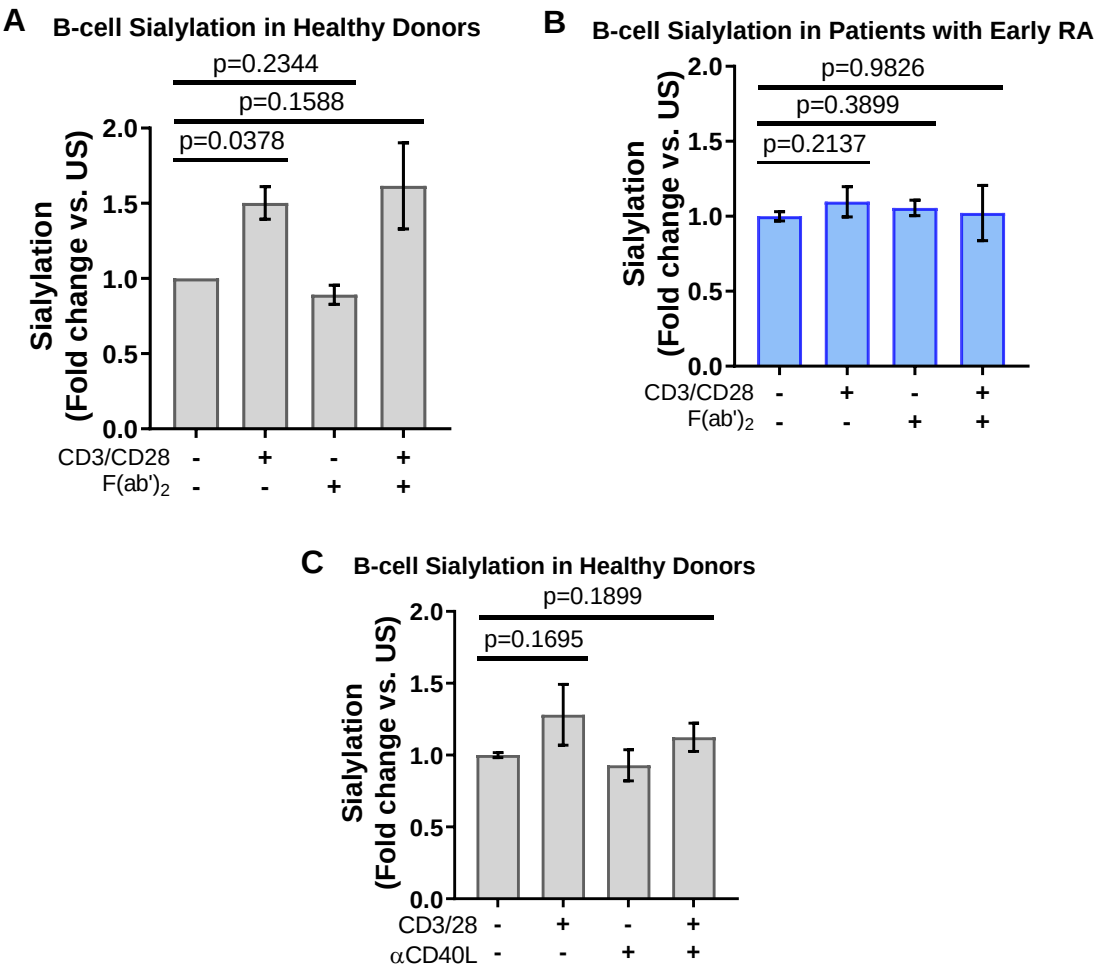


Figure 3.13 Influence of activated T-cells on B-cell sialylation in B-cells from healthy donors and patients with ERA

B-cells from healthy donors (n=3) **(A)** or patients with early RA (ERA) (n=5) **(B)** were co-cultured with PBMCs from the same donor, ± T-cell activation beads (CD3/CD28) and ± F(ab')$_2$. **(C)** B-cells and T-cells from healthy donors (n=4) were co-cultured in the presence of CD3/28 beads ± anti-CD40L antibody (αCD40L). Sialylation was measured after 24 hrs. Graphs show mean with SD of B-cell SNA FITC MFI, expressed as a ratio relative to SNA FITC MFI of unstimulated (US) samples. One-way ANOVA and Tukey's multiple comparisons test were used to generate p values. Differences were statistically significant where $p<0.05$.

3.2.7 Expression of α2,3-sialic acid in activated B-cells

Despite much of the literature reporting on the importance of α2,6 sialylation, it is reasonable to hypothesise that other SA linkages – such as α2,3 sialylation - may also be impacted by the same mechanisms as α2,6 sialylation. Therefore, the impacts of B-cell activation via TLRs, BCR crosslinking and CD40L co-stimulation on α2,3 sialylation were also studied. Expression of α2,3-SA was measured using biotinylated Maackia Amurensis (bMAA) lectin which preferentially binds α2,3-SA.

3.2.7.1 Optimisation of MAA lectin staining for flow cytometry by titration and neuraminidase digestion of sialic acid

As described previously, use of lectins in flow cytometry requires several optimisation steps. Titration of bMAA staining was carried out, using streptavidin-PE, along with Neu treatment of cells to determine the specificity of binding. It was determined that the best concentration of staining was 20 µg/ml (Figure 3.14), and Neu treatment showed a reduction in bMAA binding, however the extent of the decrease in staining was lesser than that seen in cells treated with neuraminidase and stained with SNA (Figure 3.2C). For the following experiments, bMAA staining was conducted with streptavidin-APC as a secondary molecule, to allow expression of α2,3 and α2,6 SA to be measured in the same cells, using 20 µg/ml bMAA.

3.2.7.2 Changes to expression of α2,3-sialic in B-cells in response to activation is highly variable

To investigate the regulation of α2,3 SA expression by B-cell activation, B-cells isolated from healthy donors (n=3) were stimulated with either CpG, $F(ab')_2$, CD40L or $F(ab')_2$ + CD40L for 48 hrs, then α2,3-sialylation measured by flow cytometry using bMAA. Expression of α2,3-SA was unchanged in cells stimulated with CpG, CD40L and $F(ab')_2$ + CD40L (Figure 3.15). α2,3-sialylation appeared to increase in cells stimulated with $F(ab')_2$ in isolation, however, there was a large margin of error within these results, making them somewhat hard to interpret (Figure 3.15).

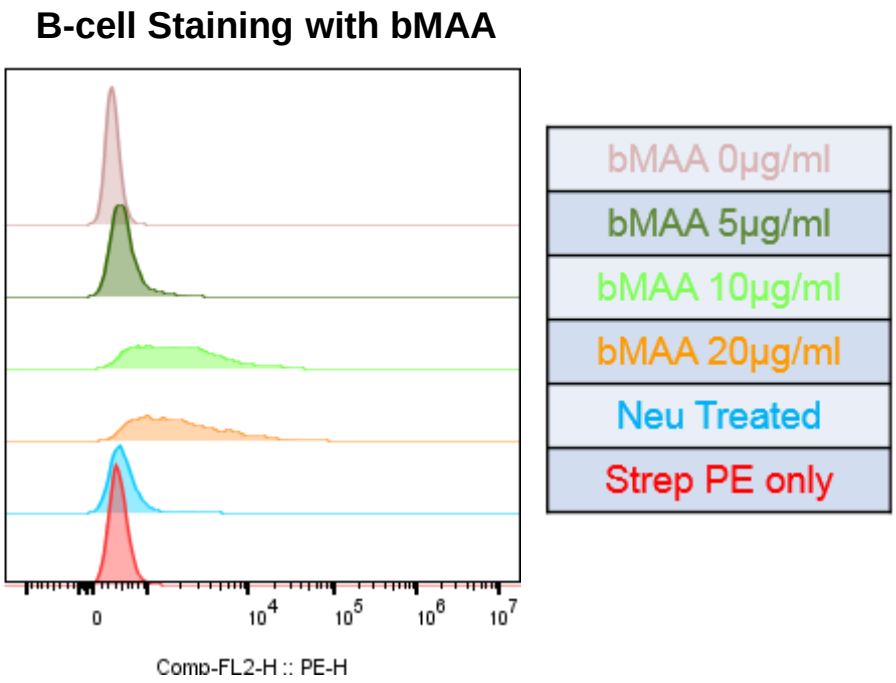


Figure 3.14 Optimisation of flow cytometry staining with *Maackia Amurensis* Lectin

PBMCs from a healthy donor were stained with increasing doses (0 - 20 µg/ml) of biotinylated *maackia amurensis* lectin (bMAA). A sample of cells were also treated with neuraminidase (Neu) to remove surface sialic acid. Cells stained with bMAA were then stained with streptavidin-PE then fluorescence measured, with histogram overlay displaying PE MFI.

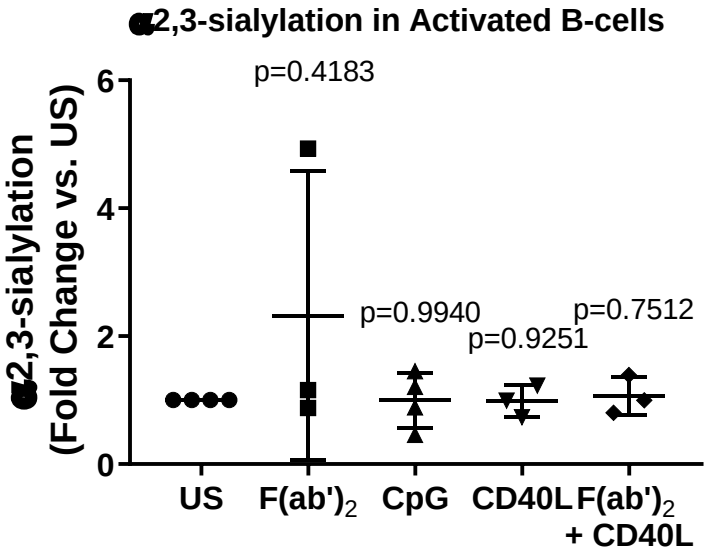


Figure 3.15 Expression of α2,3-sialic acid in activated B-cells from healthy donors

B-cells from healthy donors (n=3) were stimulated for 48 hrs with increasing doses of CpG (1 - 5 µg/ml) **(A)**, F(ab')$_2$ (5 – 15 µg/ml) **(B)**, CD40L (2 – 10 µg/ml) (n=2) **(C)** or F(ab')$_2$ + CD40L (n=4) **(D)**. After 48 hrs α2,3 sialylation was measured by flow cytometry. Graphs show mean with SD of APC MFI, expressed as a ratio relative to APC MFI of unstimulated (US) samples. One-way ANOVA and Tukey's multiple comparisons tests were used to generate p values, based on comparison with unstimulated control sample mean.

Taken together, these results show that α2,3-SA may be affected by stimulation with F(ab')$_2$. However, variance in the data made it difficult to draw any conclusions from these experiments. Considering there was no change in α2,3-SA expression induced by CD40L or CpG, it suggests that α2,3-SA is less susceptible to changes induced by B-cell activation, or that perhaps the response is more variable in cells from healthy donors.

3.2.8 Expression of ST6Gal1 and NEU1 in activated cells from patients with RA and healthy donors

There are likely several factors which determine the level of SA which is displayed on the cell surface. One potential factor may be the level of expression of enzymes which may add or remove SA from protein chains within the cell. ST6Gal1 is the Golgi enzyme responsible for the addition of α2,6 SA to glycan chains[163] and NEU1 is able to cleave α2,6 SA from protein chains[170]. Little is known about how the expression of these genes is regulated in B-cells. Therefore, experiments were carried out to determine if activation of B-cells leads to changes in enzyme mRNA expression in line with levels of cell surface SA expression.

3.2.8.1 Patterns of expression of NEU1 over 48hrs in B-cells from patients with RA differs compared to cells from healthy donors

To determine if activation of B-cells had an impact on the level of expression of NEU1 and ST6Gal1 mRNA and to determine if gene expression correlated with surface SA expression, B-cells from healthy donors (n=3), or patients with ERA (n=3) were stimulated for 48 hrs with CpG or F(ab')$_2$. Expression of NEU1 and ST6Gal1 were measured by RT-qPCR at baseline, after 24 and 48 hrs - relative to expression of HPRT1.

The results showed that in B-cells from healthy donors, after 24 hrs, expression of NEU1 decreased in cells stimulated with CpG compared to baseline expression levels (0.168 ± 0.060 vs. 0.418 ± 0.033, p=0.0218 – ΔCT values) (Figure 3.16A). Expression then increased slightly between 24 and 48 hrs (0.168 ± 0.060 vs. 0.236 ± 0.062, p=0.4334). Unstimulated and F(ab')$_2$-stimulated cells showed no change in expression after 24hrs (0.407 ± 0.063, p=8278 and 0.393 ± 0.143, p=0.9396 respectively, vs. 0.418 ± 0.033), and a slight decrease in

expression in F(ab')$_2$ stimulated cells after 48 hrs (0.380 ± 0.080, p=0.9408). At 48 hrs expression of NEU1 was lower in cells stimulated with CpG than in unstimulated and F(ab')$_2$-stimulated cells (0.236 ± 0.062 vs. 0.417 ± 0.022, p=0.0935 and 0.380 ± 0.080, p=0.1980 respectively) (Figure 3.16B) – concurrent with the increase in expression of surface SA in cells stimulated with CpG compared to those stimulated with F(ab')$_2$ and unstimulated cells (Figure 3.8A).

Unlike in B-cells from healthy donors, in B-cells from patients with ERA, after 24 hrs expression of NEU1 was increased in both stimulated and unstimulated cells – with the largest increase in the unstimulated cells (US: 0.531 ± 0.143, p=0.0443; CpG: 0.335 ± 0.194, p=0.4211; F(ab')$_2$: 0.237 ± 0.039, p=0.2605 vs. 0.143 ± 0.052) (Figure 3.17A). Expression decreased in unstimulated (0.531 ± 0.142 vs. 0.471 ± 0.145, p=0.7424) and CpG stimulated (0.335 ± 0.194 vs. 0.257 ± 0.218, p=0.0773) cells between 24 and 48 hrs, however expression increased in F(ab')$_2$ stimulated cells (0.237 ± 0.039 vs. 0.305 ± 0.085, p=0.5310). At 48 hrs, expression of NEU1, similar to the observations in cells from healthy donors, was decreased in CpG-stimulated compared to unstimulated and F(ab')$_2$-stimulated cells (0.257 ± 0.218, p=0.5726 vs. 0.471 ± 0.145, p=0.9351 and 0.305 ± 0.085 respectively) (Figure 3.17B). This pattern of NEU1 expression does not concur with the expression of surface SA, which was broadly equal across all conditions of activation at 48 hrs in B-cells from patients with RA (Figure 3.8C).

3.2.8.2 Patterns of expression of ST6Gal1 over 48hrs in B-cells from patients with RA differs compared to cells from healthy donors

In terms of expression of ST6Gal1, in cells from healthy donors, expression of ST6Gal1 was decreased in both stimulated and unstimulated cells after 24 hrs compared to baseline, with the largest decrease in CpG stimulated cells (US: 2.160 ± 0.483, p=0.0135; CpG: 0.993 ± 0.135, p=0.0110; F(ab')$_2$: 1.683 ± 0.184, p=0.0156 vs. 3.095 ± 0.370 – ΔCT values) (Figure 3.16C). Sialylation increased between 24 and 48 hrs in F(ab')$_2$ (1.683 ± 0.184 vs. 2.015 ± 0.279, p=0.3479) and CpG stimulated cells (0.993 ± 0.135 vs. 1.507 ± 0.254, p=0.0367), with a slight decrease in unstimulated cells (2.160 ± 0.483 vs. 1.566 ± 0.420, p=0.0070), and at 48 hrs expression of ST6Gal1 was lower in CpG stimulated and unstimulated cells than in F(ab')$_2$-stimulated cells (US: 1.566 ± 0.420; CpG: 1.507 ± 0.254; F(ab')$_2$: 2.015 ± 0.279) (Figure 3.16D). The decrease in expression of

ST6Gal1 in all cells, including stimulated cells is in contrast to the increase in expression of SA on the cell surface induced by stimulation, particularly with CpG (Figure 3.8A). These results may suggest that at this particular time-point, expression of ST6Gal1 mRNA does not directly correlate with expression of surface SA. There may be several other determining factors for surface sialylation, or the kinetics of the response may be such that an increase in ST6Gal1 expression may occur prior to the time-points measured here. These results also suggest that CpG stimulation may reduce the expression of both ST6Gal1 and NEU1 to a greater extent than stimulation with $F(ab')_2$.

In cells from patients with ERA, expression of ST6Gal1 was increased in unstimulated (2.784 ± 0.279 vs. 5.517 ± 1.835, p=0.1829) and CpG stimulated cells between baseline and 24 hrs (2.784 ± 0.279 vs. 3.872 ± 1.255, p=0.3300), however expression decreased in $F(ab')_2$ stimulated cells (2.784 ± 0.279 vs. 1.779 ± 0.526, p=0.0436) (Figure 3.17C). Between 24 and 48 hrs, expression of ST6Gal1 remained high in unstimulated cells (5.517 ± 1.835 vs. 5.274 ± 0.747, p=0.9582), but decreased in CpG stimulated cells (3.872 ± 1.255 vs. 2.110 ± 0.886, p=0.2475) and increased in $F(ab')_2$ stimulated cells (1.779 ± 0.526 vs. 2.874 ± 0.334, p=0.0226), resulting in slightly higher expression in $F(ab')_2$ stimulated cells than in CpG stimulated cells at 48 hrs (2.874 ± 0.334 vs. 2.110 ± 0.886, p=0.4679) (Figure 3.17D), similar to expression patterns in cells from healthy donors. Although, unlike in cells from healthy donors, both were significantly lower than in unstimulated cells.

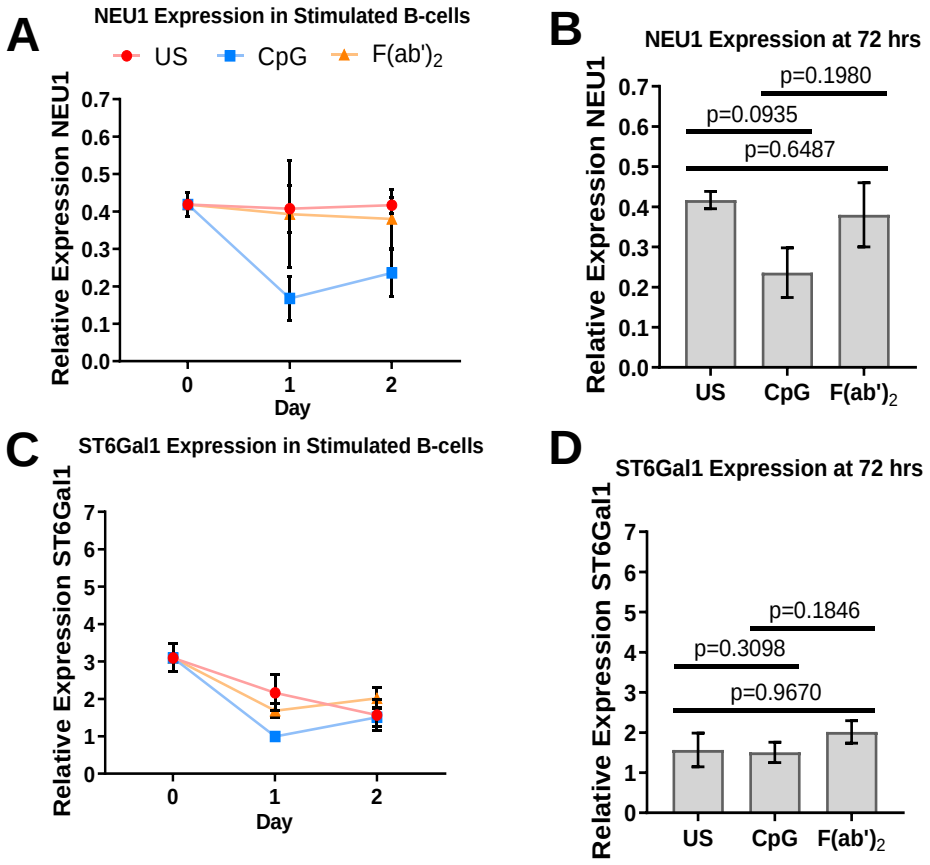


Figure 3.16 Expression of ST6Gal1 and NEU1 in activated B-cells from healthy donors

B-cells from healthy donors (n=3) were stimulated for up to 48 hrs with CpG or F(ab')$_2$ or cultured without and additional stimuli (US). Expression of NEU1 **(A-B)** and ST6Gal1 **(C-D)** were measured by RT-qPCR at baseline, after 24 and 48 hrs. Graphs **A** and **C** show changes to expression over the 48 hr period. Graphs **B** and **D** show expression of NEU1 or ST6Gal1 at 48 hrs in different treatment conditions. ΔCT values were calculated relative to expression of HPRT1. Graphs show ΔCT mean with SD. ANOVA with Tukey's multiple comparisons tests were used to analyse statistical significance. Differences were statistically significant where $p<0.05$. (**(A, C)** p values quoted in main text where relevant).

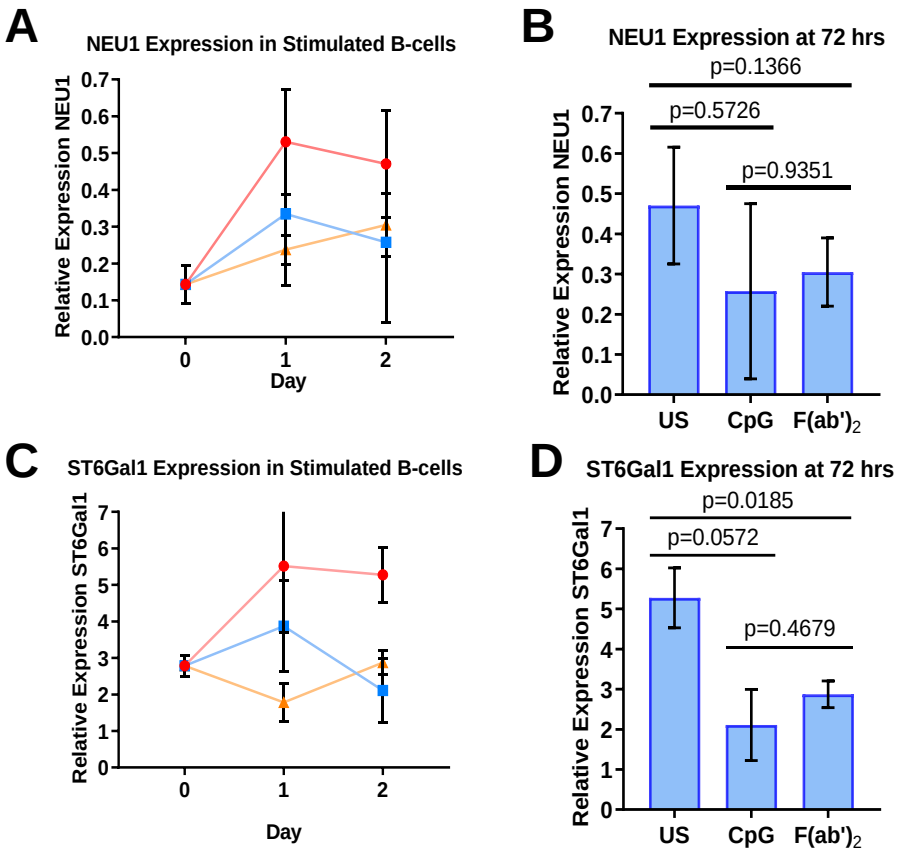


Figure 3.17 Expression of ST6Gal1 and NEU1 in activated B-cells from patients with ERA

B-cells from patients with early RA (ERA) (n=3) were stimulated for up to 48 hrs with CpG or F(ab')$_2$ or cultured without and additional stimuli (US). Expression of NEU1 **(A-B)** and ST6Gal1 **(C-D)** were measured by RT-qPCR at baseline, after 24 and 48 hrs. Graphs **A** and **C** show changes to expression over the 48 hr period. Graphs **B** and **D** show expression of NEU1 or ST6Gal1 at 48 hrs in different treatment conditions. ΔCT values were calculated relative to expression of HPRT1. Graphs show ΔCT mean with SD. ANOVA with Tukey's multiple comparisons tests were used to analyse statistical significance. Differences were statistically significant where $p<0.05$. (**(A, C)** p values quoted in main text where relevant).

3.2.8.3 Patterns of expression of NEU1 within the first 24 hrs following stimulation differ in B-cells from healthy donors and B-cells from patients with RA

To further examine the early changes in expression of NEU1 and ST6Gal1 in response to stimuli, B-cells isolated from healthy donors (n=3) and patients with ERA (n=3) were stimulated for 24hrs with CpG, $F(ab')_2$, or $F(ab')_2$ + CD40L. Expression of ST6Gal1 and NEU1 was measured at baseline and after 1, 4 and 24 hrs.

In cells from healthy donors, after 1 hr, NEU1 expression increased in cells stimulated with $F(ab')_2$ (0.557 ± 0.231 vs. 0.359 ± 0.126, p=0.2010 – ΔCT values) and $F(ab')_2$ + CD40L (0.449 ± 0.122 vs. 0.359 ± 0.126, p=0.0355), with a slight increase in unstimulated cells (0.385 ± 0.173 vs. 0.359 ± 0.126, p=0.9168) and a decrease in expression in CpG stimulated cells (0.278 ± 0.148 vs. 0.359 ± 0.126, p=0.2296) (Figure 3.18A). Between 1 and 4 hrs, expression in unstimulated cells continued to increase (0.385 ± 0.173 vs. 0.472 ± 0.272, p=0.5552), and expression in CpG stimulated cells continued to decrease (0.278 ± 0.148 vs. 0.234 ± 0.092, p=0.6957), however expression in $F(ab')_2$ (0.557 ± 0.231 vs. 0.265 ± 0.084, p=0.1892) and $F(ab')_2$ + CD40L (0.557 ± 0.231 vs. 0.169 ± 0.062, p=0.0377) stimulated cells sharply decreased. Between 4 and 24 hrs, expression remained low in stimulated cells, and slightly increased in unstimulated cells. At 24 hrs, expression in stimulated cells was far lower than in unstimulated cells, however there was no discernible differences in expression in the stimulated samples (CpG: 0.175 ± 0.044, p=0.0670; $F(ab')_2$: 0.206 ± 0.091, p=0.0879; $F(ab')_2$ + CD40L: 0.185 ± 0.066, p=0.0577 vs. US: 0.483 ± 0.134).

In cells from patients with ERA, there was also an increase in expression of NEU1 in $F(ab')_2$ (0.297 ± 0.205 vs. 0.557 ± 0.231, p=0.2479) and $F(ab')_2$ + CD40L (0.297 ± 0.205 vs. 0.449 ± 0.122, p=0.1774) stimulated cells after 1 hr, with a slight increase in unstimulated (0.297 ± 0.205 vs. 0.385 ± 0.173, p=0.7956) and CpG-stimulated cells (0.297 ± 0.205 vs. 0.234 ± 0.092, p=0.8117) (Figure 3.18B). Following this, between 1 and 24 hrs, expression decreased in $F(ab')_2$ (0.557 ± 0.231 vs. 0.206 ± 0.091, p=0.1417) and $F(ab')_2$ + CD40L stimulated cells (0.449 ± 0.122 vs. 0.185 ± 0.066, p=0.0778), remained steady in unstimulated cells (0.385 ± 0.173 vs. 0.483 ± 0.134, p=0.6958) and decreased slightly in CpG

stimulated cells (0.234 ± 0.092 vs. 0.175 ± 0.044, p=0.6081). Cells from healthy donors and patients with ERA followed similar patterns of expression in cells stimulated with $F(ab')_2$ and $F(ab')_2$ + CD40L, in that there was an upregulation of NEU1 expression in the first hour following stimulation, which then decreased. The initial response to $F(ab')_2$ and $F(ab')_2$ + CD40L appeared to be stronger in patients with RA, as it induced a greater fold change compared to unstimulated cells than in samples from healthy donors (3.01 and 3.04 vs 1.55 and 1.25 respectively). The response differed however, in the fact that after 24 hrs in cells from healthy donors expression of NEU1 in unstimulated cells was higher than in stimulated cells (Figure 3.18A), whereas in cells from patients with RA, expression in unstimulated cells was nearly equal to stimulated cells (Figure 3.18B).

3.2.8.4 Patterns of expression of ST6Gal1 within the first 24 hrs following stimulation differ in B-cells from healthy donors and B-cells from patients with RA

As described previously, ST6Gal1 was also measured in cells stimulated for 24 hrs at baseline, 1, 4 and 24 hrs. In cells from healthy donors, expression of ST6Gal1 decreased after 1 hr in unstimulated cells (3.405 ± 1.113 vs. 4.906 ± 2.705, p=0.6093 – ΔCT values), with very little change in stimulated cells (CpG: 4.577 ± 1.710, p=0.9889; $F(ab')_2$: 4.929 ± 2.077, p=0.9999 and $F(ab')_2$ + CD40L: 5.067 ± 2.671 vs. 4.906 ± 2.705, p=0.9403) (Figure 3.18C) Between 1 and 4 hrs, expression increased in unstimulated samples (3.405 ± 1.113 vs. 4.719 ± 2.768, p=0.7843), and decreased in all stimulated cells, with the sharpest decrease in $F(ab')_2$ + CD40L-stimulated cells (CpG: 4.577 ± 1.710 vs. 3.686 ± 2.017, p=0.0557; $F(ab')_2$: 4.929 ± 2.077 vs. 3.168 ± 1.250, p=0.7133; and $F(ab')_2$ + CD40L: 5.067 ± 2.671 vs. 1.450 0.726, p=0.4918). At 24 hrs, there was no change in expression between stimulated cells (CpG: 2.449 ± 1.824; $F(ab')_2$: 2.822 ± 0.744; and $F(ab')_2$ + CD40L: 2.595 ± 1.540), although expression was marginally lower in stimulated cells than in unstimulated cells (US: 3.951 ± 3.798) (Figure 3.18C).

In cells from patients with ERA, expression of ST6Gal1 showed little variation after 1 hr, with a slight decrease in $F(ab')_2$ + CD40L (2.921 ± 0.227 vs. 2.985 ± 1.041, p=0.9997) and unstimulated cells (2.836 ± 1.185 vs. 2.985 ± 1.041,

p=0.9990), and a slight increase in CpG (3.179 ± 0.753 vs. 2.985 ± 1.041, p=0.8260) and F(ab')$_2$ stimulated cells (3.756 ± 0.724 vs. 2.985 ± 1.041, p=0.6256) (Figure 3.18D). Between 1 and 4 hrs expression increased slightly in all samples (US: 2.836 ± 1.185 vs. 3.491 ± 0.898, p=0.9216; CpG: 3.179 ± 0.753 vs. 3.443 ± 0.249, p=0.9512; F(ab')$_2$: 3.756 ± 0.724 vs. 4.010 ± 1.119, p=0.4807; F(ab')$_2$ + CD40L: 2.921 ± 0.227 vs. 3.241 ± 1.193, p=0.9357), then decreased slightly in all stimulated samples, with unstimulated cells remaining unchanged between 4 and 24 hrs. After 24 hrs, expression was slightly lower in stimulated samples (CpG: 2.899 ± 0.041; F(ab')$_2$: 2.642 ± 0.600; F(ab')$_2$ + CD40L: 2.812 ± 0.733) than in unstimulated samples (3.401 ± 0.821), similar to pattern of expression in cells from healthy donors after 24 hrs (Figure 3.18C). The timeline of expression of ST6Gal1 in cells from patients with RA differed from that in healthy donors in that expression in cells stimulated with F(ab')$_2$ increased between 1 and 4 hrs before decreasing between 4 and 24 hrs (Figure 3.18C), whereas in cells from healthy donors, expression slowly decreased after 1 hr (Figure 3.18D).

Taken together, these results suggest that although there may be subtle changes in expression of ST6Gal1 following stimulation, there are likely other factors at play which determine expression of SA on the cell surface. Expression of surface SA may, however, be related to expression of NEU1, as this tended to be lower in CpG stimulated cells from healthy donors which previously showed the highest levels of SA expression (Figure 3.8A). Expression of ST6Gal1 and its relationship to expression of surface SA appears to be more nuanced, since gene expression did not appear to correlate with surface SA expression, rather an increase in SA expression was accompanied by a decrease in ST6Gal1 expression. It could be hypothesised that expression of surface sialic acids may be determined by dynamic expression of both NEU1 and ST6Gal1, rather than a direct relationship existing between surface sialic acid and expression of either enzyme.

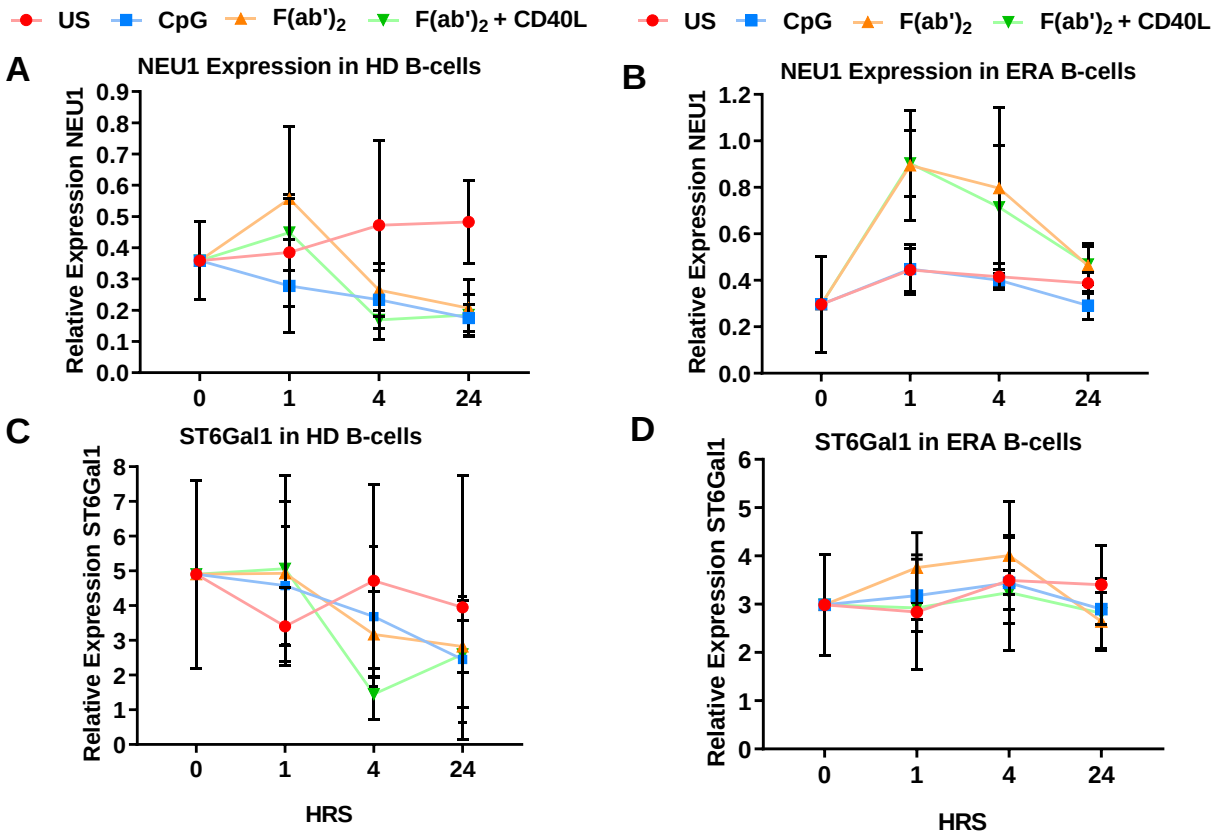


Figure 3.18 Time-course of expression of ST6Gal1 and NEU1 in activated B-cells from healthy donors and patients with ERA over 24 hrs

B-cells from healthy donors (n=3) **(A,C)** and patients with early RA (ERA) (n=3) **(B,D)** were stimulated for up to 24 hrs with CpG, F(ab')$_2$ or F(ab')$_2$ + CD40L or cultured without any additional stimuli (US). Expression of NEU1 **(A-B)** and ST6Gal1 **(C-D)** were measured by RT-qPCR at baseline, after 1, 4 and 24 hrs. ΔCT values were calculated relative to expression of HPRT1. Graphs show ΔCT mean with SD. ANOVA with Tukey's multiple comparisons tests were used to analyse statistical significance (p values quoted in main text where relevant).

3.3 Discussion

Despite the long-established finding that Fc sialylation is reduced in ACPA-IgG in RA[227], it was only recently described that sialylation of the plasmablast surface may also be altered, thought to be an indication of overall reduced sialyltransferase activity in B-cells[235]. The implications of reduced antibody Fc sialylation mostly relate to the impact on Fc receptor binding, however, the potential implications of reduced B-cell surface sialylation are not yet well understood. In this study, the results supported the previous finding that sialylation of the plasmablast surface is reduced in RA, along with the additional finding that surface sialylation is also reduced in memory and naïve B-cells from patients with RA.

The results of this study showed a trend for decreased SNA binding in B-cells from patients with ERA and PRA. However, data did not reach statistical significance - likely due to the small sample size, which was limited to due to availability of patient samples. There was a clear trend for decreased binding however, and this data would benefit from repetition in a much larger cohort of patients and healthy donors. The current data, however, are enough to validate the previously reported data[235], and suggest that the decrease in binding occurs in memory and naïve B-cells in addition to plasmablasts.

When conducting these experiments, difficulties were encountered in standardising the measurement of SNA binding, using MFI of PE-streptavidin as an output. It was found that MFI measurements were highly variable when B-cell sialylation was measured in freshly isolated cells. This was possibly due to technical variations in the flow cytometer, slight differences in staining conditions or human error in pipetting. The use of molecules of equivalent soluble fluorescence (MESF) beads to standardise measurement of MFI across different experiments was also trialled, but could not successfully resolve this issue. To overcome this, it was decided that samples of PBMCs isolated from peripheral blood would be frozen, then sialylation measured in one large batch experiment. Handling and storage of samples was standardised in order to minimise bias introduced by the freezing process.

Factors which may determine sialyltransferase activity in B-cells were largely unknown, therefore, the impact on sialylation of several factors which led to B-cell activation were investigated. B-cells from healthy donors upregulated sialylation in response to activation, particularly in response to TLR ligands - suggesting that upregulated sialylation may be associated with B-cell activation, increased cell survival and proliferation. B-cells from patients with RA, however, showed unaltered sialylation in response to TLR ligands. Activation markers were upregulated in cells from patients with RA in response to TLR ligands, indicating that activation was occurring, however activation markers were increased slightly less than in cells from healthy donors. This indicated that regulation of sialylation in response to TLR signalling may be disrupted in RA, and the response to TLR stimulation may be dampened. Further to this, cells from patients with PRA also showed no upregulation of sialylation in response to TLR ligands, suggesting that this disruption may occur in the pre-RA phase and may be associated with the development of autoimmunity.

In order to assess B-cell activation, expression of one marker of B-cell activation – CD69 - was measured in stimulated cells. It would be of value to include other markers of cell activation and functions such as antibody and cytokine production in future experiments. Although measuring sialylation with SNA lectin staining has uncovered a net increase in sialylation in activated cells, this method cannot be used to determine which particular surface proteins may have increased in expression or increased in sialylation. It would be beneficial in future to measure upregulation of particular sialylated proteins by flow cytometry, or to use a technique such as lectin blotting – a modified Western blotting technique where lectins are used in place of antibodies to (semi) quantify protein sialylation - and immunoprecipitation to investigate changes to sialylation of individual proteins. During *in vivo* B-cell activation, cells require two signals for complete activation, however, in the current experiments the impact of TLR ligands was measured only in isolation. This would be taken into consideration for future experiments, which would combine TLR activation with BCR ligation, to give a fuller picture of B-cell activation in this manner. It is interesting to note that co-stimulation with BCR crosslinking and CD40L had similar results in terms of sialylation to each individual stimuli, despite the increase in CD69 expression when cells were subject to dual stimulation.

As well as B-cells from patients with RA failing to upregulate sialylation in response to TLR ligands, neither did these cells upregulate sialylation in response to activated T-cells. Stimulation with activated T-cells lead to increased sialylation in the absence of other B-cell stimuli in experiments using healthy donor cells. This suggested that there may be an overall lack of response to activation in RA in terms of regulation of sialic acid expression. However, with current data it is not possible to conclude whether this observation is due to intrinsic B-cell factors or a lack of activation in T-cells. To get a more complete picture of the activity both B-cells and T-cells in RA it would be beneficial to measure activation in these cells, including regulation of CD40L expression in T-cells. The upregulation of B-cell sialylation in healthy donor cells was in part attributed to CD40/CD40L signalling. When a blocking antibody was applied, the increase of B-cell sialylation was impaired, but was still higher than in cells cultured with unstimulated T-cells. There are a number of factors which may influence this, firstly a potential lack of efficiency of the blocking antibody allowing some CD40/CD40L interactions to take place. Th cell production of cytokines such as IL-4, IL-10, IFN-γ or TGF-β, which are produced by Th2 and Th1 subsets[272], may also have had an impact on B-cell sialylation.

The main limitation of these current data is that it explores only a snapshot of the B-cell response to stimulation. An interesting follow-up experiment would be to conduct a time-course and follow the changes which occur shortly following activation, through longer periods of exposure to the stimuli. It would also be of interest to include some patients with other autoimmune conditions which share similar mechanisms in further experiments. Furthermore, it would be beneficial to establish if particular sialylated proteins are downregulated in RA or if there is indeed reduced overall sialyltransferase activity.

Since cell surface sialylation is not restricted to α2,6-linkages, α2,3-linkages were also investigated. However, it was found that in this study, measurement of α2,3-sialylation by flow cytometry delivered highly variable results, suggesting this form of sialic linkage may be more variable in nature, or the detection methods used less reliable. There were also some changes in α2,3-sialylation in response to stimuli, however these were largely negligible in comparison to changes in α2,6-sialylation and were difficult to interpret due to variability between samples. It was decided therefore that the rest of the study would focus on the measurement of

α2,6-sialylation, in line with its widely reported importance in autoimmune disease and inflammation.

Little is known about how expression of enzymes which regulate sialylation – ST6Gal1 and NEU1 – is controlled in B-cells. In this study, expression of ST6Gal1 and NEU1 mRNA were studied in cells from healthy donors and patients with RA. The results presented a complicated picture of gene expression patterns. NEU1 was generally downregulated in activated cells from healthy donors compared with unstimulated cells, in line with increased expression of surface SA, suggesting a correlation between NEU1 activity and sialylation. Expression of NEU1 was particularly low in B-cells measured in these experiments, potentially leading to less accurate readings, making it challenging to draw strong conclusions from this work. Despite the low expression, there were some clear trends which emerged although future experiments would benefit from higher starting concentrations of RNA within the samples.

In addition to NEU1 downregulation, ST6Gal1 also tended to be downregulated in activated cells, suggesting a more complicated relationship between its expression and surface sialylation. In cells from patients with RA, gene expression patterns were contrasting, with a slight increase NEU1 and a decrease in ST6Gal1 expression, which could suggest that NEU1 could be in part responsible for the lack of upregulated sialylation. However, to further complicate matters, expression of NEU1 and ST6Gal1 were both increased to a high degree in unstimulated cells after 48 hrs in culture media. This observation was unexpected, and may be explained by an experimental anomaly, however it may also be an indication of gene expression changing when cells are cultured *ex vivo*, removed from *in vivo* factors which may suppress gene expression. This warrants further investigation, and would benefit from increased n numbers to solidify conclusions drawn. There was a relatively high degree of variability within the samples, given this and the low expression of NEU1, it would have been useful to include an internal positive control for each experiment to further validate these results. The use of multiple housekeeping genes may also prove useful in determining more meaningful results.

A limitation of the current data is the assumption that mRNA expression of each enzyme is directly correlated with enzyme activity output, which may not be the

case. To further investigate the link between mRNA and activity, it would first be helpful to establish the link between protein expression and mRNA expression – measuring ST6Gal1 and NEU1 protein levels in the cell by Western blot under the same conditions of activation. Furthermore, it may be of interest to measure levels of CMP-SA and free CMP within the cell, to determine if SA metabolism and transport increases when B-cells are activated. It would also be interesting to note whether enzymes which control galactosylation (galactosyltransferases) are also affected by cell activation, since sialylation requires a galactosylated substrate glycoprotein. In addition to this, since n numbers were modest and error margins were relatively high, it was difficult to establish differences in gene regulation between cells from healthy donors and patients with RA. Therefore repeating these experiments on a larger scale may assist in identifying any differences in regulation driven by pathology.

In summary, the findings of this chapter have shown that B-cell surface sialylation can be influenced by activation, and the mode of activation may determine the extent of the surface sialylation. It is hypothesised that increased sialylation may be a mechanism to promote B-cell survival and proliferation, and that greater surface sialylation is required in cells which quickly differentiate to short-lived ASCs in response to TI antigens. It was also shown that B-cells from patients with RA did not upregulate sialylation in response to activation, particularly with TLR stimuli, suggesting impaired response to TLR stimuli. It is hypothesised that response to B-cell stimuli may contribute to sialylation in peripheral B-cells. However, as well as these B-cell intrinsic factors which were investigated, it is also possible that B-cell extrinsic factors, such as cytokines and other serum proteins may influence peripheral B-cell sialylation, and such factors will be investigated in the following chapters.

Chapter 4
Impact of B-cell differentiation and exposure to cytokines on B-cell surface sialylation

4.1 Introduction

Since previous data have shown that sialylation is reduced in plasmablasts from patients with RA and Pre-RA[235], it was hypothesised that events during B-cell activation may influence plasmablast sialylation in differentiated cells. In the previous chapter, the early events in B-cell activation were studied in detail in cells from healthy donors, and patients with pre-RA and/or RA. However, in order to understand the impact of early activation events on sialylation in ASCs, B-cells were differentiated *in vitro* to plasma cells. This was not only to determine the impact of activation conditions on ASC sialylation, but to determine how regulation of sialylation changes during B-cell differentiation.

The previous results of this study have shown that B-cell sialylation may be altered by the activation status of B-cells, and that the mode of activation is important in determining overall surface sialylation. As well as activation via the BCR and TLRs, B-cell function can also be influenced by soluble proteins such as cytokines and chemokines. It was hypothesised that the presence of such factors in the extracellular environment may also influence B-cell sialylation. As mentioned previously, little is known about what regulates B-cell sialylation, and the impact of exposure to particular cytokines on B-cell expression of SA is unknown. In this chapter the impact of exposure to key inflammatory cytokines in RA on B-cell sialylation will be studied.

Cytokines play a crucial role in driving inflammation in RA. A complex network of inflammatory cytokines can be present from the onset of synovial inflammation, and can help determine cell trafficking, phenotype and function[63]. Cytokines are a popular target for therapy, and the key roles of TNF and IL-6 have been illustrated by the wide successes of anti-TNF and anti-IL-6 targeted therapies, compared with relative failures in treatment with anti-IL-1 targeted therapies[7].

TNF is a driving factor behind several inflammatory processes in RA. It is produced by numerous cell types and is involved in promoting lymphocyte activation and migration, expression of chemokines, activation of stromal cells and osteoclast functioning. TNF acts as an autocrine growth factor in B-cells, and is produced when cells are activated. TNF signalling mainly takes place via two receptors, TNF receptor 1 and 2 (TNFR1 and TNFR2). TNFR1 is ubiquitously expressed in most tissues, whereas TNFR2 is mainly expressed by lymphocytes and is better activated by membrane bound TNF than soluble TNF[273]. In B-cells signals are transduced via MAPK/JNK signalling pathways to drive expansion of activated cells[274].

The role of IL-6 in RA is similar to that of TNF, however it also plays a role in driving the acute phase response in the liver, which leads to the release of C-reactive protein, serum amyloid A, fibrinogen and haptoglobin[275]. IL-6 can also be produced by numerous cell types, including fibroblasts and endothelial cells in response to IL-1 or TNF[275]. In B-cells, IL-6 signals via the JAK/STAT pathway[73], and has been shown to promote plasmablast maturation to plasma cells[259] and promote antibody production[276]. IL-6 also drives the generation of Tfh cells, which may also promote the formation of spontaneous germinal centres in autoimmunity[277].

Although Th17 cytokines are thought to play an important role in RA, like in many autoimmune diseases, treatment of patients with RA with anti-IL-17 therapies has proven less effective than in treating conditions such as psoriasis and psoriatic arthritis[7]. The presence of Th17 cells in the blood[60], and Th17 cytokines in synovial fluid[61] has been detected in patients with RA, and IL-17 is able to induce the production of inflammatory cytokines including IL-6, IL-8 and G-CSF in synovial fibroblasts[62]. In addition to this it can stimulate the production of matrix metalloproteinases[60], and stimulate osteoclastogenesis[61], both of which can lead to tissue destruction in the joint. In B-cells, it has been shown that, *in vitro*, IL-17 is able to induce proliferation and differentiation to plasma cells, via IL-17RA[278], and has also been associated with the formation of spontaneous ectopic germinal centres in models of autoimmune disease[279]. Th17 cytokines including IL-17 have also been shown to play a role in the production of desialylated IgG antibodies in murine models[235], suggesting a role for Th17 cells in RA during the development of autoimmunity, prior to the onset of synovial inflammation. It was hypothesised

that sialyltransferase activity, and potentially surface sialylation may be downregulated in B-cells in response to exposure to IL-17.

Although not typically associated with inflammation and inflammatory disorders, *in vivo*, exposure to IL-4 drives B-cell proliferation and survival, and is produced by germinal centre cells and T-cells in the lymph node. It was important to include IL-4 in this study to be able to investigate the impact of a non-inflammatory cytokine on B-cell sialylation. Furthermore, in previous optimisation experiments it was found that inclusion of IL-4 in cultures led to increased sialylation, which was an intriguing finding that warranted further investigation. In light of the finding that B-cell activation also led to increased sialylation, it was hypothesised that upregulated sialylation may be induced in proliferating cells, and may be upregulated to promote B-cell survival upon activation.

To study the impact of cytokine exposure on B-cell sialylation, cells were stimulated with TNF, IL-6, IL-17 or IL-4 and sialylation measured after a short exposure *in vitro*. As well as studying the short-term effects of acute exposure to cytokines, the impact of cytokine exposure prior to differentiation to ASCs was also studied.

4.2 Results

4.2.1 B-cell phenotypic changes and changes to sialylation during *in vitro* differentiation to plasma cells

Thus far, the results of this study have shown that B-cells from healthy individuals upregulate sialylation when activated – particularly in response to TLR stimulation. Following activation *in vivo*, B-cells go on to differentiate to long or short-lived ASCs or memory cells. There are currently no data on the changes to sialylation which occur during this differentiation, nor any evidence that the changes to sialylation seen during activation with various stimuli would translate to terminally differentiated cells. Therefore, in order to study the changes in B-cell sialylation during differentiation to plasma cells, B-cells isolated from the peripheral blood of healthy donors and patients with ERA were differentiated *in vitro* to plasma cells via a three step differentiation protocol. A detailed description of each step is provided in Chapter 2. In short, B-cells are first activated via BCR crosslinking and co-stimulation provided by CD40L expressing cells in the presence of IL-2 and IL-21 in order to fully activate the cells and prime them for differentiation, replicating the dual stimulation received by B-cells in the lymph nodes *in vivo*. Following this step, the activating and co-stimulatory factors are removed, allowing for differentiation to plasmablasts. Lastly, cells are cultured with IL-21, IL-6 and IFN, and IL-2 is withdrawn so that differentiate to plasma cells can occur[259]. Several measurements were taken at each stage of differentiation, including expression of cell surface phenotype markers and surface sialylation - measured by flow cytometry, expression of ST6Gal1 and NEU1 mRNA by RT-qPCR and concentration of IgM, as measured by ELISA, in cell culture supernatants.

4.2.1.1 Mitogens and cytokines in combination can drive the differentiation of B-cells to plasma cells *in vitro*

To determine the phenotypic changes experienced by B-cells during the process of differentiation, and to track the expression of markers which imply cell differentiation, cells were assessed by flow cytometry at days 0, 3, 6 and 13. Cells were stained with a viability dye, and with CD19, CD20, CD27, CD38 and CD138 antibodies. A representative example of staining is shown in Figure 4.1.

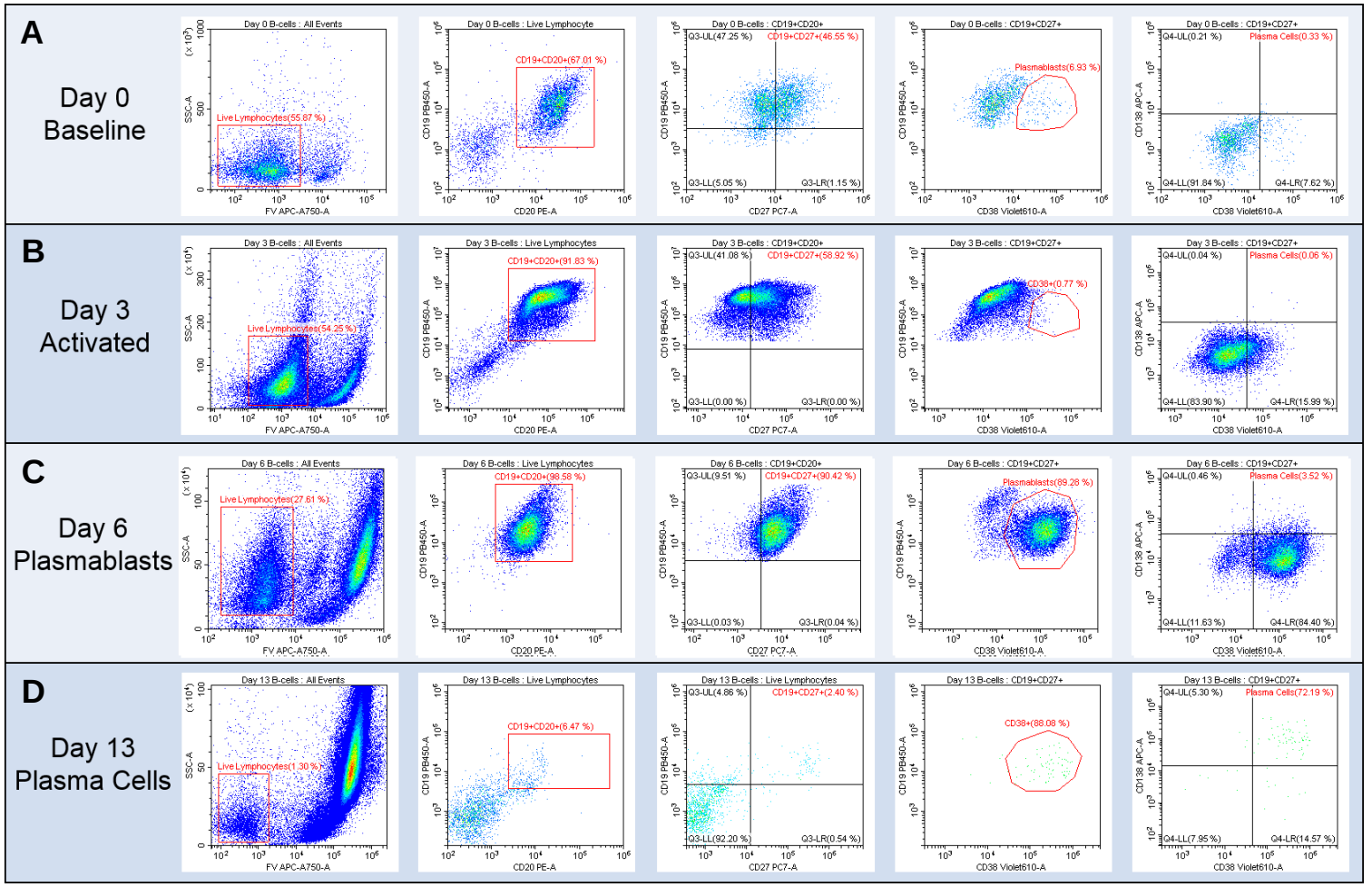


Figure 4.1 Changes to B-cell phenotype during *in vitro* differentiation to plasma cells

B-cells undergoing *in vitro* differentiation to plasma cells were analysed by flow cytometry at four stages of differentiation – baseline B-cells isolated from peripheral blood **(A)**, activated B-cells at day 3 **(B)**, plasmablasts at day 6 **(C)** and plasma cells at day 13 **(D)**. At each stage cells were stained with a viability dye, CD19 BV421, CD20 PE, CD27 PE-Cy7, CD38 BV605 and CD138 APC. Cells were first gated based on the viability stain, to exclude stained dead cells. Cells were then gated on expression of CD20 and CD19 to identify the total B-cell population. Based on expression of CD27 and CD38, $CD27^{-}CD38^{-}$ naïve B-cells, $CD27^{+}$ memory B-cells, and $CD27^{+}CD38^{++}$ plasmablasts can be identified. Plasma cells can then be identified as $CD19^{+/-}CD27^{+}CD38^{++}CD138^{+}$.

At day 0 the population of B-cells mainly consists of either naïve or memory B-cells, with a small percentage of plasmablasts and very few plasma cells (Figure 4.1A). By day 3 the phenotype of the cells changes dramatically, and cell populations expand by up to four-fold (unreported observations). The forward and side scatter profile of the cells changes dramatically with a large increase in side scatter. Similarly to day 0, the majority of cells express markers of naïve or memory B-cells, with a greater percentage of cells expressing CD27 than at day 0. Plasmablasts are no longer detectable and there are no plasma cells (Figure 4.1B). At day 6, side scatter increases further. Viability of cells in the culture is reduced, and the majority of cells express CD27 and CD38 – suggesting a plasmablast phenotype (Figure 4.1C). At day 13, cell viability is once again reduced (~30-50% viable cells). Expression of CD19 and CD20 is variable and the majority of cells are $CD38^{+}CD138^{+}$, indicating cells have adopted a plasma cell phenotype (Figure 4.1D).

4.2.1.2 Variations exist in sialylation, expression of ST6Gal1 and NEU1 mRNA, and IgM production between differentiating B-cells from patients with RA and from healthy donors

As well as changes to cell phenotype being measured during *in vitro* B-cell differentiation, expression of SA on the B-cell surface was also measured by flow cytometry at day 0, 3, 6 and 13 – to detect changes to sialylation accompanying phenotypic changes. In B-cells from healthy donors (n=6) expression of SA increased between days 0 and 3 during the activation phase (9.700 ± 7.867, p=0.1139 – fold change relative to baseline samples). It then decreased between days 3 and 6 (6.88 ± 0.263 vs. 9.700 ± 7.867, p=0.0863), and between days and 6 and 13 (6.88 ± 0.263 vs. 0.149 ± 0.263, p=0.0366), with greatly reduced expression in plasma cells compared to day 0 B-cells (Figure 4.2A). The increase in sialylation in activated B-cells between day 0 and day 3 is in line with previously observed results which showed increased B-cell sialylation upon stimulation in Chapter 3. Fold change in sialylation was much higher in this case (up to 18 fold increase vs. 2 fold increase recorded previously (Figure 3.8A). This is possibly reflective of the high dose of stimuli delivered to cells, in this case via $F(ab')_2$ and CD40L expressing L-cells, as well as potentially the influence of cytokines

included in these cultures. The reduction in sialylation between day 3 and day 13 may be due to reduced requirement for expression of sialylated proteins, previously it was observed (data not shown) that unstimulated *ex vivo* plasmablasts express lower levels of SA than naïve and memory B-cells, which is reflected in these results.

Previous results had showed that sialylation was dysregulated in cells from patients with ERA, and was not upregulated in response to activation to the same extent as in cells from healthy donors. To determine the impact of dysregulated expression of sialic acid on differentiation, B-cells from patients with ERA (n=3) were also isolated and differentiated to plasma cells. It was found that, similar to cells from healthy donors, expression of SA increased between day 0 and 3 (1.706 ± 0.487, p=0.2888 – fold change relative to baseline samples), however this increase was less pronounced in cells from patients with ERA than in cells from healthy donors (1.706 ± 0.487 vs. 9.700 ±7.867, p=0.2472). Of note, the absolute MFI values were highly variable in the cells from healthy donors at both day 0 and day 3, making it difficult to draw any strong conclusions from these data. Sialylation also decreased between day 3 and 6 (1.706 ± 0.487 vs. 0.908 ± 0.519, p=0.2537) and day 6 and 13 (0.908 ± 0.519 vs. 0.010 ± 0.014, p=0.0463), similar to patterns of expression seen in cells from healthy donors (Figure 4.2A). The relatively low fold increase in sialylation between day 0 and day 3 in B-cells from patients with ERA could be in line with previous results which showed that B-cells from patients with ERA did not respond to activation with an increase in sialylation (Figure 4.2B). Despite the potentially dampened response to stimuli and lack of increase in sialylation within the first 3 days, B-cells from patients with ERA were still able to differentiate to plasma cells, suggesting that the fluctuation in SA expression may not be a requirement for B-cell differentiation in cells from patients with RA.

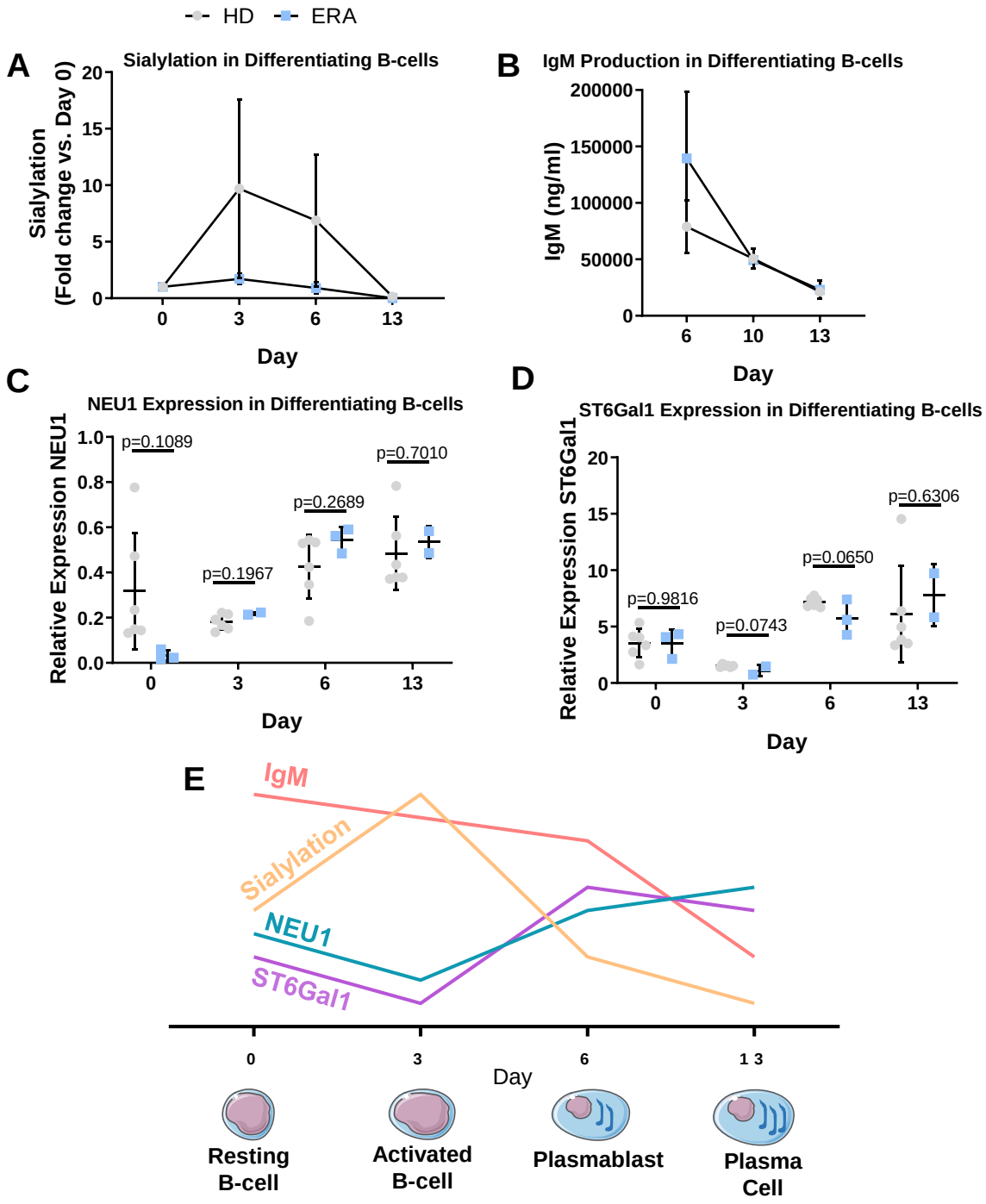


Figure 4.2 Changes to sialylation, expression of ST6Gal1 and NEU1, and production of IgM during *in vitro* B-cell differentiation in B-cells from healthy donors and patients with ERA

B-cells isolated from healthy donors (HD) (n=6) or from patients with early RA (ERA) (n=3) were differentiated to plasma cells *in vitro*. At baseline, day 3, day 6 and day 13 expression of SA was measured by flow cytometry **(A)**. IgM in cell culture supernatants was measured by ELISA at day 6, day 10 and day 13 **(B)** and expression of NEU1 and ST6Gal1 mRNA were measured by RT-qPCR **(C-D)**. Schematic summary of B-cell phenotypic and gene expression changes in B-cells during differentiation to plasma cells **(E)**. Graphs show mean with SD. Unpaired t tests were used to generate p values, comparing HD and ERA at each time point.

Further to measuring expression of SA, expression of ST6Gal1 and NEU1 mRNA were also measured by RT-qPCR. Expression of ST6Gal1 and NEU1 mRNA followed similar patterns of expression, with a trend for decreased expression of both enzymes between day 0 and day 3 (NEU1: 0.318 ± 0.259 vs. 0.181 ± 0.035; p=0.6644; ST6Gal1: 3.538 ± 1.274 vs. 1.518 ± 0.109, p=0.0367 – ΔCT values) then an increase between day 3 and day 6 (NEU1: 0.181 ± 0.035 vs. 0.427 ± 0.141, p=0.0224; ST6Gal1: 1.518 ± 0.109 vs. 7.170 ± 0.421, p<0.0001) (Figure 4.2C, 4.2D). In contrast, expression of ST6Gal1 was decreased in plasma cells at day 13 compared to plasmablasts at day 6 (6.094 ± 4.294 vs. 7.170 ± 0.421, p=0.9115) (Figure 4.2D). Expression of NEU1 however, increased between day 6 and day 13 (0.427 ± 0.141 vs. 0.485 ± 0.163, p=0.8441), reaching the highest expression in plasma cells (Figure 4.2C). These results also agree with previous observations which described a decrease in expression of both NEU1 and ST6Gal1 with increasing expression of SA (Section 3.2.8).

In patients with ERA, expression of NEU1 increased in cells from patients with RA between days 0 and 3 (0.034 ± 0.023 vs. 0.219 ± 0.007, p=0.0243) (Figure 4.2C), then increased between day 3 and 6 (0.219 ± 0.007 vs. 0.546 ± 0.055, p=0.0894) and remained constant between day 6 and 13 (0.546 ± 0.055 vs. 0.534 ± 0.069, p=0.4766). This was similar to the pattern of expression seen in cells from healthy donors, however – in contrast – in the cells from healthy donors, expression decreased between day 0 and day 3 (Figure 4.2C).

Expression of ST6Gal1 also showed a similar pattern of expression in cells from patients with ERA as in cells from healthy donors (Figure 4.2D). Expression decreased between days 0 and 3 (3.517 ± 1.194 vs. 1.104 ± 0.521, p=0.2081) then increased between day 3 and 6 (1.104 ± 0.521 vs. 5.761 ± 1.575, p=0.1678). In contrast to cells from healthy donors however, expression of ST6Gal1 mRNA increased in cells between days 6 and 13 (5.761 ± 1.575 vs. 7.781 ± 2.762, p=0.4738) whereas a decrease was seen in cells from healthy donors (Figure 4.2D). Expression of NEU1 and ST6Gal1 was higher in plasmablasts and plasma cells than baseline and activated B-cells in cells from both healthy donors and patients with ERA. These results are consistent with previous results which showed that higher expression of NEU1 and ST6Gal1 occurs when surface sialylation is lower (Figure 3.16). It would appear that in differentiating cells, as

sialylation decreases following initial cell activation, expression of both NEU1 and ST6Gal1 increases in cells from healthy donors and patients with ERA.

Production of IgM by differentiating cells was also measured by ELISA in supernatants collected at day 6, 10 and 13. Production of IgM declined as cells from healthy donors and patients with ERA differentiated from plasmablasts to plasma cells (Figure 4.2B). This is most likely indicative of a switch to IgG producing cells induced by differentiation, and is in line with previously described data[259]. Concentration of IgM was initially higher in day 6 supernatants from cultures of cells from patients with ERA than in cells from healthy donors (139456 ± 83637 vs. 78894 ± 56654, p=0.2765 IgM concentration ng/ml), but concentration then decreased dramatically between day 6 and day 10 in cultures of ERA cells and was at a similar level to cultures of cells from healthy donors at both day 10 and day 13 (Day 10: HD 50548 ± 21543 ERA 49207 ± 3907, p=0.9363; Day 13: HD 21238 ± 7439 ERA 23158 ± 11382, p=0.7846 ng/ml) (Figure 4.2B). These results could indicate that plasmablasts from patients with ERA intrinsically produce more IgM. However, since the concentrations were approximately equal in ERA and healthy donor cultures by day 10, it may indicate that there was a delay in class switching to IgG producing cells in the cells from patients with ERA, which then occurred between days 6 and 10.

4.2.2 Impact of TLR stimulation on B-cell phenotypic changes during *in vitro* differentiation to plasma cells

In Chapter 3 it was determined that changes to B-cell sialylation in response to activation were dependent on the type of stimuli received (Section 3.2.5). Results showed that stimulation with TLR ligands led to a greater increase in sialylation of activated B-cells, than in those activated by BCR crosslinking (Figure 3.8A). It was therefore hypothesised that changes to sialylation induced by activation stimuli may translate to differential expression of SA in differentiated plasma cells, as a potential mechanism of reduced B-cell sialylation in RA.

4.2.2.1 Stimulation with TLR ligands may alter production of IgM in plasmablasts from healthy donors and patients with RA

In order to determine if changes to sialylation in plasma cells and plasmablasts may be primed by a pre-differentiation stimulus, B-cells isolated from the blood of healthy donors (n=2) and patients with RA (n=2) were differentiated to plasma cells under one of two initial conditions of stimulation – either $F(ab')_2$ or CpG + R848 in combination with CD40L stimuli. Sialylation, along with expression of NEU1 and ST6Gal1 and production of IgM were first measured in plasmablasts at day 6 of the differentiation protocol. In the cells from healthy donors, it was found that sialylation did not significantly differ in cells stimulated with $F(ab')_2$ and those stimulated with TLR ligands at day 0, showing only a very slight decrease in expression of SA in the TLR stimulated cells (0.871 ± 0.111, p=0.3463 – fold change relative to $F(ab')_2$ stimulated cells) (Figure 4.3A). It was also found that there was a very slight increase in expression of NEU1 (0.476 ± 0.075 vs. 0.542 ± 0.051, p=0.1585 – ΔCT values) (Figure 4.3B) and a slight decrease in ST6Gal1 mRNA expression (6.813 ± 0.143 vs. 5.838 ± 0.344, p=0.2164) (Figure 4.3C). Production of IgM was very slightly increased in cells stimulated with TLR ligands (140276 ± 147101 vs. 32068 ± 3926, p=0.4958 IgM concentration, ng/ml), however there was also a large degree of variation in IgM production in TLR-stimulated cells (Figure 4.3D). In cells from a patients with ERA, expression of SA did not significantly differ other than a very slight increase in expression in TLR ligand stimulated cells (1.147 (n=1 due to insufficient cells at day 6 and day 13 to carry out measurements by flow cytometry and qPCR in samples from one patient)) (Figure 4.3E). Expression of NEU1 and ST6Gal1 were also both slightly reduced in cells stimulated with TLR ligands than with $F(ab')_2$ (0.354 vs. 0.485 and 4.107 vs. 5.603 respectively) (Figure 4.3F 4.3G). In contrast to cells from healthy donors, production of IgM was reduced in cells treated with TLR ligands (63724 ± 21363 vs. 139456 ± 83637 ng/ml) (Figure 4.3H), suggesting the cells from patients with RA may respond differently to TLR ligands in terms of IgM production at the plasmablast stage.

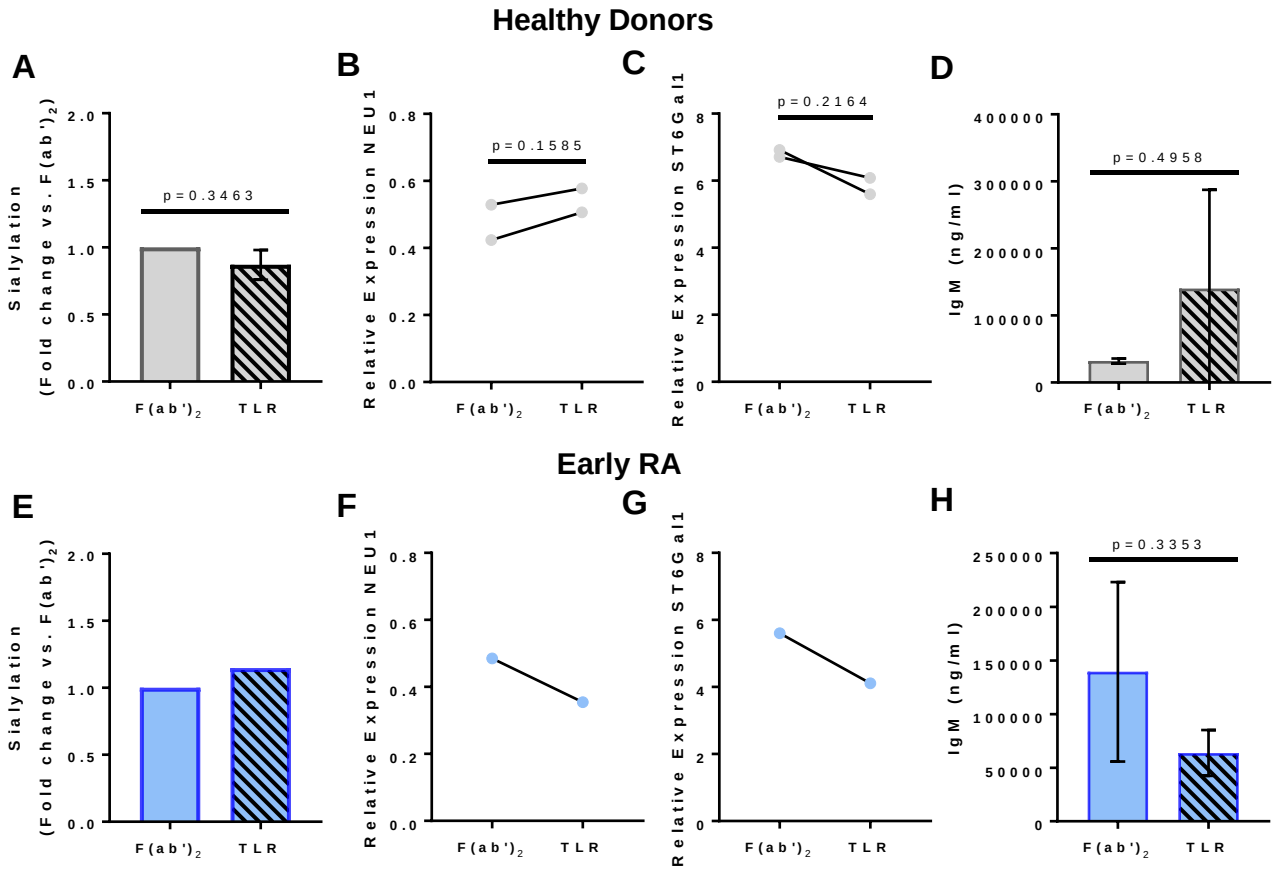


Figure 4.3 Changes to sialylation, expression of ST6Gal1 and NEU1 and production of IgM in plasmablasts after exposure to TLR stimuli in B-cells from healthy donors and patients with RA

B-cells isolated from healthy donors (n=2) **(A-D)**, or from patients with early RA (ERA) (n=2) **(E-H)** were differentiated to plasma cells *in vitro*. Between day 0 and 3, cells were stimulated as per protocol with CD40L L-cells and either 4 µg/ml $F(ab')_2$ (US) or 1 µg/ml CpG + 1 µg/ml R848 (TLR). Plasmablasts at day 6 were analysed and expression of SA was measured by flow cytometry **(A, E)**, expression of NEU1 **(B, F)** and ST6Gal1 **(C, G)** mRNA were measured by RT-qPCR and IgM in cell culture supernatants was measured by ELISA **(D, H)**. Graphs show mean with SD. Paired t tests were used to calculate p values. Differences were statistically significant where $p<0.05$.

4.2.2.2 Stimulation with TLR ligands may alter production of IgM in plasma cells from healthy donors and patients with RA

As well as measurements taken at the plasmablast stage at day 6, sialylation, gene expression and production of IgM were also measured at day 13 when cells had differentiated to plasma cells. In cells from healthy donors, it was found that expression of SA was increased in cells treated with TLR ligands compared with those stimulated with $F(ab')_2$ (1.386 ± 0.465, p=0.4490 – fold change relative to $F(ab')_2$ stimulated samples) (Figure 4.4A). Expression of both NEU1 and ST6Gal1 mRNA was also increased slightly in cells stimulated with TLR ligands (0.554 ± 0.007 vs. 0.471 ± 0.130, p=0.5118 and 6.082 ± 0.884 vs. 5.104 ± 1.792, p=0.9778 respectively, ΔCT values) (Figure 4.4B 4.4C). Most strikingly, production of IgM was increased in cells treated with TLR ligands compared to those stimulated with $F(ab')_2$ (55808 ± 6852 vs. 23158 ± 11382, p=0.1778 – IgM concentration, ng/ml) (Figure 4.4D). In cells from patients with RA, expression of SA was slightly lowered in TLR-stimulated cells compared to $F(ab')_2$-stimulated cells (0.712 fold change relative to unstimulated sample) (Figure 4.4E). Expression of NEU1 and ST6Gal1 mRNA were largely unchanged in cells stimulated with TLR ligands compared to those stimulated with $F(ab')_2$ (0.498 vs. 0.486 and 6.638 vs. 5.828 respectively) (Figure 4.4F, 4.4G). Similar to the observations in cells from healthy donors, production of IgM was increased in cells stimulated with TLR ligands (55808 ± 6852 vs. 23158 ± 11382 ng/ml) (Figure 4.4H). These results may suggest that priming with TLR ligands leads to continued production of IgM in plasma cells, perhaps indicating a delay in or lack of class-switching to IgG producing cells in these cultures.

4.2.3 Influence of cytokines on B-cell sialylation

As discussed previously, there are several factors which may lead to changes in B-cell sialylation, involving activation of B-cells either via TLR signalling or BCR crosslinking with CD40L co-stimulation (Chapter 3). Aforementioned inflammatory cytokines TNF and IL-6 are known to play key roles in RA, with involvement in numerous inflammatory processes, including leukocyte activation, adhesion and migration, and chemokine expression. IL-6 has similar functions to TNF in the synovium, as well as driving the acute phase response[63]. IL-17 is also

associated with a number of autoimmune and inflammatory diseases, however its exact role in RA remains to be fully elucidated[280]. IL-17 has also been previously linked to the reduction in sialylation of autoantibodies produced by B-cells in RA, in a murine model of disease[235].

It was hypothesised that exposure to certain cytokines, particularly those which may be increased in the serum of patients with RA, could contribute to the changes in sialylation in B-cells from patients with RA described in Section 3.2.2. B-cells from healthy donors and patients with ERA were cultured *in vitro* in the presence of these TNF, IL-6 and IL-17 - as well as IL-4 – to establish if the response to cytokines differs between health and disease, and to determine the possible impact of exposure to cytokines on B-cell sialylation.

4.2.3.1 Changes to sialylation in response to IL-4 may be different in B-cells from patients with RA to cells from healthy donors

B-cells from healthy donors (n=5), patients with ERA (n=5) and patients with PRA (n=4) were cultured for 48 hrs with 20 ng/ml of either IL-4, TNF, IL-6 or IL-17. Sialylation was measured after 48 hrs. In B-cells from healthy donors, exposure to TNF led to trend of slightly decreased expression of SA (0.883 ± 0.349, p=0.9224 - fold change compared to unstimulated samples), IL-4 led to a slight increase in SA expression (1.342 ± 0.418, p=0.2328), though these changes were not statistically significant. Additionally there was no change in sialylation in response to IL-6 or IL-17 at 20 ng/ml (0.976 ± 0.295, p=0.9998 and 1.018 ± 0.213, p=0.9999 respectively) (Figure 4.5A). In B-cells from patients with ERA, exposure to TNF, IL-4 and IL-6 did not lead to any change in sialylation (1.029 ± 0.247, p=0.9987; 0.980 ± 0.214, p=0.9997 and 1.059 ± 0.337, p=0.9803 respectively), whereas IL-17 led to a very slight decrease in SA expression, though not statistically significant. (0.892 ± 0.113, p=0.8797) (Figure 4.5B). In B-cells from patients with PRA, similar to results seen in cells from patients with ERA, there was no change in sialylation in B-cells stimulated with TNF and IL-6, and these cells also showed no change in response to IL-17 (1.075 ± 0.053, p=0.9991; 1.024 ± 0.057, p=0.9821 and 1.030 ± 0.066, p=0.9910 respectively) (Figure 4.5C). In contrast to cells from patients with ERA, cells from patients with PRA did show a significant upregulation of sialylation in response to IL-4 (1.556 ± 0.136, p=0.0011).

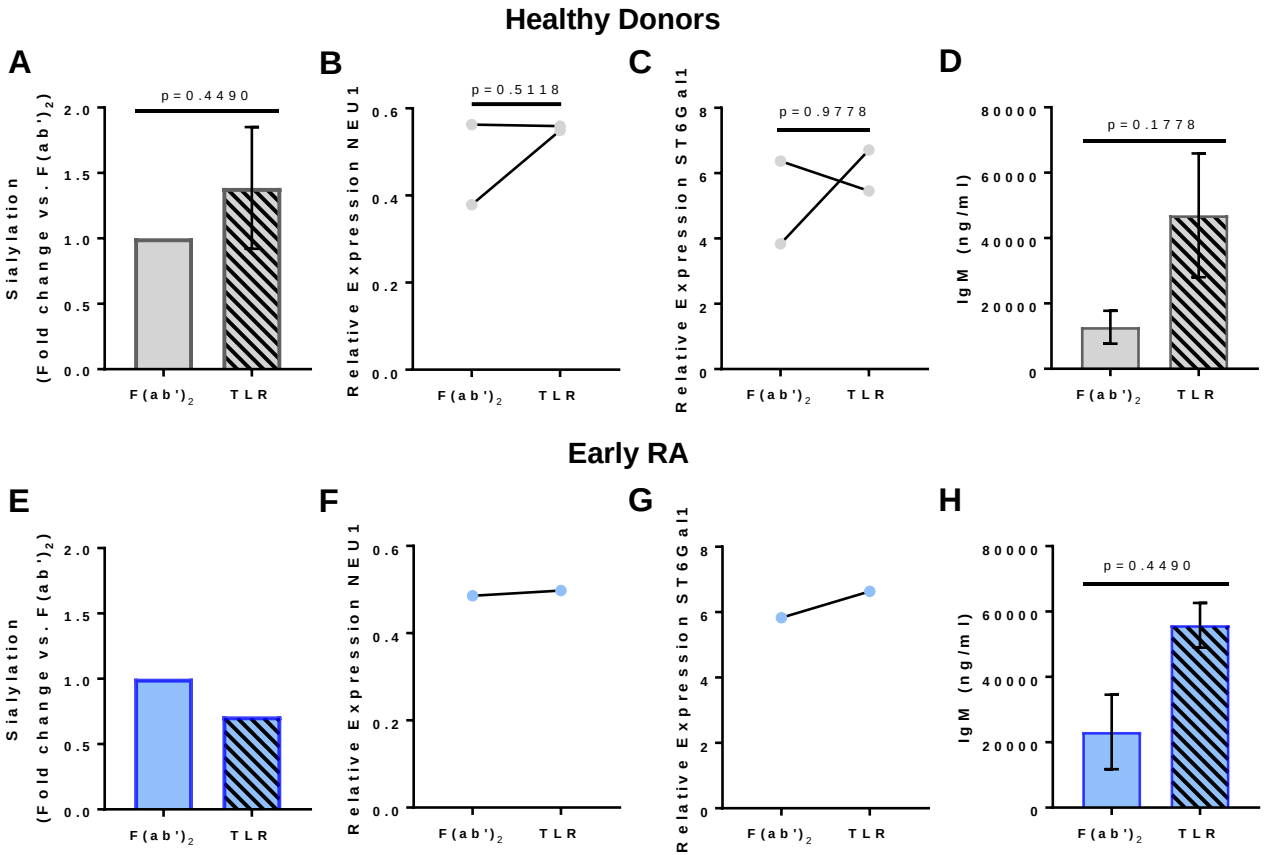


Figure 4.4 Changes to sialylation, expression of ST6Gal1 and NEU1 and production of IgM in plasma cells after exposure to TLR stimuli in B-cells from healthy donors and patients with RA

B-cells isolated from healthy donors (n=2) **(A-D)**, or from patients with early RA (ERA) (n=2) **(E-H)** were differentiated to plasma cells *in vitro*. Between day 0 and 3, cells were stimulated as per protocol with CD40L L-cells and either 4 µg/ml F(ab')$_2$ (US) or 1 µg/ml CpG + 1 µg/ml R848 (TLR). Plasma cells at day 13 were analysed and expression of SA was measured by flow cytometry **(A, E)**, expression of NEU1 **(B, F)** and ST6Gal1 **(C, G)** mRNA were measured by RT-qPCR and IgM in cell culture supernatants was measured by ELISA **(D, H)**. Graphs show mean with SD. Paired t tests were used to calculate p values. Differences were statistically significant where $p<0.05$.

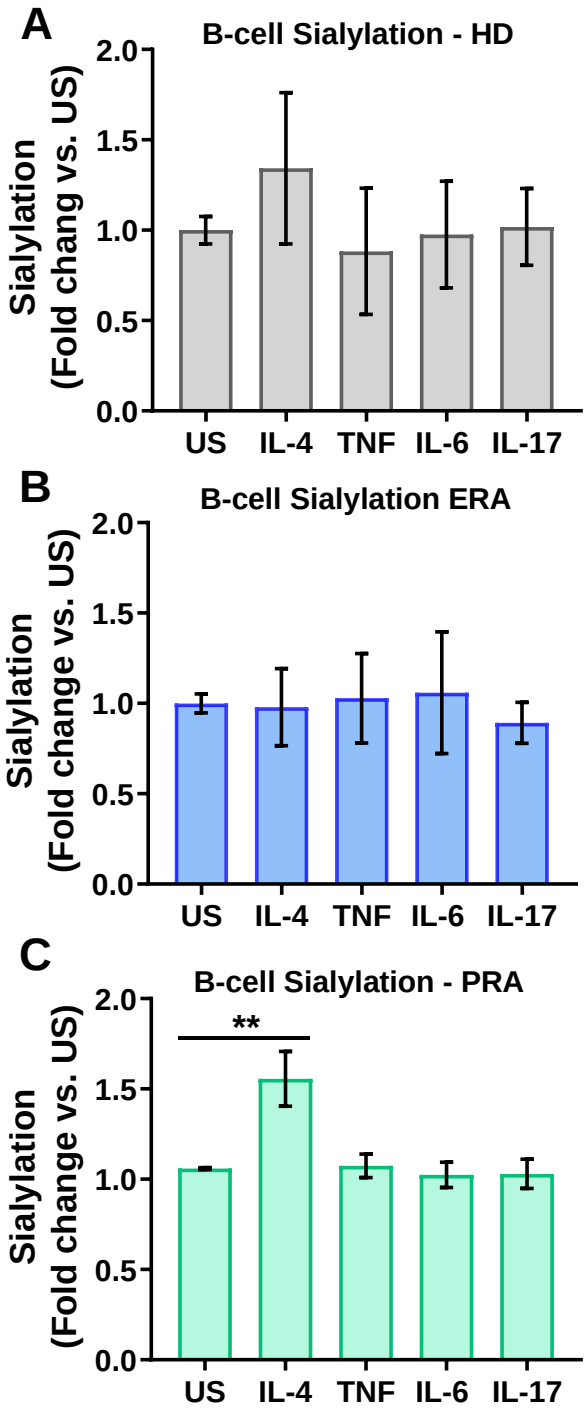


Figure 4.5 Influence of cytokines on B-cell sialylation in cells from healthy donors and patients with ERA or PRA

B-cells from healthy donors (n=5) **(A)**, patients with early RA (ERA) (n=5) **(B)** or patients with pre-RA (PRA) (n=4) **(C)** were cultured for 48 hrs with 20 ng/ml IL-4, TNF, IL-6 or IL-17. Sialylation was measured by flow cytometry. Graphs show mean with SD of SNA FITC MFI, expressed as a ratio relative to mean MFI of unstimulated (US) samples. One-way ANOVA followed by Dunnet's multiple comparisons tests were used to analyse statistical significance, with relevant statistically significant comparisons highlighted on graphs. ** $p<0.005$.

4.2.3.2 Response to cytokines *in vitro* varies according to dose

To examine the dose response to cytokines on B-cell sialylation, B-cells from healthy donors (n=5) and from patients with ERA (n=5) were stimulated for 48 hrs with increasing doses of IL-4, TNF, IL-6 and IL-17 (10, 20 or 50 ng/ml). Sialylation was measured after 48 hrs and it was found that - as described previously - in cells from healthy donors, IL-4 led to an increase in sialylation at all doses, with the strongest response to 10 ng/ml (1.542 ± 0.207, p=0.0050 – fold change relative to unstimulated samples) (Figure 4.6A). In cells from patients with ERA, there was no change in sialylation in cells stimulated with 10 ng/ml (1.206 ± 0.448 vs. 1.542 ± 0.207) (Figure 4.6B). Furthermore, at higher doses of IL-4 there was very little change in sialylation in B-cells from patients with ERA. In B-cells from healthy donors stimulated with TNF, there was a trend for decreased sialylation at all three doses (Figure 4.6C). In cells from patients with ERA, there was no change in sialylation in response to 10 and 20 ng/ml, and even a slight increase in sialylation in some samples at 50 ng/ml (Figure 4.6D). IL-6 at a dose of 10 ng/ml led to a slight increase in sialylation in cells from healthy donors (1.182 ± 0.109, p=0.0786), with little change at higher doses (20 ng/ml: 0.976 ± 0.295, p=0.9961 and 50 ng/ml: 0.904 ± 0.198, p=0.6369) (Figure 4.6E). In contrast, in cells from patients with ERA, there was a trend for a slight increase in sialylation at higher doses of IL-6, however this increase was not statistically significant (50 ng/ml: 1.120 ± 0.135, p=0.2846) (Figure 4.6F). In response to IL-17, cells from patients with ERA showed a decrease in sialylation at 10 ng/ml (0.832 ± 0.073, p=0.0406) (Figure 4.6G, 4.6H). In cells from healthy donors, at 20 and 50 ng/ml, sialylation was similar to unstimulated controls (1.018 ± 0.213, p=0.9945 and0.820 ± 0.305, p=0.4890) (Figure 4.6G). In cells from patients with ERA, sialylation was slightly reduced at both 20 (0.892 ± 0.113, p=0.2986) and 50 ng/ml (0.950 ± 0.375, p=0.9849) (Figure 4.6H), though these changes were not significant. These results suggest that the response to certain cytokines may be altered in patients with ERA compared to healthy donors.

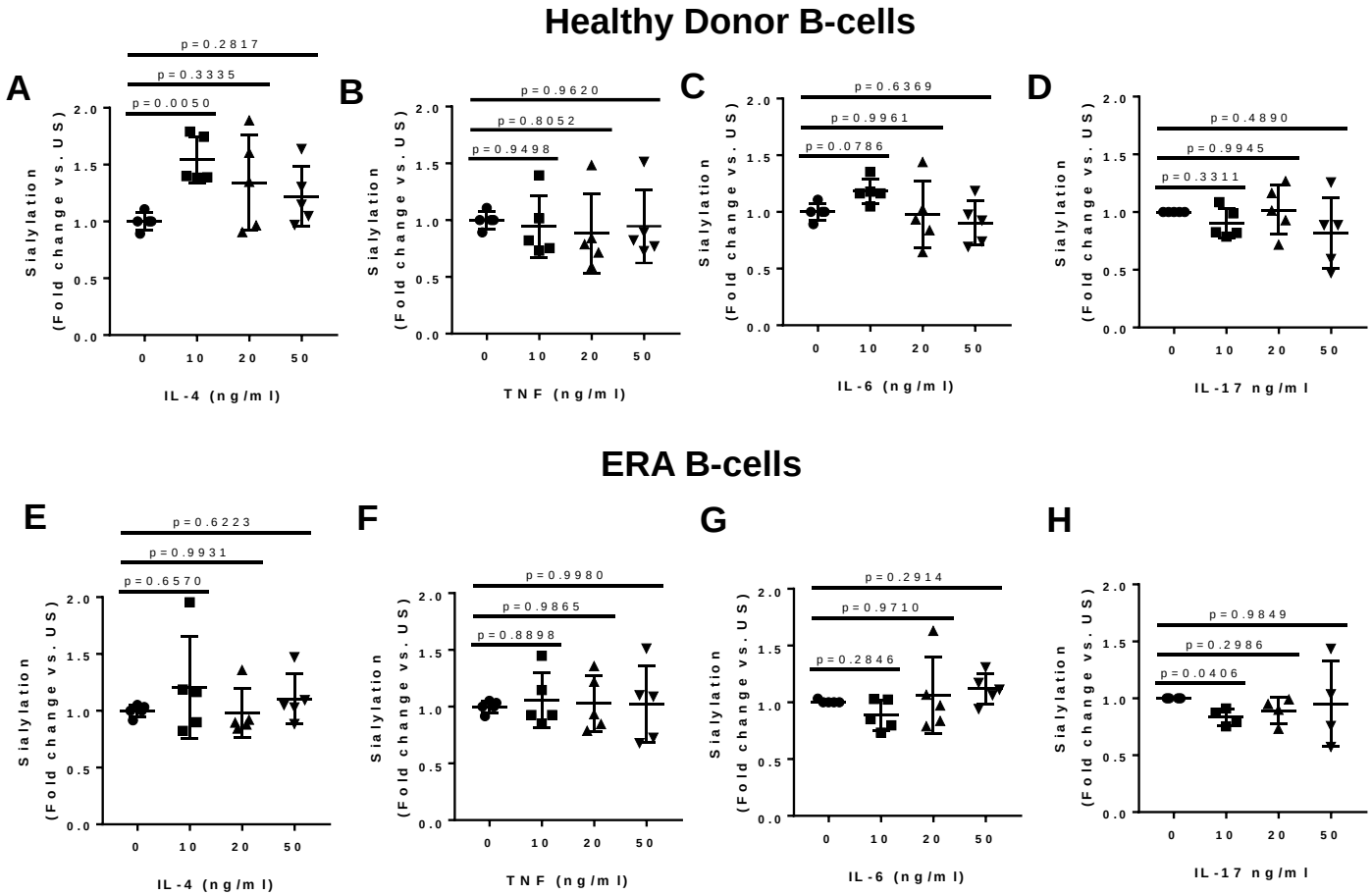


Figure 4.6 Influence of increasing doses of cytokines on B-cell sialylation in cells from healthy donors and patients with ERA

B-cells from healthy donors (n=5) **(A, C, E, G)**, patients with early RA (ERA) (n=5) **(B, D, F, H)** were cultured for 48 hrs with 10, 20 or 50 ng/ml IL-4, TNF, IL-6 or IL-17. Sialylation was measured by flow cytometry. Error bars show mean with SD of SNA FITC MFI, expressed as a ratio relative to mean MFI of unstimulated (US) samples. One-way ANOVA and Dunnett's multiple comparisons tests were used to generate p values. Statistically significant differences are indicated where $p<0.05$.

IL-4 leads to an increased in sialylation in cells from healthy donors (Figure 4.5A) as well as in cells from patients with PRA (Figure 4.5C), however, in cells from patients with ERA, there was no change in sialylation in response to IL-4 (Figure 4.5B, 4.6B). This suggested that the response to IL-4 is disrupted in RA when disease progresses to the active symptomatic stage, but not during the asymptomatic autoimmune phase. It may be hypothesised therefore, that this disruption in IL-4 response is due to chronic inflammation, and may not be associated with autoimmunity. The results also suggested that there may be a small decrease in sialylation in response to IL-17. This is interesting considering previous findings which showed decreased sialylation in autoantibodies produced by B-cells exposed to Th17 cytokines[235]. It is possible that expression of sialylated surface proteins may also be affected by B-cell exposure to IL-17.

4.2.3.3 Exposure to IL-4 and TNF in activated B-cells inhibits upregulation of sialic acid

To assess the impact of exposure to certain inflammatory cytokines on B-cell surface sialylation during activation, B-cells from healthy donors (n=6) were stimulated for 48 hrs with $F(ab')_2$ + CD40L ± 20 ng/ml IL-4, TNF or IL-6. Sialylation was measured after 48 hrs. As described previously in Section 3.2.4, it was found that stimulation of B-cells with $F(ab')_2$ + CD40L led to an upregulation of SA expression (1.407 ± 0.254, p=0.0073 – fold change relative to unstimulated samples) (Figure 4.7). When cells were stimulated with $F(ab')_2$ + CD40L with TNF (1.295 ± 0.624, p=0.3752) or IL-4 (1.244 ± 0.521, p=0.3953), sialylation was slightly increased, however, the changes were not significant and were less than the increase in sialylation induced by $F(ab')_2$ + CD40L alone. It was also found that cells stimulated with $F(ab')_2$ + CD40L and IL-6 showed increased expression of sialic acid, comparable with $F(ab')_2$ + CD40L only samples (1.464 ± 0.444 p=0.0048 and 1.407 ± 0.254, p=0.0073). Results in TNF-stimulated cells are consistent with previous findings that exposure to TNF tended to decrease sialylation (Figure 4.5A 4.6C) and exposure to IL-6 tended to lead to an increase in sialylation (Figure 4.5A, 4.6E). However, in unstimulated cells we previously reported that exposure to IL-4 led to an increase in sialylation in B-cells from healthy donors (Figure 4.5A, 4.6A). This discrepancy may indicate that IL-4 has varying effects on B-cells dependent on the activation status of the cells, which

has been described previously[281]. These results suggest that exposure to TNF and IL-4 in combination with B-cell activating factors may inhibit the upregulation of sialylation.

4.2.3.4 Exposure to IL-4 and TNF does not significantly affect B-cell expression of NEU1 and ST6Gal1

Since activation of B-cells via TLR and BCR signalling pathways was previously shown to have an impact on both expression of SA on the cell surface (Section 3.2.3) and on expression of NEU1 and ST6Gal1 mRNA (Section 3.2.8), the impact of exposure to cytokines on gene expression was also studied. B-cells from healthy donors (n=2) were stimulated for 48 hrs with increasing doses (10, 20 or 50 ng/ml) of either IL-4 or TNF, since these cytokines appeared to have opposing effects on B-cell surface sialylation. After 48 hrs, expression of ST6Gal1 and NEU1 mRNA was measured by RT-qPCR. It was found that expression of NEU1 was slightly upregulated in samples exposed to 50 ng/ml IL-4 versus unstimulated samples (0.299 ± 0.018 vs. 0.240 ± 0.031, p=0.5161 – ΔCT values) (Figure 4.8A). In contrast, exposure to 50 ng/ml IL-4 led to downregulation of ST6Gal1 expression compared to unstimulated samples (1.270 ± 0.552 vs. 1.977 ± 0.050, p=0.4544) (Figure 4.8C) however, due to small study size these changes did not reach statistical significance.

NEU1 expression slightly decreased at all doses of TNF (Figure 4.8B), and expression of ST6Gal1 tended to be slightly increased with increasing doses of TNF (Figure 4.8D), although there were no statistically significant differences. These results are somewhat surprising, since surface sialylation tended to be increased in cells stimulated with IL-4 (Figure 4.5A, 4.6A) and decreased in cells stimulated with TNF (Figure 4.5A, 4.6C). It was therefore hypothesised that ST6Gal1 and NEU1 would both decrease in cells stimulated with IL-4 and vice versa in TNF-stimulated cells, in line with results shown previously in Chapter 3, however, small study size makes it difficult to draw a solid conclusion from this current data.

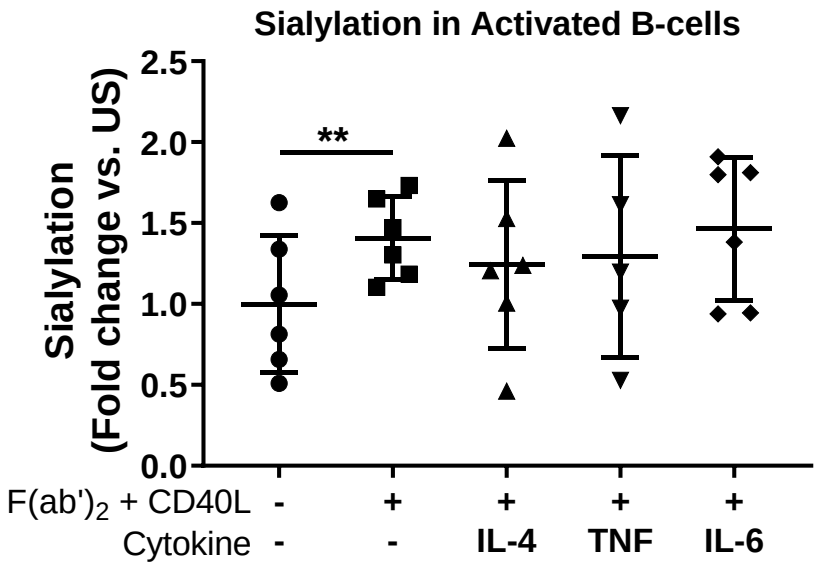


Figure 4.7 Influence of cytokines on activated B-cells from healthy donors

B-cells from healthy donors (n=5), were stimulated for 48 hrs with 20 ng/ml IL-4, TNF or IL-6 ± $F(ab')_2$ + CD40L. Sialylation was measured by flow cytometry. Graphs show mean with SD of SNA FITC MFI, expressed as a ratio relative to mean MFI of unstimulated (US) samples. One-way ANOVA and Tukey's multiple comparisons tests were used to asses statistical significance, with relevant statistically significant comparisons highlighted on graphs (**$p<0.005$).

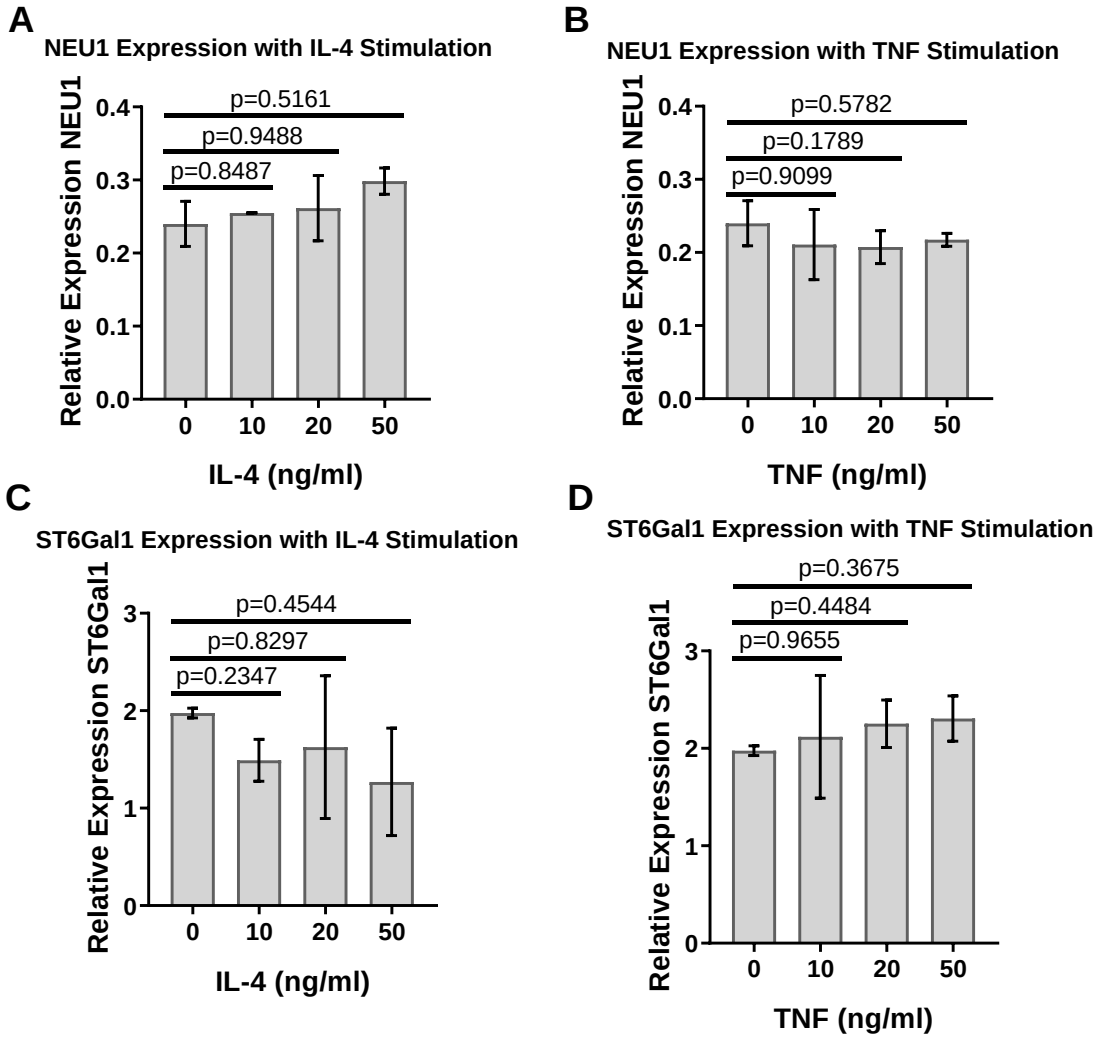


Figure 4.8 Influence of IL-4 and TNF on expression of ST6Gal1 and NEU1 in B-cells from healthy donors

B-cells from healthy donors (n=2) were stimulated for 48 hrs with 20 ng/ml IL-4 or TNF and expression of ST6Gal1 **(A, C)** and NEU1 **(B, D)** were measured by RT-qPCR after 48 hrs. ΔCT values were calculated relative to expression of HPRT1. Graphs show ΔCT mean with SEM. One-way ANOVA and Dunnett's multiple comparisons tests were used to generate p values.

4.2.4 Impact of cytokine stimulation on B-cell phenotypic changes during *in vitro* differentiation to plasma cells

Results in this chapter have suggested that, in cells from healthy donors, exposure to low doses of TNF may lead to a decrease, and exposure to IL-6 may lead to an increase in sialylation after 48 hrs *in vitro* (Figure 4.5). It was hypothesised that exposure to cytokines *in vivo* in the serum or in the synovial fluid, may contribute to the observed decrease in B-cell sialylation in cells from patients with RA. To determine if B-cell priming with cytokines could alter plasmablast and plasma cell sialylation following differentiation, cells from healthy donors were stimulated with 20 ng/ml of either TNF or IL-6 between day 0 and day 3, then differentiated as per protocol.

4.2.4.1 Stimulation with TNF did not alter sialylation, gene expression or production of IgM during *in vitro* differentiation to plasma cells

B-cells isolated from the blood of healthy donors (n=4) were stimulated with $F(ab')_2$ and CD40L L-cells, ± 20 ng/ml TNF at day 0. Expression of SA, along with expression of NEU1 and ST6Gal1 mRNA and production of IgM were all initially measured at day 6 in generated plasmablasts. It was found that exposure to TNF at day 0 did not have an effect on sialylation at day 6 in plasmablasts (1.017 ± 0.240, p=0.8953 – fold change relative to untreated samples) (Figure 4.9A), nor did it appear to have an impact on expression of NEU1 and ST6Gal1 (0.446 ± 0.084 vs. 0.460 ± 0.095, p=0.8415 and 6.851 ± 0.626 7.355 ± 0.397, p=0.0810 respectively – ΔCT values) (Figure 4.9B, 4.5C) or the production of IgM in plasmablasts (102054 ± 70687 vs. 102306 ± 56140, p=0.9936 IgM concentration ng/ml) (Figure 4.9D).

Sialylation, expression of NEU1 and ST6Gal1, and production of IgM were also measured in day 13 plasma cells that were treated ± TNF at day 0. Similar to results in day 6 plasmablasts, there were no observed differences in surface sialylation (0.991 ± 0.383, p=0.9698), expression of NEU1 (0.480 ± 0.108 vs. 0.492 ± 0.196, p=0.8472) and ST6Gal1 mRNA (5.737 ± 3.529 vs. 6.589 ± 5.355, p=0.4246) or IgM production (26569 ± 12983 vs. 25480 ± 3436, p=0.8629) in cells exposed to TNF compared to untreated cells (Figure 4.10). These results suggested that despite the initial trend for a decrease in sialylation observed in B-cells after a short exposure to TNF that was reported previously (Figure 4.5A),

the same dose does not appear to have a priming effect on these cells during differentiation, and does not translate to decreased plasmablast or plasma cell sialylation.

4.2.4.2 Stimulation with IL-6 may lead to decreased production of IgM in plasma cells differentiated *in vitro*

As in Section 4.2.4.1, B-cells isolated from the blood of healthy donors (n=4) were stimulated as per protocol with $F(ab')_2$ and CD40L L-cells, ± 20 ng/ml IL-6 at day 0. Expression of SA, along with expression of NEU1 and ST6Gal1 mRNA and production of IgM were all initially measured at day 6 in generated plasmablasts. It was found that exposure to IL-6 at day 0 led to increased expression of SA in day 6 plasmablasts, though this change was not statistically significant (1.592 ± 0.980, p=0.3135 – fold change relative to untreated samples) (Figure 4.11A). However, there was very little change observed in expression of NEU1 or ST6Gal1 mRNA (0.498 ± 0.064 vs. 0.460 ± 0.095, p=0.4624 and 8.160 ± 1.533 vs. 7.335 ± 0.397, p=0.3199 respectively – ΔCT values) (Figure 4.11C). Production of IgM was found to be slightly decreased in the plasmablasts which were primed with IL-6 (74996 ± 44135 vs. 102306 ± 56140, p=0.0921 - IgM concentration ng/ml) (Figure 4.11D).

Similarly, sialylation, expression of NEU1 and ST6Gal1 and production of IgM were measured in day 13 plasma cells, and expression of SA was only slightly increased in cells exposed to IL-6 at day 0, and the change was not statistically significant (1.296 ± 0.366, p=0.4574)(Figure 4.12A). Parallel to results at day 6, there was very little change observed in expression of NEU1 and ST6Gal1 mRNA in day 13 plasma cells (0.526 ±0.113 vs. 0.492 ± 0.196, p=0.5030 and 7.260 ± 4.478 vs. 6.589 ± 5.355, p=0.4504 respectively) (Figure 4.12B, 4.8C). Production of IgM was also found to be largely unchanged in cells stimulated with IL-6 compared to unstimulated cells in day 13 plasma cells (27416 ± 10588 vs. 25480 ± 3436, p=0.7224) (Figure 4.12D).

These results suggested that exposure to IL-6 prior to differentiation may increase sialylation and decrease production of IgM in plasmablasts, but changes to sialylation and IgM production may not be maintained when cells further differentiate to plasma cells. This agrees with previously reported results which showed that exposure to low doses of IL-6 tended to lead to upregulated

sialylation in cells from healthy donors (Figure 4.6E). However, despite reports of increased levels of IL-6 in the serum of patients with RA[282], resting peripheral B-cell sialylation is decreased. Results showed that when B-cells from patients with RA were exposed to the same low dose of IL-6 *in vitro*, sialylation did not increase (Figure 4.6F). It was hypothesised that chronic exposure to IL-6 may increase the threshold for activation in these cells. Taken together, these results suggest that acute IL-6 exposure may lead to increased B-cell sialylation, however this effect may be dampened by chronic exposure or increased dose.

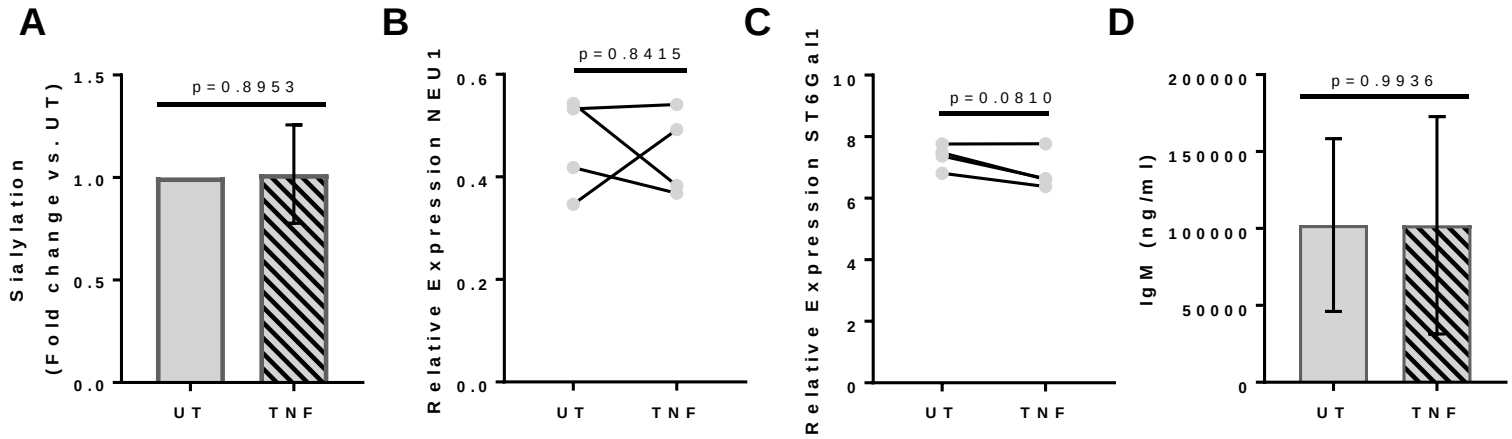


Figure 4.9 Changes to sialylation, expression of ST6Gal1 and NEU1 and production of IgM in plasmablasts after exposure to TNF in B-cells from healthy donors

B-cells isolated from healthy donors (n=4) were differentiated to plasma cells *in vitro*. Between day 0 and 3, cells were stimulated as per protocol with CD40L L-cells + 4 µg/ml $F(ab')_2$ with 20 ng/ml TNF (TNF) or without (UT). Plasmablasts at day 6 were analysed and expression of SA was measured by flow cytometry **(A)**, expression of NEU1 **(B)** and ST6Gal1 **(C)** mRNA were measured by RT-qPCR and IgM in cell culture supernatants was measured by ELISA **(D)**. Graphs show mean with SD. Paired t tests were used to generate p values, with statistically significant differences indicated where $p<0.05$.

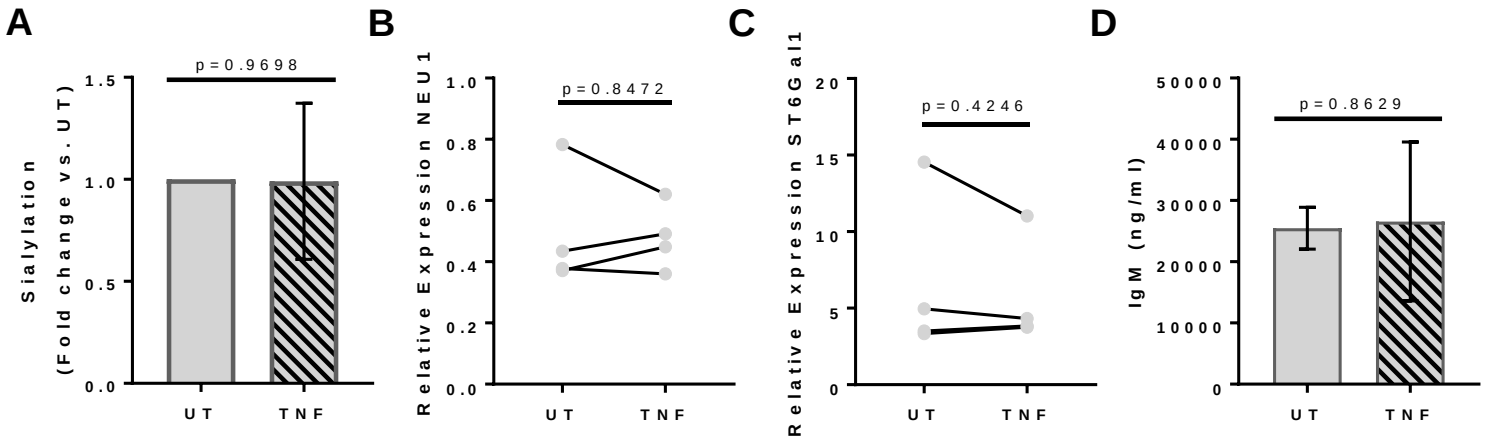


Figure 4.10 Changes to sialylation, expression of ST6Gal1 and NEU1 and production of IgM in plasma cells after exposure to TNF in B-cells from healthy donors

B-cells isolated from healthy donors (n=4) were differentiated to plasma cells *in vitro*. Between day 0 and 3, cells were stimulated as per protocol with CD40L L-cells + 4 µg/ml $F(ab')_2$ with 20 ng/ml TNF (TNF) or without (UT). Plasma cells at day 13 were analysed and expression of SA was measured by flow cytometry **(A)**, expression of NEU1 **(B)** and ST6Gal1 **(C)** mRNA were measured by RT-qPCR and IgM in cell culture supernatants was measured by ELISA **(D)**. Graphs show mean with SD. Paired t tests were used to generate p values, with statistically significant differences indicated where $p<0.05$.

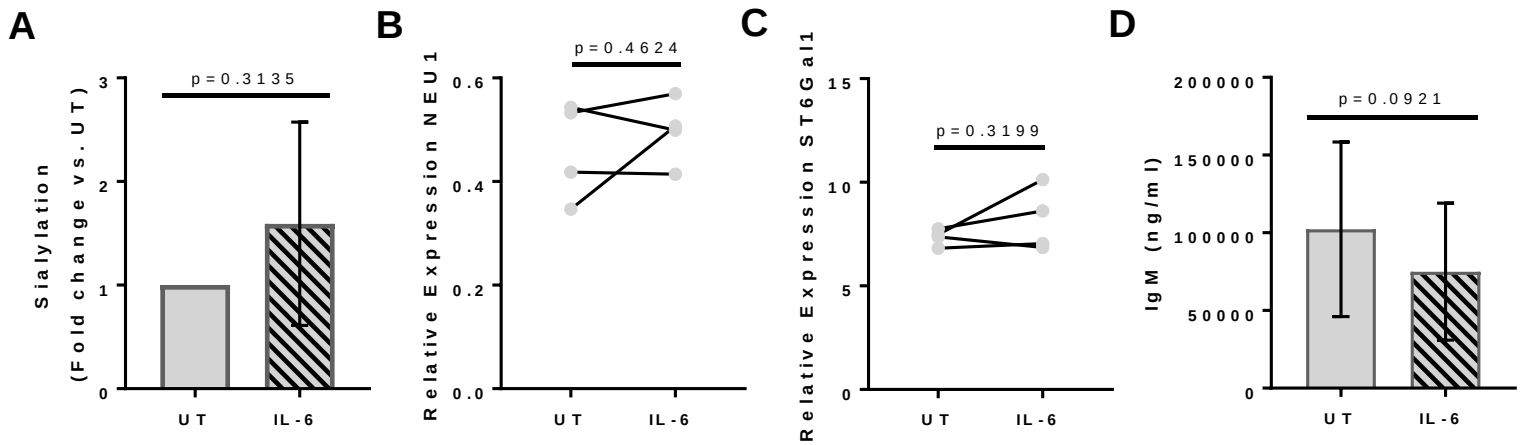


Figure 4.11 Changes to sialylation, expression of ST6Gal1 and NEU1 and production of IgM in plasmablasts after exposure to IL-6 in B-cells from healthy donors

B-cells isolated from healthy donors (n=4) were differentiated to plasma cells *in vitro*. Between day 0 and 3, cells were stimulated as per protocol with CD40L L-cells + 4 µg/ml $F(ab')_2$ with 20 ng/ml IL-6 (IL-6) or without (UT). Plasmablasts at day 6 were analysed and expression of SA was measured by flow cytometry **(A)**, expression of NEU1 **(B)** and ST6Gal1 **(C)** mRNA were measured by RT-qPCR and IgM in cell culture supernatants was measured by ELISA **(D)**. Graphs show mean with SD. Paired t tests were used to generate p values, with statistically significant differences indicated where $p<0.05$.

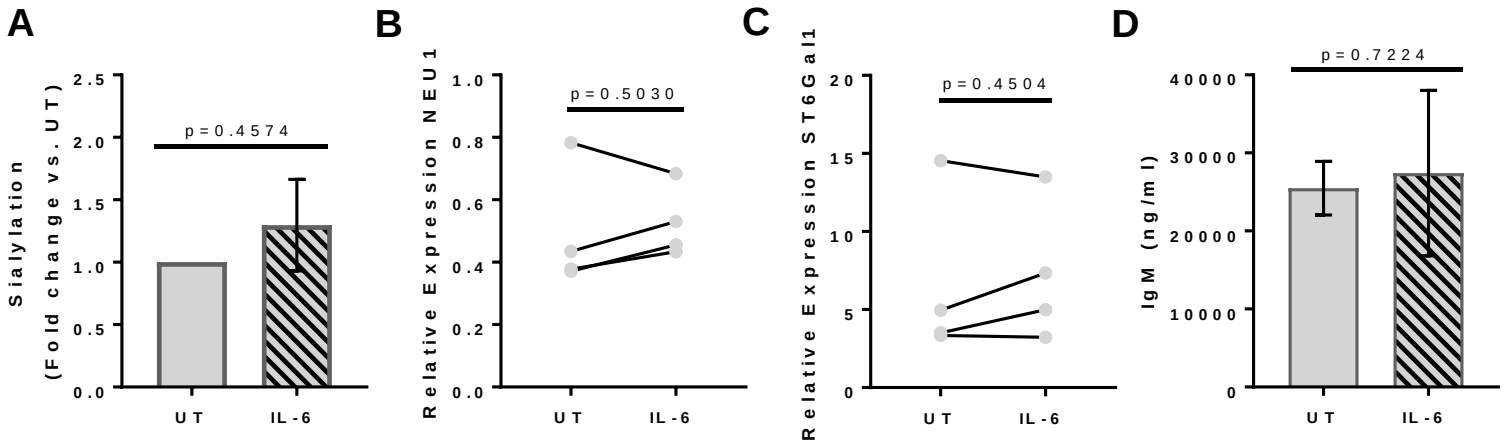


Figure 4.12 Changes to sialylation, expression of ST6Gal1 and NEU1 and production of IgM in plasma cells after exposure to IL-6 in B-cells from healthy donors

B-cells isolated from healthy donors (n=4) were differentiated to plasma cells *in vitro*. Between day 0 and 3, cells were stimulated as per protocol with CD40L L-cells + 4 µg/ml $F(ab')_2$ with 20 ng/ml IL-6 (IL-6) or without (UT). Plasma cells at day 13 were analysed and expression of SA was measured by flow cytometry **(A)**, expression of NEU1 **(B)** and ST6Gal1 **(C)** mRNA were measured by RT-qPCR and IgM in cell culture supernatants was measured by ELISA **(D)**. Graphs show mean with SD. Paired t tests were used to generate p values, with statistically significant differences indicated where $p<0.05$.

4.3 Discussion

Thus far, sialylation had been investigated only in the initial stages of B-cell activation, but following activation *in vivo*, B-cells undergo differentiation to ASCs. The impact of sialylation on the differentiation process was previously unknown. When B-cells from both healthy donors and patients with RA were differentiated, it was found that sialylation was first increased during the initial phase of activation, as seen previously, then decreased as cells differentiated to ASCs, with greatly reduced expression in plasma cells compared with memory and naïve B-cells isolated from peripheral blood. When cells were activated with TLR ligands instead of by BCR stimulation as in previous differentiations, it was found that TLR-stimulated cells may generate plasma cells with increased sialylation and sustained production of IgM. This may be an indication of reduced class-switching in these cells, as TLR-stimulated cells *in vivo* generally differentiate to short-lived, IgM-producing ASCs.

Due to the small sample size, which was limited due to availability of samples and a requirement for a large number of starting cells in order to conduct the experiments, the data would benefit from adding a number of biological repeats to improve statistical power and strengthen any conclusions which might be drawn. In these experiments, the measurement of sialylation was carried out by flow cytometry, which may have introduced a degree of variation in MFI measured at different time-points, potentially influencing the results. It may be of benefit to use a more stringent method of measuring cell sialylation, which is less susceptible to technical variation and more easily standardised. Quantifying sialylation by lectin blot may prove effective in this case. Despite these challenges, the increase in sialylation between cells at day 0 and day 3 was obvious, and there was a clear reduction in sialylation in all samples measured at days 6 and 13 despite these potential confounding factors.

Production of cytokines by immune cells is an important driving factor for inflammation in RA. Illustrated by the large successes of treatment of patients with RA with anti-TNF and anti-IL-6 therapies, it is clear that these cytokines in particular are key to its pathogenesis. Despite successes in treatment, however, disease relapse, non-response to therapy and drug-resistant disease are still

challenging issues in the treatment of RA, and strategies which can prevent disease from reaching the multi-drug-resistant stage are the focus of much attention in RA research. It is clear that TNF and IL-6 promote and propagate chronic inflammation in RA, but their specific effects on aspects of B-cell autoimmunity are not well understood. It was therefore important to study the effects of cytokine exposure in the context of B-cell sialylation, to determine whether there may be some causal factor in cytokine exposure which leads to altered sialylation in RA. It was found that exposure to TNF *in vitro* tended to lead to reduced sialylation in cells from healthy donors. It would be interesting to speculate therefore, that extended exposure to TNF may contribute to decreased surface expression of sialylated proteins in B-cells from patients with RA. However, when cells from patients with RA were exposed to TNF *in vitro*, there was no change in sialylation. It was hypothesised that this may indicate a lack of response - induced by chronic over-exposure to TNF - leading to the induction of a negative feedback loop and reduced response to prevent over-activation. Despite the lack of response to TNF from B-cells from patients with RA, the impact of TNF on B-cell sialylation *in vivo* cannot be discounted, and is an interesting factor to consider which may, in combination with other factors alter B-cell surface sialylation in RA.

On exposure to IL-6 *in vitro*, sialylation tended to be increased in cells from healthy donors, but similarly to TNF, this effect was absent in cells from patients with RA at the same dose. There was, however, a mild increase in sialylation at a higher dose in cells from patients with RA, indicating that there may be a similar mechanism of negative feedback to prevent over-activation. It is interesting that these cytokines should have opposing effects, considering the wide-ranging effects of each cytokine are largely similar. It could be hypothesised that, in RA, a combination of cytokines may contribute to changes in sialylation, and the impact on sialylation may change over time with chronic exposure. Despite recent evidence that IL-17 may also play an important role in RA, treatment of patients with anti-IL-17 has proven less effective than other cytokine therapies[7]. A previous study linked exposure to Th17 cytokines with reduced expression of ST6Gal1 in murine B-cells, leading to production of IgG with reduced Fc sialylation[235]. In this study, IL-17 was found to decrease B-cell sialylation in low

doses in B-cells from patients with RA, potentially linking IL-17 with reduced B-cell sialylation in autoimmunity.

Although not considered an inflammatory cytokine, IL-4 drives B-cell proliferation, and it was found that following exposure to IL-4 *in vitro*, sialylation was increased in cells from healthy donors and patients with PRA, but not in those with ERA. These results suggest that increased B-cell sialylation may be associated with proliferation, and that response to IL-4 may be lost upon progression to chronic inflammation in RA.

Despite the initial decrease in sialylation observed in B-cells from healthy donors upon exposure to TNF, when cells were activated and differentiated following exposure, there was no change in sialylation. Interestingly, exposure to IL-6 did lead to increased production of IgM in plasmablasts and slightly increased sialylation. This is an interesting observation, and a potential indication of a role for IL-6 in determining sialyltransferase activity during differentiation. However, small numbers in this study of differentiation make it more difficult to draw solid conclusions, and the data would benefit from increased biological repeats.

When interpreting *in vitro* data involving cytokine exposure, it is important to consider the dose used experimentally in relation to physiological dose. The doses which B-cells are exposed to *in vivo* are likely to vary widely depending on the tissue environment. A range of cytokine doses were tested in this study to determine any dose-sensitive effects on B-cell sialylation. In RA, B-cell cytokine exposure *in vivo* may also vary according to stage of disease. It has been suggested that Th17 cytokines may play a role during the onset of autoimmunity, before TNF and IL-6 become more prominent during the onset of synovial inflammation[63]. It is also important to note that B-cells in the synovium are likely to be exposed to several cytokines at once, which may have separate opposing or cumulative effects on sialylation. Further study could include several cytokines in combination, and a wider variety of doses to reflect changing physiological conditions. As well as the cytokines investigated in this study, there are several other cytokines which are involved in the pathogenesis of RA which may have an impact on B-cell sialylation such as B-cell activating factor (BAFF), IFN and GM-CSF[63], which could also be studied in future experiments. For further studies, it would also be useful to add a measure of response to each cytokine such as

proliferation in response to IL-4, to determine if the response to each cytokine is altered or if there is an intrinsic alteration in sialylation in B-cells from patients with RA.

To summarise, the findings of this chapter have shown that B-cell sialylation varies during B-cell differentiation and is lowest in plasma cells. It was also shown that exposure to cytokines could potentially influence B-cell sialylation, and could account for some of the alterations to B-cell sialylation observed in baseline peripheral B-cell samples, in combination with B-cell intrinsic factors which were studied in Chapter 3. Although cytokines are an important driving factor for inflammation in RA, and concentrations are often altered in the serum or RA patients, there may yet be other B-cell extrinsic factors in serum which may also contribute to altered sialylation. Further serum proteins and their impact on sialylation will be studied in Chapter 5.

Chapter 5
Serum neuraminidase activity and consequences of reduced B-cell sialylation in RA

5.1 Introduction

The results of the previous chapters have described several potential candidates for B-cell intrinsic and extrinsic factors which may influence sialylation. Thus far each potential factor has been investigated more or less individually, without taking into account the dynamic presence of certain molecules such as cytokines in different body compartments, and without considering the impact of exposure to multiple cytokines and inflammatory markers in both the serum and in synovial fluid at disease sites in RA. Thus far, cytokines have been considered as potential serum factors which may alter sialylation, however there is potential for many other molecules to cause a shift in glycosylation. For example, free ST6Gal1 has been shown to be present in the bloodstream during inflammation, as it is released by hepatocytes as part of the acute phase response[283]. Free neuraminidase can also exist in the serum[284] and its increased presence in the serum has been shown to be associated with inflammation in some cases[285]. In this chapter, serum factors in RA were considered as a whole, and cells from healthy donors were exposed to serum from patients with RA during activation and differentiation *in vitro*.

Since the previous results of this study showed that sialylation was reduced on the surface of B-cells from patients with RA compared to healthy donors, the consequences of reduced sialylation were also considered in this chapter. There is a large body of research in several areas of pathology which suggests that altered sialylation can contribute to pathogenesis. In several cancers, increased sialylation promotes tumour cell survival, invasiveness and promotes resistance to chemotherapy[286]. In cardiovascular pathologies, during plaque formation in atherosclerosis, sialylation is downregulated in vessel wall integrins, facilitating monocyte transendothelial migration and promoting vessel wall inflammation[173,191]. The potential consequences of reduced B-cell sialylation in RA are as yet unknown, as are the direct consequences of reduced B-cell sialylation on function.

The overall impact of a shift in the glycocalyx of the B-cell surface has been examined by several groups in relation to the ability of the B-cell to activate T-cells[209-211]. It was observed that when B-cells were treated with Neu to digest surface sialic acid, the ability of resting B-cells to stimulate T-cells was greatly increased[209]. This was dubbed the “neuraminidase effect” and was thought to be a consequence of removing sialic acids which were masking co-stimulatory molecules on the B-cell surface. However, only one group reported that B-cell sialylation was also reduced in response to activation[211]. This research was carried out in mouse splenocytes, and thus far no follow-up experiments to determine the sialylation of primary human B-cells under differing conditions of activation have been published, nor have any further consequences for B-cell function been reported.

As mentioned previously, several studies have shown that increased sialylation promotes chemoresistance in cancer cells[197,198,201,203,287]. It was therefore hypothesised that altered B-cell sialylation in autoimmunity may also have an impact on biologic drug treatment. Rituximab is an anti-CD20 monoclonal antibody which is thought to lead to B-cell depletion via several proposed mechanisms – by opsonising cells and promoting complement-dependent lysis or ADCC, or by directly triggering B-cell apoptosis[245]. Since its modes of action depend on the anti-CD20 antibody binding to CD20 on the B-cell surface, it was hypothesised that reduced SA on the B-cell surface would improve binding and also improve the efficacy of complement-dependent lysis and ADCC. An *in vitro* rituximab killing assay was used to study the impact of sialylation on rituximab B-cell killing efficacy.

5.2 Results

5.2.1 Impact of serum factors on B-cell sialylation

Previous results showed that exposure to certain cytokines may have an impact on B-cell surface expression of SA (Chapter 4). It was hypothesised that exposure to these cytokines in the serum, as well as other factors, may contribute to altered B-cell sialylation *in vivo* in RA. It was also hypothesised that serum from patients with RA may have an impact on healthy donor B-cell sialylation *in vitro*.

5.2.1.1 Exposure to serum from both healthy donors and patients with ERA leads to a reduction in B-cell sialylation

In order to determine the impact of exposure to RA serum on healthy donor B-cells *in vitro*, B-cells isolated from healthy donors (n=2) were exposed to serum from both healthy donors (n=5) and patients with ERA (n=5) in an increasing percentage in media (2.5, 5 or 10% in 200 µl media). B-cells were cultured with the serum for 48 hrs, then sialylation was measured by flow cytometry. Results showed that the expression of surface SA in the healthy donor B-cells was inversely correlated with serum concentration (Figure 5.1). This was true for serum from both healthy donors and patients with ERA, and expression of B-cell sialylation was very slightly lower in cells cultured with serum from patients with ERA than with serum from healthy donors at 2.5% and 5% (2.5%: 0.404 ± 0.118 vs. 0.443 ± 0.098, p=0.5778; 5% 0.283 ± 0.086 vs. 0.323 ± 0.102, p=0.5206 – fold change relative to untreated samples), although sialylation was slightly higher on exposure to 10% serum (0.190 ± 0.035 vs. 0.181 ± 0.041, p=0.7213). These results suggested that serum from both healthy donors and from patients with RA contains factors which led to a decrease in sialylation of B-cells from healthy donors on exposure *in vitro*.

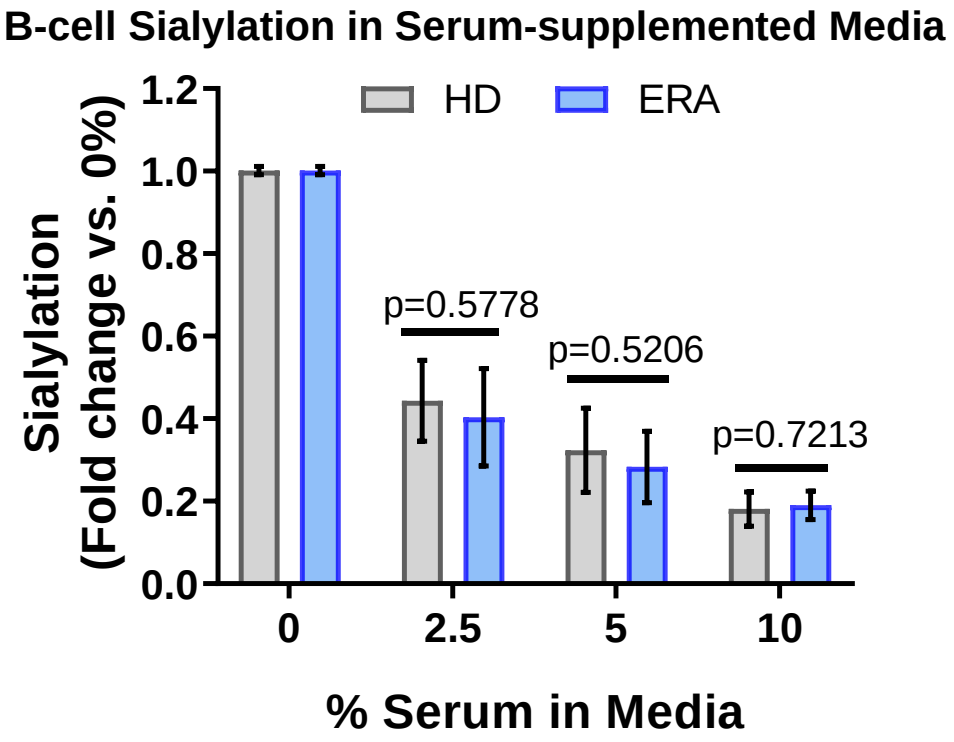


Figure 5.1 Impact of serum from patients with RA on sialylation in B-cells from healthy donors

B-cells from healthy donors (n=2) were cultured with serum from healthy donors (HD) (n=5) and patients with early RA (ERA) (n=5) in increasing percentages in RPMI (2.5, 5 or 10%). After 48 hrs, sialylation was measured by flow cytometry. Graphs show mean with SD of B-cell SNA FITC MFI, expressed as a ratio relative to SNA FITC MFI of samples cultured in 0% serum. Unpaired t tests were used to generate p values, comparing HD and ERA serum at each concentration.

5.2.2 Neuraminidase activity in serum of patients with ERA and healthy donors

It was hypothesised that neuraminidases in the serum were responsible for the reduction in B-cell expression of SA when serum was added to B-cell cultures (Figure 5.1). Studies of serum neuraminidase are few and far between, however there has been some evidence to link serum neuraminidase with inflammation. One study found that in patients with type II diabetes, levels of serum and urine neuraminidase were increased in patients with diabetic nephropathy compared to control groups[285]. A neuraminidase enzymatic activity assay was carried out to confirm the presence of active neuraminidase in serum and to determine if there was any difference in activity of neuraminidases between serum from healthy donors and patients with RA. The assay used Amplex red - a reagent which produces fluorescent molecule resorufin in the presence of H2O2[288]. H2O2 is generated by oxidation of desialylated galactose – the end product of neuraminidase activity - via galactose oxidase[289], thus the assay can be used to detect neuraminidase activity in the sample by measuring fluorescence/absorbance.

5.2.2.1 Activity of neuraminidase is increased in patients with RA compared to serum from healthy donors

Before comparing neuraminidase activity in the serum of healthy donors and patients with RA, optimal dilution of the serum in reaction buffer from the assay kit had to be established. Dilutions of serum from healthy donors (n=3) were made, containing 10 – 50% serum. Serum samples were then incubated with the rest of the kit reagents, including the fluorescent substrate, Amplex Red for up to 2 hrs at 37°C. Absorbance was measured at 30, 60, 90 and 120 mins. Activity could be detected after just 30 mins (Figure 5.2A) and remained relatively stable for the 2 hrs measured in this optimisation test (results not shown). This confirmed the presence and activity of neuraminidase within the serum, and potentially explained the reduction in SA expression observed when B-cells were exposed to serum samples in culture (Figure 5.1). A serum concentration of 30% in the reaction buffer was found to be optimal for the assay (Figure 5.2A).

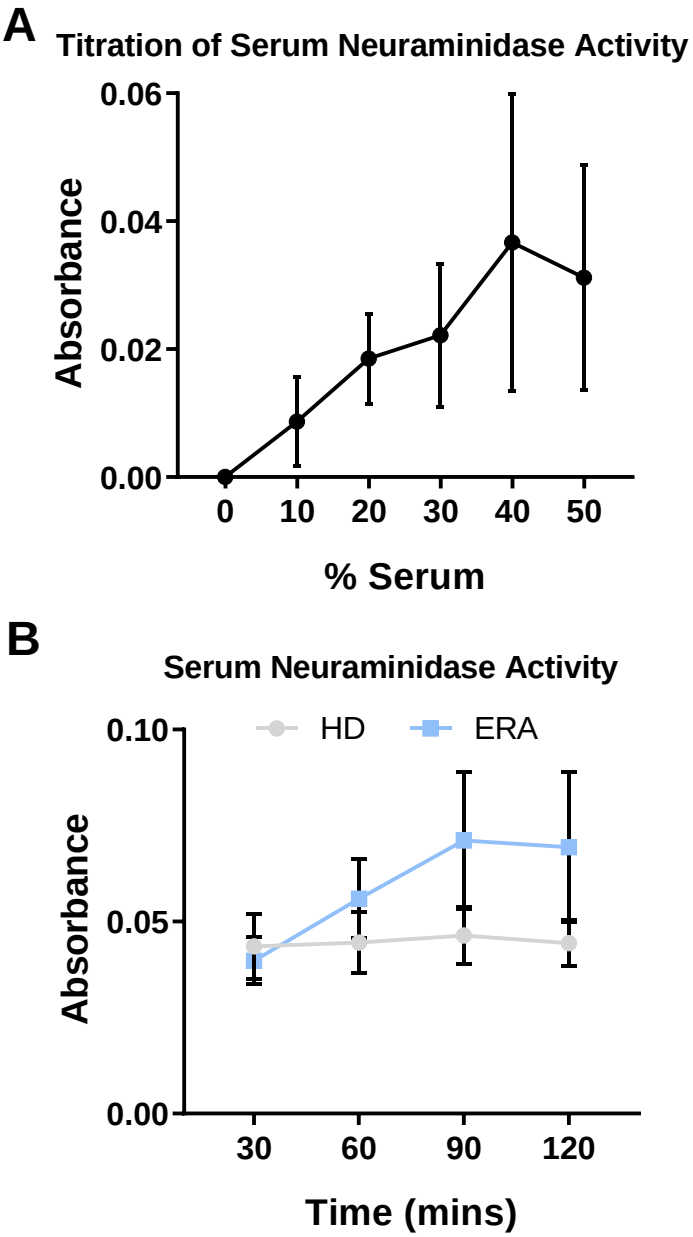


Figure 5.2 Serum neuraminidase activity in healthy donors and patients with ERA

Activity of Neu in serum of healthy donors (HD) (n=6) and patients with early RA (ERA) (n=6) were measured by Amplex Red™ fluorescence assay. To optimise serum from healthy donors (n=3) was diluted in sample diluent at 10, 20, 30, 40 or 50% and absorbance measured after 30 mins **(A)**. Serum was diluted to 30% in sample diluent and absorbance measured at 30, 60, 90 and 120 mins **(B)**. Graphs show mean absorbance with SD. Unpaired t tests were used to calculate p values, comparing HD and ERA serum at each time point – 30 mins p=0.7322, 60 mins p=0.4023, 90 mins p=2263 and 120 mins p=0.2509.

To determine if neuraminidase activity in the serum was altered in RA, samples of serum from healthy donors (n=6) and patients with ERA (n=6) were tested. Serum was diluted in reaction buffer then incubated with the kit reagents for up to 2 hrs, with absorbance measured every 30 mins. At 30 mins, absorbance, and therefore neuraminidase activity was approximately equal in the samples from healthy donors and patients with ERA (0.040 ± 0.015 and 0.044 ± 0.021, p=0.7322 absorbance respectively) (Figure 5.2B). However, between 30 and 90 mins absorbance increased in samples from patients with ERA, tapering off between 90 and 120 mins. Samples from healthy donors however, showed no change in absorbance throughout the time-course and at 120 mins absorbance was higher in samples from patients with ERA than healthy donors (0.069 ± 0.048 vs. 0.044 ± 0.015, p=0.2509). The increase in absorbance seen in the samples from patients with ERA indicates an increase in fluorescent substrate, indicating increased neuraminidase activity in these samples. These results suggested that there may be a higher level of Neu present in the serum of patients with ERA, which may have implications for the expression of SA on the B-cell surface *in vivo*.

5.2.3 Impact of exposure to serum on B-cell phenotypic changes during *in vitro* differentiation to plasma cells

As discussed previously in Section 5.2.1, when B-cells from healthy donors were exposed to serum from patients with ERA and from healthy donors - sialylation decreased, possibly due to the presence of neuraminidases in the serum. It was therefore hypothesised that exposure to serum from patients with RA, prior to *in vitro* differentiation, may influence the sialylation of plasmablasts and plasma cells generated from serum-primed B-cells.

5.2.3.1 Exposure to serum from patients with RA led to a reduction in sialylation and production of IgM in plasmablasts

B-cells from healthy donors (n=4) were differentiated under normal conditions, however between days 0 and 3, cells were cultured in media containing either 10% FBS, or 5% FBS + 5% serum from healthy donors (n=2) or patients with ERA (n=2). Following day 3, the differentiation protocol was carried out as normal, in media containing 10% FBS only. Expression of SA was measured at day 6 in generated plasmablasts, and was found to be reduced in cells cultured

with serum from patients with ERA (0.761 ± 0.130, p=0.0688 – fold change relative to no serum controls). There was also a slight increase in expression of SA in cells cultured with serum from healthy donors (1.121 ± 0.484, p=0.8769), compared to FBS only controls (Figure 5.3A). Further to this, expression of NEU1 and ST6Gal1 mRNA was also measured at day 6, and it was found that exposure to serum from healthy donors slightly increased expression of both enzymes (0.558 ± 0.167 vs. 0.460 ± 0.095, p=0.5220 and 9.117 ± 1.772 vs. 7.355 ± 0.397, p=0.2260 respectively) (Figure 5.3B, 5.3C). Exposure to serum from patients with ERA slightly increased expression of ST6Gal1 (8.150 ± 1.048 vs. 7.355 ± 0.397, p=0.3751 – ΔCT values) (Figure 5.3C) and had no effect on NEU1 (0.454 ± 0.076 vs. 0.460 ± 0.095, p=0.9772) (Figure 5.3B). Differentiating cell production of IgM was also measured at day 6, and it was found that cells exposed to serum from patients with ERA produced less IgM than FBS only controls and cells exposed to serum from healthy donors (68600 ± 45627 vs. 102306 ± 56140, p=0.3804 and 122015 ± 69712, p=0.1151 respectively IgM concentration ng/ml) (Figure 5.3D).

5.2.3.2 Exposure to serum from patients with RA led to a reduction in sialylation and production of IgM in plasma cells

As in section 5.2.3.1, expression of SA, along with expression of NEU1 and ST6Gal1 mRNA and production of IgM were also measured in day 13 plasma cells, following early exposure to serum from healthy donors and patients with RA. Similar to findings in plasmablasts, expression of SA was reduced in cells exposed to serum from patients with ERA pre-differentiation, compared to those exposed to serum from healthy donors and to FBS only controls (0.135 ± 0.027, p=0.0212 – fold change based on unstimulated samples) (Figure 5.4A). Sialylation was also slightly reduced in cells exposed to serum from healthy donors compared to FBS controls (0.734 ± 0.123, p=0.2987). Further to this, similar to results in plasmablasts, production of IgM was also reduced in cells exposed to serum from patients with ERA compared to those exposed to serum from healthy donors and FBS controls (9753 ± 3195 vs. 20248 ± 17762, p=0.5883 and 25480 ± 3436, p<0.0001 respectively, IgM concentration ng/ml) (Figure 5.4D). Contrary to results in plasmablasts, expression of NEU1 and ST6Gal1 mRNA was increased in cells exposed to serum from healthy donors and from patients with ERA compared to FBS only controls (NEU1 0.606 ± 0.101, p=0.4204 and 0.566 ± 0.228, p=0.4866 vs. 0.492 ± 0.196; ST6Gal1 7.686 ± 3.974,

p=0.6575 and 8.836 ± 0.699, p=0.7685 vs. 6.589 ± 5.355 respectively – ΔCT values) (Figure 5.4B, 5.4C). Taken together, these results suggested that exposure to serum from patients with ERA prior to differentiation leads to a reduction in sialylation, which translates to downregulated sialylation in differentiated plasmablasts and plasma cells. Production of IgM may also be reduced in cells exposed to serum from patients with RA, possibly due to decreased surface sialylation, or as a result of other cytokines and inflammatory markers which may be present in the serum.

5.2.4 Impact of exposure to serum factors on B-cell activation

Previously, it was found that when B-cells from healthy donors were activated via certain signalling pathways, there was an upregulation of sialylation. However, in cells from patients with ERA, this response was absent, despite cells showing a similar level of expression of CD69 Section 3.2.3. It has also been shown previously that several cytokines may have an effect on B-cell sialylation, with or without additional stimuli (Chapter 4). With the observation that serum from healthy donors and from patients with RA leads to a decrease in B-cell sialylation (Figure 5.1), and the findings that exposure to serum from patients with ERA prior to differentiation led to decreased sialylation in plasmablasts and plasma cells (Figure 5.3, 5.4), it was hypothesised that exposure to serum may have an impact on sialylation following B-cell activation. Therefore, B-cells from healthy donors were exposed to serum from patients with ERA, and activated via TLR stimulation and BCR crosslinking ± CD40L co-stimulation – to determine if serum factors play a role in regulating sialylation in activated cells.

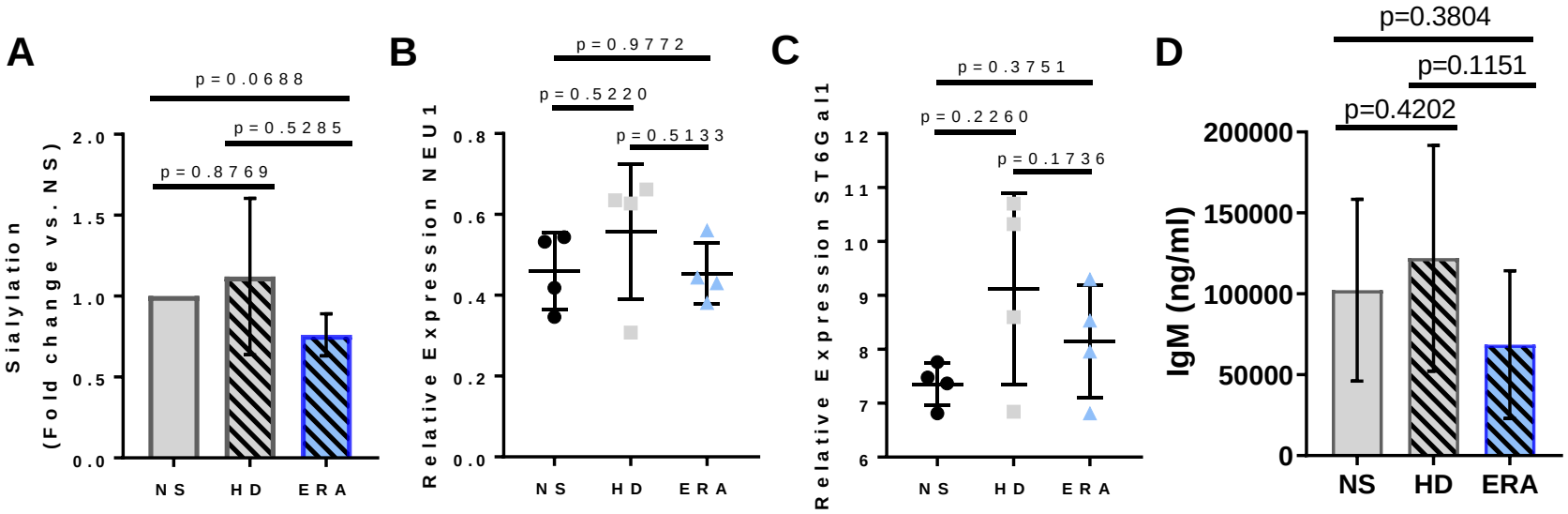


Figure 5.3 Changes to sialylation, expression of ST6Gal1 and NEU1 and production of IgM in plasmablasts after exposure to serum from patients with RA in B-cells from healthy donors

B-cells isolated from healthy donors (n=4) were differentiated to plasma cells *in vitro*. Between day 0 and 3, cells were stimulated as per protocol with CD40L L-cells + 4µg/ml $F(ab')_2$ in media with 10% FBS (NS) or 5% FBS + 5% serum from a healthy donor (HD) or a patient with ERA. Plasmablasts at day 6 were analysed and expression of SA was measured by flow cytometry **(A)**, expression of NEU1 **(B)** and ST6Gal1 **(C)** mRNA were measured by RT-qPCR and IgM in cell culture supernatants was measured by ELISA **(D)**. Graphs show mean with SD. ANOVA with Tukey's multiple comparisons tests were used to generate p values, with statistically significant differences indicated where $p<0.05$.

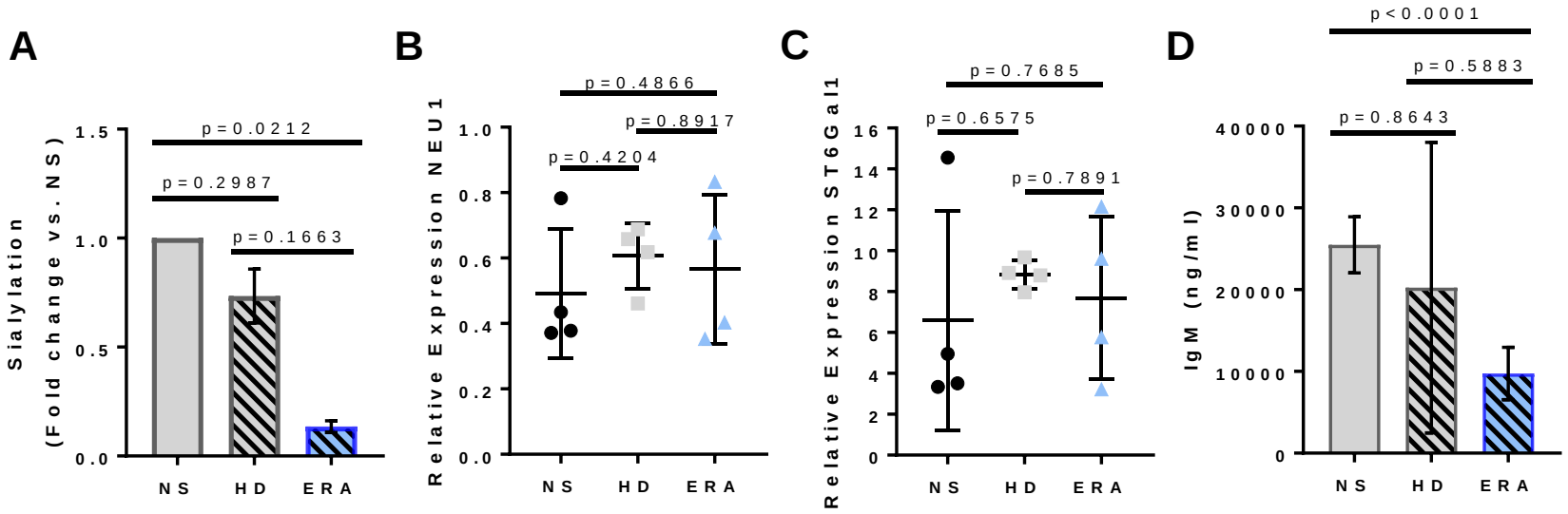


Figure 5.4 Changes to sialylation, expression of ST6Gal1 and NEU1 and production of IgM in plasma cells after exposure to serum from patients with RA in B-cells from healthy donors

B-cells isolated from healthy donors (n=4) were differentiated to plasma cells *in vitro*. Between day 0 and 3, cells were stimulated as per protocol with CD40L L-cells + 4µg/ml $F(ab')_2$ in media with 10% FBS (NS) or 5% FBS + 5% serum from a healthy donor (HD) or a patient with ERA. Plasma cells at day 13 were analysed and expression of SA was measured by flow cytometry **(A)**, expression of NEU1 **(B)** and ST6Gal1 **(C)** mRNA were measured by RT-qPCR and IgM in cell culture supernatants was measured by ELISA **(D)**. Graphs show mean with SD. ANOVA with Tukey's multiple comparisons tests were used to generate p values, with statistically significant differences indicated where $p<0.05$.

5.2.4.1 Exposure to serum from both healthy donors and patients with ERA leads to a reduction in B-cell sialylation in activated B-cells, and an overall reduction in activation

B-cells from a healthy donor were stimulated for 48 hrs ± 10 µl serum (5% in 200 µl RPMI) from healthy donors (n=2) or patients with ERA (n=2) and activated with either CpG, $F(ab')_2$ or $F(ab')_2$ + CD40L. Sialylation was measured after 48 hrs, and as shown previously, exposure to both sera from healthy donors and patients with RA led to a reduction in sialylation (Figure 5.5A). Interestingly however, in B-cells exposed to serum from patients with ERA, sialylation was lower than in cells exposed to serum from healthy donors in all stimulated and unstimulated conditions, particularly in CpG stimulated cells (0.750 ± 0.136 vs. 0.962 ± 0.204 p=0.5499) and $F(ab')_2$ + CD40L (0.3478 ± 0.010 vs. 0.610 ± 0.079, p=0.0771) compared with cells exposed to serum from healthy donors.

The impact of exposure to serum from healthy donors and patients with RA on B-cell activation was also studied, by measuring the percentage of $CD69^+$ cells after 48 hrs. It was found that the percentage of $CD69^+$ cells was markedly lower in cells exposed to both types of sera, and stimulated with $F(ab')_2$ (HC: 14.57 ± 1.46, p=0.0009; RA: 14.95 ± 1.46, p=0.0010 vs. Control: 68.99) and $F(ab')_2$ + CD40L (HC: 46.91 ± 5.83, p=0.0830; RA: 40.28 ± 7.69, p=0.0613 vs. Control: 84.43) (Figure 5.5B). However, in cells exposed to serum from healthy donors, the percentage of $CD69^+$cells following CpG stimulation was similar to the no serum control (68.52 ± 10.39 vs. 68.85, p=0.9992). However, percentage $CD69^+$ cells was slightly lower in cells exposed to serum from patients with RA than from healthy donors (55.23 ± 0.59 vs. 68.85, p=0.4436). Overall, these results suggest that exposure to serum in general leads to reduced sialylation in B-cells and a dampened response to stimuli with $F(ab')_2$. Exposure to serum from patients with ERA in particular led to a greater reduction in sialylation, potentially contributing to the greater inhibition of response to activating stimuli.

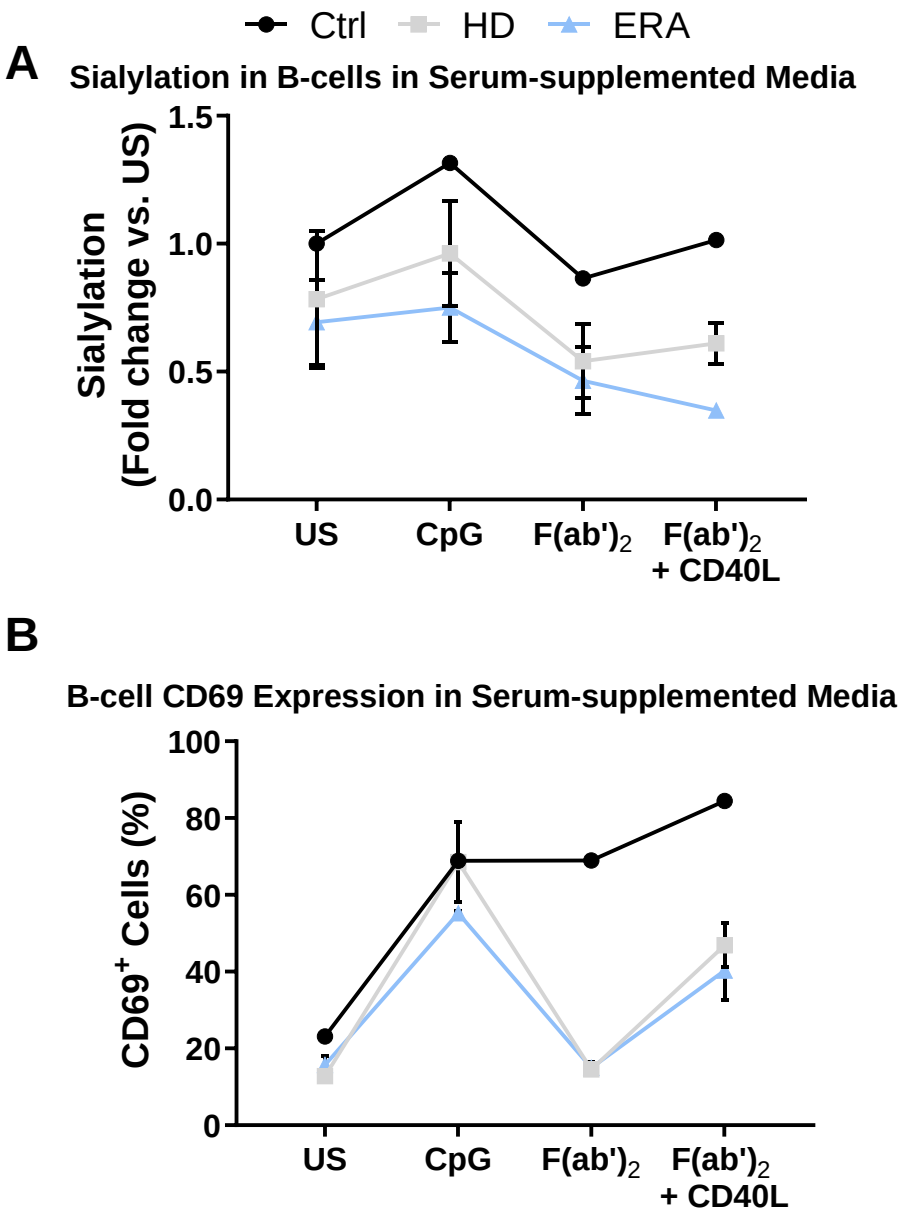


Figure 5.5 Impact of serum from patients with RA on sialylation in activated B-cells from healthy donors

B-cells from a healthy donor were cultured with 5% serum from healthy donors (HD) (n=2) and patients with early RA (ERA) (n=2) in RPMI (2.5, 5 or 10%). Cells were stimulated with CpG, $F(ab')_2$, $F(ab')_2$ + CD40L or left unstimulated (US). After 48 hrs, sialylation and % $CD69^+$ were measured by flow cytometry. Graphs show mean with SD of B-cell SNA FITC MFI **(A)**, expressed as a ratio relative to SNA FITC MFI of samples cultured in 0% serum or % $CD69^+$ cells in each condition **(B)**. ANOVA with Tukey's multiple comparison tests were used to analyse statistical significance (p values quoted in text where relevant).

5.2.5 Impact of reduced B-cell sialylation on B-cell activation *in vitro*

As described previously, (Section 3.2.2) expression of SA on the B-cell surface is reduced in patients with ERA and PRA compared to B-cells from healthy donors. It was also shown that Neu was present in the serum of healthy donors and of patients with ERA – with higher expression in samples from patients with ERA (Figure 5.2B). Furthermore, exposure to serum in culture led to a decrease in expression of SA on the cell surface (Figure 5.1) and a decrease in cell activation (Figure 5.5B). To further understand the potential consequences of reduced B-cell sialylation in B-cells in RA, the direct effect of reducing surface SA expression on B-cell function was investigated. B-cells were treated with Neu to digest SA from the cell surface, producing low SA expressing cells *in vitro* - which were used for further analyses.

5.2.5.1 Incubation of B-cells with neuraminidase leads to a reduction in surface sialic acids measured by flow cytometry

To first optimise Neu digestion of B-cell surface SA, B-cells were treated with 100 mU Neu in RMPI at 37°C for 1 – 18 hrs. Sialylation was measured after 1, 2, 4, 6 and 18 hrs by flow cytometry. It was found that even after one hour, sialylation was reduced compared to untreated cells and continued to decrease up to 4 hrs. There was no difference in sialylation between 4 and 6 hrs, however by 18 hrs sialylation was again reduced compared to 4 hrs (Figure 5.6A).

To determine if higher concentrations of Neu would achieve better digestion of SA in shorter periods of time, cells were treated with 100 or 200 mU of Neu for 4 hrs then stained with fSNA and FITC MFI recorded. Results show that there was no difference in sialylation after 4 hrs of treatment with 100 or 200 mU Neu (Figure 5.6B). Therefore, for future experiments, 100 mU of Neu were used for digestion of SA.

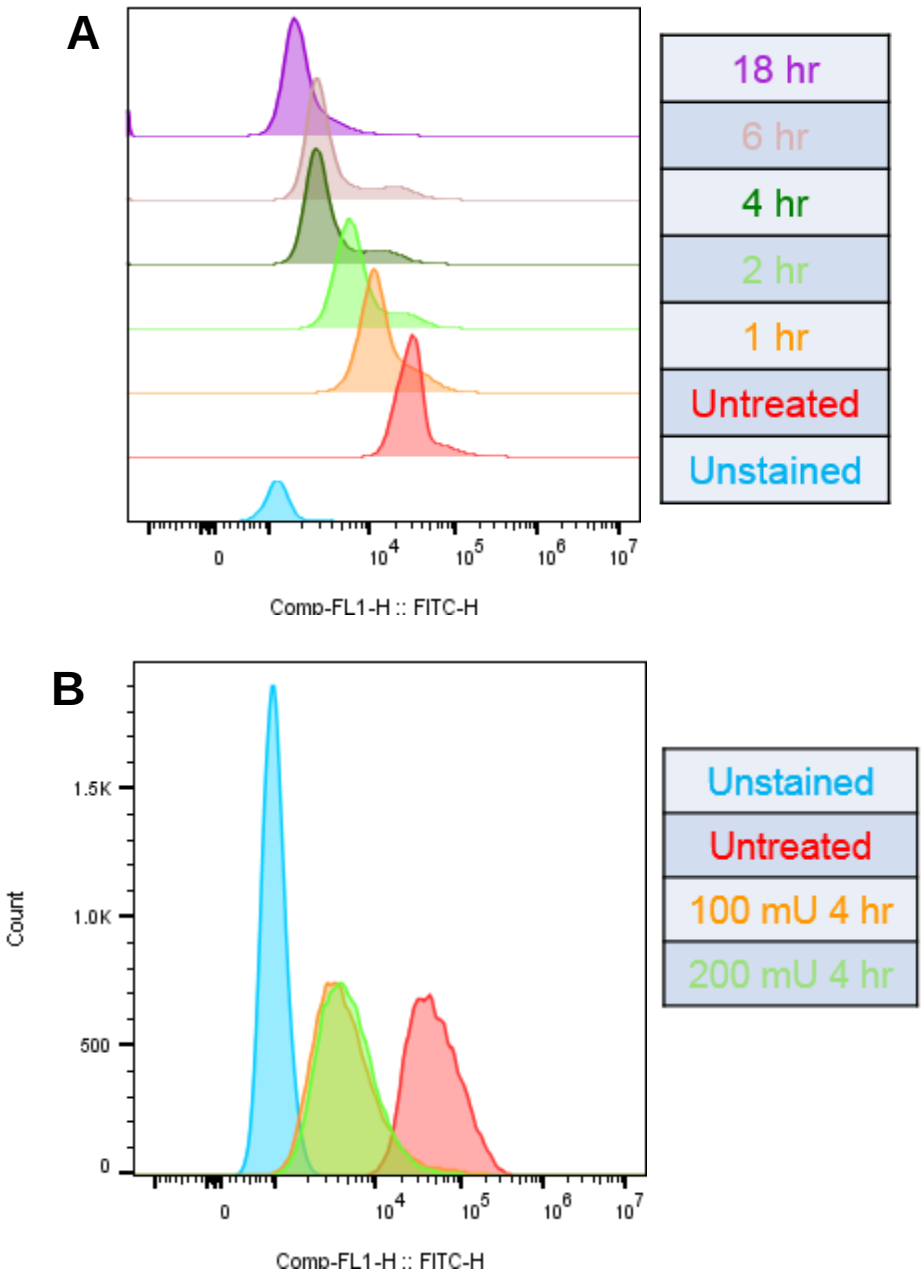


Figure 5.6 Optimisation of neuraminidase mediated B-cell surface sialic acid digestion

B-cells from a healthy donor were treated with 100 mU Neu for 1-18 hrs or left untreated. Sialylation was measured by flow cytometry at 1, 2, 4, 6 and 18hrs and compared to untreated and unstained samples **(A)**. Cells were treated with 100 mU or 200 mU Neu for 4 hrs and sialylation measured then compared to untreated and unstained samples **(B)**. Graphs show histogram overlays of sialylation in treatment conditions in one representative experiment.

5.2.5.2 Digestion of surface sialic acid by neuraminidase treatment leads to a decrease in B-cell activation potential

To directly test the consequences of reduced sialylation on B-cells, cells from healthy donors (n=5) were first treated with 100 mU Neu for 4hrs then stimulated with CpG or $F(ab')_2$ + CD40L. Activation was measured after 24 hrs of stimulation. Expression of three surface markers of B-cell activation – CD69, CD80 and CD86 were measured by flow cytometry. Activation marker expression is recorded as a ratio of MFI for each antibody relative to the unstimulated, untreated sample in each experiment. It was found that expression of all three markers of activation was decreased in cells treated with Neu, in cells stimulated with CpG and in cells stimulated with $F(ab')_2$ + CD40L (Figure 5.7). These results suggest that expression of surface SA may be important for adequate cell activation via both of these signalling pathways, and reduced surface sialylation may inhibit signalling.

5.2.6 Influence of expression of sialic acid expression on production of IgM

Since previous findings showed that treatment of B-cells with neuraminidase led to a decrease in B-cell activation potential (Figure 5.7) it was hypothesised that surface SA expression may also have an impact on further B-cell functions. A key function of activated B-cells is the production of antibodies, therefore the potential relationship between sialylation and antibody production was investigated.

5.2.6.1 Expression of sialic acid on the cell surface did not correlate significantly with production of IgM

In order to study the impact of sialylation on production of IgM, these factors were measured by flow cytometry and ELISA respectively. Measurements were taken at day 6 in plasmablasts and in day 13 plasma cells differentiated from healthy donors (n=6). Cell culture supernatant IgM concentration was plotted with cell sialylation measured at each time-point, and the correlation between the two variables was analysed. It was found that at day 6, there was no significant correlation between sialylation and IgM concentration (r -0.053, p= 0.787) (Figure 5.8A). However, at day 13 there was found to be a modest positive correlation between sialylation and production of IgM (r 0.431, p=0.036) (Figure 5.8B). This

suggested that surface sialylation may be associated with production of IgM in plasma cells, but not in plasmablasts.

5.2.6.2 Treatment of B-cells with neuraminidase prior to differentiation led to increased production of IgM in plasmablasts and plasma cells

To further study the impact of sialylation on production of IgM, cells were treated with Neu at various points during the *in vitro* differentiation protocol. Cells were treated for 1 hr with 100 mU Neu at either day 0 prior to differentiation, day 3 following initial activation, or day 6 at the plasmablast stage. Production of IgM was then measured at day 6 and day 13 in treated and untreated cells. It was found that at day 6 in plasmablasts, production of IgM was higher in cells which were treated with Neu at day 0 (40632 ± 17165 vs. 32068 ± 3926, p=0.8565 – IgM concentration ng/ml), yet unchanged in those treated at day 3 (30493 ± 2168 vs. 32068 ± 3926, p=0.9336) (Figure 5.9A). Similarly, at day 13, the cells treated with Neu at day 0 show increased production of IgM compared to untreated cells, and cells treated at day 3 and day 6 (29931 ± 15683 vs. 12754 ± 5034, p=0.6616, 12617 ± 4040, p=0.9923 and 8590 ± 7549, p=0.3964 respectively) (Figure 5.9B).

Treatment of cells with Neu at day 3 appeared to have very little effect on IgM production at day 13, whereas there was a slight reduction in IgM production at day 13 in cells treated with Neu at day 6. Taken together, these results suggested that cells treated with Neu, or cells that have reduced sialylation at day 0 prior to differentiation may go on to produce higher levels of IgM even at the plasma cell stage. It may also be an indication that pre-treatment of cells with Neu may impede the differentiation process, leading to increased survival of IgM producing cells and less class-switched cells in these cultures.

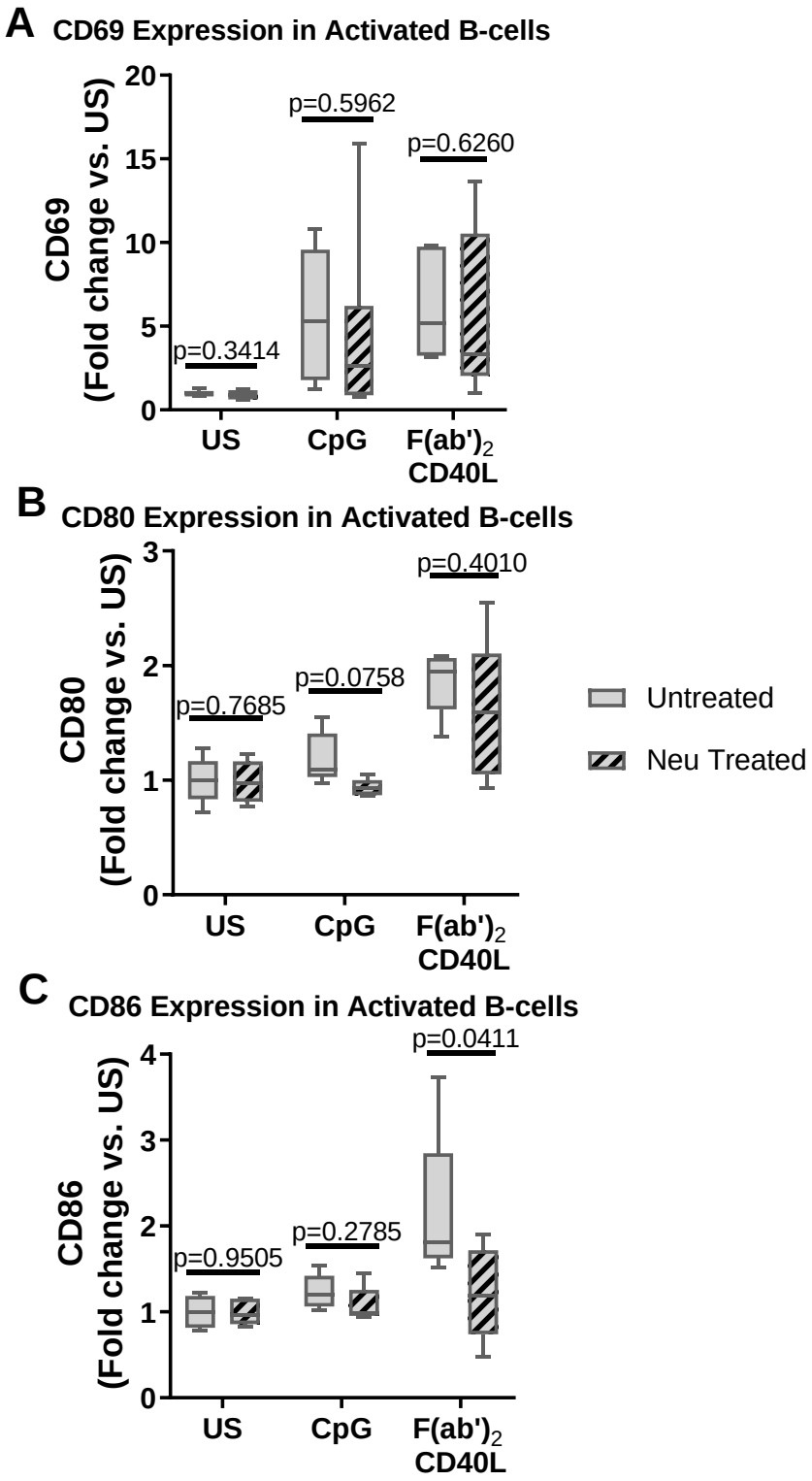


Figure 5.7 Impact of reduced sialylation on B-cell activation

B-cells from healthy donors (n=5) were treated with 10 mU Neu for 4hrs or left untreated. Cells were then stimulated with CpG, F(ab')$_2$, F(ab')$_2$ + CD40L or left unstimulated (US) for 24 hrs, then expression of CD69 **(A)**, CD80 **(B)** and CD86 **(C)** were measured by flow cytometry. Graphs show min to max expression of each surface marker, expressed as fold change relative to unstimulated (US) samples. Paired t tests were used to generate p values, comparing untreated and treated samples in each condition. Statistically significant differences are indicated where $p<0.05$.

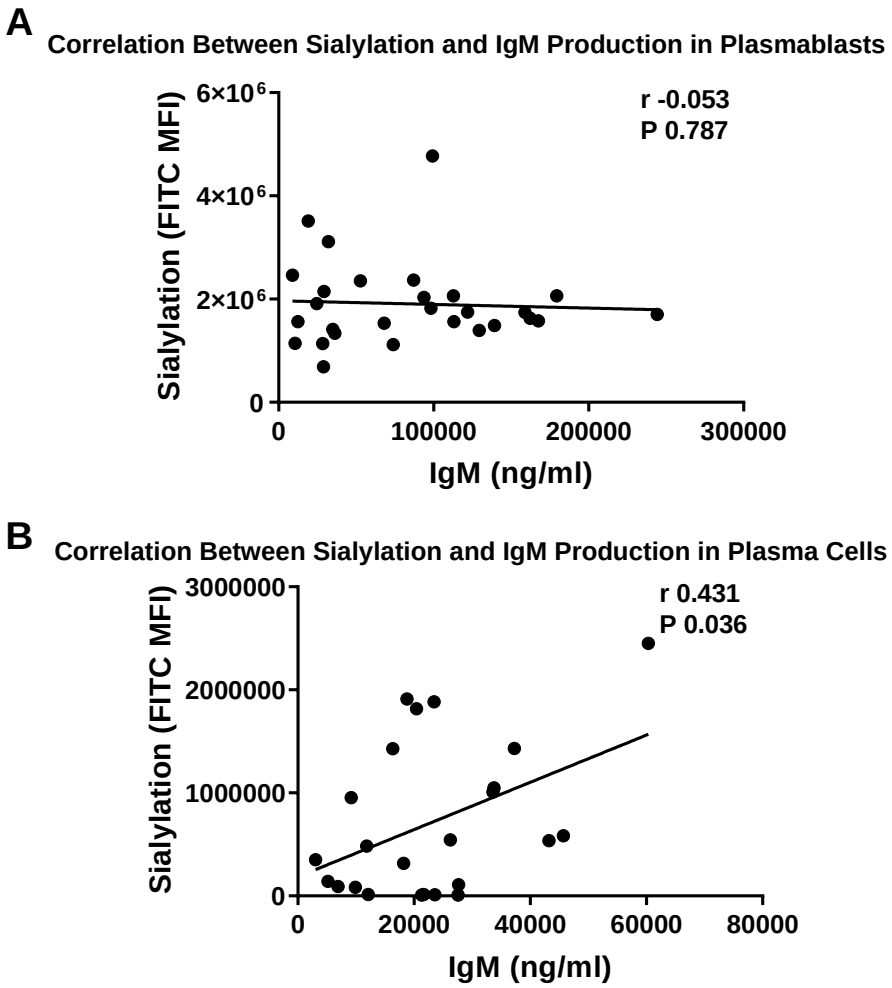


Figure 5.8 Correlation of expression of sialic acid and the production of IgM during *in vitro* B-cell differentiation

B-cells isolated from healthy donors (n=6) were differentiated to plasma cells *in vitro*. At day 6 **(A)** and day 13 **(B)** cell surface expression of SA was measured by flow cytometry and IgM in cell culture supernatants was measured by ELISA (data points include cells cultured under various conditions including TLR stimulation, exposure to TNF, IL-6 and serum). Graphs show line of best fit, and r and p values were calculated using Pearson correlation.

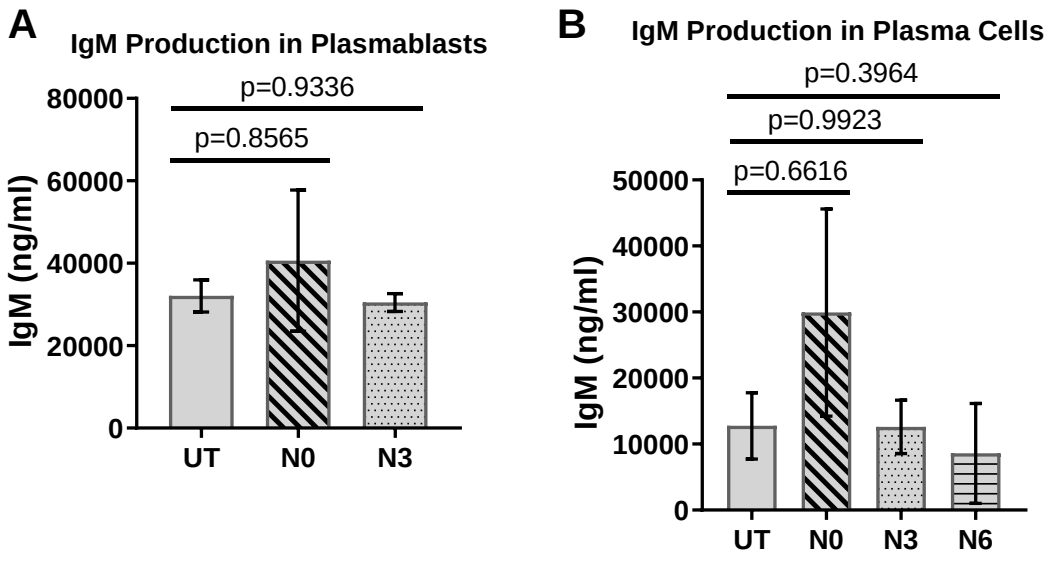


Figure 5.9 Impact of neuraminidase treatment on the production of IgM during *in vitro* B-cell differentiation

B-cells isolated from healthy donors (n=2) were differentiated to plasma cells *in vitro*. At day 0 (N0), day 3 (N3) or day 6 (N6), cells were treated with 100 mU Neu for 1 hr, or left untreated (UT). At day 6 **(A)** and Day 13 **(B)** IgM in cell culture supernatants were measured by ELISA. Graphs show mean with SD. ANOVA with Tukey's multiple comparisons tests were used to generate p values.

5.2.7 Impact of reduced sialylation on B-cell susceptibility to rituximab-induced depletion *in vitro*

As well as the potential impact of reduced B-cell expression of SA on B-cell activation potential, and potential contribution to promoting autoimmunity which has been discussed in this chapter, it was also hypothesised that B-cell surface SA expression may have an impact on response to biologic therapies. In particular, treatment with B-cell depletion therapy. A number of studies in cancer cell lines have shown that an increase in sialylation leads to increased resistance of cells to chemotherapeutic agents[197,198,203,287]. Therefore it was hypothesised that reduced sialylation in B-cells may lead to increased susceptibility to rituximab treatment. Since SAs can inhibit binding interactions due to their negative charge, it was hypothesised that reduced expression of SA would increase binding of rituximab to the B-cell surface, and therefore increase the efficiency of B-cell depletion.

5.2.7.1 Reduced sialylation leads to increased binding of a CD20 antibody

In order to determine if reduced expression of SA could increase the efficiency of rituximab binding to the B-cell surface, B-cells from healthy donors (n=3) were first treated with Neu for 4 hrs. Cells were then stained with an antibody for CD20 – CD20 eFlour 450, as a substitute for rituximab, which would allow the extent of binding to be measured by flow cytometry. It was found that the MFI of eFlour 450 was increased in B-cells treated with Neu compared to untreated cells (48671 ± 10448 vs. 28258 ± 3827, p=0.0497 MFI units) (Figure 5.10). This result suggested that binding of anti-CD20 Ab is increased in cells with lower expression of SA, potentially by “unmasking” antibody binding sites.

5.2.7.2 Reduced sialylation leads to increased susceptibility of B-cells to rituximab-induced death *in vitro*

Since reduced expression of SA led to an increase in the binding of a CD20 antibody (Figure 5.10), it was hypothesised that this may lead to an increase in B-cell depletion by rituximab. In order to study this *in vitro*, B-cell viability after treatment with rituximab was measured, (method based on a previously described assay[262]). Since it is thought that rituximab works by opsonising B-cells and inducing killing via the complement system and via clearance by phagocytosis[245], B-cells from healthy donors (n=6) were incubated with rituximab

for 4 hrs under one of three conditions: 1. B-cells in 50% healthy donor serum (in RPMI), 2. With 9:1 healthy donor PBMCs to B-cells 3. In 50% serum with 9:1 healthy donor PBMCs to B-cells (Figure 5.11A). The assay used healthy donor serum to deliver complement proteins and PBMCs to act as phagocytes. The assay was confirmed to be effective as B-cell viability reduced in cells treated with rituximab compared to untreated cells in conditions 1. and 3. As described previously (Figure 3.11B, 3.11D). The assay was less efficient in samples incubated with PBMCs alone (condition 2.), suggesting that serum, and therefore complement opsonisation was required for the most efficient response (Figure 5.11C).

To investigate the impact of reduced sialylation on the efficiency of the assay, B-cells were treated for 1 hr with Neu prior to incubation with rituximab. It was found that in samples treated with Neu, B-cell viability following treatment with rituximab was reduced in cells incubated with PBMCs (UT: 65.45 ± 29.53 vs. Neu: 54.45 ± 25.25, p=0.0541 - % viable B-cells) (Figure 5.12B) and PBMCs + serum (UT: 26.05 ± 23.15 vs. Neu: 14.29 ± 11.38, p=0.0590) (Figure 5.12C). Results were less clear in cells incubated with serum only, yet overall there was a slight decrease in cell viability in treated cells (UT: 9.58 ± 8.75 vs. Neu: 9.09 ± 9.37, p=0.8209) (Figure 5.12A). Further to this, PBMCs were pre-treated with Neu before being added to the assay with B-cells and rituximab with/without serum. Results showed that treatment of PBMCs with Neu led to decreased B-cell viability following treatment with rituximab (Figure 5.12D).These results suggested that B-cell depletion with rituximab may be more efficacious in cells with lower expression of SA, consistent with current research that suggests overexpression of SA is an important factor in drug resistance, particularly in tumour cells[197,198,203,287].

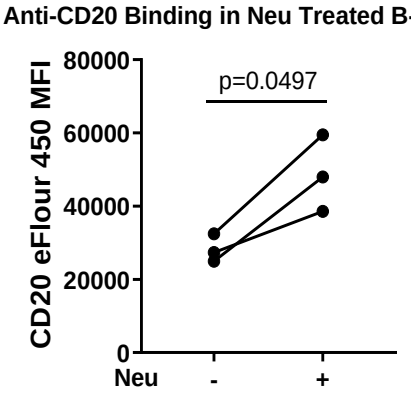


Figure 5.10 Impact of reduced B-cell sialylation on anti-CD20 Ab binding

B-cells from healthy donors (n=3) were treated with 10 mU neuraminidase (Neu) for 4hrs or left untreated. Cells were then stained with CD20 eFlour 450 antibody and binding assessed by flow cytometry. MFI of eFlour 450 was recorded for each sample. A paired t test was used to analyse statistical significance.

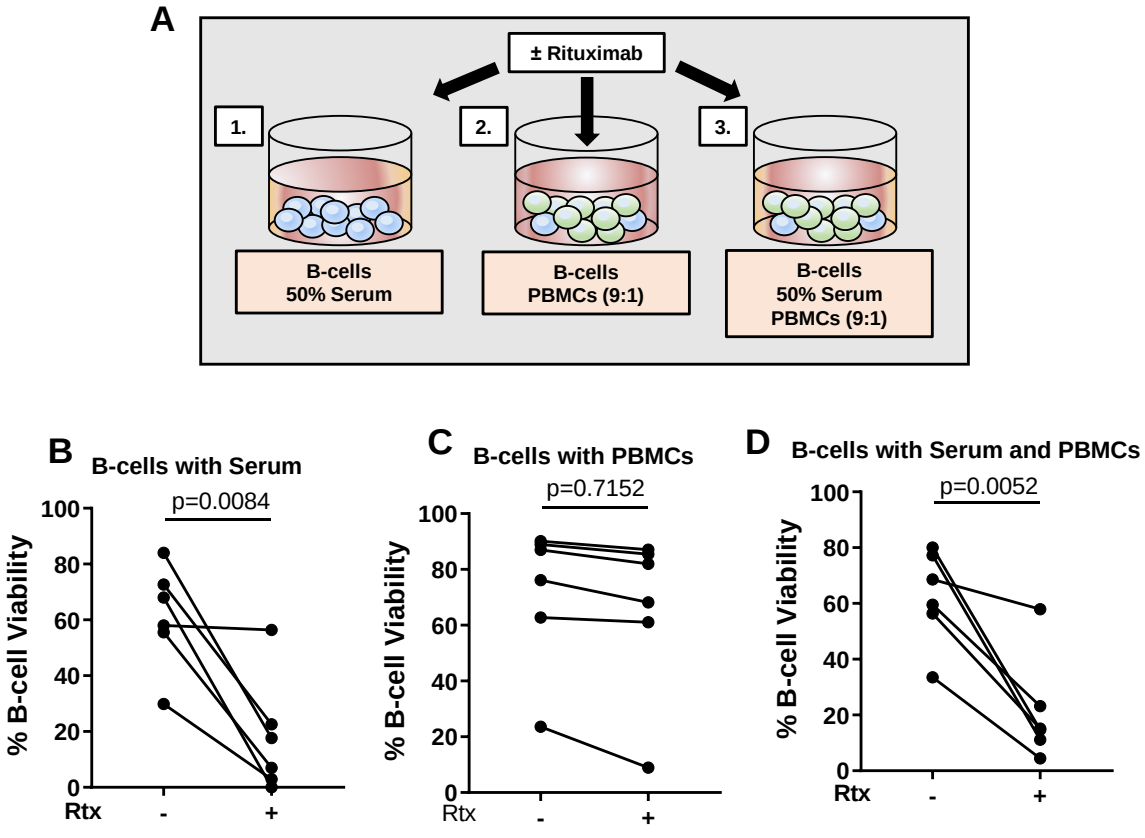


Figure 5.11 *In vitro* Rituximab B-cell killing assay

B-cells from healthy donors (n=6) were cultured ± rituximab (Rtx) for 4 hrs under one of 3 conditions **(A)**: 1. in 50% serum from healthy donors **(B)**; 2. with PBMCs from a healthy donor (9:1 PBMC:B-cell ratio) **(C)**; 3. with PBMCs from a healthy donor in 50% serum **(D)**. After 4 hrs cell viability was recorded by flow cytometry using a viability stain (FV 780). Graphs show % viable B-cells in Rtx treated and untreated samples. Paired t tests were used to generate p values, with statistically significant differences indicated where $p<0.05$.

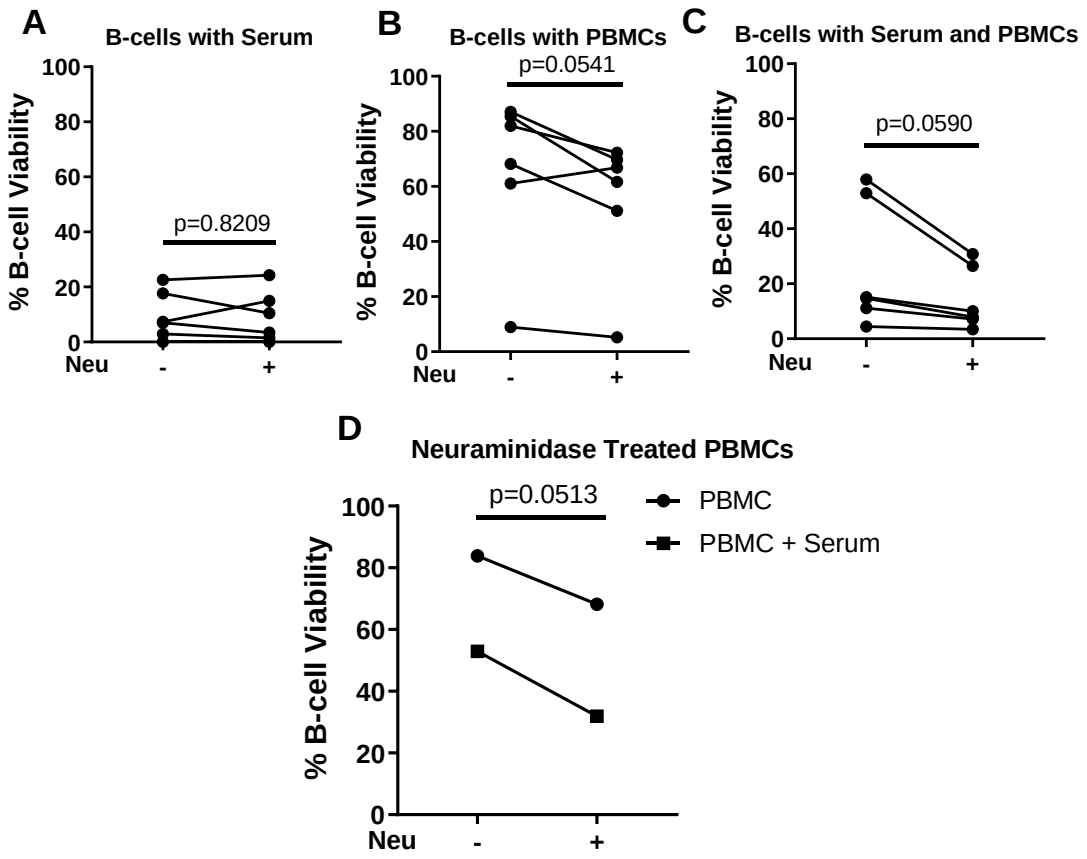


Figure 5.12 Impact of reduced sialylation on *in vitro* B-cell depletion by rituximab

B-cells from healthy donors (n=6) were cultured with rituximab (Rtx) for 4 hrs under one of 3 conditions, with or without prior treatment for 1 hr with 10 mU neuraminidase (Neu): 1. in 50% serum from healthy donors **(A)**; 2. with PBMCs from a healthy donor (9:1 PBMC:B-cell ratio) **(B)**; 3. with PBMCs from a healthy donor in 50% serum **(C)**. B-cells from a healthy donor were cultured with rituximab in 50% serum with PBMCs from the same healthy donor which were treated ± 10 mU Neu for 1 hr prior to culture **(D)**. After 4 hrs cell viability was recorded by flow cytometry using a viability stain (FV 780). Graphs show % viable B-cells in Neu treated and untreated samples. Paired t tests were used to generate p values, with statistically significant differences indicated where $p<0.05$.

5.3 Discussion

The previous results of this study showed that sialylation was reduced on the surface of naïve and memory B-cells as well as plasmablasts from the peripheral blood of patients with RA compared to healthy donors. B-cell intrinsic factors which may influence B-cell sialylation, as well as targeted cytokines involved in inflammation have been studied and it was found that there may be influence from both intrinsic and extrinsic factors on B-cell sialylation. It was hypothesised that the serum environment in RA may be a key player in determining surface sialylation, and the results of this chapter found that exposure to serum *in vitro* led to reduced sialylation in healthy cells. However, this phenomenon was observed when healthy cells were exposed to serum from healthy donors and from patients with RA, suggesting that factors – most likely neuraminidases - were present in both groups of sera which had the potential to influence sialylation. Serum neuraminidase activity was measured and was found to be increased in serum from patients with RA compared with serum from healthy donors – providing the first potential link to reduced cell surface sialylation in RA patients.

The main limitation in this data is the modest number of samples which were analysed. Study size was limited due to availability of patient samples, and these preliminary results would be strengthened by validation on a larger scale. It would also be pertinent to measure Neu activity in serum samples from patients with PRA, to determine if serum Neu activity is increased at the onset of autoimmunity, at the time when decreased B-cell surface sialylation can first be detected. It would also be interesting to investigate paired samples of peripheral blood B-cells and serum, to determine if serum Neu correlates with B-cell surface sialylation in patients with RA.

Although the focus of this current study was on B-cell sialylation, in light of these findings, it would also be interesting to compare sialylation of other immune cell types in RA to cells from healthy donors, to indicate whether increased neuraminidase activity could have wide-ranging pathological implications. As well as serum neuraminidases potentially reducing B-cell sialylation, there are several other factors present in serum which may lead to decreased sialylation, and other factors in RA serum which can reduce healthy donor B-cell activation potential *in*

vivo. These could include cytokines, antibodies, hormones, acute phase proteins etc. It would be helpful to profile RA serum for the presence of such factors, to identify any other possible candidates which may reduce serum sialylation. As well as Neu activity, it would also be useful to measure serum ST6Gal1 activity. This may be useful in the context of autoantibody sialylation, but less so in B-cell surface sialylation, since it has been previously found that soluble ST6Gal1 in serum could sialylate soluble proteins but not surface bound molecules[166].

It was also observed that when cells from healthy donors were exposed to serum from patients with RA, sialylation was upregulated less when cells were activated via TLR or BCR stimulation, than when cells were exposed to serum from healthy donors and in no-serum controls. Further to this, when cells were exposed to serum in general, they were far less responsive to BCR stimuli, and activation marker upregulation was impaired. It was hypothesised that desialylation induced by serum exposure may have an impact on B-cell activation potential. Cells were treated directly with Neu to investigate this and it was found that activation via both TLR9 and the BCR was reduced in Neu treated cells. It was then hypothesised that surface sialylation played a role in signalling via both pathways, most likely mediated by the Siglec CD22, which is known to regulate both BCR[180] and TLR signalling[290].

One limitation of this current data is that serum neuraminidases and exogenous Neu which was added to could target more than one SA linkage – therefore the consequences of reduced sialylation which were observed may be impacted by several different SA linkages. To combat this would be a difficult task, as no naturally occurring sialidases target only α2,6-linkages. However, one group have engineered a sialidase which targets α2,6-linkages specifically, which may have interesting potential for use in studying the impact of this type of linkage in isolation[291]. According to the results of this study, certain antibodies may be able to bind to cells more efficiently when SA is removed from the cell surface – therefore a potential confounding factor in these experiments may have been changes to antibody binding capabilities. However, in unstimulated samples there was no discernible increase in antibody binding, which helps to rule out this possibility. The three markers of B-cell activation which were selected, CD69, CD80 and CD86 were selected as expression can be easily detected by flow cytometry. However, it would also be useful to use other measures of B-cell

activation such as cytokine production, antibody production and proliferation, to further determine the impact of reduced sialylation on B-cell function.

As well as the impact on B-cell activation it was hypothesised that desialylation may also have an impact on B-cell differentiation, since it was previously shown that increased sialylation was an important first step in the differentiation process. It was found that Neu pre-treatment of cells prior to differentiation led to increased production of IgM in plasma cells, which may suggest that class switching was impaired in these cells, potentially due to an impaired response to the initial stimuli. In differentiation experiments, small n numbers made some of the data difficult to interpret, and introduced large error margins. It would be advantageous to repeat these experiments in a larger cohort. In further samples, B-cell activation should also be measured after initial activation phase at day 3, along with production of IgG to confirm if class switching is still able to occur in Neu treated cells.

Reduced B-cell activation in desialylated cells is an interesting concept with potential implications for treatment of B-cell disorders, potentially utilising neuraminidase to abrogate B-cell activation. However, it was also hypothesised that sialylation may have an impact on current therapeutic strategies – namely rituximab-induced B-cell depletion. B-cells were pre-treated with neuraminidase then subject to an *in vitro* rituximab killing assay. Pre-treated cells were more susceptible to depletion, suggesting that removing surface sialylation may allow better drug binding, increased complement binding or increased recognition by phagocytes.

The current data cannot tell us whether removal of SA from CD20 molecules on the cell surface is responsible for the increase in binding, or if it is a combined effect of overall reduced sialylation on the cell. First determining if CD20 is a direct target for ST6Gal1 would be a logical next step to investigate this further. It would also be interesting to compare the susceptibility of cells from patients with RA, which already express reduced SA with cells from healthy donors. One limitation of the current study was in the high variability of B-cell viability following rituximab depletion, likely due to donor variability. In an attempt to lessen this effect, the same experiments were conducted in a B-cell line, however the cell lines were particularly susceptible to rituximab treatment – achieving almost 100% cell death

under both conditions, making it difficult to discern any potential effects of changes to sialylation (results not shown). The findings of the *in vitro* rituximab assay experiments could be strengthened by replication on a larger scale, with the inclusion of patient samples to investigate intrinsic differences in susceptibility.

In summary, the data in this chapter has shown that the increased activity of neuraminidase in RA serum may contribute to reduced B-cell sialylation. It has also shown that as a consequence of reduced activation, cells may be less active, likely influenced by CD22 signalling. It was also observed that rituximab treatment may be more efficient in cells which have reduced sialylation. There are two avenues through which this observation may prove useful for further research – either in its potential use as a predictive factor for response, or as a potential therapeutic adjuvant to increase effectiveness of the drug. Use of neuraminidase as an adjuvant is not a novel concept and has been tested in breast cancer cell lines, and proven effective[292] in early studies. There are a number of situations in which this may prove to be of benefit, including RA, but also in chemoresistant cancers, which often express increased levels of SA.

Chapter 6
General Discussion

6.1 Summary of Principal Findings

Initially, the finding that sialylation, as detected by lectin flow cytometry, is reduced on the B-cell surface in patients with RA and asymptomatic PRA, was validated in the Leeds cohort. In addition, it was found that sialylation was also reduced in peripheral memory B-cells and naïve B-cells from patients with RA and PRA.

Next, mechanisms which may control sialylation in B-cells were investigated. It was found that when B-cells from healthy donors were activated via stimulation with TLR ligands, surface sialylation increased. Surface sialylation also increased when cells were stimulated by BCR crosslinking, by CD40L co-stimulation and by both in combination, though to a lesser extent than stimulation with TLR ligands. This effect did not appear to be solely due to B-cell activation status, since dual stimulation with CD40L and BCR stimuli produced the most robust response in terms of activation – measured by expression of surface activation markers - yet TLR ligands led to the greatest increase in sialylation. It was also found that B-cell sialylation was increased in B-cells from healthy donors by activated T-cells, which was, at least in part, driven by CD40/CD40L signalling. However, when the same stimuli were given to B-cells from patients with ERA and PRA it was found that sialylation was not increased in response to TLR ligands. In response to BCR crosslinking and CD40L co-stimulation there was an increase in sialylation in cells from patients with PRA, but not ERA – suggesting that the response to TLR ligands (in terms of regulation of sialylation) was impaired from the onset of autoimmunity, whereas the response to BCR activation/co-stimulation may be affected by the transition to active disease.

To further understand the influence of activation status on B-cell sialylation, the impact of stimuli on regulation of expression of two enzymes involved in α2,6-sialylation was investigated. Expression of ST6Gal1, the Golgi enzyme which adds SA to protein chains and NEU1, the enzyme which cleaves SA from glycoprotein chains were measured by RT-qPCR following B-cell activation with

TLR or BCR stimuli. It was found that activation via either signalling pathway tended to lead to a decrease in expression of both enzymes, which suggested that the downregulation of NEU1 may play a role in increased surface sialylation following activation, however the relationship between surface sialylation and expression of ST6Gal1 mRNA appears to be more nuanced.

To determine if the conditions surrounding activation could impact the surface sialylation of terminally differentiated cells, B-cells were differentiated to plasma cells *in vitro*. It was found that during differentiation sialylation increased when cells were first activated, then decreased when cells differentiated to plasmablasts and plasma cells. When cells were stimulated with TLR ligands instead of BCR stimuli prior to differentiation there was an increase in sialylation of plasma cells, and an increased production of IgM.

As well as factors which directly activate B-cells, the effect of cytokines - which are present in the blood and synovium during active RA - on B-cell sialylation was investigated. The impact of *in vitro* exposure to IL-4, TNF, IL-6 and IL-17 was tested and it was found that IL-4 led to an increase in sialylation in cells from healthy donors and patients with PRA, but not patients with ERA. Exposure to TNF tended to decrease sialylation in cells from healthy donors and, in contrast, IL-6 led to an increase in sialylation at low doses. However, this differed in cells from patients with ERA as TNF had little effect on sialylation, and only the highest tested dose of IL-6 led to an increase in sialylation.

The impact of cytokine exposure on differentiating B-cells was also investigated, and it was found that when cells were exposed to TNF during activation prior to differentiation, there was no change in sialylation of plasmablasts or plasma cells, compared with TNF-naïve cells. Exposure to IL-6, however, did result in a slight increase in sialylation of plasmablasts and plasma cells.

To determine the combined effect of factors in the extracellular environment in RA on B-cell sialylation, serum from patients with ERA was added to cultures of B-cells from healthy donors and the impact on sialylation was measured. Surprisingly, exposure to serum from both healthy donors and patients with ERA led to a decrease in sialylation in these cells. A neuraminidase activity assay was carried out, which detected neuraminidase activity in both healthy donor and ERA

samples, and also showed increased activity in the samples from patients with ERA. This identified a possible contributing factor to the decreased sialylation in B-cells in RA.

The impact of exposure to serum from patients with ERA on healthy donor B-cells prior to differentiation was also investigated, and it was found that exposure to serum from patients with RA led to decreased plasma cell sialylation, and reduced production of IgM compared with exposure to serum from healthy donors and no-serum controls.

In addition to reduced sialylation following exposure to serum *in vitro*, when the serum-exposed cells were stimulated with TLR ligands and BCR stimuli, activation was found to be dampened compared to no-serum controls. This response was particularly clear in cells exposed to serum from patients with ERA. It was hypothesised that this may be due to the reduction in cell surface SA, therefore the direct impact of reduced sialylation on activation was investigated by treating B-cells from healthy donors with neuraminidase before stimulating with TLR ligands or BCR stimuli. It was found that treatment with neuraminidase led to reduced activation in response to both types of stimuli, suggesting surface sialylation plays a part in transmission of signals in both pathways of activation. Production of IgM was also found to be loosely associated with surface sialylation in plasma cells. When B-cells were treated with neuraminidase prior to differentiation there was an increase in production of IgM compared to untreated controls, suggesting that these cells may not have undergone class switching during differentiation.

Finally, the potential impact of sialylation on response to B-cell depletion therapy was investigated. It was found that when cells were treated with neuraminidase, there was increased binding of an anti-CD20 antibody used as a surrogate for rituximab. Treatment with neuraminidase also improved the efficacy of B-cell killing in an *in vitro* rituximab killing assay, suggesting that reducing SA expression may increase the availability of binding sites for rituximab, and may increase B-cell susceptibility to death via complement lysis or via phagocytosis.

6.2 Discussion

There is a well-established link between autoimmunity and reduced IgG Fc sialylation. However, evidence has emerged that there are other areas of pathology in which sialylation may be influential. Recently it has been described that Fab sialylation is increased in ACPA antibodies in RA, and that decreased sialylation can be detected on the B-cell surface in RA and PRA. Despite this well-established association between sialylation and autoimmunity, there remains a number of unknowns as to how sialylation is regulated, and as to the diverse roles it may play in regulating autoimmunity. The aim of this research was to further investigate the changes to B-cell surface sialylation in RA, and to investigate the potential consequences of reduced sialylation for B-cell function. This research has found that there are several factors which may contribute to sialylation changes in autoimmunity, and that there are diverse consequences for B-cells depending on the cell subtype and extracellular environment.

6.2.1 B-cell sialylation is reduced in B-cells in patients with Rheumatoid Arthritis

Reduced IgG-Fc sialylation has been recognised as a feature of RA for more than three decades[227]. Only in the last few years has evidence emerged that increased IgG-Fab sialylation is also a feature of RA[230]. Changes to Fc sialylation are also known to be present in patients with asymptomatic autoimmunity[236] and have been associated with progression to symptomatic inflammation[235,236]. The use of SNA lectin staining as a surrogate for measuring sialyltransferase activity in plasmablasts has been described previously[235] along with the findings that SNA lectin staining was reduced in patients with RA and PRA compared with healthy donors[235]. The results of this current work have validated these findings, showing reduced plasmablast sialylation in cells from patients with PRA and ERA, however it was also found that sialylation is reduced in memory and naïve B-cells from the same patients, compared to the healthy donor cohort.

These results could imply that there is overall reduced sialyltransferase activity in B-cells from patients with RA and PRA. This reduction in activity may be reflected in the production of antibodies with reduced Fc sialylation and increased inflammatory activity. However, there are also some caveats to this interpretation. The relationship between B-cell SNA lectin staining and sialyltransferase activity

was previously validated in ST6Gal1 knockout mice[235], in which staining was absent. Although confirming ST6Gal1 activity is required for staining, it does not directly describe the relationship between ST6Gal1 activity and surface expression of SA. Furthermore, it would be anticipated that ST6Gal1 activity would be increased to produce sialylated Fab glycans, which is not reflected in peripheral B-cells from patients with RA if we assume surface sialylation is an indication of ST6Gal1 activity.

Neuraminidase enzymes further complicate the picture, as they are able to cleave sialic acids from glycoprotein chains. NEU1 can be expressed within the plasma membrane or within intracellular compartments such as lysosomes[293] and its activity in serum can be accurately measured by fluorescent assay[294]. Therefore it cannot be ruled out that NEU1 may have an influence on overall surface sialylation. It may be more accurate therefore, to consider SNA lectin binding more holistically as a dynamic interaction of overall activity of sialyltransferases and sialidases within the cell and in the extracellular environment.

Regardless, the observation that overall B-cell sialylation is reduced in patients with RA and PRA is an interesting one, and begs the question of whether sialyltransferase activity is lower, or perhaps sialidase activity is intrinsically higher in these patients. A further possibility is the reduction of expression of a particular sialylated protein or a number of proteins found on the surface of cells from patients with RA, a line of investigation which warrants further investigation. It also possible that these changes to B-cell surface sialylation may be unrelated to antibody sialylation, and may be a feature of B-cell autoimmunity in itself. The implications of this will be discussed later in this chapter.

6.2.2 B-cell sialylation increases response to B-cell activation via TLR and BCR signalling

A previous study had shown that stimulation of murine splenic B-cells with anti-IgM led to a decrease in surface expression of SA, but found that stimulation with anti-CD40L had no impact[211]. Since this study in 1999, there have been no further studies to support/challenge these findings, and no further data to suggest how sialylation may be controlled in B-cells. The results of this current study have shown that B-cell sialylation is generally increased when B-cells are activated. This response is particularly pronounced in cells stimulated with TLR ligand CpG

compared with cells stimulated by BCR cross-linking plus CD40L co-stimulation, in which the upregulation of sialylation still occurs but is lesser. It was also found that activated T-cells could induce a level of upregulation of B-cell sialylation which was higher than induced by free CD40L but lower than CpG.

Taking the results of this study into account, it would be tempting to speculate that, in general, B-cell activation would lead to upregulated expression of surface sialic acid. Increased surface expression may occur as a consequence of upregulated sialyltransferase activity or by upregulated expression of particular sialylated surface proteins. However, this hypothesis does not account for the differences observed in cells stimulated with TLR ligands compared with those stimulated via BCR ligation. Furthermore, surface sialylation did not correlate with expression of CD69, the selected measure of B-cell activation. This therefore suggests that the activation of distinct signalling pathways may determine surface sialylation following stimulation.

ST6Gal1 has been shown to be associated with increased survival in cancer cells and overexpression is associated with invasiveness, metastasis and chemoresistance[197,203]. Taking this into account, it could be hypothesised that the increase in sialylation induced by TLR9 stimulation by CpG could be a mechanism to promote B-cell survival. Interestingly, a previous study found that when cells were stimulated with anti-IgM alone, it induced mitochondrial dysfunction and reduced cell viability compared with cells stimulated with CpG or CpG plus anti-IgM[295]. Therefore, differences in regulation of sialylation could be attributed to differences in metabolic shifts induced by TLR or BCR signalling, with increased sialylation potentially contributing to preserved B-cell viability in CpG-stimulated cells. Another study has found that BCR recognition of antigens which also contain TLR9 ligands leads to an initially strong proliferation response, followed by a period of cell cycle arrest and apoptosis[296], which could support the hypothesis that this early upregulation of sialylation is a B-cell survival mechanism and also suggests it may be associated with proliferation.

TD antigens, which require a second signal from T-cells in order to fully activate B-cells, induce a period of proliferation and antibody production in short-lived ASCs, as well as triggering germinal centre formation and the generation of long-lived plasma and memory cells. It may be hypothesised that the dampened

increase in sialylation in anti-IgM/G and CD40L stimulated cells, could promote germinal centre formation. However, sialylation has been found to be required for GC formation in ST6Gal1 knockout mice studies[185]. Taken together these results suggest that ST6Gal1 and α2,6- sialylation are important for efficient B-cell function, and α2,6- sialylation is tightly regulated in activated cells to promote effector function dependent on the type of stimulus received.

6.2.3 B-cells from patients with RA are unresponsive to certain stimuli

Despite previous studies showing that cells from patients with ERA have lower B-cell surface sialylation[235], no studies have explored sialylation in activated cells versus “resting” cells in patients with RA and healthy donors. In this study it was found that B-cells from patients with RA did not upregulate sialylation in response to stimulation with TLR ligands, neither did cells from patients with PRA. This suggested an intrinsic mechanism related to autoimmunity which inhibits the TLR response.

The differences in response to $F(ab')_2$ and CD40L between cells from patients with RA/PRA and healthy donors is less clear cut. Cells from patients with PRA showed a comparable upregulation of sialylation to healthy donor cells, however there was generally very little alteration in sialylation in cells from patients with RA. This suggested that increased sialylation in response to BCR ligation and co-stimulation may be preserved in PRA but lost as disease progresses to symptomatic inflammation – suggesting this may be more of a feature of chronic inflammation rather than autoimmunity. Loss of response to BCR ligation and co-stimulation may also contribute to the development of joint inflammation during the pre-RA stage.

Studies have suggested that markers of activation are increased in peripheral blood B-cells in RA, suggesting these cells have a more “active” phenotype at baseline[297]. It could be hypothesised that these cells may require a higher dose of stimuli to overcome an increased threshold for activation, potentially accounting for some of the observed differences in response to different stimuli between cells from healthy donors and patients from RA. The results of this study showed that expression of CD69 was increased to a similar degree in HD and ERA B-cells in response to stimulation with $F(ab')_2$ and CD40L, however, it was

slightly less in cells from patients with ERA than in healthy cells in response to TLR ligands.

In systemic lupus erythematosus (SLE), it has been found that there is a defective response to TLR9 ligand CpG, characterised by reduced upregulation of activation markers and impaired cytokine production[298]. This is particularly significant in SLE, as autoreactive B-cells mount a response to double stranded DNAs (dsDNAs)[299], which are ligands for TLR9. It is also thought that TLR9 activation is a peripheral checkpoint to prevent autoimmunity[296], and the lack of response to TLR9 in SLE may lead to the development and persistence of anti-dsDNA antibody producing B-cells[298]. Although SLE and RA share certain similarities, this lack of response to CpG has not yet been reported in RA B-cells. The findings of the current study showed that expression of CD69 following activation with CpG was impaired in cells from patients with ERA and this was even more pronounced in cells from patients with PRA. It is interesting to speculate that there may be a shared mechanism of autoimmunity which involves impaired TLR9 responses. It would be interesting to investigate this on a wider scale in a larger cohort of patients with ERA and PRA to confirm these findings.

Despite their mechanistic similarities, RA and SLE have widely different disease phenotypes, and it would be of value to examine the differences in response to TLR9 antigens which may lead to development of SLE in some individuals and RA in others, likely due to a combination of genetic and environmental factors which take part in the “multi-hit hypothesis” of autoimmune diseases. It would interesting to study B-cells from patients with SLE and compare response to TLR9 ligands in terms of changes to sialylation. Since IgG-Fc sialylation has also been found to be reduced in SLE[300], it would be tempting to speculate that these cells would also have impaired upregulation of sialylation following activation. Furthermore, in one study, CpG stimulation was found to increase IgG1 Fc galactosylation, which in turn can lead to increased sialylation[233], further supporting a role for TLR9, and increased sialylation in protecting against the development of autoimmunity.

Another interesting finding in this study was that activated T-cells from patients with ERA did not lead to upregulated B-cell sialylation, as was the finding in healthy cells. Since lymphocytes in RA can be considered to be in a more “active”

state at baseline, it is possible that the T-cells from patients with RA were not sufficiently activated, resulting in a lack of response in B-cells. However, the lack of response in B-cells is in agreement with earlier results which showed that despite a similar level of activation being achieved in healthy donor and ERA B-cells stimulated with CD40L, sialylation was much lower in cells from patients with ERA – suggesting a potential B-cell intrinsic mechanism which prevents upregulated sialylation upon activation.

6.2.4 Gene expression and sialylation

Despite the extensive study of the impact of α2,6-sialylation in a number of pathological contexts, little is known about what determines the extent of cell surface or secreted protein sialylation. α2,6-sialylation is known to be mediated by the enzyme ST6Gal1[163] however, the regulation of this enzyme in B-cells in healthy individuals and in those with autoimmune conditions is not well understood. NEU1 is one of 5 neuraminidase enzymes expressed in human cells, which removes SA from glycan chains. Regulation of NEU1 is also poorly understood. The results of the current study showed that NEU1 mRNA was decreased when cells were activated, particularly with CpG, concurrent with a strong upregulation of surface sialylation. ST6Gal1 however, was also downregulated in these cells, suggesting the relationship between ST6Gal1 mRNA and cell sialylation may be more nuanced than a direct correlation between expression and surface sialylation.

NEU1 is abundantly expressed by B-cells and is found in the plasma membrane as well as in lysosomes[293]. It was hypothesised that decreased expression of NEU1 could increase surface SA expression due to less cleavage of SA from glycan chains. However, there may also be other, cell extrinsic factors which can influence cell surface sialylation - such as soluble neuraminidases. Interestingly, a study has found that activation of cells via TLR4 leads to NEU1 relocation to the cell surface and desialylation of TLR4 which in turn activates the receptor[301] – suggesting a potential role for NEU1 during cell activation. Furthermore, contrary to the results of the current study, NEU1 has been found to be upregulated upon activation in several other cell types, including T-cells and monocytes[293]. Despite this, given the current evidence, downregulating NEU1 cannot be ruled out as a mechanism for increased B-cell sialylation upon

activation. Especially when the relationship between ST6Gal1 mRNA expression and surface sialylation is also considered.

It is somewhat surprising that ST6Gal1 mRNA expression was also found to be reduced in CpG activated cells, despite the robust increase in sialylation of surface proteins, and suggested that the relationship between the two outputs was more complex than initially anticipated. There are a number of factors which may explain this discordance, for example ST6Gal1 activity may have been increased, perhaps by changing expression of a limiting factor, without a corresponding increase in gene expression – however direct measurement of enzyme activity would be required to test this hypothesis. Since gene expression was only measured at specific time-points there is also a chance that the true kinetics of the response may have been clearer outside of the time-points which were measured here. Since ST6Gal1 expression also decreased in unstimulated B-cells over 48 hrs, another potential hypothesis could be that ST6Gal1 expression does not play an important role in deciding cell surface sialylation. Instead, surface SA may be determined by sialidase activity and cell-extrinsic factors. Previously it has been found that in mice with B-cell specific ST6Gal1 knockdown, IgM molecules were still sialylated – indicating B-cell extrinsic measures of protein sialylation exist, thought to be driven by ST6Gal1 released from hepatocytes during the acute phase response[283]. However, another study found that serum ST6Gal1 had an effect on soluble proteins only, and not on surface proteins[166].

The results of this study identify a potential link between B-cell activation and sialyltransferase/sialidase enzyme mRNA expression. There are some other proposed mechanisms of ST6Gal1 regulation, with several studies finding that ST6Gal1 expression is influenced by hormones. Both androgens[206] and oestrogen exposure led to increased expression of ST6Gal1, as well as the latter leading to an increase in IgG-Fc sialylation[234]. Some further evidence suggests that ST6Gal1 activity may be determined by the generation of the SA substrate N-Acetyl-D-mannosamine (ManNAc) - increased flux in sialic acid metabolism led to increased sialylation of certain glycoproteins[302]. It was also shown in pancreatic cancer, that high fructose in the extracellular environment led to an increase in generation of ManNAc and increased expression of ST6Gal1[207]. This evidence further supports a role of cell metabolism in changes to sialylation, however both

of these studies describe increased expression of ST6Gal1 leading to increased cell sialylation[207,302]. Taken together with the results of this study, this suggests that regulation of NEU1 may be more important in determining B-cell surface sialylation in the case of B-cell activation. Further study of both NEU1 and ST6Gal1 and their impact on cell signalling pathways and B-cell function would be of interest, and would likely add to the understanding of the role of each enzyme in determining overall sialylation.

6.2.5 B-cell sialylation in response to cytokines

Considering the important role of cytokines in driving inflammation in RA, the potential impact of exposure to cytokines on B-cell sialylation was studied. Notably, *in vitro* exposure of B-cells to TNF led to decreased sialylation in cells from healthy donors but not patients with ERA, and IL-6 led to increased sialylation at lower doses in B-cells from healthy donors, but required a much higher dose to upregulate sialylation in B-cells from patients with RA. IL-4 was also found to increase sialylation in B-cells from healthy donors and from patients with PRA, but not ERA, and IL-17 led to a decrease in B-cell sialylation in lower doses in patients with ERA and healthy donors.

A previous study of the impact of cytokines on IgG1 galactosylation showed that exposure to IL-21 led to an increase in galactosylation, but exposure to TNF, IL-6, IL-4 or IL-17 had no significant effect[233]. However, another previous study showed that in murine B-cells, exposure to IL-21 and IL-22 produced by Th17 cells led to decreased expression of ST6Gal1 and reduced IgG sialylation[235]. Taken together, these results suggest that cytokines may have varied impacts on B-cells depending on other environmental or phenotypic factors, or may have specific effects on different isoforms of IgG. It is also difficult to determine from these studies whether reduced IgG sialylation is induced by exposure to these cytokines *in vivo* or if there are other instigating factors at work. Neither study considered the impact of these cytokines on B-cell surface sialylation. The results of the current study found that IL-17 could decrease B-cell surface sialylation, however, the impact of IL-21 and 22 on surface sialylation was not investigated.

Downregulated sialylation induced by TNF in peripheral memory and naïve B-cells *ex vivo* has interesting implications for the mechanism of decreased sialylation in peripheral B-cells from patients with RA – considering TNF is known

to be a key player in driving RA pathogenesis and is increased in the serum in RA[63]. It was hypothesised that TNF exposure may contribute to reduced B-cell sialylation in RA, possibly in combination with other factors. B-cells produce TNF upon activation, and there is some evidence that it may act as an autocrine growth factor, stimulating expansion of activated cells[274]. Another result of the current study showed that decreased sialylation led to reduced B-cell activation, which could suggest that downregulation of sialylation in B-cells exposed to TNF could form part of a negative feedback loop to prevent over-activation. This could be supported by a study which found that exposure TNF led to decreased response to TLR4 stimulation in murine B-cells[303].

TNF and IL-6 are known to be key players in driving pathogenesis in RA, and exert similar effects in terms of general immune cell activation[63]. Therefore, the observation that low doses of IL-6 led to increased B-cell sialylation was interesting. However, in B-cells TNF and IL-6 signal via different pathways – MAPK/JNK and JAK/STAT respectively[73], which may have opposing effects on B-cell sialylation. In addition, IL-6 is able to signal via surface bound receptor, as well as soluble receptors which associate with gp130 on the cell surface[304]. IL-6 classical signalling via membrane bound receptors has been associated with anti-inflammatory effects, whereas trans signalling via soluble IL-6R has been shown to promote pro-inflammatory activity[305]. IL-6 signalling and its role in anti/pro-inflammatory activity depends on a dynamic interaction between both membrane and soluble receptor and ligand concentration[306], making its true effects *in vivo* difficult to replicate in in vitro studies.

Further to this, IL-6 mediates the acute phase response in the liver. Part of the acute phase response involves the secretion of ST6Gal1 from hepatocytes, thought to be due to increased demand for sialylated proteins[283]. It could therefore be hypothesised that the mechanisms of inflammation induced by IL-6 could promote upregulated sialylation. It could be speculated that the lack of response to TNF and IL-6 in cells from patients with RA may be due to increased activation thresholds induced by chronic exposure. This is supported by the finding that higher doses of IL-6 were able to induce an increase in sialylation cells from patients with RA. Despite the decrease in sialylation observed in cells exposed to TNF, when cells primed with TNF were differentiated to plasmablasts and plasma cells, sialylation was not downregulated compared with TNF-naïve

cells. However, IL-6 exposure pre-differentiation, led to increased sialylation in plasma cells. It could be hypothesised that the impact of TNF in B-cells is transient, or only relevant in naïve or memory cells, but the impact of IL-6 exposure is sustained, however these results require further validation in a larger cohort. Overall, these results further suggest that a balance of factors are likely to form the picture of B-cell surface sialylation, and that the decrease in sialylation in RA may not be attributable to just one factor alone.

Despite previous results showing some promising findings in Th17 cytokines and the regulation of sialylation, only a mild change in sialylation was observed when B-cells were exposed to IL-17. However, in this case only IL-17A was tested, whereas previous studies showed IL-21 and IL-22 to have the greatest impact on antibody sialylation[233,235], but did not agree on whether exposure increased or decreased Ab sialylation. The role of IL-17 in RA is less well defined than that of TNF and IL-6, and treatment of RA with anti-IL-17 therapies has been shown to be less effective than anti-IL-6 and anti-TNF in clinical trials[307]. However, preclinical studies suggest that perhaps anti-IL-17 therapy may prove more beneficial in patients with pre-RA or early RA[235]. More study may be needed therefore, to determine the exact role of Th17 cytokines in RA, in order to further understand their contribution to autoimmunity, and sialylation in particular.

Although IL-4 is not usually associated with pathology in RA, it plays an important role in driving B-cell proliferation[*308*]. The finding that exposure to IL-4 led to increased sialylation in cells from healthy donors and from patients with PRA, but not ERA was interesting, and further suggests that increased sialylation is associated with B-cell proliferation and survival, as discussed previously. The lack of response in cells from patients with RA could suggest that a number of pathways are dysregulated in these cells. It may indicate that there is a lack of response to IL-4, preventing upregulated sialylation and an increase in proliferation. However, this cannot be confirmed by current results as proliferation was not studied. Another possible explanation is a normal response to IL-4, but a lack of upregulated sialylation, further indicating disrupted pathways of sialylation in RA. Further study of B-cell proliferation and activation in response to IL-4 would likely further elucidate this mechanism of dysregulated response.

6.2.6 Serum neuraminidase is increased in patients with RA

Neu activity was detected in serum samples from both healthy donors and patients with ERA, and, interestingly, it was found that there was increased activity in serum from patients with ERA. It was also found that exposure to ERA serum led to marginally reduced B-cell sialylation compared with exposure to healthy donor serum, an effect which was amplified when cells were activated. In activated B-cells exposed to serum from patients with ERA, sialylation was lower than in those exposed to HD serum and cells expressed less CD69, suggesting that desialylation, may contribute to the lack of upregulated sialylation and reduced activation observed in B-cells from patients with ERA. It was also found that when B-cells from healthy donors were exposed to serum from patients with ERA during the initial activation phase of differentiation, sialylation was decreased in differentiated plasmablasts and plasma cells, suggesting that exposure to serum factors in RA may prime cells for reduced expression of SA in differentiated cells. Increased serum Neu has previously been linked to inflammation, with increased serum levels in patients with type II diabetes mellitus who develop glomerular nephritis compared with those without nephritis[285]. It has also been found to be increased in the serum of patients with several cancers[284]. However, previous study of Neu activity in the serum of patients with RA has not been identified.

The potential contribution of serum neuraminidase to reduced B-cell sialylation in RA is interesting, and suggests a potential mechanism for reduced sialylation across B-cell subtypes. The paper which links serum Neu to glomerular nephritis suggests that there was a strong association between increased serum neuraminidase activity and increased serum and urine SA, suggesting its release from proteins[285]. In studies of cancer, increased serum Neu is implicated in removing SA from the surface of cancer cells, and increasing invasiveness and promoting cancer progression[284]. The source of increased Neu in serum is unclear, though may possibly be shed from the cell surface in the presence of gangliosides[284]. Despite finding that serum Neu activity was increased in patients with ERA, when cells from healthy donors were initially exposed to serum *in vitro*, there was only a marginal difference in sialylation between cells exposed to serum from patients with ERA versus serum from healthy donors. However, when cells were then activated, decreased sialylation in cells exposed to serum from

patients with ERA was apparent, and the activation potential of these cells was also noticeably lower. Therefore it may be hypothesised that there are additional factors in RA serum which may contribute to impaired upregulation of surface SA and decreased cell activation. Without taking these other potential factors into consideration, there are potential implications here for increased serum neuraminidase as a contributing factor to drive inflammation or autoimmunity in RA. Measuring serum neuraminidase activity in patients with other, similar autoimmune conditions, as well as inflammatory and non-inflammatory arthritic conditions would be useful in determining if serum Neu activity may have potential as a biomarker to detect inflammation/autoimmunity.

6.2.7 Consequences of reduced sialylation in B-cells

The initial finding that exposure to serum in culture leads to dramatically reduced B-cell sialylation was an unexpected observation. As was the finding that serum exposure also led to decreased B-cell activation. Initially it was hypothesised that decreased sialylation would lead to an increase in B-cell activation, due to the removal of the inhibitory negative charge of sialic acids. These two results combined challenged this hypothesis and suggested that reduced sialylation, or another factor present in serum reduced B-cell activation potential. To investigate this new hypothesis, the direct effect of reduced sialylation on B-cell activation was measured by treating cells with neuraminidase, and it was found that Neu treatment led to decreased activation potential when cells were stimulated with CpG or with BCR crosslinking plus CD40L co-stimulation. It was also found that pre-treating with Neu before B-cells were differentiated led to increased production of IgM in plasmablasts and plasma cells, which may indicate a lack of class-switching during the differentiation process. Sialylation was also loosely related to production of IgM in plasma cells, with a weakly positive correlation between sialylation and IgM concentration in culture supernatants.

The finding that reduced sialylation led to impaired activation implied that surface sialylation is important for effective signalling via both TLR and BCR signalling pathways. Lectin receptor CD22 is an important regulator of BCR signalling, the ligand for which is α2,6-SA. Following BCR activation, CD22 is phosphorylated and attenuates calcium signalling within the cell via SHP-1[176]. Generally, CD22 molecules on the cell surface are thought to exist as multimers, with each

molecule ligated by SA on neighbouring molecules[183]. This method of cis signalling limits the availability of CD22 for trans signalling[176]. Studies have shown however, that trans signalling of CD22 provides a robust inhibitory signal, and further increases the threshold for B-cell activation[180]. It was therefore hypothesised, that removal of sialic acid from the cell surface frees CD22 for trans signalling, and this leads to downregulated B-cell activation, as observed in this study (summarised in Figure 6.1). B-cells also express siglec-10 which can bind α2,6-SA, but also has affinity for α2,3-SA[309]. The influences of cis and trans ligand binding in siglec-10 signalling are less clear but would be interesting to investigate in this context, as it could be hypothesised that both CD22 and siglec-10 signalling may influence B-cell signalling by binding ligands in trans.

Decreased surface sialylation also reduced B-cell responses to TLR9 signalling, however the mechanism of this reduction is less clear. Given the link between defective CD22 signalling and autoimmunity, it could be hypothesised that reduced B-cell sialylation may lead to increased inhibition of B-cell activation via CD22, and that this may lead to the survival of autoimmune B-cells, normally regulated via TLR signalling. In mice, it has been shown that both CD22 and Siglec-G[290] (murine analogue of Siglec-10) could inhibit TLR signalling, with $CD22^{-/-}$ cells showing hyperactivation in response to TLR3, 4 and 9 ligands which was linked to the induction of suppressors of cytokine signalling (SOCS) SOCS1 and SOCS3[189]. It has also been shown that targeting CD22 with a therapeutic antibody can prevent B-cell activation by TLR7 ligands, which may help to maintain tolerance to T-independent antigens in autoimmunity[310]. Although the exact mechanism of Siglec-G inhibition of TLR signalling is not yet clear, it is thought that CD22 may be recycled between the cell surface and endosomes, which may account for the inhibition of endosomal TLRs such as TLR9[311]. However, previously it has been shown that CD22 ligands are not directly involved in CD22 regulation of TLR signalling, as $ST6Gal1^{-/-}$ mice showed normal responses to CpG[312]. Taken together, this evidence suggests a potential role for CD22 in regulating TLR activation, however the increased inhibition of TLR9 signalling in desialylated cells may occur via a mechanism which is distinct from CD22 trans signalling.

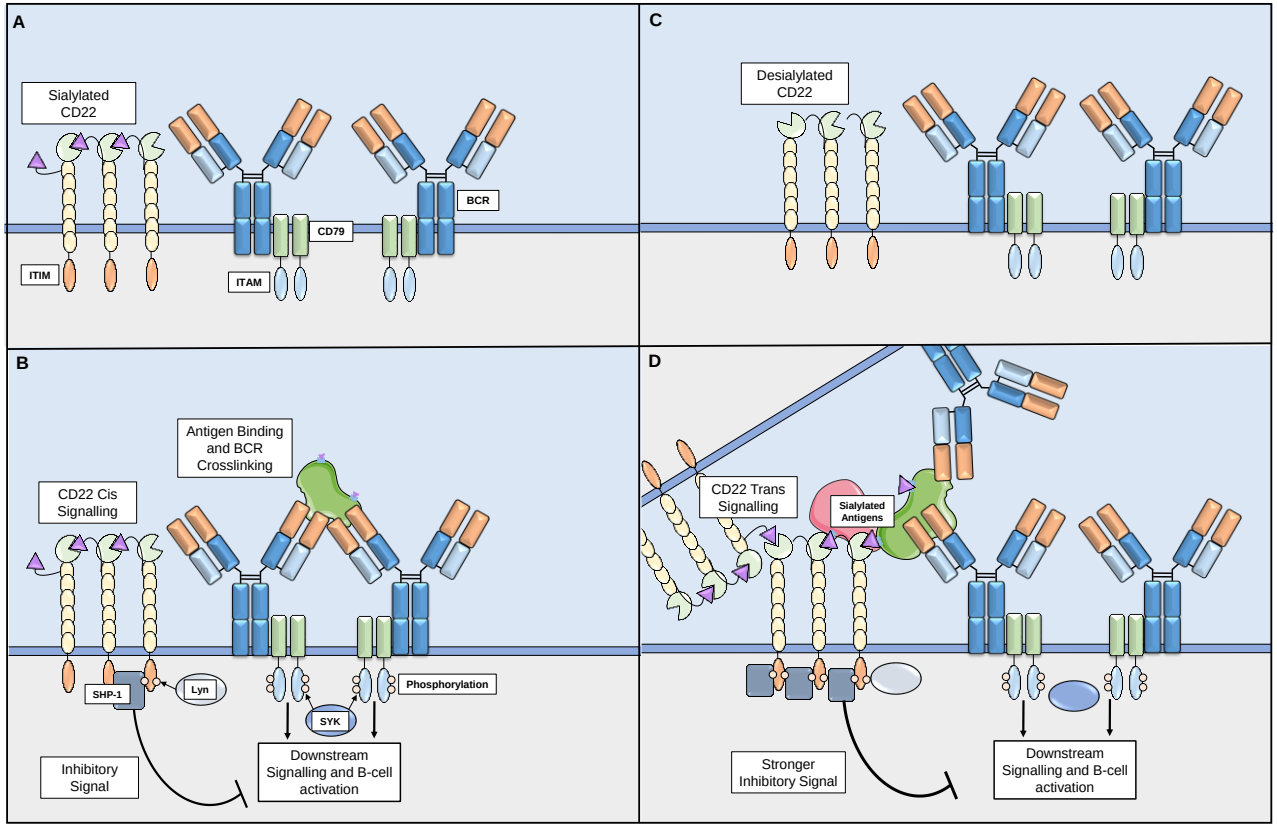


Figure 6.1 CD22 signalling in cis and trans inhibits B-cell activation via the BCR

Key events in CD22 inhibition of BCR signalling are depicted. **(A)** Under normal conditions of cell sialylation, CD22 molecules on the cell surface form multimers which are ligated by α2,6 sialic acid on neighbouring molecules. **(B)** When the BCR is crosslinked by antigen binding, kinase Syk is recruited and phosphorylates CD79 ITAM and begins the cascade of kinase activation which leads to B-cell activation. Kinase Lyn is also recruited to CD22 ITIM which recruits SHP-1 and this leads to inhibition of the BCR signalling cascade. **(C)** When surface sialic acid molecules are removed, CD22 molecules exist in an open confirmation and are available to bind sialylated ligands. **(D)** Desialylated CD22 molecules can bind α2,6- sialic acid on other cells or on antigens which may also engage the BCR. This triggers a stronger inhibitory signal than CD22 signalling in a cis manner. The exact mechanism of increased inhibition is currently unknown. This figure was created using templates from Servier Medical Art which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

6.2.8 Changes to sialylation during B-cell differentiation

Changes to sialylation which occur during B-cell differentiation to plasma cells were previously unknown. The results of this study found that B-cell sialylation initially increased greatly during activation, then decreased during differentiation to plasmablasts and further decreased during terminal differentiation to plasma cells. It was observed previously that sialylation was lower in plasmablasts isolated from peripheral blood compared with memory and naïve B-cells (unreported observations). Accordingly, expression of NEU1 and ST6Gal1 mRNA also tended to increase as cells differentiated to plasmablasts and plasma cells, in line with previously reported results which suggested that surface sialylation and expression of both NEU1 and ST6Gal1 are inversely related.

The initial increase in sialylation which was observed following the first three days of exposure to a strong activation stimuli is in line with our previous results which showed that sialylation increased in B-cells from healthy donors when activated. This further indicated that upregulated sialylation is important during activation and may promote proliferation and survival to allow for B-cell differentiation. The corresponding decrease in sialylation which follows as cells differentiate to plasmablasts and plasma cells could be due to a number of factors. Previously, the results of this study found that a decrease in cell sialylation was associated with reduced activation, associated with CD22 trans signalling. However, these tests were performed in naïve and memory B-cells and the baseline set-point for plasmablast or plasma cell sialylation may be much lower than in naïve or memory cells. Further to this, expression of CD22 is known to increase in activated B-cells then decline as cells differentiate to plasma cells[313], suggesting less of an influence for CD22 and surface sialylation in determining activation in terminally differentiated ASCs. Decreased expression of CD22 in plasma cells could also contribute to reduced expression of surface sialylation, as the molecule itself contains SA.

As well as changing surface marker expression, ASCs are phenotypically distinct from naïve and memory B-cells, and have altered functional requirements. They are much larger in size and have to adapt to a greater secretory load by

upregulating genes which can counteract endoplasmic reticulum stress[259]. Plasma cells also exit the cell cycle and become non-dividing cells[314]. This may be another indication that increased sialylation is important in B-cells during the proliferative stage of differentiation, and is then downregulated as cells move towards a non-dividing phenotype.

As mentioned previously, the increase in expression of both NEU1 and ST6Gal1 during differentiation whilst sialylation decreased, was in line with the previous results of this study. A similar upregulation of NEU1 expression was previously observed in differentiating monocytes, and its expression was associated with macrophage cytokine production and phagocytic capacities[315]. It could be hypothesised increased NEU1 expression may also be important during B-cell differentiation to plasma cells. The increase in ST6Gal1 could be a reflection of increased requirement for sialylation of proteins for secretion, particularly the greatly increased production and secretion of IgG antibodies, which are a known target of ST6Gal1 activity[212]. It is interesting to note that ST6Gal1 expression in differentiated plasma cells from patients with ERA was slightly higher than in cells from healthy donors. It was initially hypothesised that ST6Gal1 expression would be reduced in these cells – as a reflection of reduced antibody sialylation which is a well-established feature of RA[227]. However, since cells from healthy donors and patients with ERA were differentiated under identical condition *in vitro*, this may be an indication that the environment in which cells differentiate has an important impact on ST6Gal1 activity and sialylation of secreted antibodies, rather than an intrinsic difference in ST6Gal1 activity. Taken together, these results highlight the need for further investigation in this area, paying particular attention to the relationship between NEU1 and ST6Gal1 mRNA expression and B-cell surface sialylation. Further study of the relationship between antibody sialylation and events which may alter expression and activity of ST6Gal1 would also be of great benefit, and increase the potential for development of therapeutics which may be able to target events which alter sialylation and enhance autoantibody pathogenicity.

6.2.9 Reduced sialylation may lead to improved response to B-cell depletion therapy

There is a large body of research in oncology which suggests that sialylation status of tumour cells has an important influence on response to chemotherapies. The majority of studies find that increased tumour cell surface sialylation promotes chemoresistance[197,198,201,203,287], and may be a strategy utilised by cancerous cells to avoid detection by immune cells[179,286]. It was therefore hypothesised that cell-surface sialylation may also be important in the treatment of autoimmune diseases. B-cell depletion by rituximab is a commonly used treatment for RA, therefore the impact of B-cell sialylation on response to rituximab was investigated. It was found that cells with reduced sialylation were more susceptible to death in an *in vitro* rituximab assay.

There are a number of factors which may contribute to this finding. When B-cells were treated with neuraminidase, a CD20 antibody (used as a surrogate for rituximab) could more easily bind to the cell surface. SAs often function to block receptor-ligand interactions due to their negative charge, and it would appear that removal of this inhibition and charge allows for easier binding to CD20. There are several mechanisms which are thought to contribute to rituximab-induced B-cell depletion, which include opsonisation - leading to complement-mediated lysis and phagocytosis, and by directly inducing apoptosis in B-cells[245]. However, the mechanism by which rituximab can directly induce B-cell death by binding to CD20 is not well defined, and has not been convincingly shown to occur *in vivo*[316]. Reduced sialylation could potentially have a bearing on all of these mechanisms, by facilitating interactions between cells and by increasing binding of the drug to the cell surface.

The findings of the current study have potentially important implications for prediction of response to therapy and for targeted delivery of neuraminidase as a potential therapeutic adjuvant. Any potential consideration of neuraminidase as a means of increasing response would have to be carefully targeted, considering the wide-ranging roles that sialylation plays in different cells and tissue types. One study describes targeted delivery of a recombinant sialidase conjugated to an antibody targeting HER2+ breast cancer, which enhanced ADCC during in vitro experiments[292]. This line of investigation may prove to be fruitful in both

cancer and autoimmunity therapeutics. Alternatively, work has already been carried out to investigate the use of small molecule inhibitors of SA metabolism, which have proven effective in early *in vitro* studies[317].

An interesting next step would be to investigate the potential benefits of measuring sialylation as a predictor of response to therapy. It would be a challenging task to measure the impact on initial response, since rituximab infusions commonly led to near total B-cell depletion, to the point where B-cells are undetectable by conventional flow cytometry[253]. It may, however, be useful to investigate sialylation in patients who do not achieve complete depletion in the first instance. A pressing issue with rituximab treatment is the high rate of disease relapse, particularly when B-cells repopulate following depletion[318]. It would be interesting to study whether baseline sialylation has an influence on relapse rates, or whether sialylation is altered when B-cells repopulate and if this may have a bearing on disease relapse.

6.3 Future Direction

Much of the work in this thesis has presented opportunities for interesting future studies. The initial work has provided preliminary data that could form the basis of three key lines of investigation which are detailed below.

6.3.1 Regulation of sialylation in B-cells in health and in autoimmunity

Firstly, the mechanisms which regulate sialylation in B-cells from healthy donors warrant further investigation. The results of this study have shown that sialylation is increased in response to activation, particularly via TLR stimulation. It is important to next investigate how sialylation is altered – i.e. are naturally sialylated proteins upregulated and if so which ones, or is sialylation of particular surface proteins increased. To do this, B-cell surface molecules which are ligands for ST6Gal1 would have to be identified, and their expression monitored, as well as investigating sialic acid content of particular surface proteins – possibly utilising mass spectrometry to do so. Throughout this study it was hypothesised that sialylation was upregulated to promote proliferation and cell-survival, and it would be useful to confirm this experimentally, potentially by treating cells with neuraminidase following activation.

Further to this, investigating which signalling molecules and transcription factors are involved in regulating sialylation would be of great interest. Studying molecules in the signalling pathways utilised by the BCR and TLRs using small molecule inhibitors may of benefit in identifying potential candidates for further study. As well as this, the involvement of gene regulation of ST6Gal1 and surface sialylation was not clear in this study, warranting further investigation. It would be beneficial to initially measure expression of ST6Gal1 protein in activated and resting cells, and determining how this relates to surface sialylation, and mRNA expression of ST6Gal1.

It was found that in cells from patients with RA, TLR stimulation did not induce upregulation of sialylation, whereas in some cases, stimulation via the BCR was able to induce a degree of upregulation similar to healthy donor cells. It would be pertinent to examine the link between autoimmunity and TLR response in RA, given the association between SLE and impaired TLR response. Firstly, it would

be interesting to measure other indicators of B-cell activation in RA cells in response to TLR stimuli such as cytokine production, antibody production and proliferation. Next it would be important to repeat such experiments in cells from patients with other autoimmune diseases to determine if reduced response to TLR stimuli, and lack of upregulation of SA may be a shared mechanism of autoimmunity.

6.3.2 Serum Neuraminidase changes

Secondly, further investigation of serum neuraminidase activity would likely be of great benefit. Validating preliminary data described in this study in a much larger cohort of patients would be a logical first step, as would the inclusion of samples from patients in the pre-RA “at-risk” category and patients with other autoimmune diseases such as SLE and Psoriatic Arthritis. Once this data has been validated on a larger scale it would be interesting to study serum neuraminidase in relation to disease activity in RA, to determine any potential correlation.

Further to these studies, in light of this data, it would be interesting to study the sialylation of other immune cells in RA in relation to cells from healthy donors. Sialylation in T-cells and monocytes from peripheral blood could be measured in the first instance, to determine if other cell types may be impacted by increased serum neuraminidase. It would also be pertinent to study the relationship between neuraminidase activity and B-cell sialylation in patients with RA. In addition, since neuraminidase cleaves sialic acid from glycoprotein chains, it may also be useful to study free sialic acid in the serum, as another potential marker of increased neuraminidase activity.

To further investigate the consequences of reduced sialylation on B-cell function, it is important to establish the mechanisms which led to greater inhibition of B-cell activation when cells are desialylated. It was hypothesised that CD22 signalling in a trans manner may be responsible for the increased inhibition, however this remains to be confirmed experimentally. To do so, utilising CD22-specific knockouts would provide further confirmation of this, potentially combined with the use of sialylated ligands which also bind the BCR. To determine the link between TLR signalling and reduced sialylation may be a more challenging task, but may be investigated through the use of tracking CD22 recycling to endosomes, and the influence of surface sialic acid on this process.

Exploring further consequences for B-cell function of reduced sialylation would also be of benefit, such as measuring cytokine production, proliferation and possibly interactions with other cells.

6.3.3 Impact of sialylation on therapies

Lastly, there is scope to investigate the potential use of sialylation status as a marker of response to rituximab therapy. Initial work in this area could involve a pilot study in rituximab-naïve patients with RA, with sialylation measured at baseline prior to treatment. Patients would then be followed up after their first rituximab infusion, and efficacy of B-cell depletion measured by flow cytometry to assess any correlation between B-cell sialylation and poor initial response to rituximab depletion. It may also be interesting to investigate the relationship between serum Neu activity and rituximab response in these patients. Investigating the impact of sialylation on other therapies used to treat RA could also prove interesting. Drug candidates could be selected based on their mechanism of action, targeting drugs such as ipilimumab which bind the T-cell surface.

Additional study of the use of neuraminidase as a targeted therapeutic adjuvant is another interesting prospect with potential to prove useful in a number of clinical situations. In the case of B-cell depletion therapy, the delivery of neuraminidase with the drug could potentially increase binding and increase efficacy of killing by phagocytosis and complement lysis.

6.4 Concluding Remarks

Studying B-cell sialylation is a challenging task. The phenotypic and functional diversity of B-cell subsets, as well as the difficulties of studying sialylation itself can often lead to data which is complicated to interpret. However, the findings presented here have some potentially exciting implications for the study of B-cell sialylation in the future, along with some interesting implications for the treatment of B-cell mediated pathologies. Although this work has made great inroads into understanding the complicated picture of the regulation of B-cell sialylation, there is much which still remains to be understood.

Arguably the most important current challenge facing patients with RA, and clinicians treating patients with RA, is in establishing a treatment regimen which achieves long-lasting disease remission and greatly improves quality of life. The issue of non-response to treatment is a most pressing one, and the solutions will most likely depend on effective prevention rather than cure, as is the case in many pathologies. Therefore, understanding the key events in the process of progression from asymptomatic autoimmunity to development of chronic joint inflammation in RA could be key to improving outcomes for patients worldwide. Without doubt sialylation plays a role in driving autoimmunity, and further understanding this role will undeniably assist in planning future areas of therapeutic research, and help to identify the most pertinent time for effective intervention.

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