Understanding the Variability in Clinical and Biological Response to B-Cell Depletion Therapy in Rheumatoid Arthritis and Systemic Lupus Erythematosus

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Declaration

I, Venkata Ranga Reddy Indluru confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Factors including pharmacokinetics, B-cell internalisation of anti-CD20 monoclonal antibodies (mAbs) and disease-associated defects in complement system, NK cells and macrophages may influence the efficiency of rituximab, a Type I anti-CD20 mAb disposed to internalisation by B cells, and contribute to variable clinical and biological response in patients with Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE). The work presented in this thesis investigated the potential of Obinutuzumab, a commercially available, mechanistically different Type II anti-CD20 mAb with an afucosylated Fc portion not disposed to internalisation, as an alternative B-cell depleting agent in RA and SLE.

In patients with SLE, the duration of B-cell depletion was shorter and serum rituximab levels were significantly lower compared to RA. Hypogammaglobulinemia in the SLE cohort was mostly limited to the IgM isotype and was associated with lower baseline IgM levels, sequential therapy with mycophenolate mofetil and lower frequency of IgD+CD27+ unswitched memory B cells. Anti-dsDNA antibodies in those with high pretreatment levels remained elevated in the long-term, a potential mechanism of poor response to rituximab. Obinutuzumab was at least two-fold more efficient than rituximab at inducing cytotoxicity in B cells from patients with RA and SLE in whole blood assays. B cells from patients with RA and SLE internalised obinutuzumab to a significantly lower extent than rituximab, which was significantly more efficient than obinutuzumab at evoking complement-dependent cellular cytotoxicity of isolated B cells. In contrast, obinutuzumab was significantly more efficient at inducing NK cell activation, an indirect measure of antibody-dependent cell cytotoxicity, in RA and SLE; and also activated neutrophils, an indirect measure of antibody-dependent cell phagocytosis, more efficiently than rituximab in SLE. Obinutuzumab was also more efficient at inducing direct cell death in CD19+ B-cells and switched (IgD-CD27+) memory B cells specifically, a higher frequency of which is associated with poor clinical response to rituximab.

Thus, increased clearance and/or internalisation of rituximab may impair its efficiency in RA and SLE. Regardless, obinutuzumab was more efficient than rituximab at inducing B-cell cytotoxicity in vitro in both RA and SLE samples mediated by superior $Fc\gamma R$-dependent and -independent effector mechanisms with greater ability to remain at the cell surface following CD20 engagement despite inferior ability to evoke complement-dependent cellular cytotoxicity. These data provide compelling mechanistic reasons for expecting better outcomes with obinutuzumab as an alternative B-cell depleting agent in RA and SLE.

Acknowledgements

The work presented here was inspired by the pioneering work of Professors Edwards, Cambridge, Isenberg and Dr Leandro demonstrating the efficacy of rituximab-based B-cell depletion therapy in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). It has been a great privilege to work with the group whose insightful knowledge of the area encompassing clinical and basic science matched only by their work ethic provided a great source of inspiration.

I was drawn to the area of B-cell depletion as I was curious to understand the reasons for the clear disparity in clinical response based on serological status of patients with RA but also to understand the reasons for poor clinical response in some seropositive RA patients and variability in responses in SLE patients in an attempt to address the unmet need. The disparate responses suggested potential mechanistic pathways for exploring toward optimising B-cell depletion therapy.

I am deeply indebted to the kindness and generosity of Dr Leandro for agreeing to supervise and support my intentions to research in this area that rekindled her own long-standing interests, a natural follow-up from her previous work. Dr Leandro's insightful clinical knowledge and experience of using rituximab to treat RA and SLE patients complemented by wealthy knowledge of B-cell biology was fundamental to guide the line of enquiry. Dr Leandro's skills of supervision, providing guidance, critical questioning of ideas mindful of time constraints was fundamental for timely completion of the project successfully. Her continuous encouragement, guidance, approachability and support extending beyond regular working hours with a great degree of flexibility were essential for writing up of the thesis.

This work would not have been possible without the collaboration established by Dr Leandro with Professors Cragg and Glennie, the brains behind the idea of exploring internalisation of rituximab as a resistance mechanism. Professor Cragg's acute scientific approach at every stage ensured the experimental plan remained clear, focused and comprehensive. His generous support in providing materials, advice, clear and logical construction of arguments in manuscript preparation was incisive and instructive. His seemingly boundless knowledge of B-cell biology and monoclonal antibodies complemented by tactful approach to translate theoretical knowledge toward clinically meaningful benefit provided perpetual motivation. All that said remains an understatement.

Aligning with the values described in the Bhagavad Gita, Chapter 2, Verse 47; and as interpreted by Swami Vivekananda; helped my persistence without focusing on the results:

karmany evadhikaras te
ma phalesu kadacana
ma karma-phala-hetur bhur
ma te sango 'stv akarmani

The interpretation of the verse —

To work you have the right, but not to the fruits thereof
Let not the fruits of action be your motive, nor let your attachment be to inaction.

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List of Abbreviations

ACR   American College of Rheumatology

ADCC   Antibody-dependent cell cytotoxicity

ANA   Antinuclear antibody

APC   Allophycocyanin

APRIL   A proliferation ligand

BAFF   B-cell activating factor

BCDT   B-cell depletion therapy

BCR   B-cell receptor

BILAG   British Isles Lupus Assessment Group

BLyS   B lymphocyte survival factor

AZT   Azathioprine

CD   Cluster of differentiation antigens

CDR   Complementarity determining region

CDC   Complement-dependent cellular cytotoxicity

CHO   Chinese Hamster Ovary

CLL   Chronic lymphocytic leukemia

CRP   C-reactive protein

CTI   Cytotoxicity Index

CYC   Cyclophosphamide

CS   Corticosteroids

DCD   Direct Cell Death

DHOH   Dihydroorotate dehydrogenase

DMARDs	Disease modifying anti-rheumatic drugs
DNA	Deoxyribose nucleic acid
ELISA	Enzyme linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
Fab	Antigen binding fragment
FBS	Fetal bovine serum
Fc	Fragment crystallisable
Fv	Variable fragment of immunoglobulin
FcγR	Fc receptor for IgG
FITC	Fluorescein isothiocyanate
HACA	Human anti-chimeric antibodies
HC	Healthy controls
HCQ	Hydroxychloroquine
HIS	Heat inactivated serum
HLA	Human leucocyte antigen
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IQR	inter-quartile range
IFN-g	Interferon g
IL	Interleukin
IQR	Inter-quartile range
LN	Lupus nephritis

LFN	Leflunomide
mAbs	Monoclonal antibodies
MFI	Mean fluorescence intensity
MTX	Methotrexate
N/A	Not applicable
ns	not significant
NHL	Non-Hodgkins lymphoma
NHS	Normal healthy serum
NK	Natural killer
NRs	Non responders
OBZ	obinutuzumab
PBMC	Peripheral
PE-Cy5	Phycoerithrin-indodicarbocyanine or Cy-chrome
PerCP	Peridinin-chlorphyll a complex protein
PTPN22	Protein tyrosine phosphatase non-receptor type 22 gene
RA	Rheumatoid Arthritis
RPMI	Roswell Park Memorial Institute medium
RTX	Rituximab
RF	Rheumatoid factor
SF-36	Short form (36)
SLE	Systemic Lupus Erythematosus
SSZ	Sulphasalazine
TNF	Tumor necrosis factor

μl	Microlitre
μg	Micrograms
mL	Millilitre
L	Litre
UCL	University College London
UCLH	University College London Hospitals

Chapter 1   Introduction

Background

Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) are chronic, multisystem inflammatory autoimmune conditions that affect joints and vital organs leading to significant morbidity and mortality (Rahman and Isenberg 2008, Scott, Wolfe et al. 2010). Currently, there are no cures for either. Patients with RA have worse morbidity and mortality compared with the general population, despite treatment with conventional therapies (Scott, Wolfe et al. 2010) and biologics (Pollard, Choy et al. 2005). The risk of mortality in SLE is also increased compared to the general population, due to persistent disease activity and infections that occur in the first five years after diagnosis, whereas cardiorespiratory causes contribute significantly to late deaths (Abu-Shakra, Urowitz et al. 1995). Biologics appear to be superior to conventional therapies in reducing mortality risk in RA (Listing, Kekow et al. 2015). Cardiovascular risk is increased independent of traditional risk factors, in both RA (Scott, Wolfe et al. 2010) and SLE (Manzi, Meilahn et al. 1997). Consequently, the socio-economic impact is substantial both for RA (Scott, Wolfe et al. 2010) and SLE (Rahman and Isenberg 2008).

Rheumatoid Arthritis

The prevalence of RA varies from 0.5-1.1% with a 3-fold increase in women, and more often presents in the fifth decade (Tobon, Youinou et al. 2010). Chronic inflammation of the synovium, which typically affects the small joints of hands and feet in a symmetrical distribution leads to joint erosions and irreversible deformities (Scott, Wolfe et al. 2010). A characteristic cluster of symptoms and signs including joint swelling, morning stiffness, rheumatoid nodules and the presence of Rheumatoid Factors (RFs), which are autoantibodies directed against the Fc portion of Immunoglobulin (Ig) were major components of the 1987 classification criteria for RA (Arnett, Edworthy et al. 1988).

The pathogenesis of RA is complex (Feldmann, Brennan et al. 1996, Scott, Wolfe et al. 2010, McInnes and Schett 2011). Genetic factors, including HLA DRB1 and tyrosine phosphatase PTPN22 polymorphisms, and smoking, are strongly associated with its development (Scott, Wolfe et al. 2010, Tobon, Youinou et al. 2010). Both B cells and T cells play important roles in the pathogenesis. A complex interplay of genetic and environmental factors culminates in synovial inflammation, predominantly mediated by macrophages (Yanni, Whelan et al. 1994, Scott, Wolfe et al. 2010) through secretion of proinflammatory cytokines TNF and IL-6 (Feldmann, Brennan et al. 1996, Scott, Wolfe et al. 2010, McInnes and Schett 2011).

RFs, first described by Waaler in 1939 (Waaler 2007) and highly specific anti-citrullinated peptide antibodies (ACPA) (Schellekens, de Jong et al. 1998) have long been linked to RA and may be detectable before the onset of clinical disease (Rantapaa-Dahlqvist, de Jong et al. 2003). RFs and ACPAs interact with FcγRs on macrophages leading to secretion of TNF-alpha (Abrahams, Cambridge et al. 2000, Mathsson, Lampa et al. 2006, Sokolove, Johnson et al. 2014), the dominant pro-inflammatory cytokine in RA (Scott, Wolfe et al. 2010). Thus, RF and ACPAs are not just of diagnostic value but are potential mediators of a key immune effector pathway through stimulation of effector cells such as macrophages. Together, these findings implicate a role for B cells in RA.

A better understanding of the pathogenic mechanisms and concurrent technological advances in the field of monoclonal antibodies helped to develop a number of biological therapeutic strategies. So far, direct T-cell targeted therapies have not shown to be effective in RA (Isaacs 2008) whereas diverse biological strategies targeting pro-inflammatory cytokines such as TNF, IL-6 and also co-stimulation blockade using CTLA-4 Ig (abatacept) and B-cell depletion therapy with rituximab are effective in RA (discussed later). Tyrosine kinase inhibitors have been also shown to be effective in RA(Weinblatt, Kavanaugh et al. 2010). Fibroblast-like synoviocytes display distinct characteristics in RA and epigenetic modulators such as histone deacetylase inhibitors that potentially modulate the function of fibroblasts are being developed for use in RA (Tough, Prinjha et al. 2015). Currently, a number of biological therapies including B-cell depletion therapy are in clinical use in RA (explored in detail later).

Systemic Lupus Erythematosus

Systemic Lupus Erythematosus affects many organs resulting in arthritis, nephritis, heart disease, skin rashes, mucositis, serositis, hematologic abnormalities and central nervous system (CNS) dysfunction (Rahman and Isenberg 2008). The prevalence of SLE varies from 35-200/100,000 persons with a greater prevalence noted in women (9-fold) and in African, Asian and Hispanics when compared with Caucasians (Hopkinson 1992). Differences between ethnicities in the prevalence of biopsy-proven lupus nephritis occur with approximately 100/100,000 persons of Chinese and African descent whereas the prevalence was 20 and 5/100,000 persons in Indo-Asian and Caucasians, respectively(Patel, Clarke et al. 2006). Apparent disparities between ethnicities in the natural course of the disease and outcomes may reflect a complex influence of genetic and non-genetic factors including socio-economic effects (Uribe, McGwin et al. 2004).

The pathogenesis of SLE is particularly complex (Rahman and Isenberg 2008). Interplay between genetic and environmental factors leads to immune dysregulation and consequently, inappropriate activation of inflammatory pathways result in tissue injury (Mok and Lau 2003). Excessive accumulation of self-antigens due to defective clearance (Baumann, Kolowos et al. 2002) (Gaipl, Voll et al. 2005) also facilitates autoimmunity (Green, Moody et al. 2012). Abnormalities in T cells, macrophage functions, plasmacytoid dendritic cells, complement systems and type I Interferons (IFN) (Elkon and Wiedeman 2012) have all been implicated in the pathogenesis of SLE.

Autoantibodies directed against a range of intracellular and cell surface antigens are characteristic of SLE. Antinuclear antibodies are seen in > 95% of patients whereas the presence of antibodies against extractable nuclear antigens such as anti-Ro, anti-La, anti-RNP and anti-Sm antibodies is variable (approximately 10-40%). Autoantibodies may precede the onset of disease with anti-dsDNA antibodies being detected closer to the onset of clinical disease. They are typically present in 50-70% of patients with kidney involvement (Arbuckle, McClain et al. 2003). Antiphospholipid antibodies are linked to thrombosis and foetal loss. Autoantibodies form immune complexes, leading to the activation of complement-mediated tissue injury or alternatively autoantibodies directed against cellular antigens trigger antibody-dependent cellular cytotoxicity, (Dorner, Giesecke et al. 2011). Thus, B cells play a critical role in several autoimmune pathways characteristic of SLE. These observations reflect the underlying heterogeneity of the pathogenesis and manifestations of SLE. Therefore, it is important to take into consideration the disease heterogeneity when the efficacy of targeted biological agents is evaluated (discussed later).

Conventional Treatments

Medical treatment with immunosuppressants and/or immunomodulatory agents is the main therapeutic strategy for a majority of patients with RA and SLE.

Rheumatoid Arthritis

Conventional treatments commonly referred to as disease-modifying anti-rheumatic drugs (DMARDs), include Methotrexate (MTX), Sulphasalazine (SSZ), Hydroxychloroquine (HCQ), Leflunomide (LFN) and corticosteroids (CS). DMARDs are grouped as synthetic conventional (scDMARDs) drugs, biological (bDMARDs) drugs, and targeted synthetic (tsDMARDs) drugs such as Tofacitinib and scDMARDs are

often used in combination in RA (Gaujoux-Viala, Nam et al. 2014). The precise mechanisms of action of scDMARDs are not clearly understood. Most of the DMARDs were not specifically developed for the treatment of RA or SLE and their clinical utility was discovered serendipitously based on clinical observations preceding empirical use in the clinic. Most of these scDMARDs target rapidly dividing cells including lymphocytes. HCQ is an exception in that the main mechanism is through immunomodulation.

The use of corticosteroids revolutionized the treatment of RA in the 1950s (Hench, Kendall et al. 1949), however, prolonged use is associated with significant, undesired effects such as infections, diabetes, osteoporosis, hypertension and weight gain. CS bind to glucocorticoid receptors and inhibit pro-inflammatory transcription factors such as activation protein-1 and nuclear factor-kappa B (Adcock and Ito 2000). CS might also alter leucocyte migration by directly regulating the expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1 (Cronstein, Kimmel et al. 1992). Currently, MTX, which is considered to exert anti-inflammatory effects through adenosine-mediated pathway (Tian and Cronstein 2007), is used as a first line agent and is effective in controlling arthritis in up to two thirds of patients in RA (Choi, Hernan et al. 2002). SSZ, is a prodrug for 5-aminosalicylic acid (5-ASA), which is involved in scavenging of the reactive oxygen species which in turn may perturb the metabolism of arachidonic acid to prostaglandins and leukotrienes, leading to its anti-inflammatory effects in rheumatoid arthritis (Choi, Hernan et al. 2002). Leflunomide inhibits the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH), which plays a key role in the de novo synthesis of the pyrimidine ribonucleotide uridine monophosphate (Fox, Herrmann et al. 1999). HCQ increases pH within intracellular vacuoles, consequently, altering protein degradation by acidic hydrolases in the lysosome. Acidic cytoplasmic compartments facilitate processing of antigenic protein into peptides and the formation of peptide-MHC protein complexes. Thereby interfering with the function of antigen presenting cells (Fox 1993). Thus, a number of conventional therapies are in clinical use in RA, however, in at least up to a third of patients the response to scDMARDs is inadequate. Several other approaches including tyrosine kinase inhibitors are being explored for use in RA (D'Aura Swanson, Paniagua et al. 2009), but not considered relevant for the thesis and will not be discussed.

Systemic Lupus Erythematosus

In routine clinical practice, immunosuppressants and/or immunomodulatory agents are used, often in combination, for optimal control of disease activity, to prevent flares and improve patient outcomes. Commonly used immunosuppressants include CS,

azathioprine (AZT), mycophenolate mofetil (MMF), MTX, cyclophosphamide (CYC) and less commonly cyclosporine or tacrolimus for refractory cases.

The immunomodulatory agent, HCQ, is also commonly used. HCQ is effective for SLE and improves survival (Alarcon, McGwin et al. 2007). MMF inhibits inosine monophosphate dehydrogenase, a rate limiting enzyme in de novo synthesis of guanosine nucleotides, and preferentially affects T and B cells (Allison 2005). AZT is a prodrug and its metabolites 6-mercaptopurine and 6-thioguanine mediate immunosuppression through inhibition of purine nucleotide synthesis particularly targeting lymphocytes (Lennard 1992). CYC is an alkylating agent and its metabolites, derived via the cytochrome-P450 enzymatic system, phosphoramide mustard and acrolein inhibit DNA synthesis by adding alkyl group to guanine base of DNA and depletes lymphocytes and to some extent macrophages (Foley, Friedman et al. 1961). Despite the use of immunosuppressants, damage may continue to accrue due to persistent disease activity and also as a consequence of the adverse effects associated with treatments.

Thus, conventional therapies non-selectively suppress different immune cells to control disease activity in a majority of the patients with RA and SLE. However, many patients suffer adverse effects and continue to have active disease (Scott, Wolfe et al. 2010).

Biological therapies

A better understanding of the pathogenesis of RA and SLE informed the development of targeted biological therapies. Broadly, biological therapies target specific cytokines or cells. Biological therapies are now increasingly used to treat active RA and SLE refractory to conventional therapies.

Rheumatoid Arthritis

As previously discussed B cells, T cells and pro-inflammatory cytokines TNF and IL-6 are implicated in the pathogenesis of RA. Biological therapies targeting these pathways are now routinely used in RA. Anti-TNF agents are effective in RA, however, clinical response is not attained and/or maintained in a significant proportion (approximately 25-30%) of patients (Hyrich, Watson et al. 2006). Tocilizumab (a recombinant monoclonal antibody directed against the IL-6 receptor) is also effective, however, again up to a third of patients either do not or respond poorly. Similar observations have been made with the co-stimulation blocking agent abatacept, which is also effective in refractory RA (Nam, Ramiro et al. 2014). Alternatively, Rituximab, a

chimeric anti-CD20 monoclonal antibody (mAb) that depletes B cells is also effective in the treatment for refractory RA (Nam, Ramiro et al. 2014) and, is superior to conventional therapies in reducing the risk of mortality (Listing, Kekow et al. 2015) (discussed later). However, not all patients respond to a single biological agent.

Systemic Lupus Erythematosus

Unlike RA, in routine clinical practice, biological therapies are not in widespread use for SLE. A number of biological agents targeting different pathways in SLE are being explored (discussed later). Belimumab an anti-BAFF (B lymphocyte Activating Factor also known as BLyS, B lymphocyte stimulator) monoclonal antibody was found to be effective for SLE in two randomized controlled clinical trials (Merrill, Neuwelt et al. 2010, Rovin, Furie et al. 2012) and licensed for use in active refractory autoantibody positive SLE (EMEA 2011). The success of Belimumab in clinical trials supports a role for B cells in the pathogenesis of SLE.

B-cell depletion therapy using Rituximab continues to be used in routine clinical practice to treat refractory SLE. Many uncontrolled studies report a notable efficacy and safety in real 'life' setting. However, two randomized controlled clinical trials failed to demonstrate the efficacy of Rituximab in SLE (Merrill, Neuwelt et al. 2010, Rovin, Furie et al. 2012) although a number of reasons including the trial design may, at least partly, explain the apparent negative results (discussed later). Importantly, there were some clear signals of efficacy in these trials and moreover the success of Belimumab in clinical trials provides the impetus to explore the underlying reasons for the variability in clinical response to B-cell depletion therapy in SLE.

Rationale for B-cell depletion as a therapeutic strategy in RA and SLE

Autoreactive B cells in RA and SLE

The success of B-cell targeted therapies in RA and SLE provide direct evidence implicating B cells in the pathogenesis of these diseases. B cells promote autoimmunity through both antibody-dependent and antibody-independent mechanisms (Dorner, Giesecke et al. 2011). B cells are the precursors of antibody-secreting plasma cells and autoantibodies contribute to autoimmunity by both type II antibody-dependent cytotoxicity and also type III immune response mediated by immune complexes including chromatin-IgG-complexes involving TLR-9 and MyD88

pathways resulting in pro-inflammatory cytokine secretion such as TNF by dendritic cells (Boule, Broughton et al. 2004, Giltiay, Chappell et al. 2012). Autoantibody-independent B-cell functions include antigen presentation and secretion of cytokines, chemokines and lymphangiogenic growth factors (such as IL-10, IL-6, IFN-γ and lymphotoxin-α). However, evidence about the relative contribution of autoantibody-independent roles for B cells in SLE was derived from murine models of lupus (Chan, Hannum et al. 1999). B cells reactive against self-antigens or auto-antigens are known as 'autoreactive B cells'. Our immune system has robust mechanisms (see below) to limit the development of autoreactive B cells, but some autoreactive B cells may escape these mechanisms and contribute to autoimmunity (Giltiay, Chappell et al. 2012).

Autoreactive B cells evade stringent checkpoints in RA and SLE (Mandik-Nayak, Ridge et al. 2008, Meffre and Wardemann 2008, Shlomchik 2008) during B-cell development in the bone marrow and/or B-cell maturation in the periphery. First, naïve B cells develop from pluripotent stem cells in the bone marrow evolving through a series of clearly defined phases. B cells express B-cell receptors (BCRs) that recognize a diverse range of antigens with remarkable specificity. In humans, a majority (55-75%) of immature B cells and only a minority (6-20%) of mature B cells are autoreactive (Wardemann, Yurasov et al. 2003) suggesting that a significant proportion of immature autoreactive B cells are removed during maturation (Figure 1.1). During different phases of BCR expression, cells are screened for reactivity toward self-antigens and those with high-affinity reactivity for self-antigens are eliminated by a process known as 'clonal deletion', whereas B cells with low-affinity reactivity toward self-antigens may evade this 'checkpoint-1' of 'negative selection' by three different mechanisms (Figure 1.1). First, a process known as receptor editing, where autoreactive B cells may rearrange their receptors and express non-self reactive BCRs and evade negative selection. The other two mechanisms, 'ignorance' and 'anergy' operate in autoreactive B cells with BCRs with low affinity for self-antigens, which may evade removal by apoptotic cell death (checkpoint 2) and emigrate from the bone marrow into peripheral circulation (Wardemann, Yurasov et al. 2003, Meffre and Wardemann 2008). Thus, despite two stringent and efficient check points, autoreactive B cells may evade negative selection during the first stage of development in the bone marrow known as loss of central self-tolerance (Meffre and Wardemann 2008).

A third check point of tolerance occurs during B-cell maturation and/or differentiation in the periphery. The presence of relatively low frequency of plasma cells secreting autoantibodies compared to the frequency of autoreactive switched memory B cells in

the periphery suggest that a proportion of autoreactive memory B cells are removed as they differentiate into plasma cells (Tiller, Tsuiji et al. 2007, Scheid, Mouquet et al. 2011) (Meffre and Wardemann 2008). In the periphery, the phase of differentiation of immature and mature naïve B cells may be fostered by B cell activation factor (BAFF) and/or interferonγ–STAT mediated signaling. Plasma blasts (short-lived) and plasma cells (short- and long-lived) represent the terminal stage of differentiation and are source of potentially pathogenic autoantibodies shown in Figure 1.1.

Rheumatoid Arthritis

In treatment naive RA patients, the specificity of recombinant antibodies cloned from single B cells revealed that autoreactive B cells were present in significantly higher proportion of up to 52% of mature naïve B-cell pool compared with 20% in healthy individuals (Samuels, Ng et al. 2005). The loss of self-tolerance was evident in at least two stages: first, in the new emigrant B cells, the proportion of kappa light chains with > 11 amino acids long complementarity determining region (CDR3), a majority (> 75%) of which are self-reactive, was higher in RA patients at 5.7-17.9% compared with 0 to 4.1% in healthy individuals. This variance was due to alterations in V(D)J recombination; second, > 20 % of recombinant antibodies from new emigrant B cells were polyreactive (reactive against at least 2 of 4 antigens in the ELISA assay including single-stranded DNA, double-stranded DNA, insulin and LPS) compared to 7-9% in healthy individuals. Further, a proportion of recombinant antibodies from both new emigrant B cells and mature naïve B cells reacted with immunoglobulins and cyclic citrullinated peptides suggesting that loss of self-tolerance of B cells also occurs in the periphery during the process of maturation of new emigrant B cells into mature B cells RA (Samuels, Ng et al. 2005).

B cells expressing the 9G4 idiotype use the VH434 gene and upon differentiation secrete antibodies that selectively bind to N-acetyllactosamine residues including those expressed on B-cell antigens such as CD45, and also i and I blood group antigens and are therefore autoreactive (anjalireddy, Pugh-Bernard et al. 2001). Cambridge et al. have recently demonstrated that a greater frequency of germ line coded 9G4 positive autoreactive B cells is detectable in peripheral blood of patients with RA than undifferentiated inflammatory arthritis in both early and established disease (Cambridge, Moura et al. 2014). These findings clearly demonstrate loss of B-cell self-tolerance, potentially, increasing the prospects for activation of autoreactive B cells.

Autoreactive B cell Fate

	Development in the Bone marrow			Differentiation in the periphery	

autoreactive B cells (%): 75   25-40   10-20

targeted by anti-CD20mAbs
CD20+

check point 3
Follicular and extra-follicular

not directly targeted by anti-CD20mAbs

Immune B cell → New emigrant B cell → Mature naïve B cell → Memory B cell

CD20-
plasma blast
autoantibodies
Y Y Y Y Y Y Y
Y Y Y Y Y Y

CD20-
plasma cell
Y Y Y Y Y Y YY
Y YY Y Y Y

targeted by proteasome inhibitors (Bortezomib)
BAFF and APRIL inhibition (Belimumab and TACI-Ig)

antibody-dependent cellular cytotxicity

immune complex mediated activation of innate cells through TLR and MyD88 pathways and secretion of pro-inflammatory cytokines such as
-TNF-α, IFNs by dendritic cells
-IFNs by plasmacytoid dendritic cells and
-BAFF by monocytes

antigen presentation to autoreactive T cells
secretion of pro-inflammatory cytokines ? IL-6

Autoimmunity
BAFF
IFNγ

Receptor editing

Ignorance (< 5% receptor occupancy)

Anergy (5-45% receptor occupancy)

Developmental arrest

Reactive for low affinity auto-antigen

Reactive for high affinity auto-antigen

Auto-reactive B cell

Checkpoint
2
1

Competition for survival from non-autoreactive B cells

Apoptotic cell death

Figure 1.1 Autoreactive B cells

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So, how and where does activation of autoreactive B cells occur? Unlike, classical germinal centre pathway of activation of B cells, upon encounter with foreign antigens, activation of autoreactive RF+ B cells may occur in extra-follicular sites (William, Euler et al. 2002). Studies in mice suggest that interaction with IgG2a-chromatin immune complexes may drive activation of autoreactive RF+ B cells mediated by Toll-like receptor 9, suggesting a synergy between BCR signaling and TLR signaling in RA (Leadbetter, Rifkin et al. 2002). Alternatively, post-translational modification of histones associated with chromatin that occurs following apoptotic cell death may serve as a source of new self-antigens (Dwivedi and Radic 2014). For example, when infections trigger neutrophil death, the dying neutrophils actively trap microbial pathogens using their chromatin as a net, a process known as NETosis, which is regulated by peptidyl arginine deiminase (PAD) enzymes that modify histones associated with chromatin by converting arginine to citrulline. Therefore, deimination by PAD alters both the composition of protein and the charge by replacing the positively charged arginine with neutral charge citrulline, evoking more potent antibody binding. Thus, post-translational modifications of antigens increase the prospects for targeting by autoantibodies and provide a plausible explanation for the association between autoimmunity and infections in those with background predisposition (Dwivedi and Radic 2014).

Taken together, autoreactive B cells may escape central tolerance mechanisms in the bone marrow (Samuels, Ng et al. 2005) and may be activated by chromatin-Ig complexes through dual engagement of IgM and Toll-like receptors via the Myd88 pathway independent of help from T cells (Leadbetter, Rifkin et al. 2002, William, Euler et al. 2002). Thus, autoreactive B cells may evade self-tolerance mechanisms in RA.

Systemic Lupus Erythematosus

B cells unequivocally contribute to the pathogenesis of SLE. Although B cells may act as antigen presenting cells their involvement in the pathogenesis of SLE is also attributed to the production of a range of autoantibodies (Rahman and Isenberg 1994) that recognize a wide variety of antigens, prominent among them are those directed against nuclear components. Autoantibodies are detectable several years before the onset of the disease (Arbuckle, McClain et al. 2003), most notably anti-dsDNA antibodies, which are considered pathogenic (Ehrenstein, Katz et al. 1995, Ravirajan, Rahman et al. 1998), and anti-nucleosome antibodies (Ng, Manson et al. 2006). Autoantibodies may form immune complexes, which deposit in organs such as the kidney and cause damage and contribute to anemia and (Giannouli, Voulgarelis et al. 2006) cytopenias (Hepburn, Narat et al. 2010).

Autoreactive B cells have also been demonstrated in patients with SLE (Yurasov, Wardemann et al. 2005). Yurasov et al. found that, similar to RA, recombinant antibodies expressed from cDNA libraries of 25-50% of mature naïve B cells from patients with SLE were autoreactive compared with 5-20% in health individuals. Also, mature naïve B cells from patients with SLE expressed significantly higher proportion of polyreactive antibodies compared with healthy controls. In contrast, there was no significant difference in the antibodies expressed by new emigrant B cells from patients with SLE and healthy individuals (Yurasov, Wardemann et al. 2005). Furthermore, autoreactive B cells expressing 9G4 idiotype evade negative selection in the germinal centre in SLE, but not in healthy individuals or those with RA (Cappione, Anolik et al. 2005).

A series of observations suggests that abnormal germinal center (GC) and post-GC reactions are critical in the development of SLE (Grammer, Slota et al. 2003). B cells whilst undergoing somatic hypermutation may randomly express autoreactive receptors and differentiate into autoreactive IgG antibody secreting cells (Mietzner, Tsuiji et al. 2008). Further, autoreactive B cells may overcome negative selection by the FasL pathway through expression of CD95 (Jacobi, Reiter et al. 2008). Intriguingly, in a murine mouse model of SLE, it was shown that autoreactive B cells, but not non-autoreactive B cells, utilize a B-cell intrinsic type I IFNγ-STAT1 pathway to form germinal centres through induction of T-bet and recruitment of T follicular helper cells (Domeier, Chodisetti et al. 2016, Jackson, Jacobs et al. 2016). In this context, it was shown that, in vitro, defective secretion of IL-10 by regulatory B cells may allow plasmacytoid dendritic cells from patients with SLE to secrete type I IFN-alpha, a pathway that may promote the formation of memory B cells (Menon, Blair et al. 2016). Taken together, these findings suggest that peripheral check point of B-cell tolerance is defective in SLE.

Autoreactive B cells may also be rescued by cytokines BAFF and type I IFN (Mackay and Browning 2002, Kiefer, Oropallo et al. 2012) and they express lower levels of BAFF-receptor compared to non-self reactive B cells (Lesley, Xu et al. 2004, Thien, Phan et al. 2004). Therefore, increased expression of BAFF, reported in autoimmune disease such as SLE and Sjogren's syndrome, may provide a survival advantage for autoreactive B cells, probably through increased expression of anti-apoptotic protein Bcl-2 as suggested by studies in BAFF-transgenic mice (Batten, Groom et al. 2000).

Central to sustained autoreactivity in SLE is the abundant availability of self antigens which accumulate due to defective clearance of apoptotic material secondary to abnormalities in the complement system (Manderson, Botto et al. 2004) and/or

phagocytosis (Baumann, Kolowos et al. 2002) or complement-independent sera factors (Cortes-Hernandez, Fossati-Jimack et al. 2002). Accordingly, alterations in B cell signaling and the composition of B-cell subpopulations have been described in patients with active SLE (Odendahl, Jacobi et al. 2000, Dorner and Lipsky 2006) including increased frequency of activated memory B cells identified as IgD-CD27-CD95+ and CD27 (high) plasma cells (Jacobi, Odendahl et al. 2003, Wei, Anolik et al. 2007, Dorner, Jacobi et al. 2011). Thus, B cells are involved in many different pathways of SLE (Dorner, Giesecke et al. 2011) providing the rationale to target B cells for the treatment of SLE (Edwards and Cambridge 2006).

Regulatory role for B cells in RA and SLE

In addition to the 'effector role' of antibody production, it has been proposed that IL-10 secreting B cells also play a 'regulatory role' and that defects in the number and/or function of regulatory B cells, in vitro, correlates with disease activity in RA and SLE (Blair, Norena et al. 2010, Flores-Borja, Bosma et al. 2013, Menon, Blair et al. 2016). Proposed regulatory mechanisms are that CD24hiCD38hi B cells may: a) secrete anti-inflammatory cytokine IL-10 and / or; b) influence T cell differentiation toward an anti-inflammatory profile and/ or; c) fail to deter plasmacytoid dendritic cell function, as in the case of SLE, as discussed earlier. Although, these in vitro models suggest a regulatory role for B cells through plausible effector pathways, it is not clear as yet, to what extent regulatory B cells influence the outcome of clinical disease in RA and SLE. Perhaps a balance between pathogenic and regulatory B cells exists in inactive disease, which is perturbed in active disease. Regardless, the success of B-cell depletion therapy argues against a clinically relevant role for regulatory B cells in active, refractory RA and SLE, where B-cell depletion therapy is effective.

Hypothesis for B-cell targeted therapies

A simple hypothesis is that B cells contribute to the production of pathogenic autoantibodies and therefore removal of 'pathogenic' B cells would interrupt their production. In particular, the IgM isotype, which is considered to be more dependent on differentiation of B cells to new plasma cells and thus the removal of B cells, would interrupt this process, in turn increasing the prospects for clinical response.

An alternative hypothesis is the "self-perpetuating B-cell hypothesis" for RA proposed by Edwards and Cambridge (Edwards, Cambridge et al. 1999), on which basis

rituximab was first used to treat RA at University College London (UCL). The rationale behind this hypothesis are: 1) autoreactive B cells produce RFs, which in turn drive the survival of autoreactive B cells, i.e., 'antibody (RF)' is also the 'antigen'; 2) RF-related antigen-antibody (immune) complexes are small in size and do not activate complement, but are potent activators of effector cells such as macrophages; 3) macrophages in the sites of RA activity selectively overexpress the low affinity activating FcγRIIIa (Bhatia, Blades et al. 1998). Therefore, soluble RF-immune complexes, without the requirement of B cells to invade sites of inflammation, may gain entry to the sites of inflammation and activate effector cells, such as macrophages and monocytes to secrete inflammatory cytokines such as TNF (Abrahams, Cambridge et al. 2000). This study supports the notion that RFs are pathogenic and mediate a plausible effector pathway for driving inflammation in RA.

As discussed earlier, B cells contribute to autoimmunity through a diverse range of functions including giving rise to antibody secreting cells, interactions with other immune cells and forming lymphoid structures, illustrated in Figure 1.2. Therefore, B cells were targeted for therapy in RA and SLE.

B cells may contribute toward autoimmunity in several ways. Predominantly, B cells differentiate into plasma cells, which may secrete antibodies leading to the formation of immune complexes that can activate other FcγR-bearing immune cells or deposit in tissues leading to complement and/or effector cell mediated tissue injury. Autoreactive B cells may recruit and/or activate T cells and other immune cells through antigen presentation, secretion of pro-inflammatory cytokines or contribute toward formation of ectopic lymphoid architecture and aid T follicular helper cell survival.
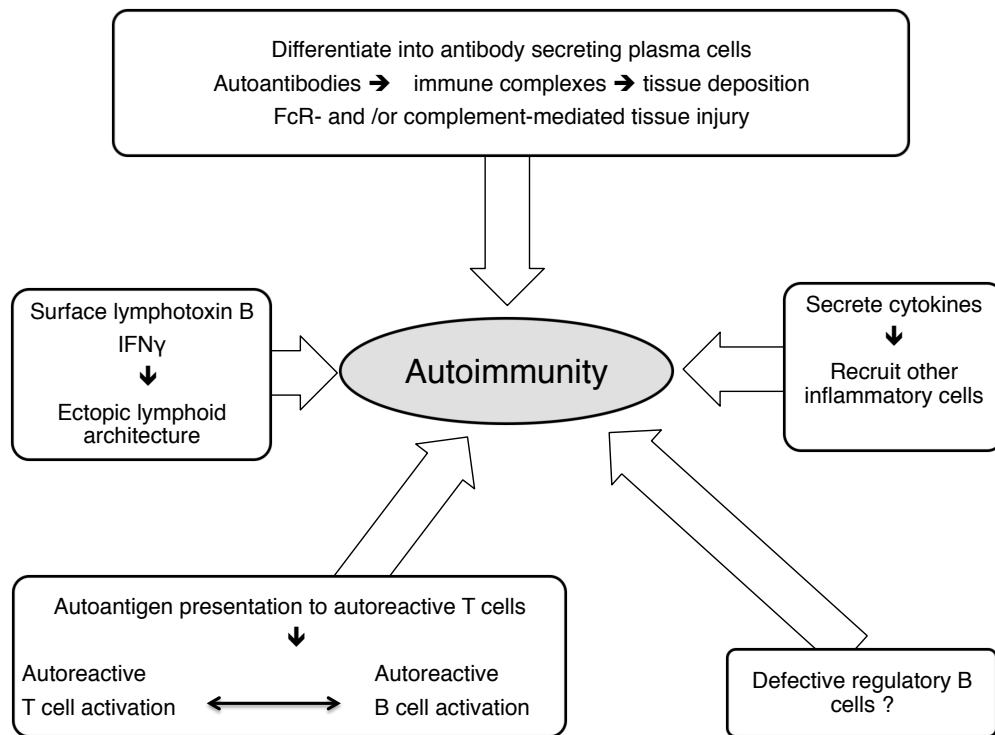
Figure 1.2. B cell pathways in autoimmunity.

Strategies to target B cells

Selective targeting of pathogenic B cells, leaving non-pathogenic and regulatory B cells intact, although desirable, remains impractical given the lack of reliable markers that clearly distinguish pathogenic from non-pathogenic B-cells. As discussed earlier, the development, differentiation and survival of B cells is influenced by a number of factors and therefore B cells may be directly targeted through modulation of their function and/or survival or through deletion. B cell survival may be modulated using anti-BAFF (anti-BLyS) mAb; Belimumab (discussed later). B cell differentiation may also be targeted by: inhibiting the interferon pathway; blocking IL-6 receptor; and interfering with their interaction with T cells by blocking co-stimulation using CTLA-4 Ig, abatacept (Merrill, Burgos-Vargas et al. 2010) or ICOSL mAb, AMG557 (Sullivan, Tsuji et al. 2016). Other strategies for targeting B cells include antagonizing the interaction of antibodies and immune complexes with FcγR-bearing effector cells (Browning 2006), illustrated in**Error! Reference source not found.** Figure 1.3.

A number of studies are in progress evaluating the safety and efficacy of anti-IFN therapy using monoclonal antibodies and/or therapies that target TLR signaling to modulate the function of plasmacytoid dendritic cell function in SLE (Kirou and

Gkrouzman 2013). In SLE, early studies using tocilizumab, a monoclonal antibody that binds the IL-6 receptor, involving a small number of patients demonstrated biological and clinical benefits (Illei, Shirota et al. 2010), further studies are warranted. Trials with Bortezomib, a proteosome inhibitor that targets plasma cells are also in progress for the treatment of refractory SLE (Alexander, Sarfert et al. 2015). Thus, a number of strategies that target B cells indirectly are ongoing and the long-term safety and efficacy of these therapies is not known, however, a significant body of evidence is available on the safety and efficacy of B-cell depleting therapies in SLE.
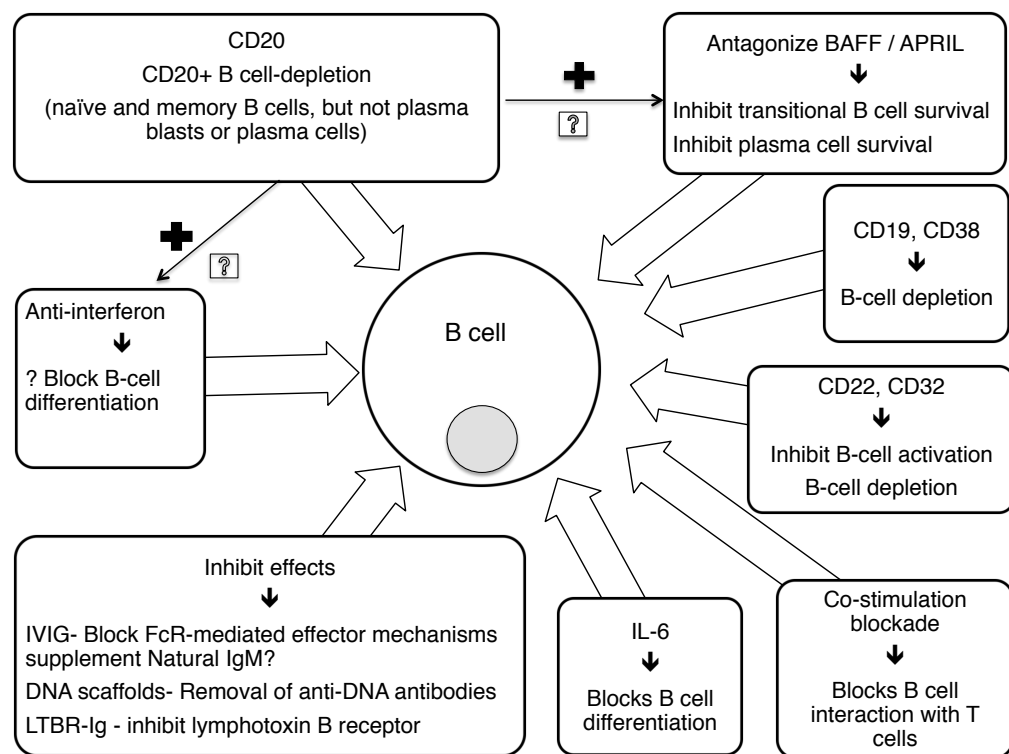
Figure 1.3. **Strategies to target B cells** Current strategies to target B cells use monoclonal antibodies directed against B-cell surface antigens: CD19, CD20 and CD38, triggering B-cell depletion; CD22 and CD32, to inhibit B-cell activation and/or induce modest B-cell depletion; or indirectly target B-cell survival by antagonizing BAFF. Anti-IL6 and anti-IFN therapies block B-cell differentiation pathways. Other strategies include: blocking co-stimulation with other immune cells using CTLA-4-Ig; or antagonizing the actions of antibodies and immune complexes on FcγR-bearing effector cells using intravenous immunoglobulins (IVIG) or by supplementing natural IgM; removing anti-DNA antibodies or inhibiting lymphotoxin β receptor using LTBR-Ig.

Experience with biological agents targeting B cell survival

In two randomized controlled trials involving > 700 patients in each trial, belimumab has met its primary end point with a 10% and 14% absolute difference in clinical response over placebo (Furie, Petri et al. 2011, Navarra, Guzman et al. 2011). BAFF seems to be important for plasma cells whereas memory B cell survival appears to be independent of BAFF and APRIL (Benson, Dillon et al. 2008), accordingly, Belimumab therapy resulted in a reduction in anti-dsDNA antibodies. The success of Belimumab in trial setting also supports the notion that B cells make a significant contribution to the pathogenesis of SLE. However, a phase III study of tabalumab, a human mAb that neutralizes membrane and soluble BAFF, was not effective in RA (Smolen, Weinblatt et al. 2015) and in SLE delivered modest clinical response rates (Merrill, van Vollenhoven et al. 2016). Further, Atacicept, an agent that targets both BAFF and another B cell survival cytokine APRIL (a proliferation inducing ligand), despite significant biological activity, did not achieve significant clinical efficacy in RA (van Vollenhoven, Kinnman et al. 2011) and SLE (Isenberg, Gordon et al. 2015). Thus, available evidence about clinical efficacy of strategies that target B cell survival through BAFF-pathways is limited to Belimumab whereas alternative agents elicited, if at all, surprisingly little clinical efficacy in both RA and SLE.

B cells have also been directly targeted using several therapeutic monoclonal antibodies (mAbs) such as those targeting B cell surface antigens CD19, CD20, CD22 and CD32 (FcγRIIb) in RA and/or SLE. Other modalities of targeting B cell antigens will not be considered here. The effects of these mAbs on B cells are variable and important to consider for optimising desired therapeutic effect. Potential effects on B cells by mAbs targeted against B cell antigens include inhibition (antagonistic) or activation (agonistic) or opsonisation to trigger their removal by other effector mechanisms (depletion) shown in Figure 1.4. Some mAbs may induce a combination of these effects. However, the efficiency of mAbs in evoking these effects is dependent on the target antigen as well as the epitope targeted by the specific mAb that collectively determine whether mAbs remain at the cell surface (opsonisation) or are internalised by target cells or shed from the cell surface. The latter two processes reduce cell surface accessible mAbs and thereby potentially reduce the efficiency of B-cell depletion by mAbs (discussed later).

Anti-CD22 mAb (Epratuzumab)

CD22, is a B cell specific antigen that negatively regulates BCR signaling (Doody, Justement et al. 1995). CD22, undergoes constitutive endocytosis, rapidly (Shan and

Press 1995), and may also function as a recycling receptor (O'Reilly, Tian et al. 2011). Therefore immune-conjugates targeting CD22 are being explored for use in B cell malignancies (Linden, Hindorf et al. 2005). Another potential mechanism of action of Epratuzumab is by altering B cell migration through trogocytosis of adhesion molecules (Daridon, Blassfeld et al. 2010). However, two phase III studies investigating Epratuzumab did not confirm efficacy in patients with SLE (Iowse MEB 2015).

Therapeutic monoclonal antibodies (mAbs) directed against B cells may: remain surface-bound triggering B-cell depletion; interfere with B cell signaling leading to either B-cell inhibition or activation. Alternatively, mAbs may be lost from the cell surface along with the target antigen (shedding) or antigen-mAb complexes may be internalised. Both shedding and internalisation are detrimental to B-cell depletion mediated by effector cells unless the mAbs are conjugated with toxins when they may induce cell death.

Anti-CD19 mAb

CD19, is another antigen that was selected for targeting B cells. CD19 associates with the B cell receptor complex (Pesando, Bouchard et al. 1989) and regulates Src protein tyrosine kinase activation in B cells (Fujimoto, Fujimoto et al. 2000) thereby influencing the threshold for BCR-mediated B cell activation (Tedder, Inaoki et al. 1997), consequently, autoantibody production (Inaoki, Sato et al. 1997). CD19 is expressed at all stages of B cell differentiation from late pro-B cells to some extent on plasma cells (Mei, Schmidt et al. 2012). Therefore, anti-CD19 mAb is thought to eliminate more diverse B cell subpopulations than anti-CD20 mAbs, at least in pre-clinical studies (Tedder 2009). In vitro studies suggest that a bi-specific mAb targeting CD19 and CD32b targets both BCR-mediated and TLR-mediated activation of B cells, potentially useful for application in RA and SLE (Karnell, Dimasi et al. 2014). However, as yet, there is no clinical evidence about the safety and efficacy of these mAbs. By contrast, there is substantial evidence about the safety and efficacy of B-cell depletion therapy in refractory RA (Edwards, Szczepanski et al. 2004) and safety data in refractory SLE.
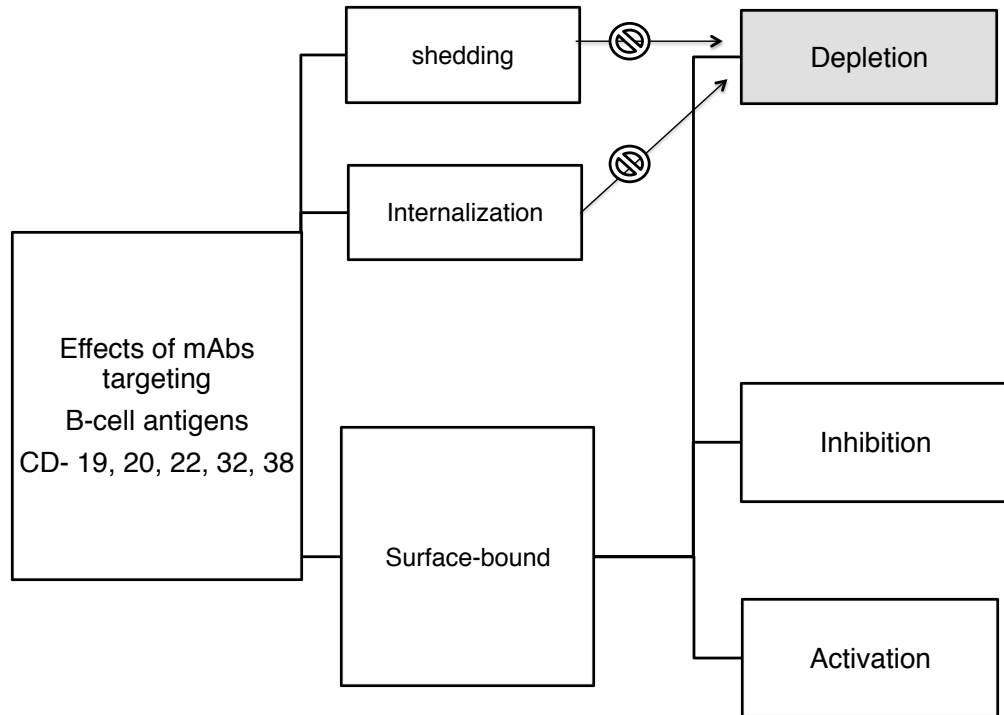
Figure 1.4 Effects of B-cell targeted mAbs.

B-cell depletion with anti-CD20mAbs: clinical and trial experience

In 1997, rituximab, a mouse-human chimeric anti-CD20 monoclonal antibody (mAb) with human IgG1 and kappa constant regions and of mouse variable regions, was licensed for use for the treatment of refractory non-Hodgkins Lymphoma (NHL), a CD20+ B-cell malignancy. It was shown that transient B-cell depletion with rituximab did not result in severe infections or excessive decrease in serum Ig levels (McLaughlin, Grillo-Lopez et al. 1998), probably because anti-CD20 mAb therapy does not deplete plasma cells as they lack CD20 expression (DiLillo, Hamaguchi et al. 2008) (discussed later). These observations provided some safety basis for the development of a rituximab-based B-cell depletion therapy protocol for the treatment of refractory RA and SLE.

Rheumatoid Arthritis

Uncontrolled studies

The first pilot study of rituximab-based B-cell depletion therapy (BCDT) in RA consisted of five patients with refractory seropositive RA who showed clear signs of clinical response (Edwards and Cambridge 2001). The treatment regimen used a combination of rituximab, cyclophosphamide and oral steroids. All five patients responded to

treatment, but even in this small study there was variability between patients in the extent of improvement noted and duration of response. The study was then extended to 22 patients with refractory RA, treated with various doses of rituximab, with or without cyclophosphamide or oral corticosteroids (Leandro, Edwards et al. 2002). The results of this study suggested that a minimum dose of rituximab required was 600 mg/m^2. These data informed the design of further studies.

Randomised controlled trials

Encouraged by the efficacy of rituximab in the preliminary uncontrolled studies, a multicenter pivotal randomized controlled trial (RCT) of 161 patients with active RA refractory to methotrexate confirmed the efficacy of rituximab-based BCDT in RA (Edwards, Szczepanski et al. 2004). At least 41% of patients receiving rituximab achieved 50% improvement in the American College of Rheumatology (ACR) responses when compared with 13% for methotrexate alone. Subsequently, rituximab was shown to be effective also in patients with RA refractory to anti-TNF agents (Cohen, Emery et al. 2006). The robust safety record of rituximab in RA, which is comparable to that of other biologics used to treat RA such as anti-TNF agents, has reinforced BCDT as an important therapeutic strategy. Consequently, rituximab was licensed and incorporated into the guidelines for routine clinical care of patients with refractory RA.

An alternative B-cell depleting agent, a humanised anti-CD20 mAb, ocrelizumab, tested in two regimens of 200mg and 500mg x 2 every six months, also showed efficacy in combination with a stable dose of methotrexate in reducing signs and symptoms and joint damage (Rigby), (Tak 2010). However, a detailed analysis of the results from 4 randomised, double-blind placebo-controlled trials investigating the safety and efficacy of ocrelizumab for RA, indicated dose-dependent increase in serious infections associated with ocrelizumab compared to placebo, particularly in Japanese patients (Emery 2010). Similarly, a fully human anti-CD20 mAb, Ofatumumab, was also shown to be effective in RA at six months after two doses of 700mg given two weeks apart (Taylor, Quattrocchi et al. 2011).

Area of unmet need

Of clear clinical importance is that a significant number of patients (up to a third) with RA do not achieve a favorable response to rituximab-based BCDT. Knowledge of factors associated with poor clinical response to rituximab in RA would help improve clinical response to BCDT and will be discussed later.

Systemic Lupus Erythematosus

Uncontrolled studies

Similar to RA, early evidence of rituximab for refractory SLE was obtained from uncontrolled studies at University College London (UCL) (Leandro, Edwards et al. 2002, Leandro, Cambridge et al. 2005, Ng, Cambridge et al. 2007), Looney's team (Looney, Anolik et al. 2004) and Eisenberg's team (Albert, Dunham et al. 2008) followed by several other groups (Ramos-Casals, Soto et al. 2009). The initial pilot open study to investigate the safety and efficacy of rituximab in six patients with refractory SLE (Leandro, Edwards et al. 2002) at UCL consisted of two 500-mg doses of rituximab and two 750-mg doses of intravenous cyclophosphamide plus prednisolone 30 mg or 60 mg for 5 days. This study showed improvements in both clinical and laboratory features of disease following treatment with rituximab in patients with refractory SLE and these observations have been supported by other non randomised studies (Leandro, Cambridge et al. 2005, Ng, Leandro et al. 2006, Smith, Jones et al. 2006, Ng, Cambridge et al. 2007, Catapano, Chaudhry et al. 2010).

In a phase I/II dose-escalation trial of the safety and efficacy of rituximab in combination with ongoing therapy in 18 patients with SLE, were studied employing three dosing regimens of rituximab as follows: 6 patients received a low dose - a single infusion of 100 mg/m^2; 6 patients received an intermediate dose - a single infusion of 375 mg/m^2; and 5 patients received a high dose (standard lymphoma dose) - 4 infusions of 375 mg/m^2 administered a week apart (Looney, Anolik et al. 2004). There was a significant improvement in the disease activity, as measured by SLAM scores, in all patients by 2 months, which persisted at 12 months, regardless of changes in anti-ds-DNA antibody and complement levels. Six of 17 patients developed human anti-chimeric antibodies (HACAs) associated with lower serum rituximab levels and inefficient B-cell depletion and less impressive efficacy. Importantly, there were no significant adverse events (Looney, Anolik et al. 2004). Further, repeat therapy also showed that improvements in disease, including remission rates, were observed upon retreatment in patients who responded to the initial treatment (Turner-Stokes, Lu et al. 2011). A review of the rituximab experience in approximately 200 patients with refractory SLE, from open studies and real clinic experience indicated that many responded at least partially to B-cell depletion (Ramos-Casals, Diaz-Lagares et al. 2009).

The UK-BIOGEAS registry study of 164 patients with refractory or relapsing lupus nephritis reported a 67% partial or complete response rate to rituximab using

standardised response criteria (Diaz-Lagares, Croca et al. 2011). Therefore, clinicians continue to use rituximab for refractory lupus nephritis as well as non-renal manifestations including the haematological, skin and central nervous system manifestations where clinically useful responses have been reported (Tokunaga, Saito et al. 2007, M Zandi 2010). Thus, there is extensive experience of rituximab from non-randomised and retrospective studies in the treatment of refractory SLE (Table1.1). A role for rituximab for this indication is supported by the consistency of the reports of improvement. However, the differences in treatment regimens; concomitant medications and end-points limit accurate comparison about the extent of effectiveness of B-cell depletion in uncontrolled studies.

Randomised controlled trials

The safety and efficacy of rituximab in SLE in a clinical trial setting, is available from two double blind, randomised, placebo-controlled trials (DBRCT) investigating renal (LUNAR trial) and non-renal (EXPLORER trial) manifestations. Both trials addressed the hypothesis that the addition of rituximab to standard of care with corticosteroids and immunosuppressants was superior to the addition of placebo for the control of SLE activity.

In the EXPLORER study, the safety and efficacy of rituximab 1,000 mg on days 1, 15, 168, and 182, in moderate-to-severe active non-renal SLE was evaluated (Merrill, Neuwelt et al. 2010). In the intent-to-treat analysis of 257 patients, there was no difference between the addition of placebo and rituximab to the standard of care in the primary and secondary efficacy end points. However, a pre-planned subgroup analysis detected a beneficial effect of rituximab in the primary end point, in the African American and Hispanic patients, a major clinical response in 13.8% and a partial response in 20% when compared with 9.4% & 6% respectively. There were significant biological effects in the rituximab treated group, with greater falls in anti-dsDNA levels with a median 76% and 55% in rituximab and standard treatment groups, respectively. Serum complement (C3) levels rose with a median 129% vs 114%, in rituximab and standard treatment groups, respectively, were also noted. Interestingly, up to 9.5% of patients did not achieve complete B-cell depletion as defined in the trial (discussed later), however, analysis without these patients did not change the primary outcome.

The second study, (LUNAR), investigated the safety and efficacy of rituximab 1,000 mg on days 1, 15, 168, and 182, as compared to placebo in addition to background therapy with high dose glucocorticoids and mycophenolate mofetil 3 g / day in 144 patients with proliferative lupus nephritis, class III and IV. The response rates for

rituximab and placebo were 26% vs 30% and 30% vs 15%, for complete response rate and partial response rate, respectively. At week 52, more patients in the placebo arm, 8 versus 0 in the rituximab arm received rescue -cyclophosphamide therapy. Improvement in proteinuria was 32% and 9% for rituximab and placebo, respectively. Although there was a greater reduction in anti-dsDNA levels in rituximab-treated group, this was a negative study in that there was no significant difference between the rituximab and the placebo group. The absolute difference in response was 11%, with 54% and 43% responding in the rituximab and placebo groups respectively (Rovin, Furie et al. 2012). Thus, despite some clear signals of efficacy and safety, this study did not meet its primary or secondary end points.

The planned efficacy margin in LUNAR was influenced by the response rates in the ALMS trial at 6 months of 56% and 53% in patients treated with MMF or cyclophosphamide, respectively. This suggested that 45% did not respond to standard of care, however, reasons for 'failure' in ALMS included death, severe adverse events, drug intolerance and patient/physician preference. It can be estimated that true treatment failure was closer to 25% than 45%. A further factor in nephritis trials is the delayed response of the outcome measure, proteinuria, to reduction in histological activity in the kidney. The true time to remission of proteinuria is up to two years. Had LUNAR aimed for a 12% efficacy difference and had a two-year duration it may well have met its end-point despite a modest sample size.

The other anti-CD20 mAb investigated in clinical trials for SLE is ocrelizumab. Two simultaneous clinical trials were initiated to study the safety and efficacy in SLE. Ocrelizumab was dosed differently than in RA with either 400 or 1000mg IV x 2 at entry with repeat, single dosing every four months on a background of high dose glucocorticoids and either mycophenolate mofetil or cyclophosphamide dosed according to the EUROLUPUS protocol. Although designed to continue for at least two years the trial was stopped early due to an imbalance in the rate of serious infections in the ocrelizumab patients receiving mycophenolate. 221 patients who had passed the 32-week treatment point were assessed. The absolute difference in renal response was 12% with 63% and 51% for the combined ocrelizumab and placebo groups prospectively. However, it is worth noting that in the subgroup analysis there was a greater treatment effect of ocrelizumab when combined with the EUROLUPUS cyclophosphamide regime (renal response of 65.7% for ocrelizumab versus 42.9 % for EUROLUPUS alone) than with MMF (renal response of 67.9% for ocrelizumab versus 61.7% for MMF alone) which was largely explained by a higher response rate in

general with MMF whilst perhaps again reflecting the outcome seen with rituximab in the LUNAR study (Mysler 2010).

Area of unmet need

Why did the DBRCTs fail to meet their end points? Thus, despite some clear indicators of efficacy and safety, these studies did not meet the primary or secondary end points (Table 1.2). Several confounding factors may have masked the ability to quantify accurately any significant, clinically meaningful, beneficial effects of rituximab (Reddy V, Jayne et al. 2013), perhaps, the most important being the aggressive background immunosuppressive therapy in the placebo and rituximab treated groups.

Thus, clinical and trial experience provides evidence of remarkable variability in clinical response to rituximab in RA and in particular in SLE. Therefore, it would be important to consider whether variability in biological response to rituximab occurs; and explore the relationship between variability in biological and clinical response to rituximab in patients with RA and SLE.

Table 1.1 The reported efficacy of rituximab in uncontrolled studies in SLE

Study reference	rituximab-regimen	Organ-specific disease	no of patients/ follow-up (months)	Method of assessment (mean disease activity score before/after BCD)
(Leandro, Edwards et al. 2002)	2-dose	No	6/6	Median BILAG score improved from 14 to 6
(Anolik, Barnard et al. 2004); Looney et al (2004)	variable	No (7 LN)	17/12	SLAM improved in patients achieving effective BCD 6.8/5.2
(Leandro, Cambridge et al. 2005)**	2-dose	No (17/19 LN)	19/6	BILAG 13.9/5
(Sfikakis, Boletis et al. 2005)	4-dose	Yes, LN	10/12	Renal parameters
(Gottenberg, Guillevin et al. 2005)	4-dose	No (4 LN)	13/8.3	SLEDAI 8/2
(Vigna-Perez, Hernandez-Castro et al. 2006)	2-dose	Yes, LN	22/3	Mexico-SLEDAI (10.8/6.8)
(Cambridge, Leandro et al. 2006)**	2-dose	No (12/15 LN)	15/6	BILAG
(Smith, Jones et al. 2006)	4-dose , re-treated with 2-dose	No	11/24	BILAG 14/2
(Gunnarsson, Sundelin et al. 2007)	4-dose	Yes, LN	7/6	SLEDAI (15/3)
(Ng, Cambridge et al. 2007)**	2-dose	No (21 LN)	32/39	BILAG (13/5)
(Tokunaga, Saito et al. 2007)	variable	Yes, NPSLE	10/(7-45)	Neurological parameters (GCS)
(Tanaka, Yamamoto et al. 2007)	2-dose	No (6 LN)	14/7	BILAG (12.5/7.1)
(Tamimoto, Horiuchi et al.	variable	No	8	SLEDAI 17.6/7.3

2008)		(4/8 LN)		
(Galarza, Valencia et al. 2008)	4-dose	No	43/12	SLEDAI (12.5/4.5)
(Jonsdottir, Gunnarsson et al. 2008)	4-dose	No (10 LN)	16/27	SLEDAI (12.1/4.7)
(Lindholm, Borjesson-Asp et al. 2008)	4-dose	No (17 LN)	29/22	Renal parameters
(Sutter, Kwan-Morley et al. 2008)	4-dose	No	12	SLEDAI (9/5)
(Reynolds, Toescu et al. 2009)	variable	No	11/10	BILAG (median reduction of 7.5)
(Lu, Ng et al. 2009)**	2-dose	No (33/45 LN)	45/39.6	BILAG (12/5)
(Pepper, Griffith et al. 2009)	2-dose +MMF maintainence	Yes, LN	20/12	Renal parameters improved in 14/18 at 12 months
(Catapano, Chaudhry et al. 2010)	4-dose (15) or 2-dose + CYC (16)	No (11 LN)	31/30	BILAG 14.5/3.5 at 24 months
(Boletis, Marinaki et al. 2009)	4-dose	Yes, LN	10/38	Renal parameters
(Melander, Sallee et al. 2009)	4-dose (10 re-treated)	Yes, LN	20/22	12/20 improved

BCD, B-cell depletion ** same cohort in these studies CYC, cyclophosphamide; LN, lupus nephritis; MMF, mycophenolate mofetil; BILAG, British Isles Lupus Assessment Group; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index

Table 1.2 A summary of the randomised-controlled trials of rituximab therapy in SLE

Study reference	Rituximab regimen	Concomitant therapy	End points (EP)	Results
LUNAR (Rovin, Furie et al. 2012)	Randomised 1:1 to receive either rituximab or placebo on days 1, 15, 168, and 182	MMF and corticosteroids	Primary EP: (i)% patients with complete or partial renal responses at week 52; secondary EP: (ii)patients with BL UPCR>3 to UPCR<1, (iii)% change from BL in anti-dsDNA and (iv)Mean change from BL in C3 (mg/dL)	Primary EPs: (i) and (ii)-no significant difference; (iii)-placebo (50) and rituximab (69) 9p=<0.01) and (iv)- placebo (25.9) and rituximab (37.5) (p<0.03). % patients requiring a new immunosuppressive agent placebo (11.1) and rituximab (1.4)
EXPLORER (Merrill, Neuwelt et al. 2010)	randomised 1:2 to receive placebo or rituximab ,	usual dose prednisolone and either Azathioprine maintaining a major, partial or no response at week 52 in each of the 8 BILAG index organ system	Primary EP: the effect of placebo or rituximab in achieving and maintaining a major, partial or no response at week 52 in each of the 8 BILAG index organ system	Primary EPs: MCR-15.9% vs 12.4% and PCR- 12.5% vs 17.2%, for placebo and rituximab, respectively. In the African American/Hispanic group: MCR- 9.4% vs

| | | methyl prednisolone 100mg and acetaminophen | either prednisolone 100-250mg/day, MMF 1-4g/day or MTX 7.5- | |

Reference	Intervention		Endpoints	Results
	and diphenhydramine or placebo on days 1, 15, 168, and 182	27.5mg/week, and additional prednisolone (0.5 mg/kg, 0.75 mg/kg, or 1.0 mg/kg), tapered beginning on day 16 to a dosage of 10 mg/day over 10 weeks and 5 mg/day by week 52.	scores. Secondary end points have been described earlier.	13.8% and a PCR-6.3% vs 20.0%, for placebo and rituximab, respectively.
(Li, Tam et al. 2009)	randomised to receive either rituximab or a combination of rituximab and cycylphosphamide 750mg on day 1 and day15, followed by i.V methylprednisolone 250mg and oral prednisolone 30mg	Other medications were stopped except for Hydroxychloroquine, oral prednisolone and statins. All patients also received angiotensin-converting enzymes inhibitors.	Primary EP: In each of the groups, % patients with complete response at week 48. Secondary EP: % patients with (i) and the duration of complete CD19+ B lymphocyte depletion, histological assessment, adverse effects or death at we4k 48.	Primary EP: no significant difference between the two groups. Overall, at week 48, 21% had a complete response, 58% achieved partial response, 11% worsened. Secondary EP: 42% patients achieved a complete response; 95% achieved effective depletion; no significant difference in the proportion of patients achieving a complete depletion at weeks 4, 8, 24 and 48 between the two groups except at week 2; a significant improvement in mean serum albumin levels

from day 2 to day 5, then 0.5mg/kg for 4 weeks and then reducing the dose by 5mg every 2 weeks to 5mg/day.	(28.1 to 39.4), changes in the concentration of serum C3 (0.55 to 0.85), ds DNA antibody (693 to 8) and immunoglobulins. At week 48, the urinary protein excretion improved and there was an improvement in ESR (62.1 to 30) and SLEDAI (9.2 to 2.5)

EP, end point; BL, baseline; UPCR, urine protein-to-creatinine ratio; MCR, major clinical response; MMF=mycophenolate mofetil; BL=baseline; MCR=major clinical response; MTX= methotrexate; BILAG, British Isles Lupus Assessment Group; SLEDAI, Systemic Lupus Erythematosus Disease Activity

Variability in Biological Response to Rituximab: What Does It Mean & Why does It Matter?

Biological response to rituximab refers to whether rituximab treatment resulted in effective depletion of B cells whereas clinical response to rituximab refers to whether a pre-defined clinical response was achieved regardless of the biological response. To understand variability in clinical response to rituximab it is fundamentally important to distinguish biological response from clinical response. Poor biological response represents resistance to rituximab whereas poor clinical response despite good biological response probably represents rituximab-refractory disease (Reddy and Leandro 2014).

Variability in peripheral & tissue B-cell depletion and relationship with clinical response

Variability in clinical response noted in early studies of rituximab in refractory RA provided some clues for understanding the potential reasons for the variability in clinical response (Table 1.3). In the early study of 22 patients with RA treated with various doses of rituximab (Leandro, Edwards et al. 2002), two patients seronegative for rheumatoid factor (RF) did not respond but had received a lower dose of rituximab. The extent of depletion correlated with clinical response with greater depletion associated with better clinical response. The time to relapse from repopulation of B cells also varied from 0 to 17 months (Cambridge, Leandro et al. 2003). Relapses were preceded by or coincided with B-cell repopulation detectable in peripheral blood even after repeated treatments suggesting a close relationship between peripheral B-cell depletion and clinical response.

B-cell depletion in the tissues also appears to be variable. In a small number of patients with RA, at 3 to 4 months after treatment, there was variability in the degree of B-cell depletion and in the proportion of CD19+ cells and of different B-cell subpopulations in the bone marrow (Leandro, Cooper et al. 2007). Several groups have reported on the variability in B-cell depletion achieved in the synovium (Teng, Levarht et al. 2007, Walsh, Fearon et al. 2008, Teng, Levarht et al. 2009, Thurlings, Teng et al. 2010). Kavanaugh and colleagues, in a small number of patients found a trend for greater synovial depletion of B cells at 8 weeks after rituximab in patients achieving ACR50 response compared to those with poor response (Kavanaugh, Rosengren et al. 2008).

Table 1.3 Variability in biological and clinical response to rituximab

Variability in parameters	Comments	Condition, number of patients and reference
Degree of B cell depletion Development of HACAs	The degree of depletion did not entirely depend on the dose of rituximab used. The development of HACAs was associated with lower rituximab levels	SLE (n=17) (Looney, Anolik et al. 2004)
Time to B cell repopulation Relationship between clinical relapse and repopulation	Time to clinical relapse from B cell repopulation varied from 0-17months	RA (n=22) (Cambridge, Leandro et al. 2003)
The decrease of anti-ds DNA antibody levels from baseline	The extent of decrease in anti-dsDNA antibody levels was greater in responders	SLE (n=16) (Cambridge, Leandro et al. 2006)
Duration of peripheral B cell depletion ranged from 3-8 months	One patient remained depleted at 4 years	SLE (n=24) (Leandro, Cambridge et al. 2005)
Bone marrow B-lineage cells at 3 months after rituximab	Variability was noted in the frequency of pro- and pre-B cells and there was a trend towards better response in patients with better depletion	RA (n=6) (Leandro, Cooper et al. 2007)
degree of depletion HACAs	BCD (< 5 cells/µl) correlated with clinical response and inversely with HACAs; HACAs were higher in 3 patients treated with low dose rituximab (100mg)	SLE (n=18) (Albert, Dunham et al. 2008)

response to immunization	no correlation between FcgRIIIa polymorphism and clinical response 9 of 14 paitents did not respond to immunization with pneumovax 7 months after treatment with rituximab	

SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; HACA, human antichimeric antibody; BCD, B cell depletion; SLEDAI, systemic lupus erythematosus disease activity index.

Although as yet there is no conclusive link between non-depleted B cells contributing to poor response in RA, it is worth noting that such a phenomenon has been implicated in chronic active antibody mediated graft rejection (renal transplant) (Thaunat, Patey et al. 2008) and in Sjogren's syndrome (Quartuccio, Fabris et al. 2008) where poor depletion was attributed to local production of BAFF considered to protect against rituximab-induced apoptosis.

Similar to RA, in SLE also clinical relapse occurred either at the time of or after B-cell repopulation. However, there was considerable variability between patients in the duration of B-cell depletion and in the interval between B-cell repopulation and clinical flare. Prolonged periods of B-cell depletion (CD19 count < 10 cells / μl for > 12months) was noted in 15 patients with one patient not having repopulated for 7.5 years after treatment with rituximab whereas another patient did not deplete well (Lu, Ng et al. 2009). Albert and colleagues also noted variability in B-cell depletion with 7 of 24 patients achieving incomplete depletion and the degree of depletion correlated with clinical response (Albert, Dunham et al. 2008). In the EXPLORER study, B-cell depletion (CD19 count < 10 cells / μl) was not achieved in 9.5% of patients (Merrill, Neuwelt et al. 2010) although there was no correlation with clinical response. Recently, in the UCL cohort (n=115), prolonged duration of B-cell depletion was shown to be associated with good clinical response (Dias, Rodriguez-Garcia et al. 2015).

Thus, there was some relationship between the degree and duration of B-cell depletion and clinical response in patients with RA and SLE.

Incomplete B-cell depletion is associated with poor response

In many studies of rituximab in RA and SLE, B-cell depletion was arbitrarily defined as peripheral CD19+ cells < 5 - 10 cells / µl (Leandro, Cambridge et al. 2005, Leandro, Cambridge et al. 2006, Smith, Jones et al. 2006, Ng, Cambridge et al. 2007, Lu, Ng et al. 2009, Merrill, Neuwelt et al. 2010, Rovin, Furie et al. 2012) based on a threshold count of up to 20,000 events in the lymphocyte gate on flow cytometry. However, the thresholds used in these studies may not be sensitive enough for defining B-cell depletion. For example, using highly sensitive flow cytometry (HSFC) (where 500,000 lymphocytes are counted instead of the usual 2,000 to 20,000) it was shown that, in both RA and SLE, the degree of B-cell depletion correlates with clinical response. A more stringent definition of B-cell depletion as a peripheral CD19+ cell count of < 1 cell / µl using HSFC is predictive of clinical response to rituximab in both RA and SLE (Vital, Dass et al. 2011) (Vital, Rawstron et al. 2011). Thus, a B-cell depletion threshold of < 1 cell / µl appears to be a good biomarker that predicts response to rituximab. Important findings of these studies show that peripheral B-cell depletion was incomplete in 18% of patients with RA (Dass, Rawstron et al. 2008) and 54% of patients with SLE (Vital, Dass et al. 2011). Thus, in RA and SLE, clinical response to rituximab was shown to depend on the degree of depletion, as assessed by HSFC.

Furthermore, poor clinical response in patients with RA who achieved incomplete B-cell depletion, regardless of the treatment with 500mg or 1g of rituximab, was improved by delivering an additional dose of rituximab (Vital, Dass et al. 2015), suggesting that inefficient B-cell depletion by rituximab in these patients is clinically relevant. Also, in a small number of patients with SLE, Prof Isenberg's team have observed better clinical response with additional doses of rituximab (personal communication) in a small number of patients with SLE who had poor response to standard dose treatment with rituximab (1g x 2, given two weeks apart) suggesting that incomplete B-cell depletion in those with partial response may benefit from extra dose of rituximab.

Thus, incomplete B-cell depletion following treatment with rituximab is associated with poor clinical response, in both RA (Vital, Rawstron et al. 2011) and SLE (Vital, Dass et al. 2011) whereas enhanced B-cell depletion achieved using either an extra-dose of rituximab in RA (Vital, Dass et al. 2015); and prolonged duration of depletion in SLE are associated with a better clinical response (Dias, Rodriguez-Garcia et al. 2015) (Figure 1.5). Taken together, achieving more complete, durable B-cell depletion may improve clinical response in both RA and SLE. Therefore, it is important to understand the basis for the variability in clinical response to rituximab.
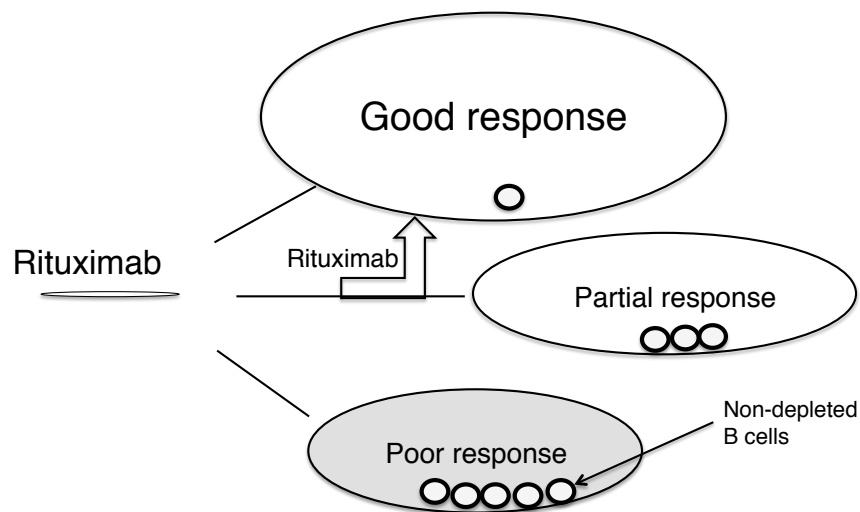
Figure 1.5 Relationship between clinical response and B-cell depletion with rituximab in RA and SLE.

Variability in depletion and reconstitution of B-cell subpopulations: relationship with clinical relapse

Based on the relative expression of IgD and CD27, B-cell subpopulations may be defined as naïve (IgD+CD27-), unswitched memory cells (IgD+CD27+), switched memory cells (IgD-CD27+) and double negative cells (IgD-CD27-). Poor clinical response to rituximab in both RA and SLE is associated with a higher number and/or frequency of CD27+ memory cells at 2-4 weeks after the first dose of rituximab (Vital, Dass et al. 2011, Vital, Rawstron et al. 2011, Adlowitz, Barnard et al. 2015) and also in the presence of IgD-CD27- (double negative, DN) B cells in RA (Adlowitz, Barnard et al. 2015) suggesting that resistance to depletion of different B-cell subpopulations is clinically relevant, reviewed by Leandro (Leandro 2013).

In RA, a better depletion of CD27+ memory B cells both in peripheral blood (Moller, Aeberli et al. 2009) and bone marrow (Nakou, Katsikas et al. 2009) appears to be associated with good response to rituximab while reconstitution with higher numbers of

C27+ memory B cells was associated with relapse (Leandro, Cambridge et al. 2006). Repopulation of B cells detectable in peripheral blood after rituximab occurs predominantly with naïve mature and transitional B cells similar to after bone marrow transplantation (Leandro, Cambridge et al. 2006, Bemark, Holmqvist et al. 2012). Patients with prolonged clinical response showed delayed reconstitution of peripheral blood CD27+ memory B cells, in some cases for years. Shorter responses seemed to be associated with repopulation with more memory B cells in RA (Leandro, Cambridge et al. 2006, Roll, Dorner et al. 2008, Anolik 2011) and in SLE (Anolik 2011, Vital, Dass et al. 2011) whereas a low frequency of CD27+ memory B cells was associated with good response to rituximab in RA (Sellam, Rouanet et al. 2011). Thus, better depletion of and reconstitution with lower frequency of CD27+ memory B cells are associated with good clinical response.

Although B-cell reconstitution after rituximab is considered to recapitulate ontogeny (Leandro, Cambridge et al. 2006, Anolik, Friedberg et al. 2007), proliferation of non-depleted B-cells (Kamburova, Koenen et al. 2012) may also contribute to repopulation after rituximab. CD27+ memory cells proliferate more readily, in vitro, than CD27- naïve cells, albeit with a considerable variation between individuals (Macallan, Wallace et al. 2005, Rouziere, Kneitz et al. 2005, van Zelm, Szczepanski et al. 2007). The majority of cells circulating during the depletion period are plasma blasts / plasma cells and memory B cells in RA and SLE (Leandro, Cambridge et al. 2006, Roll, Palanichamy et al. 2006, Vital, Dass et al. 2011). In Sjogren's syndrome also IgD-CD27+ memory B cells were also detected in higher frequency in peripheral blood and salivary glands, suggesting incomplete depletion and/or early reconstitution (Pers, Devauchelle et al. 2007). Thus, B-cell subpopulations may differ in their susceptibility to depletion with anti-CD20 mAbs (Leandro 2013). Although of clear clinical importance, how some B cells evade cytotoxic effects of rituximab remains elusive.

Anti-CD20 mAbs only target B cells that express CD20 (Edwards and Cambridge 2006). CD20 is expressed from pro-B to memory B-cells. Therefore, B-lineage cells such as plasma blasts and plasma cells that do not express CD20 are not effectively removed by anti-CD20 mAbs. Of clinical relevance, the presence of circulating plasma blasts at 6 weeks after rituximab was shown to correlate with clinical response in both RA (Vital, Rawstron et al. 2011) and SLE (Vital, Dass et al. 2011), where all non-responders were found to have detectable plasma blasts at 6 weeks after rituximab.

Discrepancy in the efficiency of B-cell depletion: relationship with B-cell subpopulations and their location

Depletion of B cells in lymphoid tissues appears to be less efficient than in peripheral blood. There are no data on the extent of B-cell depletion in secondary lymphoid tissues in relation to clinical response to rituximab in patients with RA and SLE. However, a disparity in B-cell depletion between peripheral blood and lymph nodes was reported in patients undergoing organ transplantation treated with a single dose of rituximab 375 mg/m^2 as part of induction therapy to minimise graft rejection (Genberg, Hansson et al. 2007). B-cell depletion in the peripheral blood defined by a threshold CD19+ cell count of < 5 cells/μL was achieved in around 90% of patients whereas the reduction in CD19+ cell count in lymph nodes was not significantly different between patients not treated (n=9) or treated with rituximab (n=13). Interestingly, B-cell depletion was noted in > 85% of the transplanted kidney samples in this cohort. Other studies also reported that patients who were treated with low dose (500mg) rituximab, as part of pre-conditioning before kidney transplant to inhibit graft rejection demonstrated complete B-cell depletion in peripheral blood, but had a greater frequency of switched memory (IgD-CD27+) B-cells in lymph nodes compared to those not treated with rituximab. In contrast, the frequency of naïve (IgD+CD27-) B-cells and unswitched memory (IgD+CD27+) B-cells was lower (Kamburova, Koenen et al. 2013, Wallin, Jolly et al. 2014) suggesting that both location and B-cell intrinsic mechanisms confer resistance to depletion with rituximab. Taken together, these findings suggest that rituximab depletes naïve cells and IgD+CD27+ unswitched memory cells more efficiently than IgD-CD27+ switched memory cells and DN cells, particularly in lymphoid tissues.

Interestingly, failure to deplete in lymph nodes was not due to lack of opsonisation as anti-rituximab antibodies demonstrated persistent binding of rituximab in non-depleted B cells (Kamburova, Koenen et al. 2012) (Kamburova, Koenen et al. 2013) similar to that shown in patients with B cell malignancies (Maloney, Liles et al. 1994) and in immune (idiopathic) thrombocytopenic purpura (Audia, Samson et al. 2011), suggesting that depletion by rituximab was compromised in lymph nodes. Animal model studies revealed a disparity in B-cell depletion between tissues such that splenic B cells were depleted more efficiently than B cells in lymph nodes (Vugmeyster, Beyer et al. 2005) and both the location and circulatory dynamics of B cells determine resistance to depletion with anti-CD20 mAbs (Gong, Ou et al. 2005). Further, in vitro experiments showed that non-depleted B cells in the lymph nodes of these patients expressed activation induced deaminase (AID) mRNA and retained functional capacity

to differentiate into antibody-secreting cells. Local antibody secretion by plasma cells in kidneys is an important effector mechanism in lupus nephritis (Espeli, Bokers et al. 2011) and, poor response to rituximab in patients with ITP was associated with a higher frequency of CD138- plasma cells in spleen (Mahevas, Patin et al. 2013). Thus, while achieving complete B-cell depletion in RA and SLE appears to be clinically relevant, how some B-cells evade deletion by rituximab, particularly in lymph nodes, remains elusive.

Although, B-cell depletion and reconstitution of B cells are useful to understand the variability in clinical and biological response to rituximab in RA and SLE, other factors that relate to B cell differentiation may provide further insights into resistance mechanisms to rituximab and identify potential biomarkers of response. Of these, serum immunoglobulins, autoantibodies, BAFF levels and IFN signature are of direct clinical relevance whereas other factors such as changes in T cell compartment also provide interesting insights.

Variability in changes in serum immunoglobulins, autoantibodies: relationship with clinical response

Changes in serum immunoglobulins after B-cell depletion with Rituximab

Alterations in serum immunoglobulins (Igs) after treatment with rituximab potentially represent clinically meaningful surrogate markers of B-cell depletion, reconstitution and differentiation. Whereas the effects of BCDT on serum Igs in RA are well described (Cambridge, Leandro et al. 2003), the kinetics of serum Ig levels of different isotypes in SLE are poorly understood. In patients with SLE, hypergammaglobulinemia is common, paradoxically however, hypogammaglobulinemia similar to common variable immunodeficiency occasionally occurs, and may relate to the presence of lymphocytotoxic autoantibodies (Baum, Chiorazzi et al. 1989, Fernandez-Castro, Mellor-Pita et al. 2007). Hypogammaglobulinemia may be associated with older age, low IgG at baseline, nephritis (Marco, Smith et al. 2014) and treatment with immunosuppressants including cyclophosphamide and mycophenolate mofetil (MMF) (Tsokos, Smith et al. 1986, Mino, Naito et al. 2011, Yap, Yung et al. 2014). An important concern that arises though is whether B-cell depletion increases the risk of hypogammaglobulinemia, an important adverse outcome of B-cell depletion, particularly in those patients who continue to require treatment with immunosuppressants. In this context, the probability of, and factors associated with the incidence of persistent hypogammaglobulinemia after rituximab and concurrent and/or

sequential immunosuppressants treatment in patients with SLE is not known, but such information is vital for judicious development of more efficient B-cell depletion therapies.

Autoantibodies and response to rituximab

In RA, clinical response correlated with a significant decrease in the levels of autoantibodies, notably, RF and anti-CCP antibodies (Cambridge, Leandro et al. 2003). Other studies supported the idea that seropositivity for RF and a reduction in RF and ACPA is associated with a better response to rituximab in RA (Cohen, Emery et al. 2006, Emery, Fleischmann et al. 2006, Cambridge, Isenberg et al. 2008). Further, RF-IgM and anti-CCP IgG levels in synovial fluid relative to that in the serum decreased after rituximab in patients with RA who had lymphoid aggregates on synovial histology but not in those without lymphoid aggregates. This finding suggests that local production of these autoantibodies by synovial CD138+ plasma cells and changes in CD68+ macrophages of the sub-lining infiltrate may predict a clinical response (Kavanaugh, Rosengren et al. 2008, Rosengren, Wei et al. 2008). However, it is unclear whether clinical response correlates with the extent of decrease in autoantibody serum levels.

In a study of 25 patients with SLE treated with rituximab at UCL, Cambridge and colleagues noted a variability in the extent of decrease in serum levels of anti-dsDNA both in responding and nonresponding patients at mean ± SD of 42 ± 36% and 60 ± 40% of baseline, respectively (Cambridge, Isenberg et al. 2008). An extension of the study to 50 patients with SLE also demonstrated a significant variability in biological response in terms of serum complement levels and anti-dsDNA antibodies (Lu, Ng et al. 2009). Interestingly, in SLE, antibodies against extractable nuclear antigens (ENAs) did not decrease following rituximab treatment in contrast with anti-dsDNA (Cambridge, Isenberg et al. 2008, Tew, Rabbee et al. 2010). This would suggest that rapidly proliferating clones of B cells give rise to short-lived plasma cells, which produce anti-dsDNA and anti-nucleosome antibodies and are therefore preferentially affected (Cambridge, Leandro et al. 2006). In contrast, other autoantibodies such as anti-Ro and anti-RNP or protective antibodies that develop following immunization, thought to be produced by long-lived plasma cells, remain unchanged.

In SLE, prolonged response was associated with seronegativity for autoantibodies against extractable nuclear antigens (RNP, Sm, Ro and La) and normal C3 levels (Ng, Cambridge et al. 2007). Further, it has been suggested that patients with SLE grouped according to baseline levels of anti-dsDNA antibodies demonstrate different kinetics of

B-cell repopulation, with variability in time taken to clinical relapse following treatment with rituximab such that patients with high anti-dsDNA antibodies tended to flare earlier and had a greater frequency of plasma blasts whereas those with low anti-dsDNA antibodies had a greater frequency of double negative (IgD-CD27-) cells at the time of relapse, but not in remission (Lazarus, Turner-Stokes et al. 2012).

Intriguingly, reduced levels of possibly protective natural antibodies of IgM-class have been suggested to be associated with the development of anti-dsDNA antibodies in a murine model of SLE (Ehrenstein and Notley 2010). However, the long-term effects of rituximab and/or concurrent and sequential immunosuppressive therapies on serum Igs and anti-dsDNA autoantibodies in SLE is not known, but would be of clear clinical importance, particularly, given the potential risk of hypogammaglobulinemia associated with rituximab, as shown in patients with RA (De La Torre, Leandro et al. 2012). Whether IgM hypogammaglobulinemia that may occur following treatment with rituximab is associated with elevated anti-dsDNA antibodies in patients treated with rituximab, is not known. If so, such a phenomenon could represent an important mechanism of poorer clinical response to rituximab.

Some changes in the composition of peripheral B cell phenotypes after rituximab recapitulate ontogeny (Leandro, Cambridge et al. 2006, Anolik, Friedberg et al. 2007). Therefore, the recovery of B-cell subpopulations in patients with SLE, particularly in those who develop hypogammaglobulinemia, may also relate to the extent of B-cell depletion in the short-term and, the recovery of B-cell subpopulations and/or clones may impact on serum immunoglobulin levels after B-cell depletion therapy in the long-term.

Rituximab and statins

Although, in vitro experiments suggested that statins might impair rituximab effects by inducing conformational changes in CD20 in lymphoma cell lines (Winiarska, Bil et al. 2008), two studies in patients with RA treated with rituximab and receiving concomitant statins or not, revealed conflicting results. The Dutch Rheumatoid Arthritis Monitoring (DREAM) registry reported that patients (n=23) receiving statins experienced nearly 2 months less effective period of rituximab benefit when compared with those not receiving statins (n=164) (Arts, Jansen et al. 2011). In contrast another study, involving 119 patients, did not find a difference in clinical response between patients receiving statins or not (Das, Fernandez Matilla et al. 2013). A retrospective analysis of 104 patients with RA from three randomised controlled trials also did not find a significant effect of concomitant statins on clinical response to rituximab (Lehane, Lacey et al.

2014). Given, the inconsistency in the relationship between statins and response to rituximab, this topic will not be explored further.

Do high BAFF levels reduce the efficacy of rituximab?

As discussed, persistent plasma cell activity is a potential mechanism of resistance to rituximab. In this regard, BAFF seems to be important for plasma cell survival and / or activity whereas the survival of memory B cells appears to be independent of BAFF and APRIL (Benson, Dillon et al. 2008). Although, in RA, several markers including BAFF, APRIL and IL-6 were not predictive of response to rituximab (Kavanaugh, Rosengren et al. 2008), in SLE, patients with very high BAFF levels had shorter duration of response to rituximab and relapsed with higher levels of anti-ds DNA antibodies (Carter, Isenberg et al. 2013). These findings suggest that measuring BAFF levels may be useful to identify patients who are more likely to benefit from sequential therapy with rituximab followed by an anti-BAFF agent such as Belimumab. Clinical trials are in progress investigating this strategy to target B cells.

Interferons and response to rituximab

It has been suggested that the type I IFN pathway may be activated in rituximab non-responders in RA. In RA, the expression of type 1 interferon genes in peripheral blood cells distinguished responders and non-responders (Raterman, Vosslamber et al. 2012) and the type I IFN signature was associated with a poor-response to rituximab in RA (Thurlings, Boumans et al. 2010). However, the EXPLORER study did not find a significant association between type 1 interferon expression and changes in serum complement or anti-dsDNA antibody levels to rituximab (Merrill, Neuwelt et al. 2010). Clinical trials targeting type I IFN pathway are ongoing in SLE.

Other factors predictive of response to rituximab

Rituximab has also been associated with changes in T cell compartment in both RA and SLE. Rituximab may directly deplete a small number of T cells (Leandro, Cambridge et al. 2006), which express CD20 (Hultin, Hausner et al. 1993) and these CD3+ CD20+T cells seem to be immunocompetent in that they secrete pro-inflammatory cytokines (IL-1 and TNF). Thus, it has been suggested that the response to rituximab may also be partially explained by depletion of these CD3+CD20+ T cells (Wilk, Witte et al. 2009). Rituximab treatment is also associated with reduced Th1-, Th2- and Th17-responses in RA (Yamamoto, Sato et al. 2010) and the frequency of activated T cells was shown to correlate with clinical response and relapse after rituximab in SLE (Iwata, Saito et al. 2011). Inflammatory cell recruitment into joints is

important for persistent inflammation and T cell expression of the chemokine (C-C motif) receptor CCR5 facilitates migration towards synovial cells that secrete its ligand CCL5 (Desmetz, Lin et al. 2007). Interestingly, T cell CCR5 expression positively correlated with disease activity in RA whereas CCL5 mRNA levels correlated negatively, however, after rituximab treatment a high proportion CCR5 in T cells was intracellular suggesting that CCR5 was internalised (Portales, Fabre et al. 2009). Thus, taken together, rituximab treatment is also associated with changes in T cell compartment. Thus, a number of factors summarized in Table 1.4 and Table 1.5 are associated with poor response, illustrated in Figure 1.6.

Table 1.4 Factors associated with response to rituximab in autoimmune disease

Factor associated with response	Comments	Reference
Good response correlated with a reduction in CRP and autoantibodies		RA (n=22) (Cambridge, Leandro et al. 2003)
Bone marrow B-lineage cells depletion at 3 months after rituximab	Variability was noted in the proportion of Pro- and Pre-B cells and there was a trend towards better response in patients with better depletion	RA (n=6) (Leandro, Cooper et al. 2007)
Anti-ENA +ve patients were more likely to flare earlier	Baseline autoantibody profiling may help predict poor response to rituximab in SLE	SLE (n=32) (Ng, Cambridge et al. 2007)
Incomplete depletion in the peripheral blood	Patients with incomplete B cell depletion (> or =1cell/µl) included all non responders	RA (n=80) (Vital, Dass et al. 2011)
Depletion of memory B cells in PB and BM (n=8) at 3mths	Reduction of CD19+HLADR+ activated B cells. Insignificant reduction in BM B cells	RA (n=11) (Nakou, Katsikas et al. 2009)
NRs had a higher frequency of circulating IgD+CD27+ memory B cells at the time of relapse	B cell repopulation with a higher frequency of IgD+CD27+ MCs was associated with early relapse (no differences in subsets at baseline between Rs and NRs	RA (n=17) (Roll, Dorner et al. 2008)
BAFF levels (>1011pg/ml) RF-ve Lymphocyte count >1875/ul	High BAFF levels, RF seronegativity and lymphocyte count >1875/ul were associated with poor clinical response	RA (n=138) (Ferraccioli, Tolusso et al. 2012)
FcγRIIIa F158V polymorphism	High affinity polymorphism was associated with good response	SLE (n=12) (Anolik, Campbell et al.

		2003); RA (n=177) (Kastbom, Coster et al. 2012); and RA (n=111) (Ruyssen-Witrand, Rouanet et al. 2012)
No Interferon type 1 signature	Genome-wide microarray in a small prospective RA cohort suggested IFN type 1 signature as a marker of non-response	RA (n=14) (Raterman, Vosslamber et al. 2012)
TTTT BLyS promoter haplotype in seropositive patients	The frequency of the promoter haplotype was comparable in both sero+ve and sero-ve patients, but good response to RTX was seen only in sero+ve patients with the promoter haplotype. BLyS levels weren't of predictive significance.	RA (n=152) (Fabris, Quartuccio et al. 2012)
IgJhiFCRL5lo, a combination biomarker of plasmablasts was associated with poor response	pooled samples from RCTs	(Owczarczyk, Lal et al. 2011)

RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; R, responder; NR, nonresponder; PB, peripheral blood; BM, bone marrow; MS, multiple sclerosis; BLyS, B lymphocyte survival factor; MRI, magnetic resonance imaging; RCT, randomized controlled trial

Table 1.5 Parameters associated with clinical relapse

Factor associated with early clinical relapse	Comments	Condition
Relapse was preceded by B cell repopulation and an increase in levels of autoantibodies		RA (n=22) (Cambridge, Leandro et al. 2003)
Higher numbers of CD27+ memory B cells at repopulation	Repopulation occurred mainly with naïve cells whereas relapse was associated with memory B cells	RA (n=24) (Leandro, Cambridge et al. 2006)
Higher numbers of IgD+CD27+ memory B cells at baseline and at the time of repopulation (n=17, Rs=12, NRs=6)	Variability in the frequency of IgD+CD27+ memory B cells at baseline	RA (n=17) (Roll, Dorner et al. 2008)
Anti-dsDNA antibody levels and B cell numbers, B cell phenotype were predictive factors for relapse		SLE (N=67) (Lazarus, Turner-Stokes et al. 2012)

R, responders; NR, nonresponders; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus
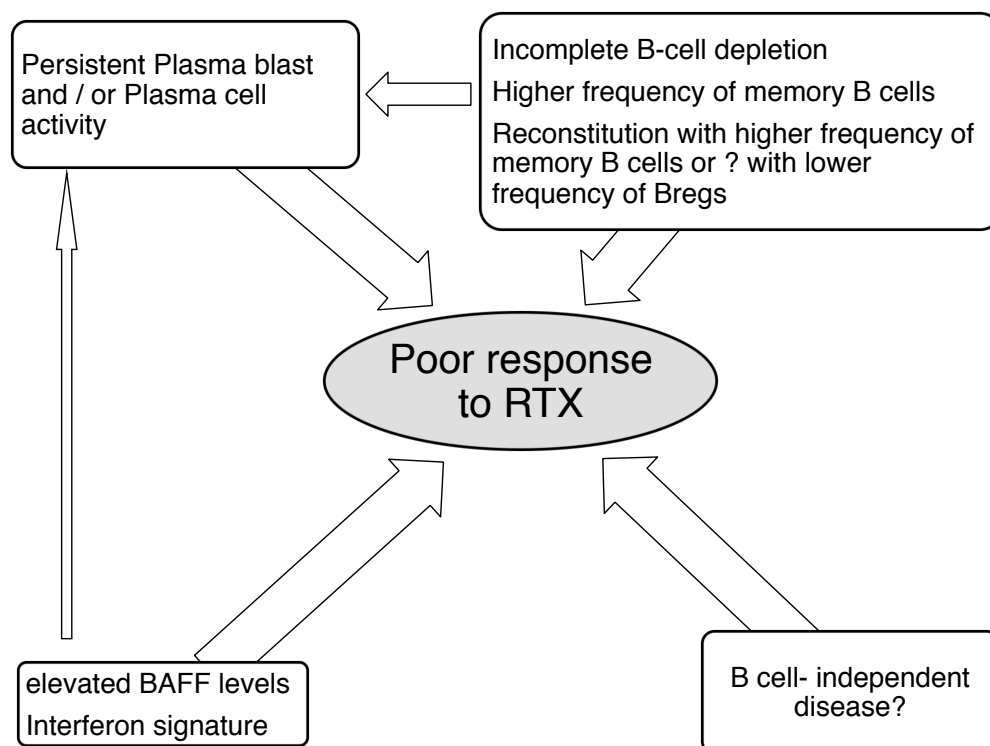
Figure 1.6 Potential mechanisms to explain poor response to rituximab in RA and SLE.

Evidence from clinical studies suggest that poor response to rituximab is associated with: incomplete B-cell depletion; a higher frequency of memory B cells prior to treatment with rituximab and/or at reconstitution after treatment with rituximab; persistent activity of plasma blasts and / or plasma cells as they are not directly affected by rituximab; if the underlying pathogenesis is not B-cell dependent; reconstitution with lower frequency of regulatory B cells?; interferon signature; and elevated BAFF levels which may promote survival of plasma cells and autoreactive cells at transitional stage.

Regardless of the effect of BAFF and Type I IFN, incomplete B-cell depletion with rituximab is associated with poor clinical response. Therefore, improving B-cell depletion may enhance clinical response. However, it should be noted that even effective depletion as measured in peripheral blood does not always result in clinical response (Vital, Rawstron et al. 2011). There are practical difficulties in understanding how B-cell depletion in peripheral blood reflects depletion in solid tissues, which may also be important. A pragmatic approach therefore is to consider inadequate clinical response despite complete depletion as 'refractory disease' whereas simply not achieving complete depletion as 'rituximab resistance'. Rituximab resistance may be related to insufficient dose or increased drug clearance, but also to resistance of

autoreactive B-cell clones to depletion mechanisms induced by binding to rituximab. A failure to achieve clinical response despite complete depletion, 'refractory disease', may occur because the disease is not B-cell dependent for initiation and/or perpetuation (Reddy and Leandro 2014). It is important to distinguish the two scenarios because it could be hypothesized that those patients who do not respond to rituximab but have achieved complete depletion and do not show any evidence of early return of B cells may not benefit from further B-cell-targeted therapies. In contrast, at least some patients from the group with poor response who failed to achieve complete depletion may benefit from alternative agents or treatment regimens that may improve B-cell depletion (Figure 1.7).
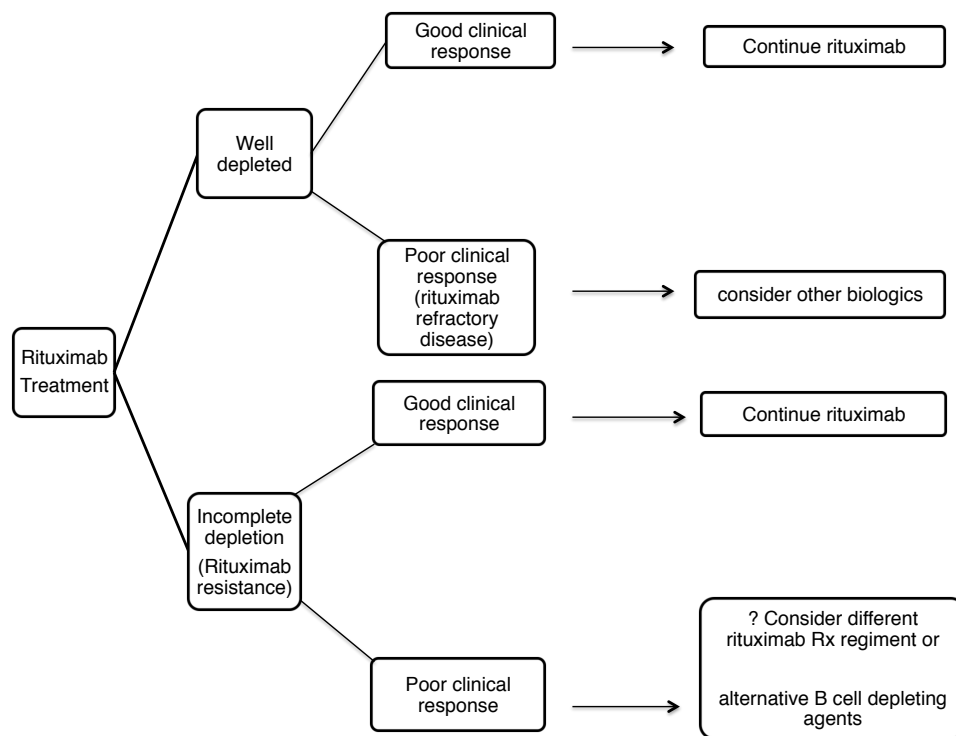
Figure 1.7 Rituximab-resistance and –refractory disease.

Pragmatically, rituximab-resistance and –refractory disease may be defined based on the efficiency of B-cell depletion and clinical response to rituximab to identify a subgroup of patients who may benefit from alternative treatment regimen and/or B-cell depleting agents.

What can we learn from the experience of using rituximab for B-cell malignancies?

The mechanisms of action of rituximab have been extensively studied in the context of B-cell malignancies informing the development of better therapies to improve patient outcomes.

Clinical response to rituximab: relationship with disease and rituximab dosing regimen

Variability in B-cell depletion with rituximab has been well documented in patients with B cell malignancies (Reff, Carner et al. 1994). In the initial Phase I, dose-ranging (10, 50, 100, 250 and 500 mg/m^2) study of rituximab in non Hodgkins lymphoma (NHL), peripheral blood B cells were specifically depleted and remained depleted for 1 to more than 3 months. Serum rituximab levels were variable and the serum half-life was 4.4 days for patients treated with doses of 100 mg/m^2 or higher. Tumor tissue examination 2 weeks after treatment showed rituximab bound to tumor cells in several of the cases, which suggests that some B cells may not be removed despite persistent binding by rituximab on their cell surface. Modest tumor responses were seen in seven of the nine patients treated with doses equal to or greater than 100 mg/m^2 (Maloney, Liles et al. 1994). Rituximab was licensed for use in refractory NHL, following the pivotal study by McLaughlin and colleagues (McLaughlin, Grillo-Lopez et al. 1998) demonstrating that almost 50% of patients with refractory low-grade or follicular lymphoma (FL) responded to rituximab monotherapy (McLaughlin, Grillo-Lopez et al. 1998). In this Phase II/III study of NHL, 166 patients with refractory NHL were treated with rituximab. The response rate was 48% after a median follow-up duration of 11.8 months. Fifteen of the 16 patients who did not deplete peripheral blood B cells to undetectable levels did not respond to treatment and extra-nodal disease was also associated with poor response in FL (Igarashi, Kobayashi et al. 2002). Response rate in FL increased to 60% with eight consecutive weekly infusions of 375 mg/m^2 of rituximab, instead of four (Piro, White et al. 1999). Thus, observations on variable B-cell depletion in early studies informed the design of more effective therapeutic regimen in subsequent studies in B cell malignancies.

Lower response rates in diffuse large B cell lymphoma (DLBCL) and mantle cell lymphoma (MCL) were also reported in another Phase II study of 54 patients with DLBCL or MCL, which reported response rates of 37 and 33%, respectively. The features that were more commonly noted in nonresponders were chemotherapy-refractory disease, MCL histology and tumor size > 5 cm in diameter (Coiffier, Haioun

et al. 1998). Response rates in small lymphocytic lymphoma (SLL) / chronic lymphocytic leukemia (CLL) were also much lower (Byrd, Murphy et al. 2001). A study of 33 patients with SLL / CLL treated with variable doses of rituximab (100 mg × 1, 250 mg/m2, weekly infusions of 375 mg/m^2 for 4 weeks) showed that the overall response rate was 45% with only 3% achieving complete response (Byrd, Murphy et al. 2001). However, the clinical response in CLL was improved using a higher dose of rituximab (O'Brien, Kantarjian et al. 2001). Thus, taken together, it appears that the clinical response to rituximab in B-cell malignancies was influenced by: the histological type, extra-nodal disease and the tumor burden; the dose of rituximab used; persistent high serum levels of rituximab; better response to prior chemotherapy; and rituximab used in combination with chemotherapy.

It is important to consider the differences between malignant and autoimmune B-cells including their number, size of the cells, location and role in pathogenesis as well as immune abnormalities associated with autoimmune disease. Therefore, careful consideration is required if we are to make progress in optimising B-cell depletion for autoimmune disease. To this end, I will appraise the available evidence to explore the potential reasons for the variability in clinical and/or biological response to rituximab in RA and SLE.

Is poor B-cell depletion simply due to low rituximab levels?

Rituximab pharmacokinetics and relationship with clinical and biological response

The pharmacokinetics of rituximab between malignancies and autoimmune disease are not directly comparable, not least due to differences in target cell number and tumour mass, but also due to differences in the dosing regimens. Moreover, patients with SLE often have proteinuria secondary to nephritis, which may also affect serum rituximab levels. Variability in rituximab pharmacokinetics has been well documented in B cell malignancies and in RA and SLE.

Tumor target cell burden was clearly shown to influence serum rituximab levels presumably because deletion of rituximab-bound target cells also eliminates rituximab (Berinstein, Grillo-Lopez et al. 1998, Harrold, Straubinger et al. 2012). Moreover, the area under serum concentration-time curve (AUC) and trough / pre-dose concentration (Ctrough), but not Cmax, was predictive of clinical response in the pivotal trial with median serum rituximab levels of 25.4 μg/mL in patients achieving good clinical

response compared to median levels of 5.9 μg/mL in non-responders (Berinstein, Grillo-Lopez et al. 1998) and also in CLL (Li, Zhi et al. 2012). In CLL, serum rituximab levels after 1 week of rituximab (375 mg/m2) treatment are typically < 1 μg/mL, significantly lower than that reported in other B cell malignancies, which show good response to rituximab (Beum, Kennedy et al. 2004), probably due to a greater load of rituximab bound to CLL tumor cells in circulation and consequently, quicker clearance. The volume of distribution of rituximab at steady state suggests that rituximab diffuses into extracellular spaces and patient's weight appears to influence rituximab clearance in the case of diffuse large B cell lymphoma (Muller, Murawski et al. 2012). Probably due to a reduction in tumor target cell burden after treatment with rituximab, serum rituximab levels increase incrementally with repeated infusions and were detectable in most patients at 3 months and serum half-life of rituximab was estimated at 18 ± 15 (mean ± SD) days (Tobinai, Kobayashi et al. 1998). Thus, rituximab pharmacokinetics in B cell malignancies follows a biphasic pattern.

After intravenous administration, rituximab binds to CD20 on target cells, subsequently, disposed either by internalisation and degradation or deletion of B cells through effector mechanisms. Alternatively, CD20:mAb complexes may also be removed by effector cells by a process known as trogocytosis, a process where only cell membrane associated proteins or complexes are removed by effector cells (Golay, Semenzato et al. 2013). However, with minimal target load, a different order of kinetics occurs. Serum rituximab half-life increases with repeated treatment and this relationship provides a model suggesting roles for target cell disposition following the initial treatment phase whereas subsequent order of kinetics, when the target cell load is lower, is probably influenced by IgG catabolism. The neonatal Fc receptor, FcRn, plays a key role in IgG metabolism including that of therapeutic monoclonal antibodies by protecting mAbs from intracellular degradation and actively transporting to extracellular space (Roopenian and Akilesh 2007). Therefore, rapid elimination by target cells after the first dose and a non-linear elimination with subsequent doses and lower target cell load may explain the biphasic pharmacokinetics in B-cell malignancies (Golay, Semenzato et al. 2013).

Alternative routes of administration of rituximab including subcutaneous routes have been explored. Unlike oral route, which can't be used because of degradation in gut, subcutaneous route provides the benefits of delivering relatively large doses (1400mg) quickly and with comparable safety to IV administration of rituximab whilst avoiding the need for hospital attendance, as evaluated by a phase IB study of patients with follicular lymphoma undergoing maintenance therapy with rituximab (Salar, Avivi et al.

2014). Further, subcutaneous administration of rituximab follows different routes of absorption and involves FcRn-mediated transport of rituximab by endothelial cells of peripheral and central vascular compartments (Deng, Meng et al. 2012, Golay, Semenzato et al. 2013).

Rituximab pharmacokinetics in RA and SLE

In RA, a phase II study involving 161 patients randomized to receive either methotrexate alone, rituximab + methotrexate or rituximab + cyclophosphamide or rituximab alone were compared. The two-compartment model seemed to best explain the pharmacokinetic patterns reported in this study with data available from 102 patients. The mean distribution time was 2.4 days and the elimination time was 20 days (Ng, Bruno et al. 2005). Similar to the observations of studies in B cell malignancies, inter-individual variability in serum rituximab levels was noted in this study, however, the terminal half-life of rituximab in these patients is not known. Two parameters including body surface area and male gender were shown to influence rituximab pharmacokinetics, which accounted for 32% and 42% of the variability of rituximab clearance and central volume of distribution, respectively. Men in this study had greater body surface area and were shown to have 17% larger central volume of distribution and 39% greater clearance rates for rituximab compared to women. Serum rituximab levels did not differ between the groups of patients treated with rituximab in combination with methotrexate or cyclophosphamide. There was no correlation between serum rituximab levels and baseline peripheral CD19+ B cell counts as all patients achieved peripheral blood B-cell depletion (but low sensitivity, < 10 cells/μl) at 2 weeks (Breedveld, Agarwal et al. 2007). In RA, serum rituximab levels did not correlate with synovial B-cell depletion or clinical response (Thurlings, Teng et al. 2010). However, the underlying reasons for the inter-individual variability in rituximab levels within each disease category remain elusive.

In SLE, the early phase I/II study investigated serum rituximab levels in patients treated with three different dosing regimens: low, a single infusion of rituximab of 100 mg/m2 ; intermediate dose, a single infusion of 375 mg/m2 ; and high dose, four doses of 375 mg/m2 given a week apart. Intriguingly, there was remarkable variability in serum rituximab levels in this cohort at 2 months after rituximab treatment. The range of serum rituximab levels was: < 0.02 – 4.8; < 0.02 – 7.8; and 0.36 – 22.5 μg/mL, in patients receiving low, intermediate and high dose of rituximab. In contrast to the phase II study in RA, B-cell depletion, as defined by a threshold CD19+ cell count < 5 cells/μL, was associated with greater geometric mean concentrations of rituximab, 2 months after the first dose of rituximab with median serum rituximab levels of 1 μg/mL,

in patients with SLE independent of the effect of FcγRIIIa genotype (Anolik, Campbell et al. 2003).

Obinutuzumab pharmacokinetics in B cell malignancies

Glycoengineering of mAbs improves engagement with effector cells (discussed later) and in this context it seems that glycoengineering as such does not affect the pharmacokinetics of glycoengineered mAbs (Leabman, Meng et al. 2013). Two treatment regimens were compared in patients with CLL: in the group that received higher dosing regimen of 1600/800mg, serum trough levels of obinutuzumab (OBZ), an afucosylated anti-CD20 mAb (discussed later), achieved a steady state at 300 μg/mL, whereas in the group that received lower dosing regimen achieved a steady state at 100-200 μg/mL, following the biphasic pattern (Salles, Morschhauser et al. 2013). However, population pharmacokinetics of obinutuzumab in four clinical trials, in histologically different B-cell malignancies suggest that the best fit model in this analysis that explained the pharmacokinetics of obinutuzumab were linear and time-dependent clearance (Gibiansky, Gibiansky et al. 2014). There were clear differences in obinutuzumab levels achieved in different histological conditions, thus a higher clearance was noted in patients with MCL compared to those with CLL while patients with DLBCL had low clearance rates. However the rate of decline of clearance was slower in CLL and MCL probably due to higher levels of target cells in circulation compared with DLBCL, where target cells are mostly located in tissues. A critical limiting factor was also that the effect of an important covariate, tumor size, was not evaluated due to the confounding effect of dosing regimen. Clearance of obinutuzumab was also slower in CLL compared to MCL, probably due to lower CD20 expression in CLL compared with MCL (Ginaldi, De Martinis et al. 1998). Thus, covariates such as tumor burden, histological type are important considerations when analyzing the pharmacokinetics of anti-CD20 mAbs, however, these data may not be applicable to non-malignant conditions where target cell count is presumed to be less variable.

Could disease-associated alterations in Ig metabolism and nephritis influence rituximab levels in RA and SLE?

Differences in CD19+ B cell counts and IgG catabolism may affect serum rituximab levels achieved in RA and SLE. Of direct relevance, differences in immunoglobulin metabolism have been reported in RA and SLE (Levy, Barnett et al. 1970). Levy et al. used radioiodinated globulins to show that patients with SLE, regardless of gastrointestinal and renal loss of intact IgG, had a higher catabolism of IgG class of

immunoglobulins with five-fold increase in synthetic rates noted in some patients compared with healthy individuals, despite having normal serum IgG concentrations. In contrast, IgM catabolism was normal in SLE patients. Interestingly, nephritis itself may also predispose to increased catabolism of IgG, but not IgM, independent of proteinuria, which has variable effects on IgG synthesis (Kaysen and al Bander 1990). RA patients have hypercatabolism of IgM, but not IgG. The mean survival half-life of IgG in patients with SLE was significantly lower than in patients with RA and normal controls at 8.2, 14 and 18 days, respectively, whereas IgG catabolic rate was 10% in SLE patients compared with 6% and 3.9% in patients with RA and normal controls, respectively (Levy, Barnett et al. 1970). However, no study has directly compared the pharmacokinetics of rituximab in patients with RA and SLE.

Is poor B-cell depletion due to the development of human anti-chimeric antibodies (HACAs)?

Patients with autoimmune diseases are more likely to develop HACAs when compared to patients with lymphoma. HACAs may antagonise rituximab and increase clearance in vivo (Melander, Sallee et al. 2009). HACAs have been reported to occur with rituximab in patients with IgM associated peripheral neuropathy (D'Arcy and Mannik 2001) and idiopathic thrombocytopenic purpura (Herishanu 2002). A recent systemic review reported that serum sickness like reactions, probably attributable to HACAs, were more common in rheumatologic conditions notably 8 of 17 cases with Sjogren's syndrome (Karmacharya, Poudel et al. 2015). In the early phase I/II study of rituximab in SLE, high titre HACAs were documented in 6 of 17 patients with SLE, more common in patients of African ancestry, higher disease activity before treatment and in those who received low dose rituximab and also had lower rituximab levels whilst achieving poor B-cell depletion (Looney, Anolik et al. 2004). Albert et al. also reported on the paradoxical incidence of HACAs in patients with SLE despite patients not mounting good response to vaccination with Pneumovax and tetanus toxoid (Albert, Dunham et al. 2008). In this study, two patients had very high HACA titers (28,700 ng/ml) resulting in a serum sickness reaction in one patient with the highest HACA levels in this cohort, who also had short lived B-cell depletion and no response to vaccination. However, the correlation between HACA formation and the duration of B-cell depletion was weak and some patients achieved full B-cell depletion despite low HACA titers (< 300 ng/ml). The underlying reasons for the discordant relationship between HACAs, duration of B-cell depletion and vaccination response is not clearly understood. Thus, early studies suggested that the development of HACAs might contribute to lack of clinical response, particularly in SLE.

The development of HACAs was < 1% in B cell malignancies whereas HACAs were noted in 26% of 169 patients with SLE over a 52-week period in the EXPLORER trial (Merrill, Neuwelt et al. 2010). In this phase III study, three of the four patients with serum sickness like reaction following rituximab had HACAs. Regardless, HACAs did not affect the outcomes of the endpoints in this study. Patients with severe serum-sickness reactions may benefit from the use of humanised anti-CD20 mAbs (Thornton, Ambrose et al. 2015). Moreover, it is not known whether all HACAs are able to neutralize rituximab. Thus taken together, in SLE, the occurrence of very high titers of HACAs are probably uncommon, but are associated with serum sickness like reaction, incomplete and/or shorter duration of B-cell depletion.

HACAs were also noted in 4.3-11% of patients with RA (Edwards, Szczepanski et al. 2004, Emery, Fleischmann et al. 2006, van Vollenhoven, Emery et al. 2010). In another study, employing two different rituximab dosing regimen of either two doses of 500mg or two doses of 1g, the incidence of HACAs was 4.2% and 2.7%, respectively, suggesting an increase in the incidence of HACAs in the lower dose treatment group. However, there were no serious adverse events associated with HACAs and a majority of patients with HACAs responded to rituximab (Emery, Fleischmann et al. 2006). Collectively, HACAs do not seem to influence the clinical response to treatment with rituximab in RA (Thurlings, Teng et al. 2010).

Furthermore, as discussed earlier, although there are no directly comparable data, humanised anti-CD20 mAbs do not appear to deliver better clinical outcomes than rituximab in autoimmune disease. Therefore, alternative resistance mechanisms need consideration in our efforts to improve B-cell depletion in autoimmune diseases.

Studies in B-cell malignancies identified several potential resistance mechanisms that contribute to ineffective B-cell depletion with rituximab (Gong, Ou et al. 2005, Glennie, French et al. 2007, Lim, Beers et al. 2010, Cartron, Trappe et al. 2011, Tipton, Roghanian et al. 2015). Therefore, a better understanding of the mechanisms of action of anti-CD20 mAbs and the potential of how immune abnormalities associated with autoimmune disease may potentially impact the efficiency of anti-CD20 mAbs may help efforts to optimise B-cell depletion therapy with anti-CD20 mAbs.

CD20 and anti-CD20 mAbs

In 1980, CD20 was first identified as a B cell specific antigen (Stashenko, Nadler et al. 1980). CD20 is a 33-37 kDa, non-glycosylated transmembrane phosphoprotein of the membrane-spanning 4-A (MS4A) family (Liang, Buckley et al. 2001) expressed on pre-

B cells, mature B cells and memory B cells, but lost at the stage of differentiation into plasma blasts and plasma cells, suggesting a role in cell-cycle regulation (Tedder and Engel 1994). Typical of the tetra membrane-spanin family, CD20 has a small and a large extracellular loop, and intracellular N- and C-terminal regions (Liang, Buckley et al. 2001). CD20 is expressed on B cell membrane as tetramers (Polyak, Li et al. 2008) and signals through lipid rafts (Deans, Li et al. 2002). CD20 appears to associate with the B-cell receptor upon engagement with some anti-CD20 mAbs leading to calcium influx (Polyak, Li et al. 2008, Walshe, Beers et al. 2008). Insights into the function of CD20 are derived from an individual with CD20 deficiency secondary to a homozygous mutation in a splice junction of the CD20 gene (MS4A1) resulting in nonfunctional mRNA species, with evidence of decreased frequency of IgD-CD27+ memory B cells and suboptimal T cell-independent immune responses (Kuijpers, Bende et al. 2010).

CD20 antigen was targeted using monoclonal antibodies to deplete B cells because CD20 is expressed by normal and malignant B cells and does not shed or was not thought to be internalised from the cell surface (Beers, Chan et al. 2010). The first CD20 mAb generated was the murine anti-CD20 mAb, B1 (Stashenko, Nadler et al. 1980). To develop rituximab, mice were immunized with a CD20+ lymphoma to generate a high affinity anti-CD20 mAb, known as 2B8 or IDEC-2B8. Subsequently, the light and heavy chain variable regions from 2B8 were amplified and inserted into a human IgG1 heavy chain and human kappa-light chain constant region using cDNA expression vector before transferring to Chinese hamster ovary cells with capacity to secrete high Ig levels, which also bound human CD20 and C1q or human effector cells, demonstrating the potential to lyse human B-lymphoid cell lines, in vitro (Reff, Carner et al. 1994), providing the pre-clinical evidence for considering clinical trials.

Relationship between CD20 expression and B-cell depletion in B cell malignancies?

In B cell malignancies, CD20 expression alone does not seem to relate to the extent of B-cell depletion, as clearly some B cells are not depleted despite being bound by rituximab (Maloney, Liles et al. 1994). B-cell expression of CD20 can be variable between different histological types of B-cell malignancies. In CLL, where clinical response to rituximab is lower compared to FL, CD20 expression in CLL cells is characteristically low (Almasri, Duque et al. 1992) and can be higher in the circulating CLL cells when compared to CLL cells found in the bone marrow or lymph nodes (Huh, Keating et al. 2001). The expression of CD20 was lowest in CLL, higher in FL, MCL and splenic lymphoma; and was highest in hairy cell leukemia. Further, using specific anti-mouse Ig antibodies it was shown that, in patients with CLL, rituximab treatment

was associated with down modulation of CD20 (Jilani, O'Brien et al. 2003). Thus, CD20 expression may, at least in part, explain the discrepancy in clinical response between the histological types. However, response to rituximab in MCL is poor despite good levels of CD20 expression (Smith 2003), suggesting that CD20 expression alone does not fully explain poor depletion. In this context, data are lacking on whether expression of CD20 in different B-cell subpopulations in autoimmune diseases may explain the variability in their susceptibility to the cytotoxic effects of rituximab.

Anti-CD20 mAbs: effector mechanisms

Anti-CD20 mAbs evoke four distinct modes of cell death: 1) complement-dependent cellular cytotoxicity (CDC); 2) antibody-dependent cellular cytotoxicity (ADCC); 3) antibody-dependent cell phagocytosis (ADCP); and 4) direct cell death (DCD) (Figure 1.8). In vitro studies have revealed differences between the efficiency of various anti-CD20 mAbs at evoking different effector mechanisms (Glennie, French et al. 2007). The mechanisms of depletion of B cells by rituximab and of resistance have been extensively studied in B-cell malignancies. In contrast, there remains a gap in our knowledge of the possible mechanisms of resistance to depletion by rituximab in the context of autoimmune diseases. Factors that could influence the outcome of triggering of these cytotoxic mechanisms include the target antigen CD20, expression of complement defense proteins involved in CDC and Fc receptors that may determine the efficiency of ADCC. The importance of these mechanisms in humans in vivo is summarised in Table 1.3.

In vitro studies suggest different rituximab pharmacodynamics for the effector mechanisms. For example, for CDC in purified cells the optimal dose appears to be 10 μg/ml (Bologna, Gotti et al. 2011) whereas other effector mechanisms such as ADCC and phagocytosis were relatively optimal at lower concentrations of 0.1 μg/ml in cell systems (Leidi, Gotti et al. 2009). However, in whole blood, ADCC and ADCP may be less efficient due to potential interfering effect of complement and excess immunoglobulins (Bologna, Gotti et al. 2013). Thus, the threshold levels of mAbs may vary for different effector mechanisms and in different locations.
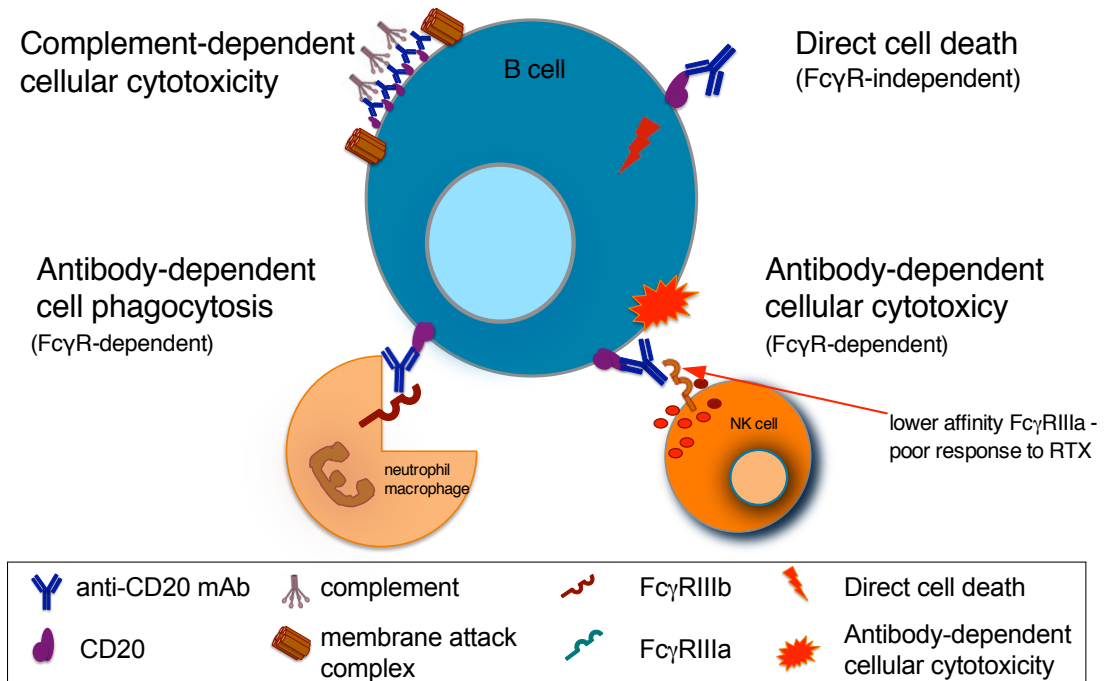
Figure 1.8 Effector mechanisms of anti-CD20 mAbs.

Anti-CD20 mAbs evoke four distinct modes of B-cell cytotoxicity including complement-dependent cellular cytotoxicity; FcγR-dependent- antibody-dependent cell phagocytosis and -antibody-dependent cellular cytotoxicity; and FcγR-independent- direct cell death.

Type I and II anti-CD20 mAbs: CD20 binding characteristics determine the effector mechanisms

Since the clinical success of rituximab, a number of alternative anti-CD20 mAbs have been developed (Lim, Beers et al. 2010, Weiner 2015). Broadly these can be categorized as type I and type II. A key characteristic that distinguishes anti-CD20 mAbs is the ability to cluster CD20 into detergent insoluble lipid rafts. Type I anti-CD20 mAbs like rituximab, are significantly more efficient at redistributing CD20 into lipid rafts compared to type II anti-CD20 mAbs like obinutuzumab (Cragg, Morgan et al. 2003, Beers, Chan et al. 2008, Lim, Vaughan et al. 2011, Tipton, Roghanian et al. 2015). The importance of this lies in the observation that redistribution of CD20 positively correlates with the ability of anti-CD20 mAbs to evoke CDC (Cragg, Morgan et al. 2003) while increasing the propensity for internalisation of CD20:CD20 mAb complexes by B cells (Lim, Vaughan et al. 2011). Whereas efficient activation of CDC is desirable for B-cell depletion, internalisation of CD20:CD20 mAb complexes is detrimental to the other effector mechanisms for B-cell depletion (Beers, Chan et al. 2008, Lim, Vaughan

et al. 2011, Tipton, Roghanian et al. 2015). A number of type I anti-CD20 mAbs have been used in clinical trials in RA and/or SLE (Table 1.6) but obinutuzumab is the only type II mAb to have entered clinical trials in SLE.

In vitro, Type I anti-CD20 mAbs (rituximab-like) redistribute target antigen CD20 into lipid rafts whereas Type II (obinutuzumab-like) do not (Niederfellner, Lammens et al. 2011). The molecular basis for this distinguishing characteristic is revealed by CD20 epitope fine mapping (Klein, Lammens et al. 2013). N-terminally located residues with 170-173 form the core epitope of rituximab whereas carboxyterminally located residues 172-178 form the core epitope of obinutuzumab (GA101). Rituximab binds to the Asn171 of CD20 whereas obinutuzumab binds to more carboxyterminally located Asn176 (Figure 1.9). X-ray crystallographic study reveals that obinutuzumab binding of native CD20 exerts steric constraints on the conformation of the large extracellular loop of CD20. Obinutuzumab binds CD20 tetramers in closed conformation whereas rituximab binds CD20 in open conformation probably leading to rituximab bound CD20 associating with different set of protein complexes compared to obinutuzumab (Niederfellner, Lammens et al. 2011).

Table 1.6 Other anti-CD20 mAbs in clinical trials or in clinical use in RA and SLE

CD20 mAb	Type	Dise-ase	Sponsor	Stage of trial	Results	Reference
Ocrelizumab	I	RA	Genentech / Roche / Biogen	Phase III	Primary end points met (48 weeks, dose-dependent serious infections)	(Rigby, Tony et al. 2012)
Ofatumumab	I	RA	Genmab AC / GlaxoSmith Kline	Phase III	Primary end points met (24 weeks, no serious adverse events)	(Taylor, Quattrocchi et al. 2011)
TRU-015	I*	RA	Trubion / Pfizer	Phase I & II	Primary end points not met	(Burge, Bookbinder et al. 2008, Hayden-Ledbetter, Cerveny et al. 2009)
Ocrelizumab	I	SLE (LN)	Genentech/ Roche/Biog en	Phase III	Not superior to placebo	(Mysler, Spindler et al. 2013)
obinutuzumab (GA101)	II	SLE	Glycart/Roc he	Phase I/II	Commenced Nov 2015	NCT02550652
Ocaratuzuamab (AME-133v,	I	RA	Mentrik Biotech/	Phase I	N/A	

LY2469298)			Eli Lilly			

* TRU-015 is a single-chain Fv (variable fragment of Fc) generated from 2H7 linked to human IgG1, but devoid of CH1 (constant heavy fragment portion 1 of immunoglobulin) and CL (constant light chain fragment of immunoglobulin) domains. The original mAb was Type I (Cragg, Morgan et al. 2003); RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; LN, lupus nephritis; and N/A, not available.
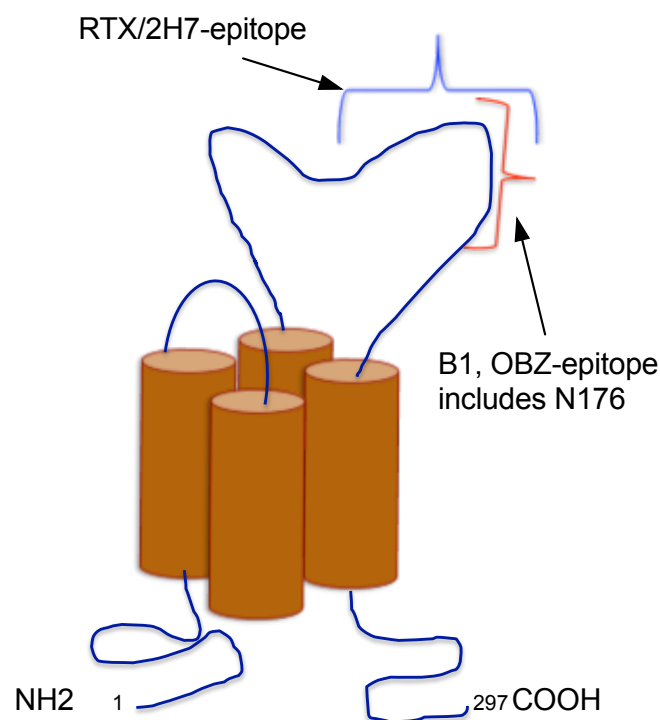
Figure 1.9 CD20 molecule and epitopes of Type I and II anti-CD20 mAbs.

CD20 has four transmembrane portions and two extra cellular loops. Type I mAbs, rituximab and 2H7 bind to the larger loop. Type II mAbs, B1 and obinutuzumab (OBZ) also bind the larger extra cellular loop but make contact with N176 position.

Remarkably, Type I and II anti-CD20 mAbs bind CD20 in 2:1 ratio, for which two potential explanations are proposed. First, the existence of two different subpopulations of CD20 (Niederfellner, Lammens et al. 2011) such that obinutuzumab binds to CD20 populations at the site of cell-to-cell contact whereas rituximab segregates CD20 into lipid rafts. An alternative model is that rituximab binds CD20 tetramers in mainly cis whereas obinutuzumab binds in trans as proposed by Klein et al (Klein, Lammens et al. 2013). Binding in cis format allows type I mAbs, but not type II mAbs, to engage with the inhibitory FcγRIIb and promote internalisation of CD20-mAb complexes, regardless of activation of FcγRIIb (Vaughan, Iriyama et al. 2014). In addition to its Type II nature, obinutuzumab has also been glycoengineered with an afucosylated Fc facilitating enhanced affinity for Fcγ-receptor IIIa (CD16a) (Mossner, Brunker et al. 2010), which may explain its superior potency in NK-mediated ADCC (Umana, Jean-Mairet et al. 1999). Differences between the two types of anti-CD20 mAbs at evoking effector mechanisms are shown in Table 1.7 and Figure 1.10.
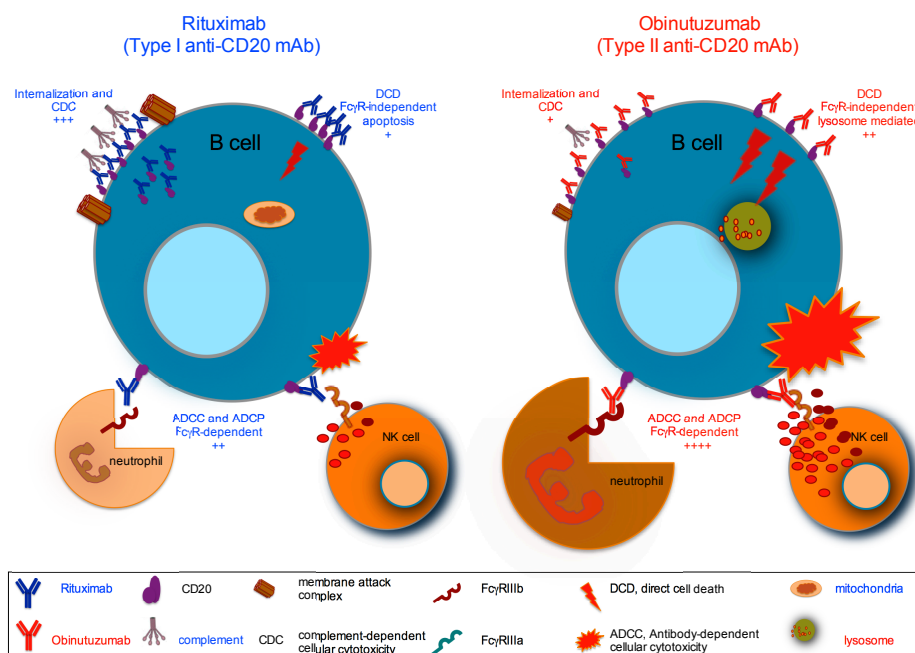
Figure 1.10 Graphical representation of the relative potency of type I and II anti-CD20 monoclonal antibodies at evoking effector mechanisms.

Table 1.7 Characteristics of type I and II anti-CD20 monoclonal antibodies

	Type I	Type II
Established mAb	Rituximab (Maloney, Grillo-Lopez et al. 1997) Ofatumumab (Teeling, Mackus et al. 2006) Ocrelizumab (Morschhauser, Marlton et al. 2010) AME-133v46 (Bowles, Wang et al. 2006) PRO131921 (Casulo, Vose et al. 2014) TRU-015 (Burge, Bookbinder et al. 2008, Hayden-Ledbetter, Cerveny et al. 2009) Velutuzumab (Goldenberg, Rossi et al. 2009)	obinutuzumab (Goede, Fischer et al. 2014) Tositumomab (Vose, Wahl et al. 2000) 11B8 (Li, Zhang et al. 2010)
In clinical or trial use in RA and/or SLE	Rituximab Ofatumumab Ocrelizumab	obinutuzumab
Redistributes CD20	Yes	No
Internalisation of anti-CD20-mAb complexes	Yes, but highly variable	To a small extent and significantly less than rituximab
Homotypic aggregation	Weak	Strong
CD20 tetramer to mAb binding ratio	1:1	2:1
CDC	potent	weak

ADCC	Yes	Yes
ADCP	Yes	Yes
DCD	Apoptosis	Non-apoptotic lysosome mediated cell death

ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cell phagocytosis; and CDC, complement-dependent cellular cytotoxicity.

Clinical efficacy of obinutuzumab in B-cell malignancies

Obinutuzumab was shown to be more effective than rituximab in CLL when both agents were used in combination with chlorambucil (Goede, Fischer et al. 2014). However, in this study rituximab and obinutuzumab were used at different doses and therefore it is not possible to exclude a dose-effect on the treatment outcomes. Recently, obinutuzumab in combination with bendamustine was more effective than bendamustine alone in patients with rituximab-refractory non-Hodgkin lymphoma (Sehn, Chua et al. 2016). This study demonstrated the efficacy of obinutuzumab in patients with CLL who had poor response to rituximab. Thus, it would appear that at least from the perspective of efficiency of B-cell depletion, obinutuzumab might overcome rituximab-resistance in depleting malignant B cells in vivo. It is also worth noting that dose-dependent differences in some clinical outcomes were noted in patients treated with standard or higher dose of obinutuzumab in a phase II trial of patients with treatment-naïve CLL (Byrd, Flynn et al. 2016). Detailed discussion of the clinical responses in B-cell malignancies is beyond the remit of this thesis, therefore, this topic will not be discussed further.

Rituximab-like type I anti-CD20 mAbs are more rapidly internalised, but evoke more potent CDC. In contrast, obinutuzumab-like type II anti-CD20 mAbs are internalised to a lower extent and are more efficient at evoking ADCC, ADCP and also FcγR-independent DCD. Further, obinutuzumab was glycoengineered to increase the affinity to bind Fcγ-receptor IIIa and thereby deliver better engagement with FcγR-bearing effector cells, consequently, evoke more efficient FcγR-mediated effector mechanisms (Mossner, Brunker et al. 2010).

However, there are no data comparing the pre-clinical activity of rituximab and obinutuzumab in RA and SLE. Such data would be of clear clinical importance to

understand whether obinutuzumab has greater potency in B-cell depletion and whether it may at least partly overcome autoimmune disease-related resistance mechanisms. Evidence for the mechanisms of B-cell depletion by rituximab in RA and SLE is limited and therefore evidence from studies in B-cell malignancies and mouse models may help to understand how disease-associated defects could impact on the efficiency of CD20 mAbs in autoimmune conditions.

Could immune abnormalities associated with autoimmune diseases impair the efficiency of anti-CD20 mAbs?

Complement-dependent cellular cytotoxicity

Complement is rapidly consumed in patients with CLL after rituximab infusion suggesting complement activation, in vivo (Kennedy, Beum et al. 2004). Replacement of complement using fresh frozen plasma enhances the efficiency of rituximab in in vitro complement assays using human CLL samples (Klepfish, Schattner et al. 2007). It was also shown that rituximab activity is dependent on complement in some animal models (Di Gaetano, Cittera et al. 2003). However, complement does not appear to be important for B-cell depletion in FL, where there is no correlation between B-cell depletion and the expression of complement defence molecules CD45, CD55 and CD59 (Weng and Levy 2003) and other studies also suggest that there is little requirement for complement for rituximab-induced B-cell depletion in vivo (Uchida, Hamaguchi et al. 2004, Beers, Chan et al. 2008). CD20 expression on malignant B cells may positively influence the extent to which mAbs may induce CDC, in vitro (Golay, Lazzari et al. 2001, Bologna, Gotti et al. 2011). It has been suggested that CDC depends on target cell expression of CD20 (van Meerten, van Rijn et al. 2006), and CDC may be enhanced by cytokine treatment to induce higher CD20 expression on B cells (Patz, Isaeva et al. 2011).

Complement has also been considered detrimental to B-cell depletion in some cases as deposition of C3b on CD20:rituximab complexes may promote their 'trogocytosis' by immune cells (Taylor and Lindorfer 2010, Taylor and Lindorfer 2015), and/or block the interaction between the Fc portion of rituximab and CD16A (FcγRIIIA) on NK cells (Wang, Racila et al. 2008), potentially inhibiting ADCC. Consistent with the concept of complement hindering the efficiency of rituximab, it was shown that FL patients with a C1qA polymorphism associated with low C1q levels displayed greater clinical responses to rituximab compared to those with high C1q levels associated with the

alternate allele (Racila, Link et al. 2008). Thus, current literature does not support a prominent role for CDC in determining rituximab efficacy.

Complement defects and homozygous C1q deficiency, in particular, are characteristic of lupus-like disease (Walport, Davies et al. 1998, Walport 2002). Could SLE-associated defects in complement impact on the efficiency of rituximab? In this regard it was shown that in murine models with C1q deficiency, B-cell depletion with rituximab was not significantly affected (Beers, Chan et al. 2008). Also, there is no direct evidence to support the view that B-cell depletion is less efficient in patients with SLE who have low complement levels and high anti-double-stranded DNA antibody levels respond well to rituximab therapy (Reddy, Jayne et al. 2013). Jones et al. showed that, in vitro, in the presence of sera from patients with seropositive RA, CDC induced by rituximab was inhibited (Jones, Shyu et al. 2013). However, whether such a phenomenon occurs in vivo, and if so, what effect this would have on the final degree of depletion and on clinical response remains to be determined, particularly because seropositive RA patients respond well to rituximab, as discussed earlier. Regardless, if CDC is a key effector mechanism for non-malignant B-cell depletion in vivo, the efficiency of type I mAbs like rituximab, but not type II mAbs like obinutuzumab, would be compromised by SLE-associated complement defects. Therefore, in this regard obinutuzumab may provide a mechanistic advantage in SLE.

Fc:FcγR dependent Effector Mechanisms

The critical importance of Fc:FcγR dependent effector mechanisms, ADCC and ADCP, for in vivo B-cell depletion was highlighted in animal models using malignant B-cell xenografts (Uchida, Hamaguchi et al. 2004). ADCC and ADCP are both dependent on the interaction between the Fc portion of mAb and FcγR on effector cells including NK cells, neutrophils, monocytes and macrophages. Current knowledge of which effector cell is key for B-cell depletion in vivo is mainly derived from studies in animal models and strengthened by indirect evidence of associations between genetic polymorphisms in patients with B-cell malignancies and autoimmune diseases and the clinical response to rituximab. Current evidence suggests that myeloid cells and likely macrophages are the key effectors, at least in mice (Dahal, Roghanian et al. 2015).

What other factors affect anti-CD20 mAb-induced ADCC/ADCP?In vitro, antigenic modulation of CD20 from the surface of normal and malignant B cells appears, at least partly, to account for the inferior efficiency of rituximab at inducing Fc:FcγR dependent effector mechanisms compared to type II anti-CD20 mAbs like obinutuzumab (Patz,

Isaeva et al. 2011, Tipton, Roghanian et al. 2015). Thus, CD20 modulation by mAbs is a key resistance mechanism specific to type I mAbs like rituximab and ofatumumab.

Antibody-dependent Cell Cytotoxicity

FcγR-mediated effector mechanisms including ADCC and ADCP are considered important mechanisms of cell death in vivo in both FL and autoimmune diseases such as RA and SLE. In FL, the high affinity FcγRIIIA 158V polymorphism and FcγRIIA 131H were shown to be associated with better clinical response (Cartron, Dacheux et al. 2002, Weng and Levy 2003). In patients with ITP, the high affinity FcγRIIIa 158V polymorphism and a reduction in platelet-associated antibodies were also predictive of a response to rituximab (Cooper, Stasi et al. 2012). In contrast, polymorphisms of activating Fc receptors (FcγRIIIA and FcγRIIA) do not predict response to rituximab in CLL (Farag, Flinn et al. 2004), which suggests that the most important effector mechanism may vary between diseases.

B cells from lupus-prone mice transgenic for human CD20 have greater resistance to depletion (Ahuja, Shupe et al. 2007), which may, at least in part, be due to an acquired defect in Ig-dependent phagocytosis (Ahuja, Teichmann et al. 2011). Murine studies suggest that B-cell depletion is dependent on both the cellular characteristics as well as the microenvironment (Gong, Ou et al. 2005). Interestingly, SLE patients from the early small phase I/II study were assessed for the genotypes of FcγRIIa and FcγRIIIa showing that clinical response and B-cell depletion was better in those with the high affinity FcγRIIIa 158VV genotype. This finding is similar to that noted in NHL, discussed earlier, and implicates the importance of ADCC as an important mechanism evoked by rituximab in vivo in patients with SLE (Anolik, Campbell et al. 2003). In RA, a retrospective study found that the high affinity FcγRIIIa 158V variant was associated with better clinical response (Kastbom, Coster et al. 2012). These studies suggest that ADCC may be the primary mechanism of depletion in vivo also in RA and SLE.

What factors associated with RA and SLE regulate ADCC? NK cells are key mediators of ADCC and defects in NK cell function are described both in RA and SLE (Neighbour, Grayzel et al. 1982, Park, Kee et al. 2009). Cytokine abnormalities associated with SLE such as increased levels of IFNα and IFNγ (Neighbour, Grayzel et al. 1982) appear to inhibit the cytotoxic function of NK cells, and consequently, ADCC (Ytterberg and Schnitzer 1984, Crow 2014). Interestingly, interferon signature is described as a marker of poor response to rituximab in patients with RA (Sibbitt, Mathews et al. 1983, Thurlings, Boumans et al. 2010, Raterman, Vosslamber et al. 2012), although alternative explanations including effects of IFNγ on the generation of plasmablasts

require consideration (Sellam, Marion-Thore et al. 2014). Thus, disease-associated elevated levels of cytokines may inhibit the cytotoxic function of NK cells, consequently, compromising the efficiency of rituximab.

In both RA and SLE the FcγRIIIa 158V polymorphism is associated with a better clinical response to rituximab (Anolik, Campbell et al. 2003, Kastbom, Coster et al. 2012) providing indirect evidence for the importance of Fc:FcγR-dependent effector mechanisms in these conditions. In ADCC assays using effector cells from healthy individuals homozygous for the lower affinity 158F FcγRIIIa polymorphism it was shown that the efficiency of rituximab was inferior to obinutuzumab (Mossner, Brunker et al. 2010). Therefore, in this context, obinutuzumab with an afucosylated Fc portion may overcome the limitation of the low affinity FcγRIIIa 158F allele in some individuals with SLE and prove advantageous.

Antibody-dependent cell phagocytosis

ADCP is considered a critical effector mechanism evoked by anti-CD20 mAbs with neutrophils, küpffer cells and macrophages serving as effectors. In mice, küpffer cells in the liver play an important role in the deletion of B cells following anti-CD20 mAb binding (Montalvao, Garcia et al. 2013). Recently, it was shown that the threshold to induce ADCP by küpffer cells was lower for obinutuzumab compared to rituximab (Grandjean, Montalvao et al. 2016). However, in humans, the data on the role of küpffer cells in the liver participating in ADCP is currently limited, and so will not be discussed further here.

With regards to other phagocytes, neutrophils are the major leukocytes in peripheral blood and whole blood assays have revealed that they can mediate phagocytosis of mAb opsonised B-cells (Shibata-Koyama, Iida et al. 2009, Golay, Da Roit et al. 2013). Given the high level of homology between FcγRIIIa and FcγRIIIb, Fc modification through afucosylation also results in higher affinity binding to FcγRIIIb on neutrophils. As a result, glycoengineered rituximab and obinutuzumab, are shown to induce greater neutrophil-mediated phagocytosis of mAb opsonised B cells in whole blood assays compared to standard non-glycoengineered rituximab (Golay, Da Roit et al. 2013). Although polymorphisms of FcγRIIIb do not correlate with clinical response in FL (Cartron, Ohresser et al. 2008), concurrent administration of GMCSF with rituximab improves clinical response (Cartron, Zhao-Yang et al. 2008). However, improvements in clinical response with GMCSF are probably not limited to effects on neutrophils, but also attributable to other effector cells such as monocytes and macrophages. Therefore, to what extent neutrophil-mediated phagocytosis determines mAb efficacy is

not clear. Furthermore, in some lupus patients, clearance of neutrophil extracellular traps (NETs) is inefficient (Leffler, Martin et al. 2012). Formation of NETs is regulated by FcγRIIIb cross linking (Omar Rafael, Nancy et al. 2016) and provides a potent and continuous stimulus for type 1 IFN release contributing to lupus pathogenesis. It will therefore be important to assess how neutrophil 'NETing' is influenced by rituximab and type II anti-CD20 mAbs.

Although different FcγR expressing effector cells are probably involved in mAb mediated ADCP, macrophages appear to be the key effectors. Evidence from animal studies using targeted deletion of effector cells, suggests that the main effector cells for in vivo B-cell depletion are macrophages, not NK cells or neutrophils (Di Gaetano, Cittera et al. 2003, Cragg and Glennie 2004, Uchida, Hamaguchi et al. 2004, Gong, Ou et al. 2005, Golay, Cittera et al. 2006, Chao, Alizadeh et al. 2010). In humans, high numbers of tumour-associated macrophages are predictive of poor prognosis in patients treated with chemotherapy alone, but not in patients treated with rituximab (Canioni, Salles et al. 2008) suggesting that rituximab treatment is facilitated by these macrophages, partly overcoming the chemotherapy-resistance effects. The correlation between FcγRIIa 131H polymorphism with clinical response to rituximab in FL (Weng and Levy 2003) also provides indirect evidence for the role of macrophages as key effectors, because the majority of macrophages and very few (if any) NK cells express FcγRIIa (Metes, Galatiuc et al. 1994). These findings suggest that macrophages are probably the key effector cells that mediate rituximab-induced B-cell depletion in B-cell malignancies.

In vitro assays suggest that high levels of immunoglobulin, often present in patients with SLE, may impair the efficacy of rituximab to a greater extent compared to obinutuzumab, presumably by inhibiting Fc:FcγR dependent effector mechanisms (Bologna, Gotti et al. 2011) and this is supported by animal studies in a lupus-prone mouse model (Ahuja, Teichmann et al. 2011). As yet there is no evidence on whether, if at all, the level of hypergammaglobulinemia described in SLE adversely affects the efficiency of anti-CD20 mAbs. Nevertheless, as afucosylation increases the affinity of IgG for FcγRIIIa on effector cells, glycoengineered mAbs such as obinutuzumab may overcome competition from the high levels of immunoglobulin resulting from hypergammaglobulinemia and perhaps yield mAb which perform better at lower concentrations in vivo, as shown in vitro (Bologna, Gotti et al. 2011, Golay, Da Roit et al. 2013, Tipton, Roghanian et al. 2015). Alternatively, engineering of mAbs with amino acid substitutions in the Fc portion (e.g. G236A) designed specifically to increase IgG1 affinity in favour of FcγRIIa relative to FcγRIIb may promote phagocytosis of mAb-

opsonised B cells by macrophages (Richards, Karki et al. 2008). However, whether such Fc engineered mAbs will increase the efficiency of mAb-mediated FcγR-mediated effector mechanisms and improve clinical response remains to be proven.

Direct Cell Death

Currently, our understanding of the importance of DCD elicited by anti-CD20 mAbs is primarily derived from studies in malignant cell-lines because evidence of DCD as an effector mechanism in vivo in humans is limited. The pathways to DCD by mAbs include induction of caspase-dependent apoptotic cell death upon hyper-crosslinking of rituximab (Szodoray, Alex et al. 2004), and lysosome-mediated cell death in the case of type II mAbs like obinutuzumab, which is activated in the absence of further crosslinking (Beers, Chan et al. 2008, Ivanov, Beers et al. 2009). In vivo, FcγR-bearing effector cells are proposed to deliver crosslinking of mAbs bound to B cells (Glennie, French et al. 2007) but whether this delivers DCD is unclear. In support, there is some evidence of caspase activation in samples from patients with CLL treated with rituximab suggesting induction of apoptosis in vivo (Byrd, Kitada et al. 2002). Expression of anti-apoptotic proteins such as MCL-1 is reported in other B-cell malignancies and may be associated with resistance to rituximab (Winter, Weller et al. 2006, Awan, Kay et al. 2009).

Type I anti-CD20 mAbs, but not type II anti-CD20 mAbs, binding of CD20 seems to trigger B cell receptor signaling pathway associated Src and Syk tyrosine kinases (Walshe, Beers et al. 2008). Although the biological significance of this is not entirely clear, it may explain the distinct modes of cell death induced by the two types of anti-CD20 mAbs. Type I anti-CD20 mAbs induce apoptotic cell death whereas type II anti-CD20 mAbs induce homotypic adhesion and evoke non-apoptotic lysosomal cell death. In this respect, differential expression of anti-apoptotic genes in B-cell subpopulations (Shen, Iqbal et al. 2004) may not impact on lysosomal DCD induced by type II anti-CD20 mAbs.

Non-malignant B-cell subpopulations differ in their expression of anti-apoptotic proteins such as Bcl-2 such that switched (IgD-CD27+) memory B-cells express relatively higher levels than naïve (IgD+CD27-) B cells (Klein, Tu et al. 2003, Ehrhardt, Hijikata et al. 2008). Therefore, switched (IgD-CD27+) memory B cells may offer greater resistance to rituximab-mediated apoptotic DCD. The regulation of apoptotic genes Bcl-2 and Bcl-xl may not only enhance the survival of autoreactive B cells contributing to the development of autoimmunity (Lopez-Hoyos, Carrio et al. 1998), but also alter their susceptibility to rituximab-induced apoptosis (Shen, Iqbal et al. 2004). Further, in

some patients with SLE, the levels of BAFF may be high and therefore BAFF-mediated anti-apoptotic effects (Craxton, Draves et al. 2005) may also offer additional resistance to rituximab. In contrast, type II mAbs like obinutuzumab induce lysosome-mediated DCD (independent of intrinsic apoptosis) and therefore the ability of obinutuzumab to induce DCD in non-malignant B-cells is less likely to be hindered by the expression of anti-apoptotic proteins.

Opsonisation with rituximab does not always induce B-cell depletion in vivo (Kamburova, Koenen et al. 2013, Wallin, Jolly et al. 2014). This raises the question as to what extent DCD serves as an effector mechanism for rituximab in vivo and whether obinutuzumab may induce greater DCD as shown to be the case in vitro (Mossner, Brunker et al. 2010). In vitro, rituximab induces modest levels of DCD in both IgD+CD27- naïve and IgD-CD27+ memory B-cell subpopulations in samples from patients with RA (Szodoray, Alex et al. 2004). One possible explanation for the differences in susceptibility to DCD may lie in the differences between B-cell subpopulations in the expression of apoptotic genes (Shen, Iqbal et al. 2004). Regardless, DCD may be an important effector mechanism particularly when other effector mechanisms such as CDC, ADCC and ADCP are compromised due to deficiencies in complement, lack of access to target cells and / or inherent or acquired defects in effector cells.

Thus, as discussed earlier, whilst a number of factors are associated with incomplete B-cell depletion with rituximab (Figure 1.11) disease-associated immune abnormalities may also potentially affect the efficiency of anti-CD20 mAbs Table 1.8.
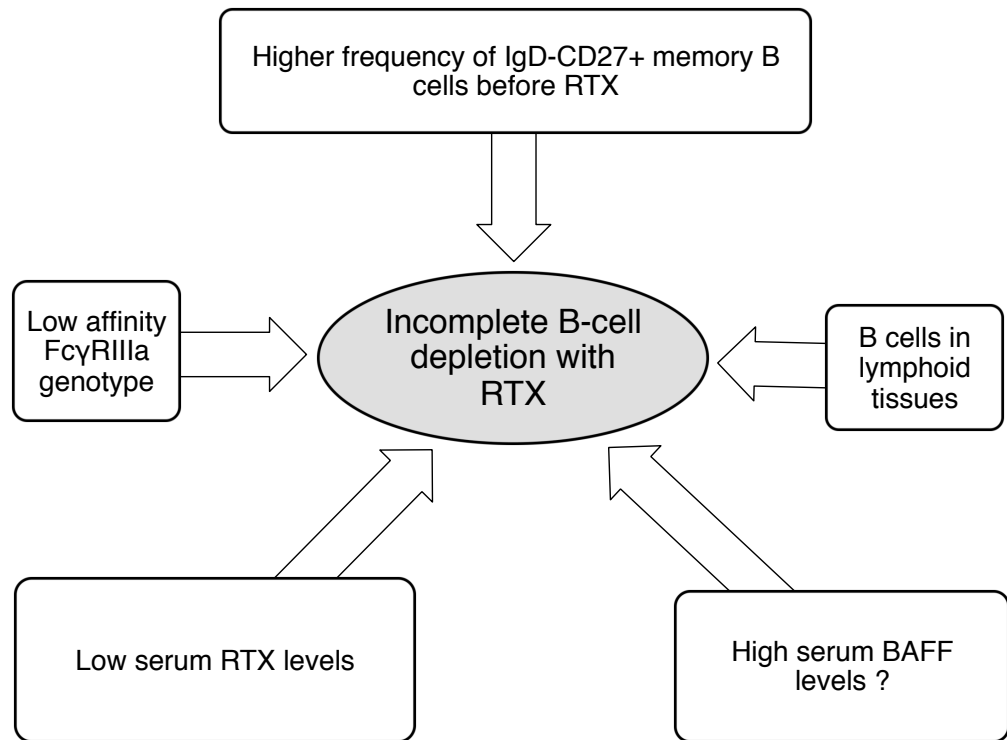
Figure 1.11 Factors associated with incomplete B-cell depletion with rituximab.

Incomplete B-cell depletion with rituximab treatment is associated with: a higher frequency of IgD-CD27+ switched memory B cells at baseline; the low-affinity FcγRIIIa genotype; low serum rituximab levels in some studies; high serum BAFF levels (limited evidence); and location of B cells in lymphoid tissues.

Table 1.8 Disease-related immune abnormalities and potential effect on the efficiency of CD20 mAb effector functions

Immune abnormality associated with disease	Effect on condition	Predicted effect on effector mechanism	Reference
C1q deficiency	Risk of SLE	CDC ↓	(Walport, Davies et al. 1998)
FcγRIIb T232	Risk of SLE	Reduced activity of FcγRIIB ADCP↑	(Clynes, Towers et al. 2000, Floto, Clatworthy et al. 2005)
FcγRIIb promoter haplotype 2B.1-386G-120T	Risk of SLE	Associated with increased expression of FcγRIIb on both lymphocytes and myeloid cells ADCC ↓ ADCP ↓	(Su, Wu et al. 2004)
FcγRIIIa 158F** (low affinity allele)	Risk of SLE	ADCC ↓ ADCP ↓	(Wu, Edberg et al. 1997, Seligman, Suarez et al. 2001, Dong, Ptacek et al. 2014)
FcγRIIIb polymorphisms and copy number variations associated with reduced expression/binding	Risk of SLE	Neutrophil ADCP ↓	(Morris, Roberts et al. 2010, Vigato-Ferreira, Toller-Kawahisa et al. 2014)
rs1143679 variant of ITGAM gene encoding the R77H	Risk of SLE	Reduced phagocytosis of iC3b opsonised targets by neutrophils,	(Rhodes, Furnrohr et al. 2012) (Zhou,

variant of CD11b		monocytes and macrophages ADCP ↓	Wu et al. 2013)
nonsynonymous SNPs rs1143678, and rs1143683 of ITGAM encoding CD11b	Risk of SLE	Reduced phagocytosis of iC3b opsonised targets by neutrophils ADCP ↓	(Zhou, Wu et al. 2013)
CD11b ↑ and CD62L ↓ on neutrophils	Risk of vasculitis associated with RA	ADCP ↑	(Haruta, Kobayashi et al. 2001)
Hypergammaglobuli naemia	Associated with SLE	ADCC / ADCP ↓	(Bologna, Gotti et al. 2011)
IFNγsignature	Risk of SLE Poor response to rituximab in RA	ADCC ↓	(Thurlings, Boumans et al. 2010, Raterman, Vosslamber et al. 2012, Crow 2014)
Reticuloendothelial Fc-Receptor-mediated clearance defects	Defect correlates with disease severity in SLE	ADCP ↓	(Frank, Hamburger et al. 1979)

RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; CDC, complement-dependent cellular cytotoxicity; ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cell phagocytosis; and DCD, direct cell death. SLE, systemic lupus erythematosus; CDC, complement-dependent cellular cytotoxicity; ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cell phagocytosis; SNP, single nucleotide polymorphism. **, In some nomenclature the 158 residue in FcγRIIIa is defined as 176.

Mechanisms of Resistance

Internalisation of mAbs and the efficiency of B-cell depletion

Internalisation of anti-CD20 mAbs may limit their efficiency (Beers, French et al. 2010). A study by Lim et al., suggests that rituximab modulates CD20, a process regulated by the inhibitory FcγRIIb on B cells. Further, clinically meaningful differences in target cell expression of FcγRIIb were noted between histological types of B cell malignancies such that FL with better response to rituximab had significantly low levels compared to CLL usually associated with poor response to rituximab (Lim, Vaughan et al. 2011). Although genetic polymorphisms in the inhibitory FcγRIIb 232I/T were found not to predict clinical outcome in FL (Weng and Levy 2009), it was shown that the target tumor expression of the inhibitory FcγRIIb was predictive of response in FL with significantly better responses seen in patients with tumor targets expressing low levels of FcγRIIb (Chern Siang Lee, Margaret Ashton-Key et al. 2013). Thus, antigenic modulation of CD20 may, at least in part, account for the inferior efficiency of rituximab at inducing Fc:FcγR dependent effector mechanisms compared to type II anti-CD20 mAbs like obinutuzumab in normal and malignant B cells (Patz, Isaeva et al. 2011, Tipton, Roghanian et al. 2015).

Early studies in lymphoma cell lines suggested differential rates of internalisation of mAbs targeting CD19, CD20 and CD22 (Press, Farr et al. 1989). Anti-CD22mAb was reported to show increased internalisation by B cells from patients with SLE than healthy individuals (Jacobi, Goldenberg et al. 2008). However, whether anti-CD19 and anti-CD20 mAbs are internalised by RA and SLE B cells is not known. Moreover, the inhibitory FcγRIIb disparately regulated internalisation of these mAbs in malignant B cells, regardless of FcγRIIb activation (Vaughan, Iriyama et al. 2014). Whether internalisation of anti-CD20 mAbs influences the efficiency of depletion of B cells from patients with RA and SLE is not known, but would be of clear clinical importance if we are to make progress in our efforts to improve B-cell depletion to enhance clinical response to B-cell depletion therapy in RA and SLE.

Alternative Resistance Mechanisms

 An alternative proposed mechanism of resistance is 'shaving of rituximab–CD20 complexes', whereby, probably due to effector cell exhaustion, rituximab treatment, instead of triggering the effector cell phagocytosis or cytotoxic degranulation to cause target cell death, leads to 'trogocytosis' of rituximab–CD20 complexes, initially demonstrated for malignant B-cells by the Taylor laboratory (Taylor and Lindorfer

2015) and also shown in samples from patients with RA (Jones, Hamilton et al. 2012). It has been suggested that using low-dose more frequent regimens (fractionated subcutaneous rituximab thrice weekly) may minimise trogocytosis and preserve CD20 on the cell surface (Williams, Densmore et al. 2006). Further studies are warranted to evaluate the relevance of this phenomenon in real clinic situations and outside the CLL context with cells with lower expression of rituximab. The relevance of this phenomenon in real clinic situation is not known and therefore will not be addressed in this thesis.

Conclusions

Thus, in RA and SLE, Rituximab-based B-cell depletion therapy results in variable biological and clinical response, at least partly, due to variability in the degree of B-cell depletion. Whilst the effects of rituximab on serum immunoglobulins in RA are well described, data about the effects of rituximab on serum immunoglobulins in SLE are poor and such data may provide insights into the factors associated with the development of hypogammaglobulinemia that would be relevant to understand the risk of adverse events and/or persistence of anti-dsDNA antibodies, which may reflect a mechanism of resistance to rituximab.

Further, whether the variability in B-cell depletion in RA and SLE might be explained by serum rituximab levels and/or B cell intrinsic mechanisms of resistance such as internalisation and/or disease associated factors including complement defects, inefficiency of antibody-dependent cellular cytotoxicity mediated by NK cells and antibody-dependent cell phagocytosis mediated by neutrophils and macrophages is not known. The efficiency of rituximab at inducing direct cell death of B cells from patients with RA and SLE remains elusive. Importantly, pre-clinical data on whether the type II anti-CD20 monoclonal antibody, obinutuzumab, provides mechanistic advantages relevant to autoimmune diseases, as an alternative B-cell depleting agent would be of clear clinical importance in both RA and SLE.

Thesis Aims

Based on the considerations, the aims of the project were to understand:

1) the mechanisms of resistance to rituximab-induced B-cell cytotoxicity in RA and SLE; and

2) whether better responses, in vitro, could be obtained with mechanistically different anti-CD20 mAbs

Therefore the focus was on the following questions:

1) Are there any differences between RA and SLE patients in the degree and duration of rituximab-induced B-cell depletion?
2) What are the long-term effects of rituximab on different serum immunoglobulin isotypes and anti-dsDNA antibodies in patients with SLE? Are pre-treatment levels of serum immunoglobulins and / or anti-dsDNA antibodies predictive of biological response to rituximab?
3) What is the relationship between CD19+ B cell counts and serum rituximab levels after treatment with rituximab in patients with RA and SLE?
4) Are type II anti-CD20 mAbs more efficient than type I at deleting B cells from patients with RA and SLE, in vitro?
5) Do B cells from patients with RA and SLE internalise rituximab? And if so, does internalisation influence the efficiency of B-cell depletion?
6) Are there any differences between B-cell subpopulations in their ability to internalise anti-CD20 mAbs? If so, what factors regulate internalisation of anti-CD20 mAbs?
7) Are there any differences between the two types of anti-CD20 mAbs in the effector mechanisms of relevance to RA and SLE? Are there any mechanistic advantages for considering type II anti-CD20 mAbs in RA and SLE?

Objectives

The main objectives were to: (i) compare the efficiency of type I and II anti-CD20 mAbs to induce B-cell cytotoxicity in in vitro whole blood depletion assay, (ii) investigate whether the percentage of surface accessible Alexa-488-labelled anti-CD20 mAbs on B cells after incubation with obinutuzumab (GA101gly, type II mAb) when compared with rituximab in samples incubated with and without anti-alexa-488 was greater than rituximab; (iii) investigate whether internalisation of mAbs correlated with the efficiency of type I and II anti-CD20 mAbs as assessed by whole blood depletion assay; (iv)

examine whether FcγRIIb regulated internalisation of anti-CD20, anti-CD22 and anti-CD19mAbs; (v) compare internalisation of type I and II anti-CD20 mAbs in B-cell subpopulations; (vi) study the effect of B-cell activation on internalisation of mAbs; (vii) compare the efficiency of type I and II anti-CD20 mAbs at inducing complement-dependent cellular cytotoxicity in vitro; (viii) compare the efficiency of type I and II anti-CD20 mAbs at inducing NK cell and neutrophil activation; and (X) compare the efficiency of type I and II anti-CD20 mAbs at inducing direct cell death by anti-CD20 mAbs.

Chapter 2

Methods and Study Design

Patients, Materials and Methods

Healthy blood donors and patients

All patients with RA satisfied the American College of Rheumatology (ACR)/European League Against Rheumatism classification criteria (Aletaha, Neogi et al. 2010) and all patients with SLE met the ACR classification criteria (Petri, Orbai et al. 2012).

Clinical and biochemical parameters

Clinical data was obtained from University College London Hospital (UCLH) electronic patient records. This was an observational and non-interventional study. Ethical approval for the study was obtained from UCLH Research Ethics Committee (references: 11/LO/1610 and 08/H0715/18). Clinical care was not influenced by the participation of the patient in the study. The data collection on clinical and biochemical parameters was guided by clinical need and constituted routine clinical care. Data about validated disease activity measures including Disease Activity Score 28 (DAS-28) and British Isles Lupus Activity Grade (BILAG) score were also collected for patients with RA and SLE, respectively, as part of routine clinical care.

Laboratorial parameters were also collected as part of routine care and were assessed by the UCLH central laboratory. Unless indicated clinical and laboratorial data were analysed retrospectively. Clinical data analysed include patient demographics: age, sex, ethnicity, disease activity indices, therapies; and serum levels of immunoglobulins and anti-dsDNA antibodies.

Peripheral Blood Sample collection and usage

Whole blood samples from all participants were anticoagulated with lithium heparin and peripheral blood mononuclear cells (PBMCs) were separated using Ficoll-Hypaque density centrifugation. PBMCs were either used fresh or stored at -80°C. B cells were isolated from PBMCs by negative selection with using EasySep™ Human B Cell Enrichment Kit (Stemcell). Using this method, the typical purity of B-cell isolation was >95%.

Serum rituximab levels

Serum rituximab levels were measured by Dr Gerona and Dr Croca in samples from patients with RA and SLE, respectively. Serum rituximab levels were measured with a capture ELISA (enzyme linked immunosorbent assay, based on anti-idiotype

antibodies that bind to rituximab, using sera diluted at a concentration of 1/40,000 were employed. This method using monoclonal antibodies directed against the rituximab idiotype provides detection to a lower limit of quantification (LOQ) of 0.005-0.01μg/mL compared to the polyclonal ELISA with a LOQ of 0.5-6.6 μg/mL.

Antibodies and reagents

A range of mAbs targeting B-cell antigens were used for different experiments, listed in Table 2.1.

Anti-CD20 mAbs

Type I Anti-CD20 mAbs

Type I ant-CD20 mAbs used here included rituximab and ofatumumab. Commercially available rituximab used in routine clinical practice was used in all experiments and was a gift from Southampton General Hospital Pharmacy. Ofatumumab was produced in house by Prof Cragg's team at the Tenovus Research Laboratory, Southampton University, U.K based on patent published sequences using Chinese Hamster Ovary (CHO) or 293F cells as vectors for transfection and protein expression. Therefore, the carbohydrate structures may differ from mAbs in clinical use.

Type II Anti-CD20 mAbs

A range of type II anti-CD20 mAbs were used. Tositumomab, the prototype murine type II anti-CD20mAb of IgG2a isotype was a kind gift from Prof T. Illidge (University of Manchester, United Kingdom). Other type II anti-CD20 mAbs used include: GA101gly (glycosylated GA101 or obinutuzumab with unmodified Fc portion produced in house by Prof Cragg's team, at the Tenovus Research Laboratory, Southampton University, U.K, based on published patented sequences); obinutuzumab (type II anti-CD20mAb with afucosylated Fc portion that is in clinical use), OBZ-WT or OBZGly (obinutuzumab-wild type, obinutuzumab with glycosylated Fc portion), OBZA-LALA (obinutuzumab with mutated Fc portion that does not engage with FcγR strongly, OBZ-LALA (Hezareh, Hessell et al. 2001). Various preparations of type II anti-CD20 mAbs were used to determine the effect of Fc modification and compare the efficiency of the type II anti-CD20 mAbs at evoking FcγR-mediated effector mechanisms.

GA101 and obinutuzumab refer to the same mAb, but different names were retained reflecting the chronology of nomenclature. GA101 or obinutuzumab was not available for the initial set of experiments described in Chapter 4. Therefore, GA101gly was

produced in-house based on patent published sequences using Chinese Hamster Ovary (CHO) or 293F cells as vectors for transfection and protein expression. Therefore, their carbohydrate structures may differ from mAbs in clinical use. For the experiments described in Chapter 5, Roche Glycart, Basel, Switzerland provided all type II anti-CD20 mAbs.

In experiments described in Chapter 5, anti-CD20 mAbs used include obinutuzumab and OBZ-WT or OBZ$_{Gly}$ and OBZ-LALA were used. This combination allowed to study the effect of Fc afucosylation on the efficiency of mAbs at evoking FcγR-mediated effector mechanisms, in vitro. Whilst obinutuzumab with afucosylated Fc would be expected to bind FcγRIIIa on effector cells with greater affinity than OBZ-WT and OBZ-LALA. The effect of Fc glycoengineering would be particularly relevant in SLE where hypergammaglobulinemia may impact on ADCC evoked by mAbs as shown for malignant B cells (Bologna, Gotti et al. 2011).

AT10 was used to inhibit FcγRIIb on B cells in the surface-fluorescence quenching assays. AT10 was produced in house by Prof Cragg's team at Southampton University, and binds both FcγRIIa and FcγRIIb (Greenman, Tutt et al. 1991). However, B cells do not express FcγRIIa and therefore the antagonistic effects of AT10 on B cells would be attributable to the effects on FcγRIIb on B cells, as described previously (Lim, Vaughan et al. 2011).

Alexa-488 and anti-Alexa-488 were purchased from Invitrogen. mAbs were labeled with Alexa-488 as per manufacturer's (Invitrogen, U.K.) instructions by Prof Cragg's team based at Southampton University. Anti-alexa-488 was used as a quenching antibody in the surface fluorescence quenching assay based on the principle that anti-Alexa-488 binds to Alexa-488-labelled mAbs on the cell surface, but not the Alexa-488-labelled mAbs that have been internalised, which would be detectable by flow-cytometry (Lim, Vaughan et al. 2011).

mAbs targeting other B-cell antigens

In some experiments mAbs directed against CD19, CD22 and CD38 were used. These mAbs were selected based on the considerations discussed in Chapter 1. Anti-CD19, anti-CD22 and anti-CD38 mAbs were produced in house by Prof Cragg's team based on patent published sequences using Chinese Hamster Ovary (CHO) or 293F cells as vectors for transfection and protein expression. Anti-CD19 and anti-CD22 are being explored for use in SLE as discussed in Chapter 1 whereas anti-CD38 was developed for use in B-cell malignancies and is considered not disposed to internalisation after

binding to anti-CD38 mAbs as shown in malignant B-cell lines (Vaughan, Iriyama et al. 2014). Therefore anti-CD38mAb was used as a positive control in internalisation experiments, described later and perhaps have the potential for use as an alternative B-cell depleting agent.

Flow cytometry

The following fluorochrome-conjugated mAbs targeting respective antigens were procured from Becton Dickinson biosciences or Biolgend, U.K. and are summarized in Table 2.2. Flow cytometry was performed using a Becton Dickinson LSR Fortessa cell analyzer using whole blood samples. Red blood cells were lysed using Becton Dikinson red cell lysis buffer, pharmlyse.

Lymphocytes were identified based on forward- and side-scatter characteristics. B cells were identified as CD19+ or CD20+, T cells as CD3+ and NK cells as CD3-56+. Neutrophils were identified based on forward- and side-scatter characteristics and CD15 positivity.

B-cell subpopulations

B-cell subpopulations were identified based on the relative expression of IgD and CD27, as described previously (Klein, Rajewsky et al. 1998). The protocol for defining B cell subpopulations was: naïve (IgD+CD27-); unswitched memory cells (IgD+CD27+); post-switched or switched memory cells (IgD-CD27+); and double negative (IgD-CD27-) DN cells; shown in Figure 2.1.

Table 2.1 Antibodies and reagents

Monoclonal antibody	Target antigen	source
AT10	FcγRIIa and FcγRIIb	Produced from published patented sequences in house at Tenovus laboratory, University of Southampton.
Rituximab	CD20	Southampton General Hospital Pharmacy
Ofatumumab	CD20	Produced from published patented sequences in house at Tenovus laboratory, University of Southampton.
Tositumomab (B1)	CD20	Gift from Prof T. Illidge, University of Manchester, United Kingdom
GA101gly (glycosylated GA101 with unmodified Fc portion, BHH2)	CD20	Produced from published patented sequences in house at Tenovus laboratory, University of Southampton
Obinutuzumab (OBZ)	CD20	Provided by Roche Glycart
OBZ-LALA (mutated Fc that does not engage with FcγR	CD20	Provided by Roche Glycart
OBZ$_{Gly}$ (obinutuzumab wild-type with glycosylated Fc, OBZ-WT)	CD20	Provided by Roche Glycart
Anti-CD19	CD19	Produced from published patented sequences in house at Tenovus laboratory, University of Southampton.

Anti-CD22	CD22	Produced from published patented sequences in house at Tenovus laboratory, University of Southampton.
Alexa-488	Labeling mAbs	Invitrogen, U.K.
Anti-Alexa 488	Alexa-488	Invitrogen, U.K.
Culture medium	RPMI 1640	gibco, Thermo Fisher Scientific
Fetal Bovine Serum supplementation	FBS	Sigma-Aldrich
Human B Cell Enrichment Kit	EasySep™	Stemcell
Red cell lysis buffer	Pharmlyse	Becton Dickinson

Table 2.2 Flow cytometry antibodies

Antigen	Fluorochrome	source
CD3	Allophycocyanin, APC	Beckton Dickinson, U.K.
CD5	Fluorescein Isothiocyanate, FITC	Beckton Dickinson, U.K.
CD11b	Phycoerythrin, PE	Biolgend, U.K.
CD15	Fluorescein Isothiocyanate, FITC	Biolgend, U.K.
CD16	Allophycocyanin-Cyanide dye 7, APC-Cy7	Biolgend, U.K.
CD19	Phycoerythrin-Cyanide dye 7, PE-Cy7 or Peridinin-Chlorophyll proteins-Cyanide 5.5, PerCP-Cy5.5 or AlexaFluor-700	Beckton Dickinson, U.K.
CD20	Fluorescein Isothiocyanate, FITC	Beckton Dickinson, U.K.
CD32	Phycoerythrin, PE	Beckton Dickinson, U.K.
CD45	Phycoerythrin, PE	Beckton Dickinson, U.K.
CD56	Phycoerythrin, PE	Beckton Dickinson, U.K.
CD62L	Allophycocyanin, APC	Biolgend, U.K.
CD107a	Brilliant Violet 421	Biolgend, U.K.
IgD	Brilliant Violet 421	Beckton Dickinson, U.K.
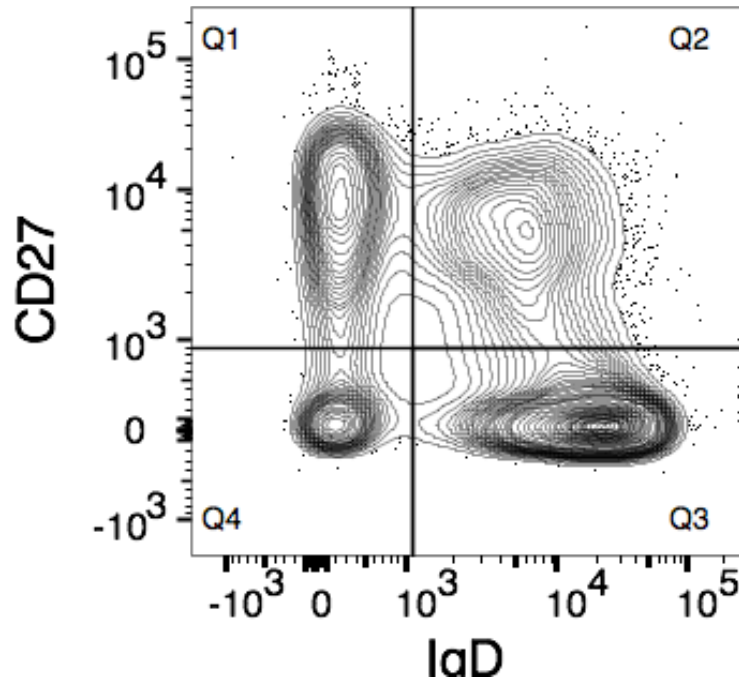
Figure 2.1 Defining B-cell subpopulations based on relative expression of IgD and CD27.

B-cell subpopulations were identified based on relative expression of IgD and CD27 as Q1, IgD-CD27+ switched memory cells; Q2, IgD+CD27+ unswitched memory B cells; Q3, IgD-CD27- double negative cells; and Q4, IgD+CD27- naïve B cells.

Whole blood B-cell depletion assay

The efficiency of anti-CD20 mAbs may be assessed in vivo or in vitro. B-cell depletion in vivo is usually assessed by CD19+ B-cell counts in peripheral circulation. In some clinical studies discussed in the chapter Introduction, B-cell depletion involving patients with RA and SLE was assessed: i) by analyzing up to 20,000 events gated on lymphocytes on flow cytometry to determine CD19+ B-cell counts, in peripheral blood; ii) by analyzing 100,000 to 500,000 events gated on lymphocytes described as highly sensitive flow cytometry to determine CD19+ B-cell counts in peripheral circulation; and iii) tissue depletion, to a limited extent, in samples from tonsils (SLE), and/or synovium (RA).

Although of clear clinical importance, B-cell depletion in the tissues may be difficult to study due to limited availability of samples and technical constraints. As yet, there are no validated imaging techniques that clearly demonstrate the extent of B-cell depletion

in lymphoid tissues in humans. In contrast, B-cell depletion may be relatively easily assessed by peripheral blood B cell counts and requires only a small amount of blood.

The main advantages of using the whole blood B-cell depletion assay to compare the efficiency of anti-CD20 mAbs are that:

1) a small amount of blood, typically less than 5 mls is adequate;
2) the assay can be performed using freshly drawn samples directly without the need for processing further, for example, by density gradient separation and centrifugation for acquiring peripheral blood mononuclear cells;
3) whole blood samples may be analysed by flow cytometry after red cell lysis and the results are rapidly available; and
4) the results represent a cumulative effect of different effector mechanisms evoked by anti-CD20 mAbs.

An important limitation though is that the results of the whole blood B-cell depletion experiments may not be extrapolated to infer on the efficiency of B-cell depletion in secondary lymphoid tissues. Nonetheless, in vitro, whole blood B-cell depletion assay represents a comprehensive method to study the efficiency of anti-CD20 mAbs at inducing cytotoxicity in B cells from patients with RA and SLE because the assay would assess the cumulative effect of all four effector mechanisms of B-cell depletion evoked by the anti-CD20 mAbs are accounted for in the assessment of the efficiency of anti-CD20 mAbs. Several groups have used whole blood B-cell depletion assays to compare the efficiency of type I and II anti-CD20 mAbs at inducing cytotoxicity of malignant B cells in vitro (Mossner, Brunker et al. 2010, Bologna, Gotti et al. 2013). Moreover, the assessment of the efficiency of anti-CD20 mAbs in different microenvironments such as in in vitro experiments using PBMCs would not account for the effect of sera factors such as complement and immunoglobulins, present in physiological concentrations in whole blood, but may potentially influence the efficiency of mAbs (Bologna, Gotti et al. 2011, Bologna, Gotti et al. 2013). However, this technique has not yet been studied in autoimmune disease such as RA and SLE, where B-cell counts would be much lower compared to B-cell malignancies.

Therefore, the in vitro whole blood B-cell depletion assay was used to compare the efficiency of different types of anti-CD20 monoclonal antibodies in a close-to physiological environment.

Here, whole blood B-cell depletion assays were used to:

1) compare the efficiency of type I and II anti-CD20 mAbs at inducing cellular cytotoxicity, in vitro;
2) assess the effect of BAFF on the efficiency of anti-CD20 mAbs; and
3) compare the ability of anti-CD20 mAbs to evoke FcγR-mediated effector mechanisms, in vitro;

The whole blood depletion assay was performed using fresh whole blood samples, as described previously (Mossner, Brunker et al. 2010, Bologna, Gotti et al. 2011, Bologna, Gotti et al. 2013). Briefly, 100µL of freshly drawn whole blood, anticoagulated with heparin, was incubated with or without mAbs at 37°C and 5% CO_2. Samples were harvested after 24 hours and stained with anti-CD3, anti-CD19 and anti-CD45 and incubated for 30 minutes before lysing red blood cells with red cell lysing buffer, BD PharmLyse. The time taken for different effector mechanisms to be activated may vary. Whilst CDC would be expected to be optimal by 30 minutes other effector mechanisms such as ADCC and ADCP may be evoked optimally over a relatively longer time scale. Therefore, the samples were analysed after 24 hours of incubation with mAbs. For each sample, 10,000 events were acquired in the lymphocyte gate and the frequency of CD19+ B cells and CD3+T cells were analysed. An example illustrating the protocol used in whole blood B-cell depletion assay to compare the efficiency of type I and II anti-CD20 mAbs, in vitro. RTX, rituximab; $GA101_{gly}$, a glycosylated type II anti-CD20 mAb shown in Figure 2.2.

In some experiments, the frequency of CD3-CD56+NK cells and CD15+neutrophils were also analysed, discussed later.
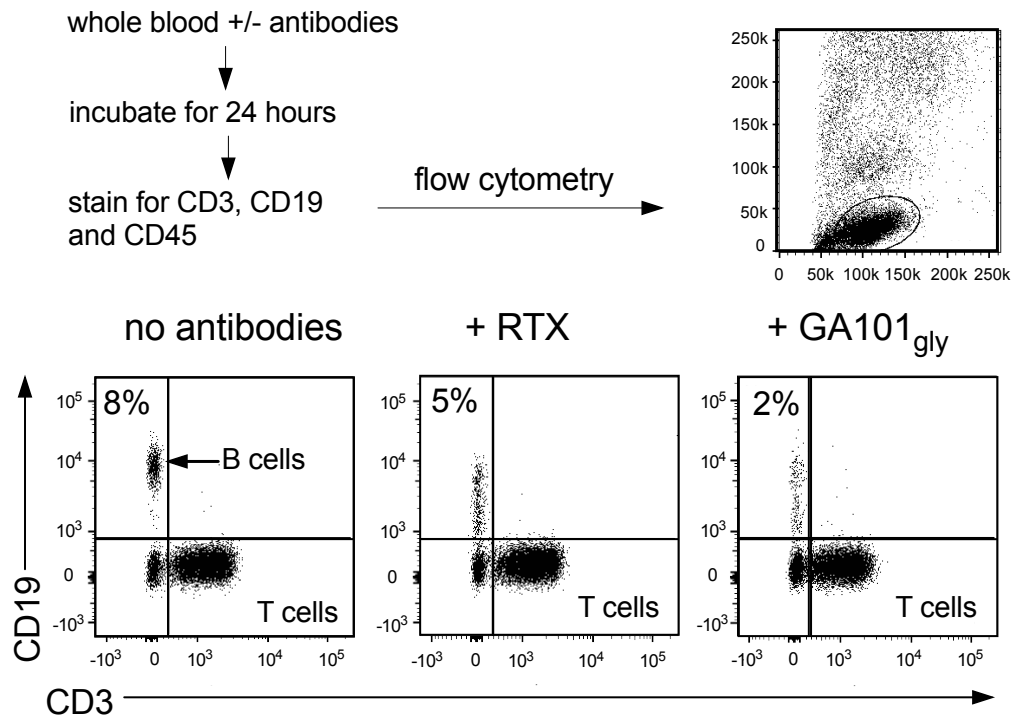
Figure 2.2 Whole blood B-cell depletion assay.

Percentage of B-cell depletion with mAbs was defined as the cytotoxicity index (CTI), which was determined using the formula:

CTI of mAb = 100 - ([100/B:T-cell ratio in sample without antibody] X [B:T-cell ratio in sample with antibody].

Mean of triplicate wells was used to calculate CTI.

Expression of CD20 and FcγRIIb on B cells

As discussed in Chapter 1, malignant B-cell expression of the target antigen, CD20, and also the inhibitory FcγRIIb may influence the efficiency of rituximab-induced B-cell cytotoxicity. Therefore, B cell expression of CD20 and FcγRIIb (CD32b) may serve as a potential biomarker of biological and/or clinical response to rituximab in RA and SLE. Here, B cell expression of CD20 and FcγRIIb was assessed by the mean fluorescence intensity (MFI). The monoclonal antibodies used are shown in table 2.2 and the flow cytometry gating strategy in Figure 2.3.
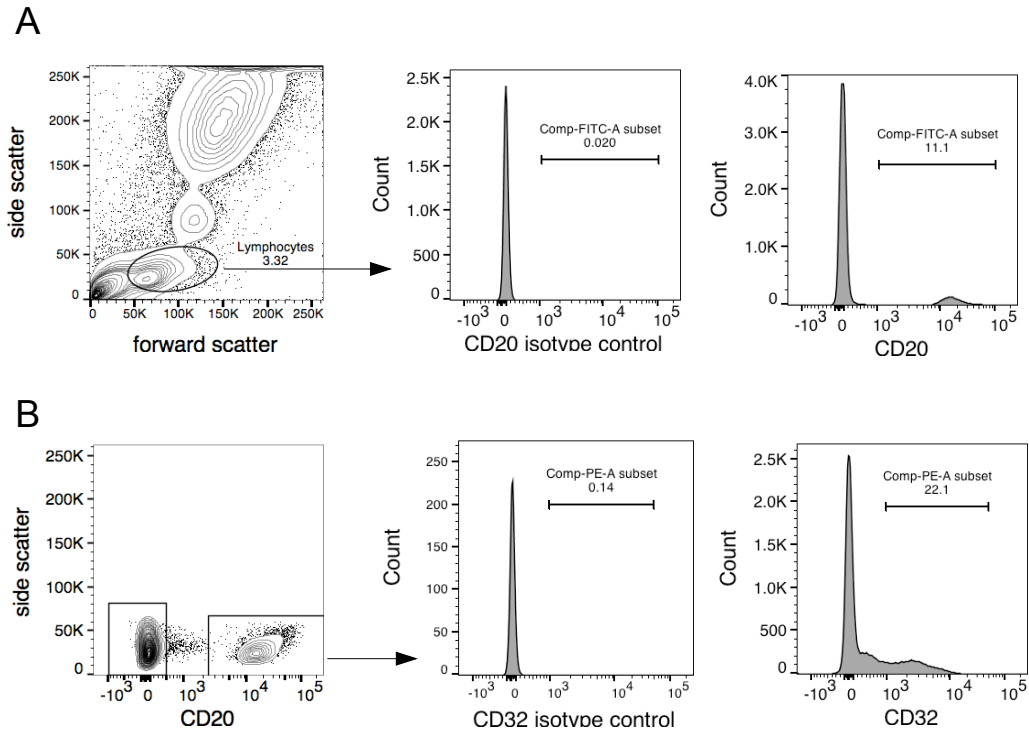
Figure 2.3 Flow cytometry gating strategy to analyse the mean fluorescence intensity of CD20 and FcγRIIb on B cells.

To assess the MFI of CD20 and FcγRIIb, 200 μL of freshly drawn whole blood samples were incubated with anti-CD20 and anti-CD32 monoclonal antibodies for 30 minutes, at room temperature. Samples were then treated with red cell lysing buffer (BD Pharmlyse) before centrifugation at 200 g for 5 minutes. Cell pellet was then resuspended in 0.5 mL of 1 X PBS (phosphate buffer solution) and analysed by flow cytometry. At least 20,000 lymphocytes were analysed. B cells were identified as CD20+ cells. The MFI of CD20 in samples incubated with isotype control mAb was used to account for background autofluorescence. The MFI of FcγRIIb on CD20+ B cells was also analysed. Flow cytometry gating strategy for assessing the MFI of CD20 and FcγRIIb are shown in Figure 2.3 A and B and the MFIs were calculated using the formulae:

MFI of CD20 = MFI of CD20 in sample incubated with fluorescence-conjugated anti-CD20 monoclonal antibody – the MFI of CD20 in sample incubated with isotype control; and

MFI of FcγRIIb = MFI of FcγRIIb in sample incubated with fluorescence-conjugated anti-CD32 monoclonal antibody – the MFI of CD32 in sample incubated with isotype control.

To assess whether B cell expression of CD20 and FcγRIIb influenced the efficiency of anti-CD20 monoclonal antibodies, the relationship between the MFI of CD20 and FcγRIIb and B-cell depletion by mAbs as assessed by the whole blood B-cell depletion assay were analysed in individual samples.

Also, the relationships between the MFI of CD20 and FcγRIIb on B cells from patients with RA and SLE were compared.

Surface fluorescence-quenching assay

The surface fluorescence-quenching assay was performed using isolated B cells, as described previously (Beers, French et al. 2010). Briefly, B cells were enriched from whole blood samples directly or harvested from PBMCs stored at -80°C to a > 90% purity. Internalisation of mAbs in the surface fluorescence-quenching assays was compared using isolated B cells for an individual experiment either from fresh or frozen PBMCs and thereby using the same sample preparation protocol for all mAbs in all experiments. At least, 2-4 x 10^5 of isolated B cells were incubated with Alexa-488-labeled mAbs at 5µg/mL at 37°C for 6 hours, a time point when optimal internalisation of mAbs was considered to have occurred (Beers, Chan et al. 2008, Lim, Vaughan et al. 2011).

IgG isotypes differentially activate FcγRIIb (Lim, Vaughan et al. 2011), therefore, all mAbs used were either human IgG1 or mouse IgG2a, which give equivalent activity in internalisation assays with anti-CD20 mAb (Vaughan, Iriyama et al. 2014). Samples were then harvested, washed twice and incubated with PE-Cy7-labeled anti-CD19, with or without the quenching antibody, anti-Alexa-488 (Invitrogen, U.K.) for 30 minutes at 4°C the quenching antibody would bind to Alexa-488-labeled mAb on the cell surface and decrease its fluorescence therefore preventing the detection of the fluorochrome-labeled mAb on the cell surface but not interfering with the detection of those that have been internalised and remain intracellular. After washing, samples were analysed by flow cytometry.

The effect of FcγRIIb on internalisation of mAbs was investigated by comparing the mean fluorescent intensity in samples with or without prior incubation with AT10, a FcγRII inhibitory mAb, at a concentration of 50 µg/mL for 30 minutes, based on experiments developed and described previously by Professor Cragg's group (Beers, Chan et al. 2008, Lim, Vaughan et al. 2011), before the addition of Alexa-488-labeled mAbs. The effect of B-cell activation on internalisation was investigated by stimulating isolated B cells with anti-IgM F(ab')^2 at 25µg/mL for 30 minutes or 6 hours before

incubating with Alexa-488-labeled mAbs. The 30 minutes time point would allow for assessment of rapid internalisation and 6 hours time point would allow for optimal internalisation of anti-CD20 mAbs to have occurred, as discussed earlier.
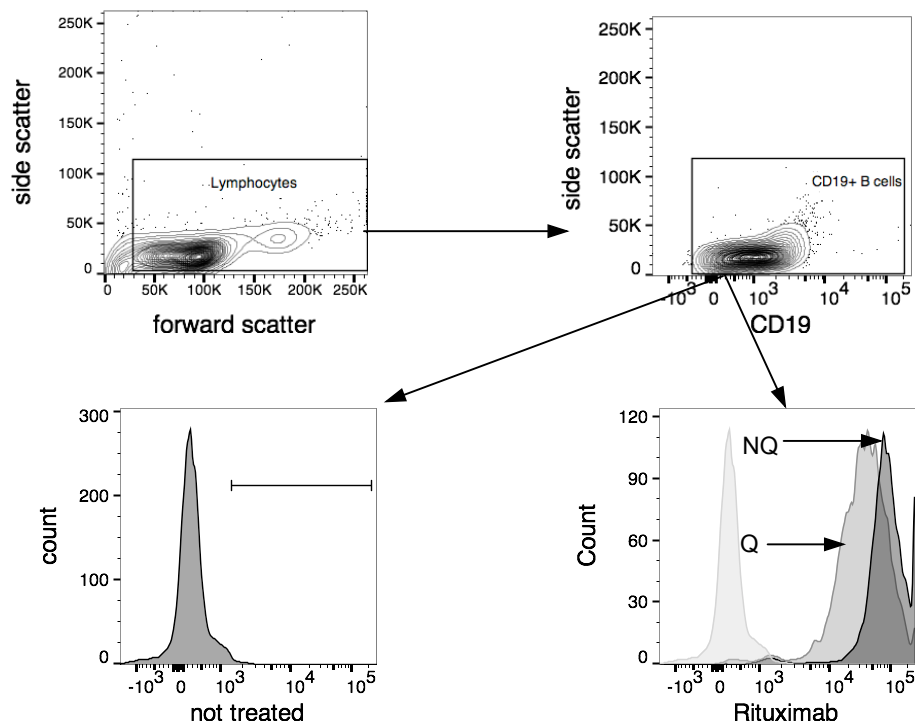
Figure 2.4 Surface fluorescence-quenching assay.

Forward- and side-scatter characteristics were used to identify lymphocytes, the purity of isolated B cells was determined by CD19 positivity. The MFI of CD20 in sample incubated without monoclonal antibodies (not treated) and the MFI of CD20 in samples incubated with Alexa-488-rituximab and subsequently with anti-alexa-488 mAb (Q) or without (NQ) or not incubated with mAbs..

MFI of Alexa-488-conjugated anti-CD20 mAb = MFI of Alexa-488-conjugated anti-CD20 mAb in sample incubated with Alexa-488-conjugated anti-CD20 mAb - MFI of Alexa-488-conjugated anti-CD20 mAb in sample incubated without Alexa-488-conjugated anti-CD20 mAb.

Surface accessible mAbs was represented by the percentage internalisation of mAbs calculated using the formula:

Internalisation of mAbs (%) = [(MFI of Alexa-488-conjugated anti-CD20 mAb in sample incubated without anti-alexa-488 - MFI of Alexa-488-conjugated anti-CD20 mAb in

sample incubated with anti-alexa-488) / MFI of Alexa-488-conjugated anti-CD20 mAb in sample incubated without anti-alexa-488] X 100

To investigate internalisation in B-cell subpopulations, samples were stained with PE-Cy7-labeled anti-CD19, BV421-labeled IgD and PE-labeled CD27 after incubation with Alexa-488 labeled mAbs and B-cell subpopulations were identified as shown in Figure 2.1.

Effector mechanisms of anti-CD20 mAbs

An important limitation of the whole blood depletion assay though is that the relative efficiency of individual mAbs at evoking different effector mechanisms is not discernible. Therefore, alternative assays such as CDC assay and DCD assay were used to study respective effector mechanisms. ADCC was analysed in the whole blood assay to account for the potential impact of complement deposition (Wang, Racila et al. 2008) and other serum factors such as immune complexes (Ahuja, Teichmann et al. 2011) on anti-CD20 mAbs interaction with FcγRs of effector cells such as NK cells.

Complement-dependent cellular cytotoxicity assays

CDC assays were performed as previously described (Cragg and Glennie 2004, Patz, Isaeva et al. 2011). B cells were isolated from PBMCs as described earlier. Isolated B cells were incubated with mAbs at a concentration of 10 μg/mL for 30 minutes at 37°C and 5% CO2. Samples were stained with fluorescence conjugated anti-CD19 antibodies, Annexin V (Av) and propidium iodide (PI) and the frequency of CD19+Av+PI+ cells assessed by flow cytometry. Freshly collected normal healthy human serum was used as a source of complement. To define the activity relating to complement, part of the serum was heat inactivated (HIS) at 56°C for 30 minutes, as described previously (Patz, Isaeva et al. 2011) and returned to room temperature to minimise heat aggregation of anti-CD20mAbs (Soltis, Hasz et al. 1979) before use in CDC assays. The ability of mAbs to activate complement was assessed by the relative frequency of CD19+Av+PI+ cells in samples incubated either with normal healthy serum or HIS. The flow cytometry gating strategy of CDC assay is illustrated in Figure 2.5.
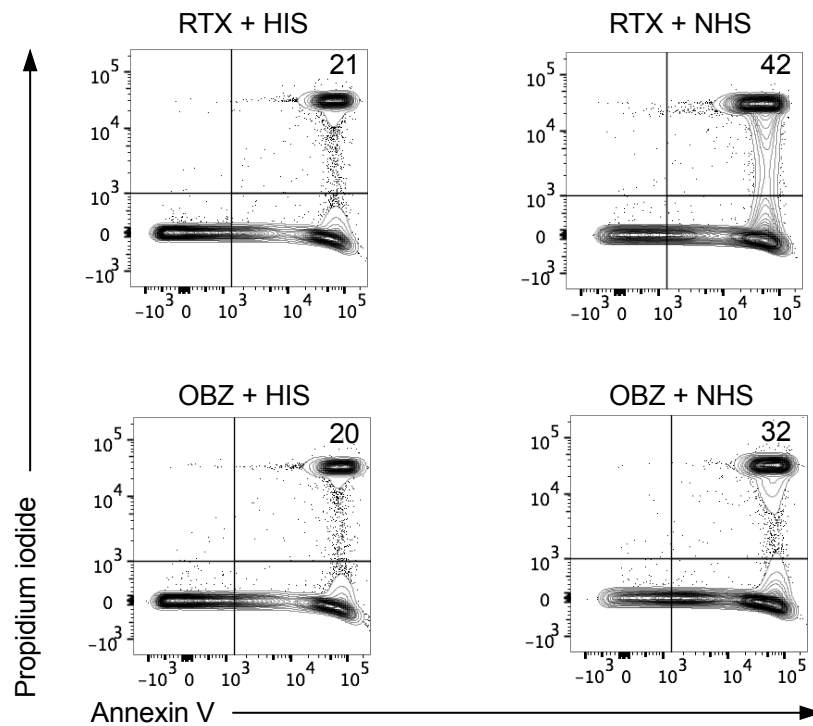
Figure 2.5 Gating strategy for complement-dependent cytotoxicity assay.

CDC by rituximab and obinutuzumab. Isolated B cells were incubated with mAbs either with NHS or HIS for 30 minutes at room temperature before analyzing by flow cytometry. The frequency of Annexin V+ PI+ cells represented cell death. HIS, heat inactivated serum; NHS, normal healthy serum; rituximab, rituximab; OBZ, obinutuzumab; and PI, propidium iodide.

Antibody-dependent cellular cytotoxicity and antibody-dependent cell phagocytosis

ADCC and ADCP were assessed in whole blood assays to allow for the potential interference of complement and immunoglobulins, as discussed in Chapter 1. Further, whole blood B-cell depletion assays would also account for the relative competitive activity of the other effector mechanisms including CDC and DCD. Therefore, ADCC, mediated by NK cells (Alter, Malenfant et al. 2004) and ADCP, mediated by neutrophils (Golay, Da Roit et al. 2013), in vitro, may be assessed by flow cytometry (discussed later). Based on these considerations, a flow cytometry based analysis of ADCC and ADCP was therefore developed to assess mAb-induced ADCC and ADCP, in whole blood B-cell depletion, indirectly.

NK cell degranulation assays

NK cell degranulation was assessed using samples from the whole blood B-cell depletion assay by measuring the expression of CD107a or LAMP-1 (lysosome associated membrane protein 1), which is up regulated upon activation of NK cells and correlates with NK cell mediated ADCC (Alter, Malenfant et al. 2004, Aktas, Kucuksezer et al. 2009). CD107a facilitates the delivery of perforin to lytic granules required for efficient cytotoxicity (Krzewski, Gil-Krzewska et al. 2013) and subsequently for NK cell protection from its own secreted cytotoxic granules (Cohnen, Chiang et al. 2013). A combination of CD3 and CD56 was used to identify NK cells in peripheral blood whereas CD57 is associated with a distinct mature phenotype of NK cells (Caligiuri 2008). Therefore, the frequency of CD3-56+107a+ NK cells in samples with mAbs was compared with that in samples incubated without mAbs. Activation of NK cells is associated with an increased activity of metalloproteinase, which cleaves CD16 reducing its expression upon NK cell activation (Romee, Foley et al. 2013). Therefore the extent of CD16 loss was also used as an indirect measure of NK cell activation (Bowles, Wang et al. 2006, Grzywacz, Kataria et al. 2007).

Here, three distinct NK-cell subpopulations were identified based on the extent of loss of CD16 after incubation for 24 hours in the absence or presence of anti-CD20 mAbs. Activated NK cells had lower expression of CD16 such that there was a hierarchy in the proportion of CD3-CD56+CD107a+ activated NK cells with CD3-CD56+CD16++ < CD3-CD56+CD16+ < CD56+CD16- NK cell subpopulations Figure 2.6. Thus, the whole blood assay used here was optimised for the assessment of NK cell activation by flow cytometry revealing the loss of CD16 and increases in CD107a in activated NK cells, in in vitro assays.
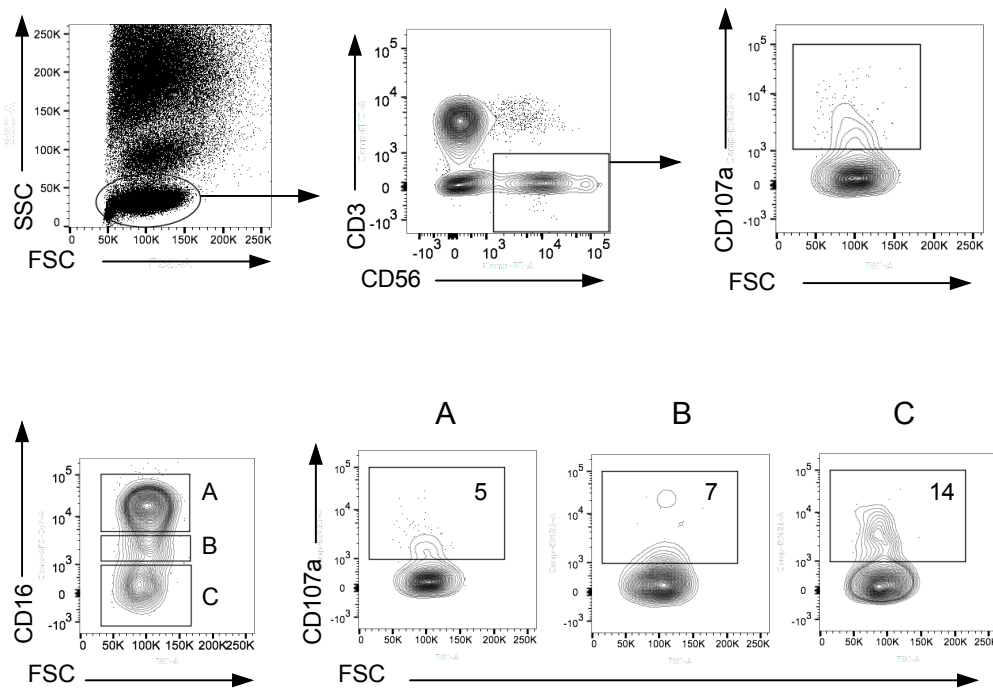
Figure 2.6. Flow cytometry-gating strategy to assess NK cell activation.

Neutrophil activation assays

Anti-CD20mAbs induce neutrophil activation as evidenced by production of reactive oxygen species in vitro (Golay, Da Roit et al. 2013, Werlenius, Riise et al. 2014). Neutrophils are the most abundant leucocytes in peripheral blood and the expression of cell surface proteins is altered upon activation whereas the expression of CD15 is retained regardless of activation and neutrophils are identified on flow cytometry as CD15+ granulocytes. Upon activation the expression of cell surface markers associated with specific function are altered. The surface expression of CD11b, which forms part of the integrin Mac-1 or complement receptor CR3 complex involved with phagocytosis, and variants of which are associated with SLE, characterised by defective phagocytosis (MacPherson, Lek et al. 2011, Rhodes, Furnrohr et al. 2012, Fossati-Jimack, Ling et al. 2013). Neutrophil activation also results in shedding of CD62L associated with neutrophil migration (Ley, Gaehtgens et al. 1991). Further, neutrophil activation is associated with an increase the expression of CD11b and decrease in the expression of CD62L with the alterations correlating with neutrophil oxidative burst response in vitro (Wittmann, Rothe et al. 2004). Therefore, a combination of CD15, CD11b and CD62L was used to investigate the ability of anti-CD20mAbs at inducing neutrophil activation, in vitro.

Neutrophil activation was assessed in whole blood B-cell depletion assays by measuring increases in the MFI of CD11b or decreases in the MFI of CD62L on CD15+neutrophils by flow cytometry (Wittmann, Rothe et al. 2004, Golay, Da Roit et al. 2013). It was also shown that neutrophils mediate phagocytosis, but not ADCC, of anti-CD20 mAb coated target cells (Golay, Da Roit et al. 2013). Hence, neutrophil activation as assessed by flow cytometry may serve as an indirect measure of ADCP. Therefore, the ability of mAbs to induce neutrophil activation was assessed by comparing the MFI of CD11b and CD62L on CD15+ neutrophils in samples incubated with or without mAbs. The MFI of CD11b and CD62L in samples incubated with mAbs were compared with samples incubated without antibodies Figure 2.7.
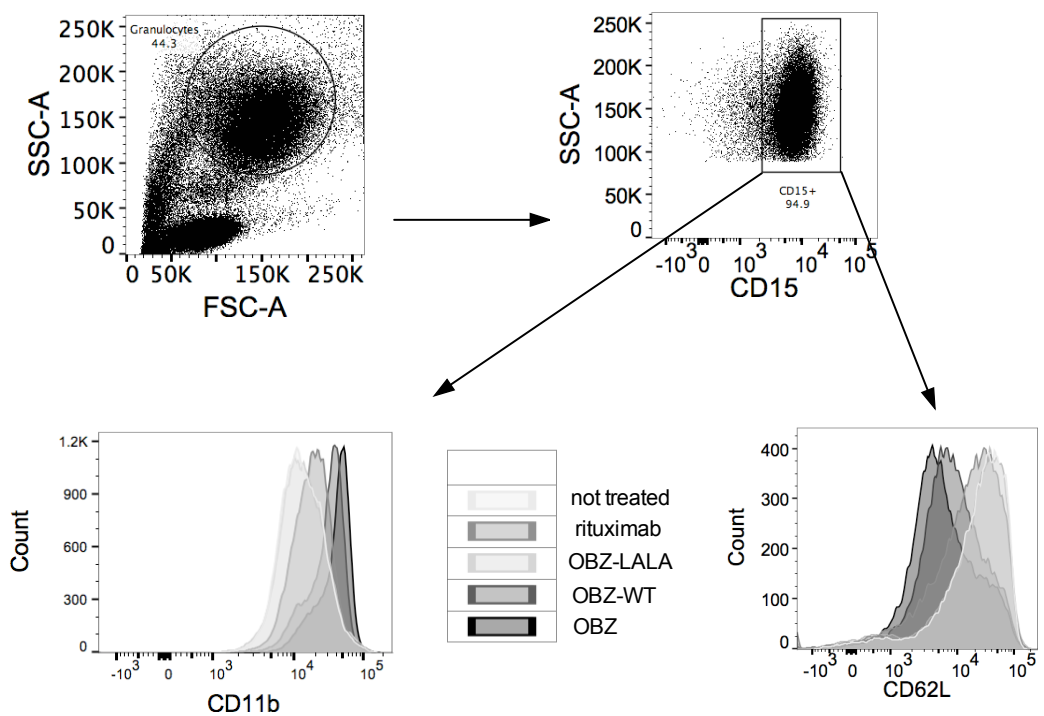
Figure 2.7 Flow cytometry-gating strategy to assess neutrophil activation.

After 24-hour incubation, whole blood samples were analysed by flow cytometry. Neutrophils were identified by forward- and side-scatter and CD15 positivity. The mean fluorescence intensity of CD11b and CD62L was analysed on gated neutrophils positive for CD15.

Direct cell death

The direct cell death assays were modified from previous studies, which evaluated the ability of anti-CD20 mAbs to induce DCD in malignant B cells and/or cell lines (Beers, Chan et al. 2008, Mossner, Brunker et al. 2010). Isolated B-cells were incubated in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% heat inactivated fetal bovine serum with or without mAbs at a concentration of 10μg/mL for 6 hours at 37°c and 5% CO_2, as previously described (Mossner, Brunker et al. 2010). The frequency of CD19+Av+ cells in samples with mAbs compared with that in samples without mAbs represented the ability of mAbs to induce DCD. The flow cytometry gating strategy for assessing DCD is illustrated in Figure 2.8.
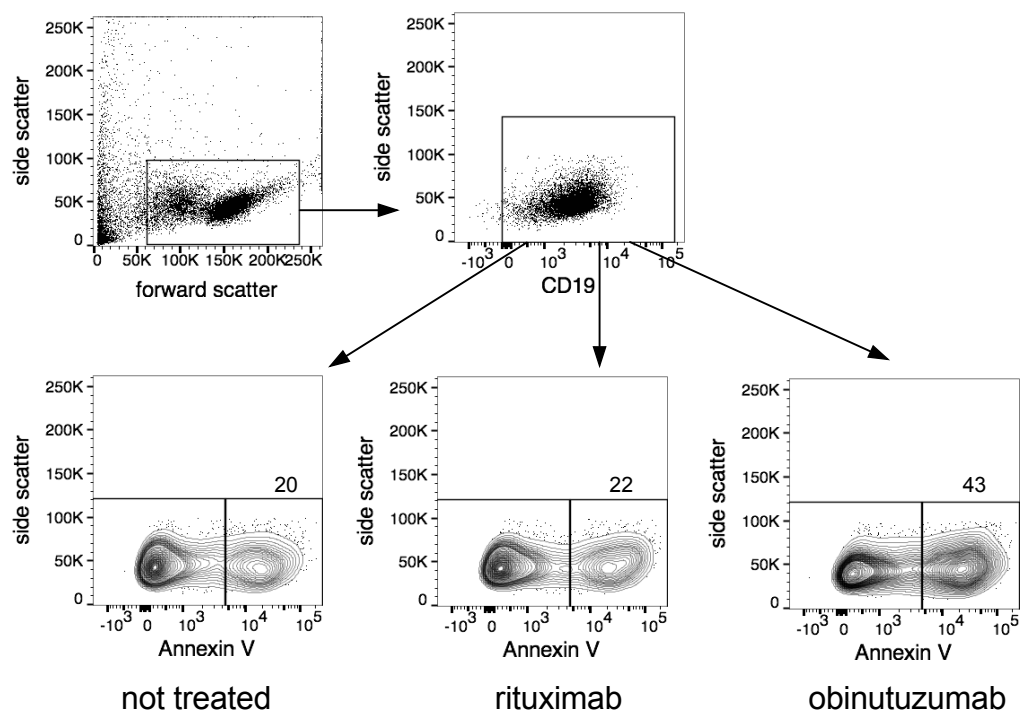
Figure 2.8 Flow cytometry-gating strategy to assess direct cell death in B-cell subpopulations.

Statistical analysis

Clinical data were analysed using Graph Pad Prism Soft ware version 5.0. Data between RA and SLE cohort were compared using Mann-Whitney U test. Data between paired samples were compared using Wilcoxon matched-pairs signed rank test. For descriptive statistics for categorical variables percentages or ratios were used and for continuous variables with normal distributions mean ±SD were used otherwise

median and interquartile range (IQR) (or range) were used. Spearman correlation coefficient r^2 was used to analyse the relationship between two parameters.

Receiver-operated-curve (ROC) analysis was performed to analyse for factors predictive of the development hypogammaglobulinemia and normalisation of serum anti-dsDNA antibody levels after treatment with rituximab.

Graph Pad Prism Soft ware version 5.0. was used for graphical representation of the data.

Chapter 3

Results: B-cell Depletion with Rituximab: Effect on Serum Immunoglobulins and Relationship with Serum Rituximab Levels

Results

The hypotheses tested in this chapter were that "*The kinetics of B-cell depletion in patients with systemic lupus erythematosus are highly variable and less predictable compared with patients with rheumatoid arthritis. Further, that the effects of rituximab on serum immunoglobulins and anti-dsDNA antibodies are variable and that baseline levels and concomitant immunosuppressive therapy may predict subsequent changes*".

The following questions were investigated in this chapter:

1) What are the kinetics of B-cell depletion and repopulation of B cells in patients with RA and SLE after treatment with rituximab;
2) What are the effects of rituximab on serum immunoglobulins and anti-dsDNA antibodies in patients with SLE;
3) What is the effect of concomitant and/ or sequential immunosuppressants on serum immunoglobulins and anti-dsDNA antibodies after rituximab in patients with SLE;
4) What is the relationship between serum IgM levels and anti-dsDNA antibodies before and after treatment with rituximab in patients with SLE;
5) Are there any differences in the relationship between B-cell counts and serum rituximab levels in patients with RA and SLE; and
6) Are there any differences in B-cell expression of CD20 and/or FcγRIIb between patients with RA and SLE.

B-cell depletion in vivo

Duration of B-cell depletion in RA and SLE

The duration of B-cell depletion was assessed in 27 patients with RA and 71 patients with SLE, treated with two doses of 1g rituximab given 1-2 weeks apart. All data was obtained and analysed, retrospectively, from the UCLH patient electronic records. CD19 + B cell counts were processed by the central laboratory at UCLH requested as part of routine clinical care of patients receiving rituximab therapy. Data about CD19+ B cell counts were analysed before and at one, three and six months after the first dose of rituximab. The kinetics of B-cell depletion and repopulation in patients with RA and SLE treated with rituximab at UCLH has been described, previously, and differences in the duration of B-cell depletion between RA and SLE was alluded to although no direct

comparison was made (Leandro, Edwards et al. 2002, Leandro, Cambridge et al. 2005, Leandro, Cambridge et al. 2006). In this chapter, the kinetics of B-cell depletion and repopulation following rituximab therapy were compared to investigate for any differences in the duration of B-cell depletion achieved between patients with RA and SLE.

Before treatment with rituximab, patients with RA had significantly higher number of CD19+ cells X 10^9/L (median and range; 0.2260 X 109/L, 0.012-0.663) in peripheral circulation, compared to patients with SLE (median and range; 0.097 X 10^9/L, 0.01-1.274) (Figure 3.1), by Mann-Whitney U test. In contrast, at both 3 and 6 months after treatment with rituximab, the number of CD19+ cells X 10^9/L was significantly lower in patients with RA compared to patients with SLE. No significant differences were noted at 1 month after rituximab although some patients with SLE had CD19+ cell counts > 0.5 X 10^9/L. These results showed that the duration of B-cell depletion was shorter in patients with SLE compared to patients with RA.
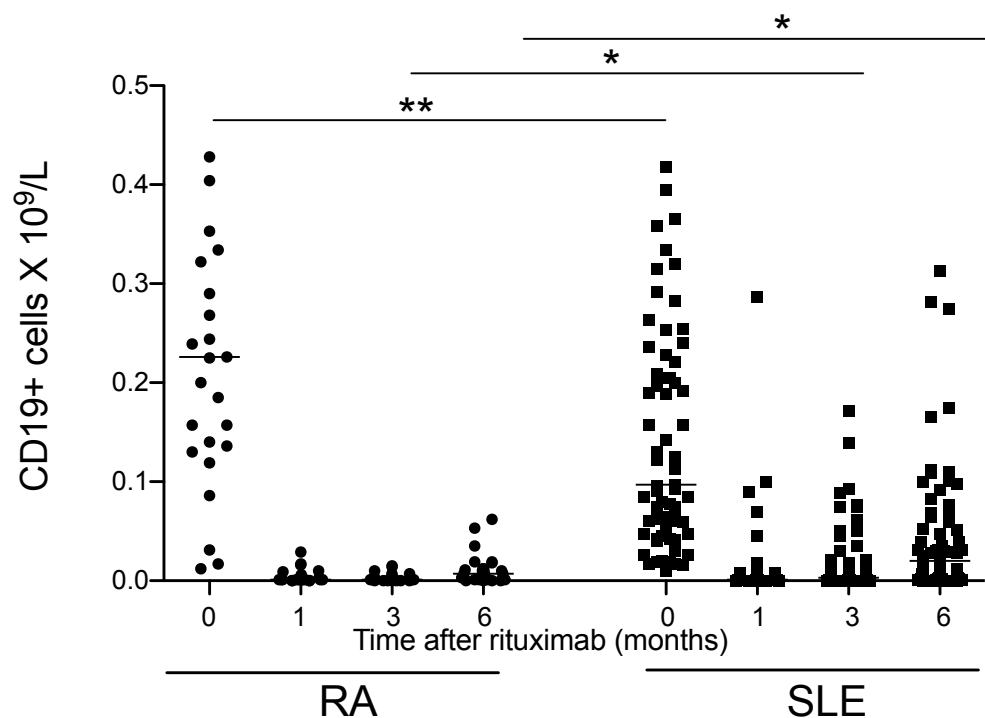
Figure 3.1. B-cell depletion with rituximab in patients with Rheumatoid Arthritis and Systemic Lupus Erythematosus.

CD19+ B-cell counts in peripheral blood before and after treatment with rituximab in patients with RA (n=27) and SLE (n=71). *, $p<0.05$. **; p, <0.001.

B-cell depletion with rituximab: effect on serum immunoglobulins and anti-ds DNA antibodies

The kinetics of serum immunoglobulins and anti-dsDNA antibodies was assessed in 53 patients with SLE treated with rituximab, at UCLH. Clinical features (including class of LN), drug therapies prior to rituximab and at most recent follow up; and serology are shown for all patients including those with low serum IgM and those retaining normal levels of IgM are shown in Table 3.1. Detailed patient demographics and clinical responses of this cohort of patients have been reported, previously (Ng, Cambridge et al. 2007, Turner-Stokes, Lu et al. 2011, Aguiar, Araujo et al. 2016). The median age at the time of the first cycle of rituximab treatment was 34 years (range 17-74 years). All patients had received previous treatment with at least 2 different immunosuppressants not including corticosteroids, which were continued at a low dose ($\leq$10 mg/day prednisolone). The median duration of follow-up was 48 months (range 13-144 months).

The kinetics of changes in serum immunoglobulins and anti-dsDNA antibody levels were analysed at 12 months and also at most recent follow-up after the first cycle of rituximab treatment. At 12 months time point, all patients who had received only one cycle of rituximab were included and most patients would have been expected to have achieved B cell repopulation therefore the changes in serum immunoglobulins and anti-dsDNA antibody levels would represent the effects of the first cycle of rituximab whereas at most recent follow-up some patients had been treated with repeat cycles of treatment with rituximab and/or sequential immunosuppressants and/or immunomodulatory therapies.

Table 3.1 Demographics and clinical details of patients with SLE

patient number	Age (years)	Ethnicity	Clinical manifestations	Rituximab-treatment rituximab	Treatment before rituximab	Treatment at most recent follow-up	Serology
1	58	C	LN (class 4)	2	CS (high)	CS (low), HCQ	Ro, La
2*	35	A	non-renal	1	CS (high), AZA	CS (low), HCQ	ANA, Ro, RNP
3	28	AC	LN (class 3)	5	AZA, MMF, MTX, CYC	CS (low), HCQ	Ro La
4	34	A	LN (class 4)	1	CS (low), HCQ MMF (2g)	CS (low), HCQ, AZA	Ro, RNP
5	33	AC	non-renal	1	CS (low), MTX	CS (low)	RNP
6	29	A	LN (class 3)	5	CS (high), HCQ	CS (low), HCQ, Tacr, MMF (1.5g)	ENA negative
7	26	Ch	non-renal	1	CS (low), HCQ, AZA	CS (low), AZA	RNP
8	31	Ch	non-renal	1	HCQ, AZA	HCQ, AZA	Sm

9	43	C	non-renal	1	CS (low), AZA	HCQ, AZA	ENA negative
10	58	C	non-renal	1	CS (high)	CS (low)	Ro
11*	56	C	non-renal	1	CS (low), AZA	nil	RNP
12	35	AC	LN (class 4)	1	HCQ, AZA	HCQ, AZA	RNP
13	29	A	LN (class 3)	4	CS (low) MMF (2.5g)	CS (low), HCQ	Ro
14	45	AC	LN (class 3)	1	CS (low) MMF (1.5g)	CS (low) MMF (0.5g), Tacr	Sm
15*	53	AC	LN (class 3)	1	CS (low)	CS (low)	RNP
16*	33	AC	LN (class 4)	2	CS (low)	CS (low) MMF (1.5g)	ENA-
17*	42	Ch	LN (class 5)	1	CS (low) MMF (3g), HCQ	CS (low) MMF (2g), Tacr RNP	Ro, La, Sm, RNP
18	75	C	non-renal	3	CS (low), MTX	CS (low)	ENA negative
19	33	A	LN (class 3)	2	CS (low), HCQ, AZA	CS (low), HCQ, MMF (2g)	Ro, Sm, RNP
20	28	C	LN (class 5)	1	CS (low), AZA	CS (low) Tacr	ENA-
21	29	C	LN (class 4)	2	CS (low), HCQ, AZA	CS (low), HCQ, AZA	Ro, Sm, RNP

No.	Age		Diagnosis		Treatment		Antibodies
22*	51	AC	LN (class 3)	2	CS (low), AZA	MMF (2g)	ENA negative
23	54	A	LN (class 4)	1	CS (low) MMF (1.5g)	CS (low)	RNP
24	29	AC	non-renal	1	CS (high)		ENA negative
25	30	C	LN (class 4)	1	CS (low), AZA	CS (low) MMF (1g), Tacr	ENA negative
26	30	Ch	non-renal	1	CS (low), MTX	IFX, MTX	ENA negative
27	29	A	LN (class 5)	1	CS (low) MMF (2.5g), HCQ	CS (low), HCQ	ENA negative
28	25	AC	LN (class 4)	2	CS (low), HCQ, AZA	CS (low), HCQ	Ro
29	42	AC	LN (class 3)	1	CS (high)	CS (low)	Ro, Sm, RNP
30*	55	C	LN (class 4)	1	CS (low) MMF (3g),	CS (low), HCQ, MMF (2g)	ENA negative
31*	50	AC	non-renal	1	CS (low), MTX	CS (low), Enbrel	Ro
32	47	C	non-renal	1	CS (low), HCQ	CS (low), HCQ	Sm
33	33	AC	LN (class 4)	3	CS (low), AZA	CS (low) MMF (2g)	Ro, rnp

34	42	AC	non-renal	2	CS (high)	CS (low)	Ro, Sm, RNP
35*	45	AC	LN (class 4)	2	CS (low) MMF (1.5g)	CS (low), HCQ	Sm
36	40	C	non-renal	1	CS (low), HCQ	HCQ	ENA negative
37	30	AC	non-renal	2	HCQ	CS (low), HCQ	Ro
38	27	AC	non-renal	1	CS (low), AZA	CS (low), HCQ	Ro
39	21	A	LN (class 4)	2	CS (low), HCQ, AZA	HCQ	Sm
40	19	AC	non-renal	2	CS (low), HCQ	CS (low), HCQ	RNP
41	51	AC	LN (class 4)	1	CS (low)	CS (low), HCQ	La, Sm
42	40	C	non-renal	2	CS (low), AZA	CS (low), HCQ	Ro, La
43*	53	AC	non-renal	1	CS (low), HCQ	HCQ	Ro, La
44	22	C	non-renal	1	HCQ, MMF (1.5g)	HCQ	Ro, RNP
45	45	AC	LN (class 4)	1	HCQ	nil	Ro, La
46	22	AC	LN (class 3)	4	CS (low), HCQ	CS (low) MMF (1.5g)	RNP

47	47	C	LN (class 4)	2	CS (low), HCQ	CS (low), HCQ, MMF (2g)	Sm, RNP
48	35	AC	non-renal	2	HCQ	CS (low)	ENA negative
49	35	AC	non-renal	2	HCQ	AZA	Ro, Sm, RNP
50	29	C	non-renal	1	CS (low), HCQ, AZA	CS (low), AZA	Sm, RNP
51*	22	AC	LN (class 3)	1	HCQ, MMF (3g)	CS (low), HCQ, MMF (3g)	Ro, La
52*	25	AC	LN (class 4/5)	1	CS (low) MMF (2g)	CS (low) MMF (2g)	Sm
53*	46	C	non-renal	2	CS (low), HCQ	nil	Ro

A, Asian; AC, Afro-caribbean; AZA, Azathioprine; C, Caucasian; Ch, Chinese; CS, corticosteroids; CYC, cyclophosphamide; CS, cyclosporine; HCQ, hydroxychloroquine; LN, lupus nephritis; MMF, mycophenolate mofetil; Tacr, Tacrolimus

* indicates patients who had persistently low serum IgM levels at most recent follow-up after rituximab treatment

Serum immunoglobulin levels and anti-dsDNA antibody levels before and at 12 months after rituximab

Changes in serum immunoglobulins and anti-dsDNA antibody levels after rituximab

Data from 53 patients with SLE were analysed at various time points from before, and at one, three, six, nine and 12 months after rituximab. Paired data was available from at least 28 patients, at each time point of analysis for serum immunoglobulins. A total of 44 patients had anti-dsDNA antibodies above the upper limit of reference range (50 IU/mL) at any given time point of analysis and, all except two of these 44 patients had baseline anti-dsDNA levels above the upper limit of reference range. Data was available from at least 25 patients for each time point of analysis for anti-dsDNA antibodies.

At baseline: seven patients had low serum IgM (< 0.4 g/L) and four had high serum IgM (> 2.3 g/L) levels; three patients had low IgG (< 7 g/L) levels and 20 patients had high serum IgG (> 16 g/L) levels; and none had low IgA (< 0.7 g/L) levels and nine patients had high serum IgA levels (> 4 g/L). At 12 months after the first treatment cycle with rituximab, paired data about serum immunoglobulins was available from 28 patients, and from 35 patients for anti-dsDNA antibodies.

Median baseline serum IgM level was 0.98 g/L (range 0.17 - 5.67 g/L), which was significantly reduced at all time points of analyses after rituximab and, at 12 months after rituximab the median serum IgM level was significantly lower at 0.69 g/L (range 0.05 - 4.54 g/L), (Figure 3.2A). At 12 months after treatment with rituximab, seven of 28 patients had low serum IgM levels of whom three had low IgM levels before treatment with rituximab. Thus, four of 28 patients developed low IgM levels at 12 months after treatment with rituximab. Data about serum IgM levels at 12 months after rituximab was available from two of four patients with high serum IgM levels at baseline, which normalised in one of these patients.

Median baseline serum IgG level was 14 g/L (range 4.8 - 33.8 g/L), which was significantly reduced at 3 and 9 months after rituximab, but similar to baseline levels at other time points (Figure 3.2B) and the median serum IgG level at 12 months was 13.1 g/L (range 4.29 - 26.79 g/L). Serum IgG levels, at 12 months after rituximab, were low in only two of 28 patients, one of whom had low IgG levels before treatment with

rituximab. Of the 20 patients with high serum IgG levels at baseline, data about serum IgG levels at 12 months time point was available for ten patients. Three of these ten patients had persistent high IgG levels, six had normal IgG levels and one patient had very low (< 5 g/L) IgG level of 4.3 g/L. Thus, at 12 months after rituximab, only one of 28 patients developed low serum IgG levels.

Median baseline serum IgA level was 3 g/L (range 0.77 – 6.2 g/L), which was significantly reduced only at 1 and 3 months, but not at other time points (Figure 3.2C) and the median IgA level at 12 months after rituximab was 2.87 g/L (range 1.37 – 5.8 g/L). At 12 months after treatment with rituximab, none of the patients had low IgA levels and seven patients had high serum IgA levels, four of whom had high IgA levels at baseline. Thus, three of 28 patients developed high serum IgA levels at 12 months after treatment with rituximab. Four of five patients with high baseline IgA levels had persistent high levels and one had normal levels.

Paired data about anti-dsDNA antibody levels at baseline and at 12 months after treatment with rituximab was available from 35 patients. The median anti-dsDNA antibody levels at baseline were 233 IU/mL (range 13 - 4912 IU/mL). One of two patients with baseline anti-dsDNA levels within the reference range had increased to abnormal range during the 12-month follow-up period after rituximab (Figure 3.2D) whereas the anti-dsDNA levels in the other patient increased to abnormal range only at most recent follow-up (discussed later). Median anti-dsDNA antibody levels at 1 and 12 months, but not at other time points, after treatment with rituximab, were significantly lower compared to baseline levels, at 173 IU/mL (range 26 – 2981 IU/mL) and 159 IU/mL (range 11 – 5169 IU/mL) vs 233 IU/mL, respectively (Figure 3.2D).

Thus, only serum IgM, but not IgG or IgA, levels, were significantly lower than baseline levels at all time points up to 12 months after rituximab and, serum anti-dsDNA antibodies were significantly lower at 12 months after rituximab compared to baseline levels (Figure 3.2).
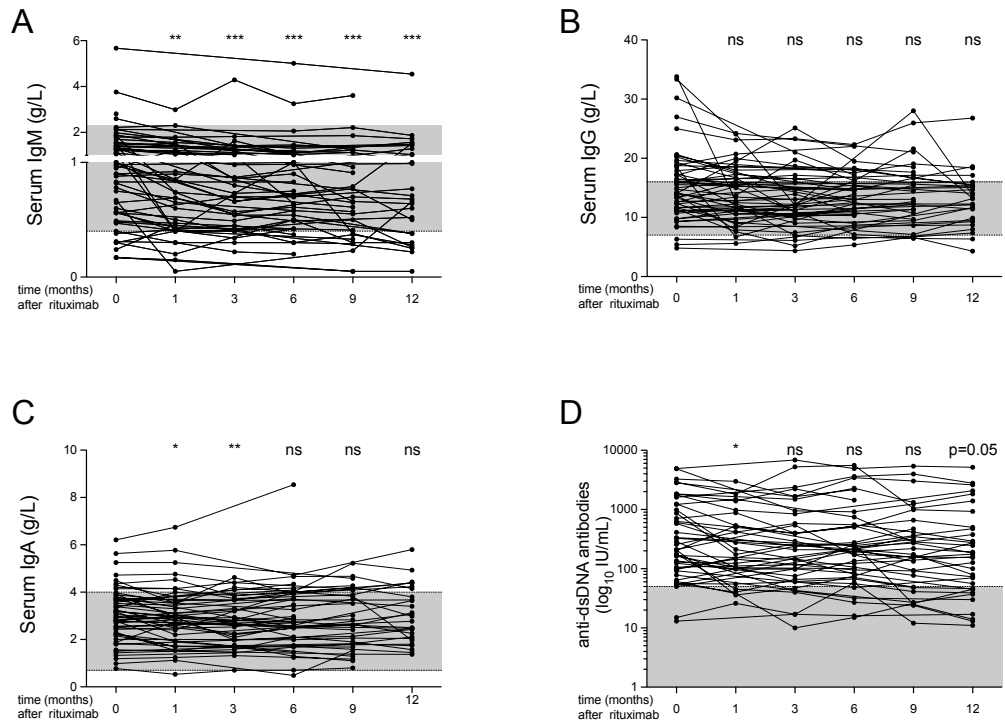
Figure 3.2 Serum immunoglobulins and anti-dsDNA levels before and after treatment with rituximab.

Shaded area represents the reference range. Ig, immunoglobulin; ns, not significant; *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

At 12 months after treatment with rituximab, the median percentage changes for immunoglobulin isotypes IgM, IgG, and IgA were -16.7 %; 0.1 %; 0.04 %, respectively (Figure 3.3 A, B, and C).

Remarkable variations in IgG anti-ds DNA levels were noted at 12 months after treatment with rituximab compared to baseline levels with the median and range in changes at -27 % and -94 % to 639 %, respectively (Figure 3.3D).
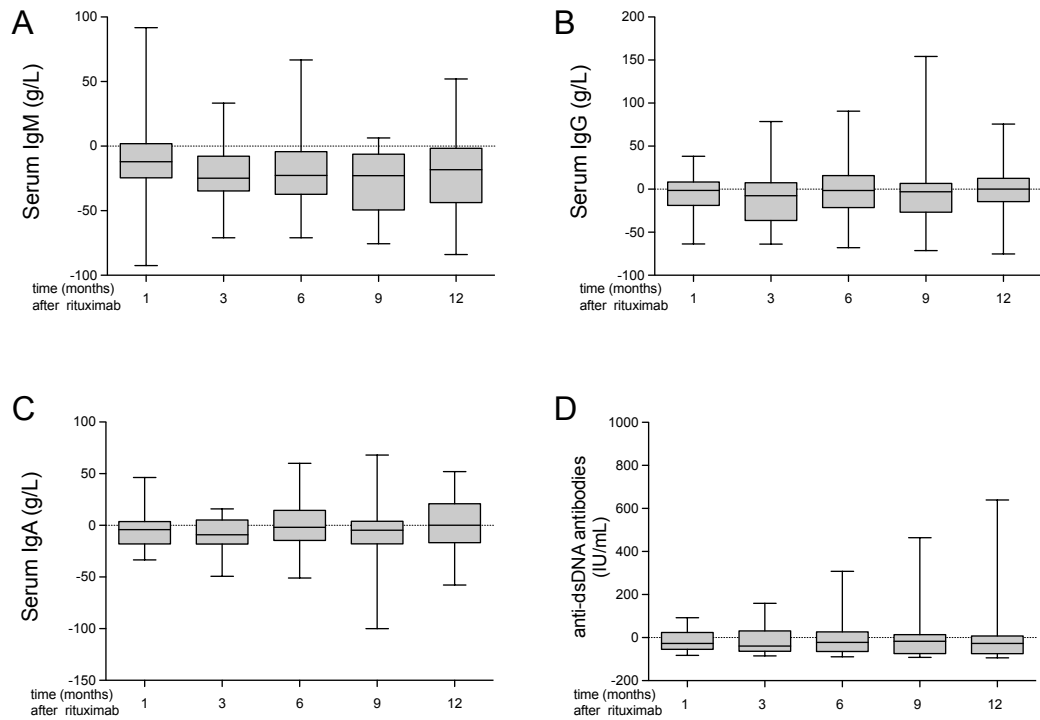
Figure 3.3 Percentage changes from baseline of serum immunoglobulins and anti-dsDNA antibodies after treatment with rituximab.

Boxes represent the inter-quartile range; the horizontal line the median; and the whiskers, the range. Ig, immunoglobulin.

Disparate fluctuations in serum immunoglobulins and anti-dsDNA antibodies

The analysis of results of serum immunoglobulin levels in the whole cohort, described so far, revealed contrasting fluctuations within the cohort for each immunoglobulin isotype and also for anti-dsDNA antibodies. Therefore, a subgroup analysis was performed to understand if baseline serum immunoglobulins and anti-dsDNA antibody levels might be useful to predict changes in the levels of immunoglobulins and anti-dsDNA antibodies after treatment with rituximab. Two subgroups of patients were identified according to whether the levels of immunoglobulins and anti-dsDNA antibodies at 12 months after rituximab were lower or higher compared to baseline levels, as subgroup A and B, respectively. The levels of immunoglobulin and anti-dsDNA antibodies at baseline and at 12 months after rituximab were compared between the two subgroups A and B.

Wilcoxon matched-pairs signed rank test was used for comparing the data with in the group and Mann Whitney U test was used for comparing the data between the groups.
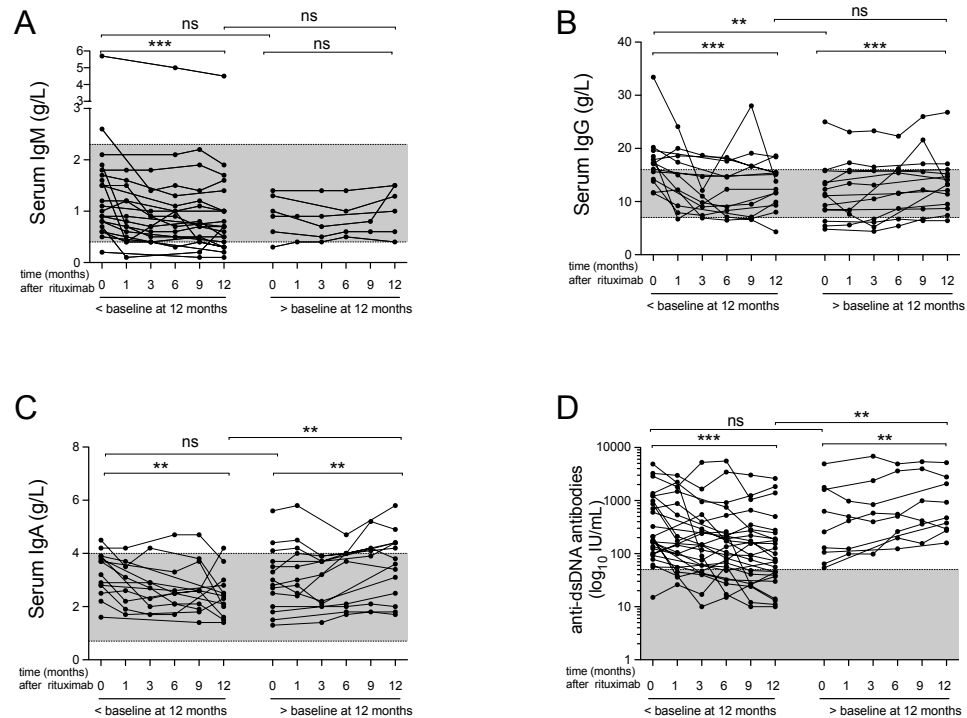
Figure 3.4 Disparate fluctuations in serum immunoglobulin and anti-dsDNA antibody levels after treatment with rituximab.

A) IgM; B) IgG; C) IgA; and D) anti-dsDNA antibodies. **, p<0.001; ***, p<0.0001.

a) Serum IgM

According to whether serum IgM levels at 12 months after rituximab were lower or higher compared to baseline levels, two groups of patients were identified, subgroup A and B, respectively Figure 3.4A. There were 21 patients in subgroup A and six in subgroup B and in one patient serum IgM levels at 12 months remained unchanged from baseline. Median serum IgM levels were significantly lower at 12 months time point compared to baseline in subgroup A at 0.7 g/L (range 0.1 – 4.5 g/L) and 1.1 g/L (range 0.2 – 5.7 g/L), respectively. No significant difference in serum IgM levels was noted between the same two time points in subgroup B. Six patients with low serum IgM levels at 12 months after rituximab belonged to subgroup A. Low baseline serum IgM levels in one patient in subgroup B increased to reference range at 12 months time point. Median serum IgM levels either at baseline or at 12 months time point were not

significantly different between the two groups. Thus, all patients with low serum IgM levels at 12 months after rituximab belonged to subgroup A.

b) Serum IgG

Two subgroups of patients were identified as A and B according to whether they had lower or higher serum IgG levels at 12 months after rituximab compared to baseline levels, respectively. There were 14 patients in each subgroup. In subgroup A, median serum IgG levels were significantly lower at 12 months time point compared to baseline at 13 g/L (range 4.3 – 18.6 g/L) and 17.1 g/L (range 11.6 – 33.4 g/L) respectively (Figure 3.4B). In subgroup B, median serum IgG levels were significantly higher at 12 months after rituximab compared to baseline levels at 13.2 g/L (range 6.4 – 26.8 g/L) and 11.2 g/L (range 4.8 – 25 g/L), respectively (Figure 3.4B). Median baseline serum IgG levels were significantly higher at 17.1 g/L in subgroup A compared to 11.2 g/L in subgroup B. High baseline serum IgG levels fell to reference range in all but two patients, one of whom had persistently high IgG levels and the other developed low IgG levels (Figure 3.4B). In subgroup B, one of the three patients with low baseline IgG levels had persistently low levels at 12 months whereas serum IgG levels reached reference range in the other two patients. Two patients in each subgroup had high IgG levels at 12 months, one from each subgroup had high IgG levels at baseline and the others had normal baseline IgG levels. However, there was no significant difference in serum IgG levels 12 months after rituximab between the two subgroups (Figure 3.4B). Therefore, only one patient in each subgroup had low serum IgG levels at 12 months after rituximab. Thus, in contrast to the changes in serum IgM levels, in most patients, serum IgG levels were within the reference range at 12 months after rituximab, and higher baseline values fell to reference range whereas lower baseline levels rose to reference range in equal number of patients.

c) Serum IgA

According to whether serum IgA levels at 12 months after rituximab were lower or higher compared to baseline levels, two groups of patients were identified, subgroup A and B, respectively. For serum IgA also there were 14 patients in each subgroup. In subgroup A, the median serum IgA levels were significantly lower at 12 months compared to baseline levels at 2.4 g/L (range 1.4 – 4.2 g/L) and 3.4 g/L (range 1.6 – 4.5 g/L), respectively, whereas in subgroup B, median serum IgA levels were significantly higher at 12 months compared to baseline levels at 3.7 g/L (range 1.7 – 5.8 g/L) and 2.9 g/L (range 1.3 – 5.6 g/L), respectively (Figure 3.4C). One patient in subgroup A had high baseline serum IgA levels that fell to reference range at 12

months after rituximab and another patient in subgroup B with high baseline serum IgA levels had persistently high levels at 12 months after rituximab (Figure 3.4C). All other patients in subgroup A had serum IgA levels within the reference range at all time points except for one patient who developed high IgA levels at 12 months after rituximab. In subgroup B, at 12 months after rituximab, seven patients had high serum IgA levels three of whom had high levels at baseline and four patients developed high IgA levels (Figure 3.4C). There was no significant difference between baseline serum IgA levels between the two subgroups. However, the median serum IgA levels in subgroup A was significantly lower at 12 months after rituximab compared to subgroup B at the same time point, at 2.4 g/L and 3.7 g/L, respectively. Thus, despite contrasting changes in serum IgA levels in the cohort, none of the patients in either subgroup had low serum IgA levels at 12 months after rituximab.

d) Serum anti-dsDNA antibodies

As noted for serum immunoglobulins, the anti-dsDNA antibodies at 12 months were either lower or higher than baseline values revealing two subgroups A and B consisting of 26 and nine patients, respectively. The median serum anti-dsDNA antibody levels were significantly lower at 12 months after rituximab compared to baseline levels in subgroup A at 76 IU/mL (range 10 – 2605 IU/mL) and 193 IU/mL (range 15 – 4870 IU/mL), respectively. Ten patients in subgroup A had anti-dsDNA antibody levels within the reference range at 12 months after rituximab, one of whom had serum anti-dsDNA antibody levels within the reference range at all-time points except at six months, when the levels were abnormal at 58 IU/mL. In subgroup B, median serum anti-dsDNA antibody levels at 12 months after rituximab were significantly higher compared to baseline values at 473 IU/mL (range 159 – 5169 IU/mL), and 257 IU/mL (range 54 – 4912 IU/mL), respectively. There was no significant difference between baseline serum anti-dsDNA antibodies between the two subgroups. In contrast, median serum anti-dsDNA antibody level at 12 months after rituximab was significantly lower in subgroup A compared to the median serum anti-dsDNA antibody level at the same time point in subgroup B at 76 IU/mL (range 10 – 2605 IU/mL), and 473 IU/mL (range 159 – 5169 IU/mL), respectively (Figure 3.4D). Therefore, despite similar median serum anti-dsDNA antibody levels at baseline, patients in subgroup A had significantly lower levels at 12 months after rituximab compared to subgroup B.

Long-term effects of rituximab on serum immunoglobulins and anti-dsDNA antibodies

Changes in serum immunoglobulins and anti-dsDNA antibody levels at most recent follow-up

Next, the long-term effects of rituximab on serum immunoglobulins and anti-dsDNA antibodies were investigated. In the previous section, the effect of rituximab on serum immunoglobulins and anti-dsDNA antibodies up to 12 months after the first cycle of treatment with rituximab was described. In the current section, extended data including most recent available data from each patient was analysed. During the extended follow-up period, based on clinical need, some patients received multiple cycles of rituximab treatment and/or concomitant therapy with sequential immunosuppressants such as MMF, AZT and corticosteroids or immunomodulatory agent, HCQ (Table 3.1). In this cohort, patients were treated with rituximab pragmatically to manage acute flares that were refractory to conventional immunosuppressants. Subsequently, disease activity was pragmatically managed with conventional therapies.

As discussed in Chapter one, Introduction, conventional immunosuppressants may also contribute to the development of hypogammaglobulinaemia. In this context, the relative long-term risk of developing hypogammaglobulinemia in patients who have been treated with rituximab and subsequently with conventional therapies is poorly understood. Therefore, analysis of the extended data may be useful in the monitoring of patients who have been treated with rituximab and sequential immunosuppressants.

Paired data from baseline and at most recent follow-up time points, available from 53 patients for serum immunoglobulins and 44 patients for anti-dsDNA antibodies are shown in Figure 3.5A-D. Median serum IgM levels at most recent follow-up were significantly lower at 0.54 g/L (range 0.05 - 4.24 g/L) compared to levels at baseline and at 12 months after rituximab at 0.98 g/L (range 0.17 - 5.67 g/L) and 0.69 g/L (range 0.05 - 4.54 g/L), respectively (Figure 3.5A). Median serum IgG levels at most recent follow-up were also significantly lower that 12.3 g/L (range 5.14 - 36.9 g/L) compared to 14 g/L (range 4.8 - 33.8 g/L) before treatment with rituximab. However, there was no significant difference between serum IgG levels at most recent follow-up and at 12 months after rituximab (Figure 3.5B). There was no significant difference in median serum IgA levels at most recent follow-up compared to baseline levels or levels at 12 months after rituximab (Figure 3.5C). Low serum immunoglobulin levels at most recent follow-up were noted in 13, four and three patients for IgM, IgG and IgA, respectively.
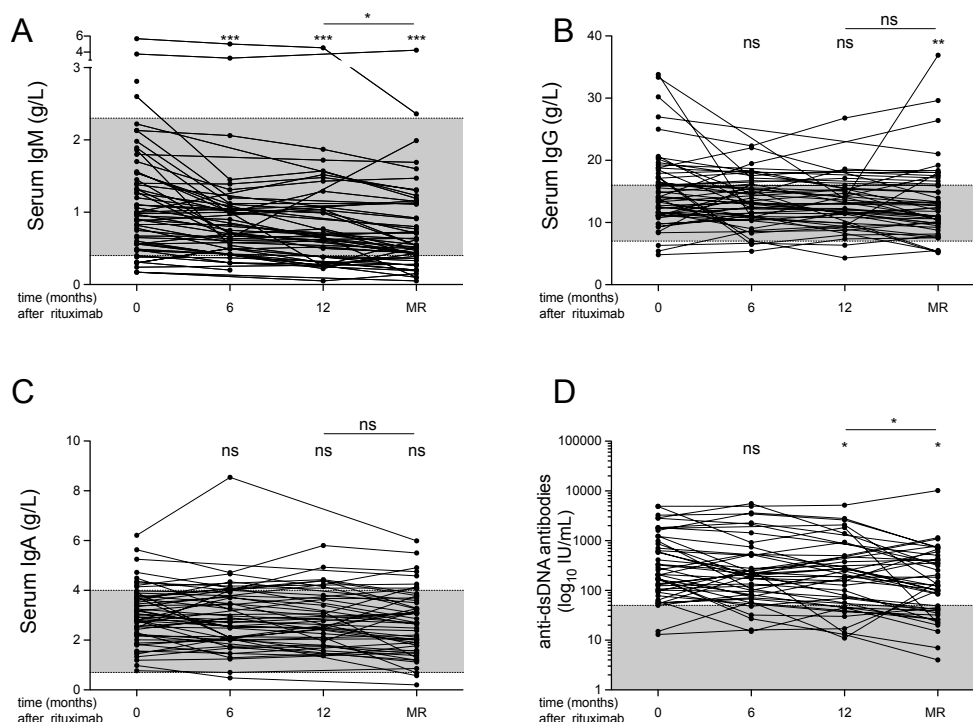
Figure 3.5 Changes in serum immunoglobulin and anti-dsDNA antibody levels in patients with SLE at most recent follow-up.

Shaded areas indicate the normal ranges for each parameter. Values for serum IgG (A), IgG-anti-dsDNA antibodies (B), IgM (C) and IgA (D) levels at baseline and at most recent (MR) follow-up are shown. *, p<0.05; **, p<0.001; ***, p<0.0001.

Median serum anti-dsDNA antibody levels at most recent follow-up, and at 12 months after rituximab, were significantly lower at 122 IU/mL (range 4 – 10171 IU/mL) and 159 IU/mL (range 11 – 5169 IU/mL) IU/mL, respectively, compared to baseline levels of 233 IU/mL (range 13 - 4912 IU/mL) (Figure 3.5D). Serum IgG levels were significantly lower at most recent follow-up compared to baseline levels. However, at most recent follow-up, the levels of serum IgM and anti-dsDNA antibodies, but not serum IgG or IgA, were significantly lower compared to both baseline levels and levels at 12 months after rituximab.

Disparate fluctuations in serum immunoglobulins and anti-dsDNA antibodies at most recent follow-up

Next, similar to the data analysis for 12-month time point, patients were identified into subgroups A and B based on whether serum immunoglobulin levels at most recent follow-up were either lower or higher compared to baseline levels, respectively.
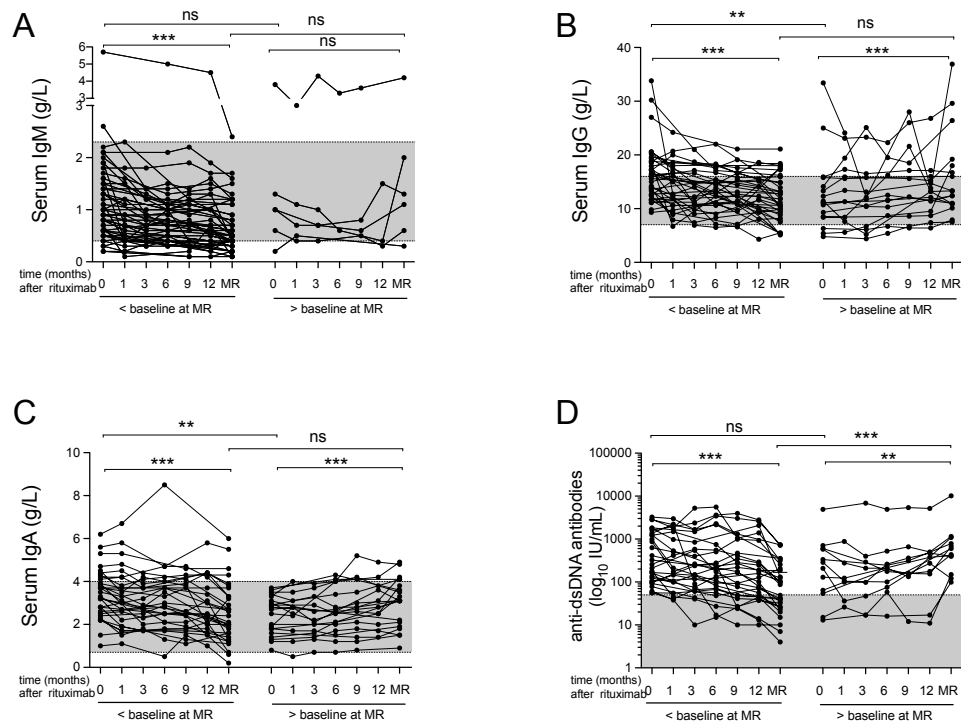
Figure 3.6 Disparate changes in serum immunoglobulins and anti-dsDNA antibodies at most recent follow-up after rituximab.

Shaded area represents the reference range for each parameter. Changes in serum A) IgM; B) IgG; C) IgA; and D) anti-dsDNA antibody at baseline and at most recent (MR) follow-up are shown. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

a) Serum IgM

There were 47 patients in subgroup A and six patients in subgroup B. Median serum IgM levels at most recent follow-up in subgroup A were significantly lower at 0.5 g/L (range 0.1 – 2.4 g/L) compared to baseline levels at 1 g/L (range 0.2 – 5.7 g/L) (Figure 3.6A). There was no such difference in subgroup B. There were no significant differences between serum IgM levels either at baseline or at most recent follow-up between the two subgroups (Figure 3.6A). All but one patient with low serum IgM levels at most recent follow-up belonged to subgroup A and the other patient to subgroup B.

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B) Serum IgG

There were 36 patients in subgroup A and 17 in subgroup B. The median serum IgG levels at baseline in subgroup A were significantly higher at 16 g/L (range 9.3 – 33.8 g/L) compared to 11.1 g/L (range 4.8 – 33.4 g/L) in subgroup B (Figure 3.6B). In contrast, there was no significant difference between median serum IgG levels at most recent follow-up between the two subgroups (Figure 3.6B). All five patients with low serum IgG levels (< 16 g/L) at most recent follow-up belonged to subgroup A whereas low serum IgG levels at baseline in all three patients in subgroup B normalised at most recent follow-up (Figure 3.6B).

c) Serum IgA

There were 32 patients in subgroup A and 21 in subgroup B. There were significant differences in median serum IgA levels at baseline and at most recent follow-up in both subgroups (Figure 3.6C). In subgroup A the median serum IgA levels fell from 3.4 g/L (range 1 – 6.2 g/L) at baseline to 2.2 g/L (range 0.2 – 6 g/L) at most recent follow-up. In contrast, median serum IgA levels rose from 2.7 g/L (range 0.8 – 3.7 g/L) at baseline to 3.1 g/L (range 0.9 – 4.9 g/L) at most recent follow-up. The median serum IgA levels at baseline in subgroup A were significantly higher at 3.4 g/L compared to 2.7 g/L in subgroup B (Figure 3.6C). In contrast, there was no significant difference between median serum IgA levels at most recent follow-up between the two subgroups (Figure 3.6C). All three patients with low serum IgA levels (< 0.7 g/L) at most recent follow up had normal serum IgA levels at baseline and belonged to subgroup A (Figure 3.6C). Nine patients had high serum IgA levels at most recent follow up, four of these patients belonged to subgroup A and the remaining five to subgroup B.

d) Anti-dsDNA antibodies

There were 33 patients in subgroup A and 11 in subgroup B. There were significant differences in median serum anti-dsDNA antibody levels at baseline and at most recent follow up in both subgroups (Figure 3.6D). In subgroup A, the median serum anti-dsDNA antibody level fell from 257 IU/mL (range 56 – 4870 IU/mL) at baseline to 84 IU/mL (range 4 – 738 IU/mL) at most recent follow-up. In contrast, median serum anti-dsDNA antibody levels rose from 287 IU/mL (range 15 – 4912 IU/mL) at baseline to 600 IU/mL (range 121 – 10171 IU/mL) at most recent follow-up. The median serum anti-dsDNA antibody levels at baseline were not significantly different between the two subgroups (Figure 3.6D). However, the median serum anti-dsDNA antibody levels at most recent follow-up in subgroup A was significantly lower at 84 IU/mL compared to 600 IU/mL in subgroup B (Figure 3.6D). Two patients from subgroup B with normal

serum anti-dsDNA antibody levels (< 50 IU/mL) at baseline had high serum anti-dsDNA antibody levels at most recent follow up (Figure 3.6D). All 15 patients with normal serum anti-dsDNA antibody levels at most recent follow-up belonged to subgroup A.

The results thus far, indicated that hypogammaglobulinemia at most recent follow-up was predominantly limited to serum IgM. Further, serum anti-dsDNA antibodies normalised in a third of patients at most recent follow-up whereas in some patients serum anti-dsDNA antibodies rose at most recent follow-up compared to baseline levels.

IgM hypogammaglobulinemia

To understand whether baseline serum IgM levels were predictive of the development of hypogammaglobulinaemia either at 12 months after rituximab or at most recent follow-up, patients were grouped according to whether serum IgM levels at these time points were lower or higher than 0.4 g/L. At 12 months after rituximab, eight patients had serum IgM levels < 0.4 g/L (range 0.1 – 0.37 g/L) and 21 had normal serum IgM levels (range 0.5 – 5.0 g/L) (Figure 3.7A). The median baseline serum IgM levels was significantly lower at 0.5 g/L (range 0.2 – 1.9 g/L) in the group with hypogammaglobulinemia at 12 months compared to 1.2 g/L (range 0.6 – 5.7 g/L) in the group that had normal IgM levels at 12 months after rituximab (Figure 3.7A). Extended data analysis revealed that at most recent follow-up, 13 patients had low serum IgM levels and 40 had normal levels (Figure 3.7B). Of the 13 patients with low serum IgM levels at most recent follow-up, seven had normal levels at baseline. Thus, seven patients had developed low serum IgM levels at most recent follow-up and the levels remained persistently low in the other six patients. The median baseline serum IgM levels were significantly lower at 0.4 g/L (range 0.2 – 1.9 g/L) in the group with hypogammaglobulinemia at most recent follow-up compared to 1.2 g/L (range 0.6- 5.7 g/L) in the group that had serum IgM levels > 0.4 g/L at most recent follow-up after rituximab (Figure 3.7B). Thus, lower baseline serum IgM levels were associated with the development of hypogammaglobulinemia.

Normalisation of anti-dsDNA antibodies

Next, to understand whether baseline serum anti-dsDNA antibody levels may predict normalisation of levels either at 12 months or at most recent follow-up after treatment with rituximab, patients were grouped according to whether the serum anti-dsDNA antibody levels at these time points were either lower or higher than the upper limit of normal range (50 IU/mL).

At 12 months after rituximab, 10 patients had serum anti-dsDNA antibody levels < 50 IU/mL and 25 had serum anti-dsDNA antibody levels > 50 IU/mL (Figure 3.7C). The median baseline serum anti-dsDNA antibody levels were significantly lower at 130 IU/mL (range 15 – 1364 IU/mL) in the group who had normal levels at 12 months compared to 602 IU/mL (range 54 – 4912 IU/mL) in the group that had persistently high levels at 12 months after rituximab (Figure 3.7C). Extended data analysis revealed that at most recent follow-up, 15 patients had serum anti-dsDNA antibody levels within the reference range (Figure 3.7D). All 15 patients with serum anti-dsDNA antibody levels with in the reference range at most recent follow-up belonged to the group with median baseline serum anti-dsDNA antibody levels that were significantly lower at 124 IU/mL (range 56 – 4870 IU/mL) compared to 591 IU/mL (range 15 – 4912 IU/mL) in the group that had persistently high anti-dsDNA antibody levels at most recent follow-up after rituximab (Figure 3.7D). Taken together, these results suggest that lower baseline levels were associated with normalisation of anti-dsDNA antibody levels after treatment with rituximab.

Figure 3.7 IgM hypogammaglobulinemia and normalisation of anti-dsDNA antibodies after rituximab: relationship with baseline levels.

Shaded area represents the reference range; boxes the inter-quartile range; the horizontal line the median; and the whiskers, the range. Ig, immunoglobulin; *, p<0.05; ***, p<0.0001.

Relationship between serum immunoglobulins and anti-dsDNA antibodies before and after treatment with rituximab

Relationship between serum IgM and anti-dsDNA antibodies

As discussed in the Chapter, Introduction, it has been suggested that low serum IgM may increase the risk of elevated anti-dsDNA antibodies. Therefore, to investigate whether IgM hypogammaglobulinemia was associated with elevated anti-dsDNA antibodies either before and/or after treatment with rituximab, the relationships between serum IgM and anti-dsDNA antibodies, before and after treatment with rituximab at most recent follow-up, were analysed.

A   B   C
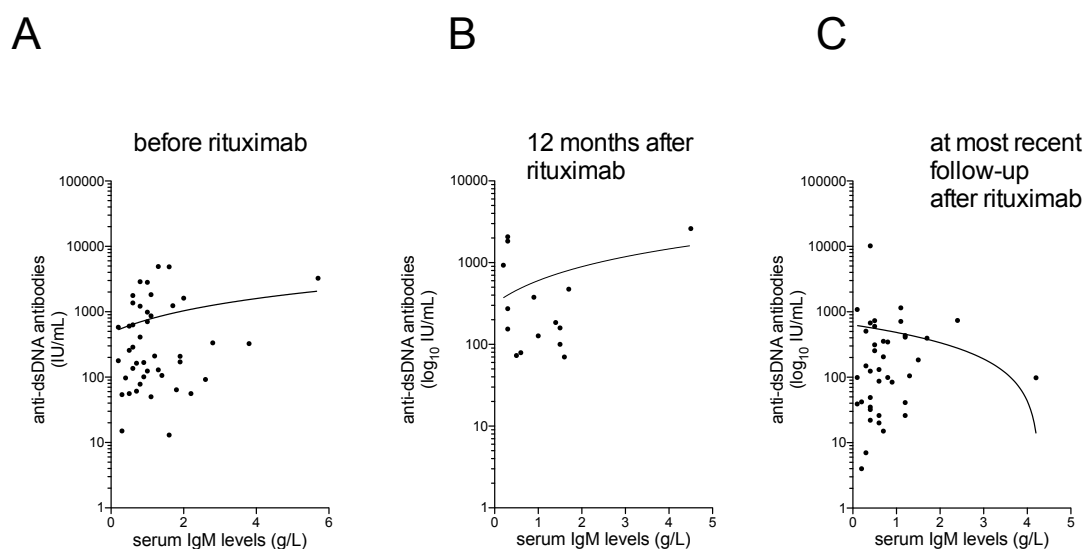
Figure 3.8 Relationship between serum IgM and anti-dsDNA antibodies before rituximab; at 12 months and at most recent follow-up after rituximab.

Relationship between serum IgM and anti-dsDNA antibodies A) before; B) at 12 months after rituximab; and C) at most recent follow-up after rituximab. Results of linear regression analysis are represented.

There were no significant relationships between serum IgM and serum anti-dsDNA antibody levels either at baseline, or at 12 months after rituximab or at most recent follow-up after rituximab with r^2 of 0.05, 0.14, 0.004, respectively (Figure 3.8 A-C). Although, serum anti-dsDNA antibodies varied remarkably between patients, there were no significant patterns of distribution in relation to serum IgM levels either before

treatment with rituximab or at 12 months and/or at most recent follow-up after treatment with rituximab, when more patients had low serum IgM levels compared to before treatment with rituximab. Thus, these results suggest that alterations in serum anti-dsDNA antibody levels occurred regardless of changes in serum IgM levels in this cohort, treated with rituximab and sequential conventional therapies based on the clinical need, in the short-term follow-up at 12 months and also at long-term follow-up after treatment with rituximab.

Disparate changes in serum IgG and anti-dsDNA antibody levels

Next, to understand whether rituximab had differential effect on total serum IgG and anti-dsDNA antibodies, the patterns of changes in total serum IgG levels and serum anti-dsDNA antibody levels were compared at 12 months after the first cycle of rituximab (Figure 3.8). Patients were grouped according to whether serum anti-dsDNA antibody levels, at 12 months after treatment with rituximab, were lower or higher compared to baseline levels, as subgroup A and B, respectively.

There were 15 patients in subgroup A and six in subgroup B. There were no significant differences in median serum IgG levels at 12 months after treatment with rituximab compared to baseline levels in either subgroup (Figure 3.9A). There were also no significant differences in median serum IgG levels either at baseline or at 12 months after treatment with rituximab between the two subgroups (Figure 3.9A). In contrast, in the same cohort, the median serum anti-dsDNA antibody levels at 12 months after rituximab were significantly lower at 73 IU/mL (range 11 – 2605 IU/mL) compared to 167 IU/mL (range 15 – 4870 IU/mL) at baseline in subgroup A (Figure 3.9B) whereas the median serum anti-dsDNA antibody levels at 12 months after rituximab were significantly higher at 425 IU/mL (range 159 – 2070 IU/mL) compared to 117 IU/mL (range 54 – 1768 IU/mL) at baseline in subgroup B (Figure 3.9B). Whilst there was no difference in median serum anti-dsDNA antibody levels at baseline between the two subgroups, the median serum anti-dsDNA antibody levels at 12 months after rituximab in subgroup A were lower at 73 IU/mL compared to 425 IU/mL in subgroup B (Figure 3.9B). Taken together, these results suggest that rituximab had disparate effects on serum IgG levels and serum anti-dsDNA antibody levels in this cohort during the 12-month follow-up after treatment with rituximab.
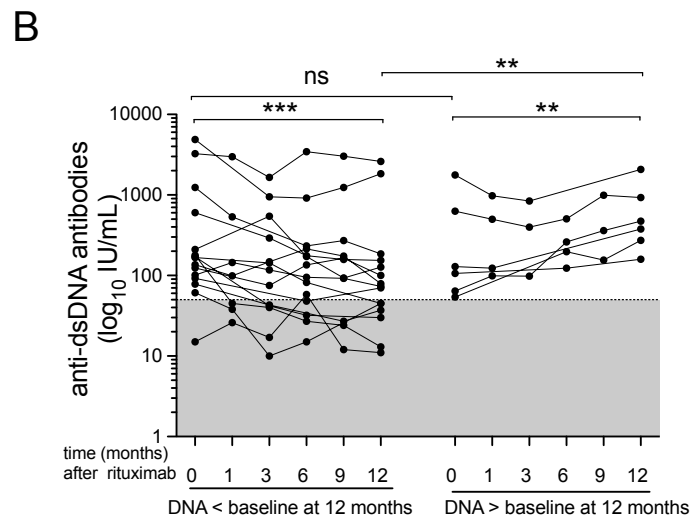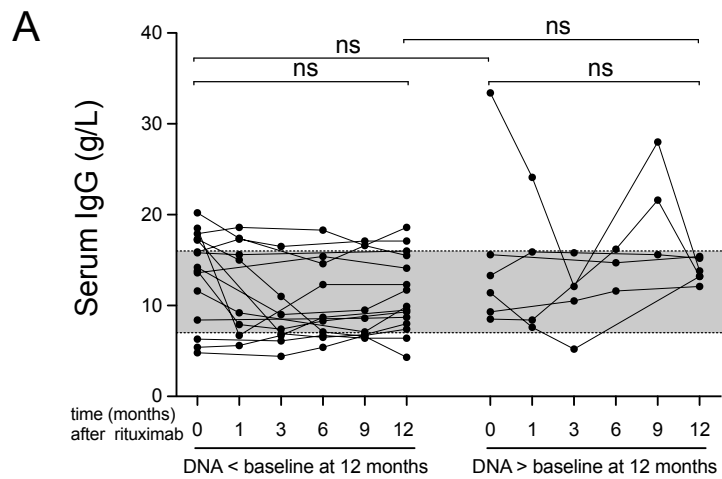
Figure 3.9 Disparate changes in serum IgG and anti-dsDNA antibody levels after rituximab: relationship with baseline levels.

Paired IgG and anti-dsDNA antibody levels at baseline and at 12 months after rituximab in 21 patients with systemic lupus erythematosus. Shaded area represents the reference range. Ig, immunoglobulin; ns, not significant; **, p<0.001; ***, p<0.0001.

Predictive factors for the development of low serum IgM after rituximab

Patients with persistent IgM hypogammaglobulinemia tended to be older with a median age of 43 years (range 22-59 years) compared to a median age of 32 years in those maintaining normal IgM levels (range 21-74 years) (Mann Whitney U test; p < 0.01).

Results described in the previous section suggested that hypogammaglobulinaemia was limited to the serum IgM isotype, particularly in patients with low baseline serum IgM levels. Therefore, a Receiver-operated-curve (ROC) analysis was performed to understand if a threshold baseline serum IgM level may predict the likelihood ratio of persistent low serum IgM levels after treatment with rituximab and subsequent immunosuppressants.

Receiver-operated-curve analysis with respect to baseline levels at 12 months after rituximab of: A) serum IgM < 0.4 g/L; B) anti-dsDNA < 50 IU/mL; and at most recent follow-up of: C) serum IgM < 0.4 g/L; and D) anti-dsDNA < 50 IU/mL.

At 12 months after rituximab, seven of 28 patients had low serum IgM levels and the ROC analysis suggested that a threshold baseline serum IgM level of 0.8 g/L had a likelihood ratio of > 4 associated with persistent low serum IgM levels at 12 months after rituximab, and a significant area under the curve of 0.9 (95% confidence interval (CI) 0.7-1.1; p=0.0002) (Figure 3.10A). Further, the ROC analysis extended to most recent follow-up data also suggested that a threshold baseline serum IgM level of 0.8 g/L had a likelihood ratio of > 4 for persistent low serum IgM levels at most recent follow-up, with a significant area under the curve of 0.9 (95% confidence interval (CI) 0.8-1.1; p < 0.0001) (Figure 3.10C). Collectively, these results suggest that baseline serum IgM level of 0.8 g/L was associated with persistent low serum IgM levels after rituximab treatment.
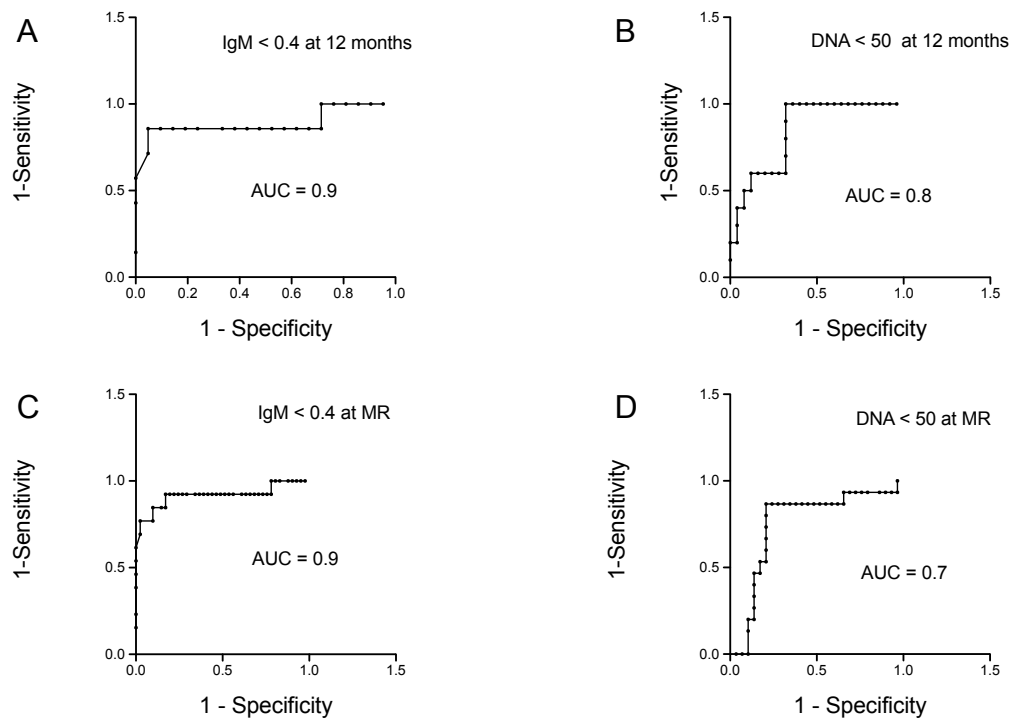
Figure 3.10 Receiver-operated curve analysis IgM hypogammaglobulinemia and normalisation of anti-dsDNA antibodies, at 12-months after rituximab and at most recent follow-up, in relation to respective baseline levels.

The results described in the previous section also suggested that in a significant number of patients serum anti-dsDNA antibody levels fell to within the normal range following treatment with rituximab. Further, the baseline median serum anti-dsDNA antibody levels was significantly lower in patients who had anti-dsDNA levels within the normal range at both 12 months and at most recent follow-up after treatment with rituximab. Therefore, ROC analysis was performed to understand if threshold baseline serum anti-dsDNA antibody levels were useful in predicting the likelihood of achieving normal anti-dsDNA antibody levels after treatment with rituximab and subsequent immunosuppressants.

At 12 months after rituximab, 25 of 35 patients had serum anti-dsDNA antibody levels > 50 IU/mL and in 10 patients the levels had normalised. ROC analysis suggested that a threshold baseline serum anti-dsDNA antibody level of 300 had a likelihood ratio > 2 associated with normal serum anti-dsDNA antibody levels at 12 months after rituximab, and a significant area under the curve of 0.8 (95% confidence interval (CI) 0.7-1.0; p = 0.002) (Figure 3.10B). ROC analysis extended to data including most recent follow-up also suggested that a threshold baseline serum anti-dsDNA antibody level of 300 IU/mL had a likelihood ratio > 2 associated with normal serum anti-dsDNA antibody

levels at most recent follow-up with a significant area under the curve of 0.7 (95% confidence interval (CI) 0.6-0.9; p = 0.002) (Figure 3.10D). Taken together, these results suggest that baseline serum anti-dsDNA antibody levels of 300 may be a useful predictor of normalising of anti-dsDNA antibody levels after treatment with rituximab and subsequent immunosuppressants. Conversely, baseline serum anti-dsDNA antibody levels > 300 IU/mL are more likely to persist in the abnormal range despite treatment with rituximab compared to baseline serum anti-dsDNA antibody levels < 300 IU/mL.
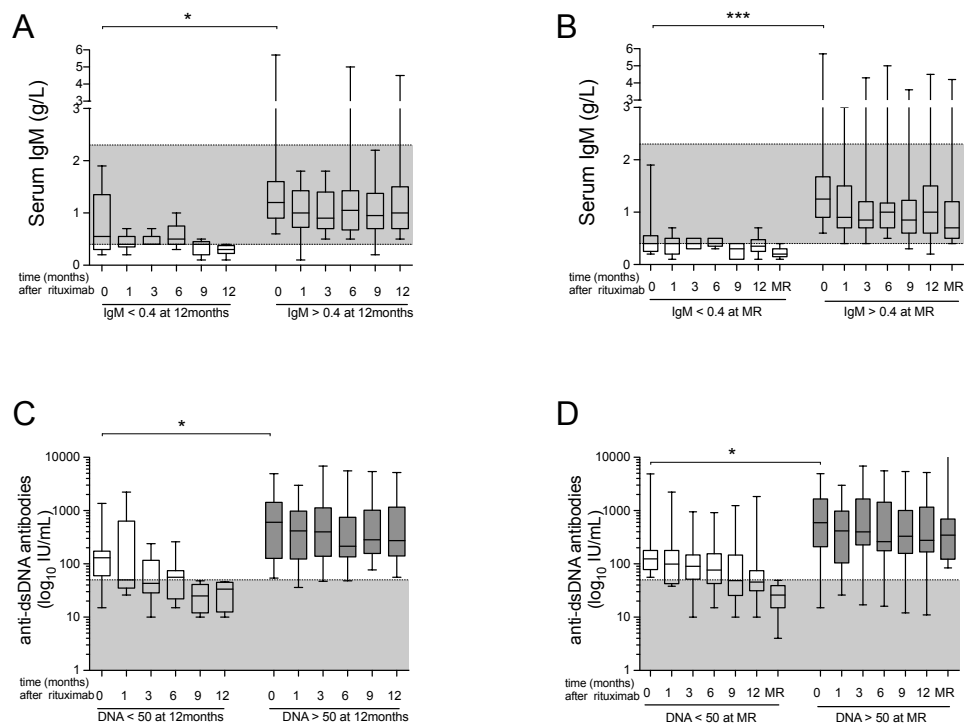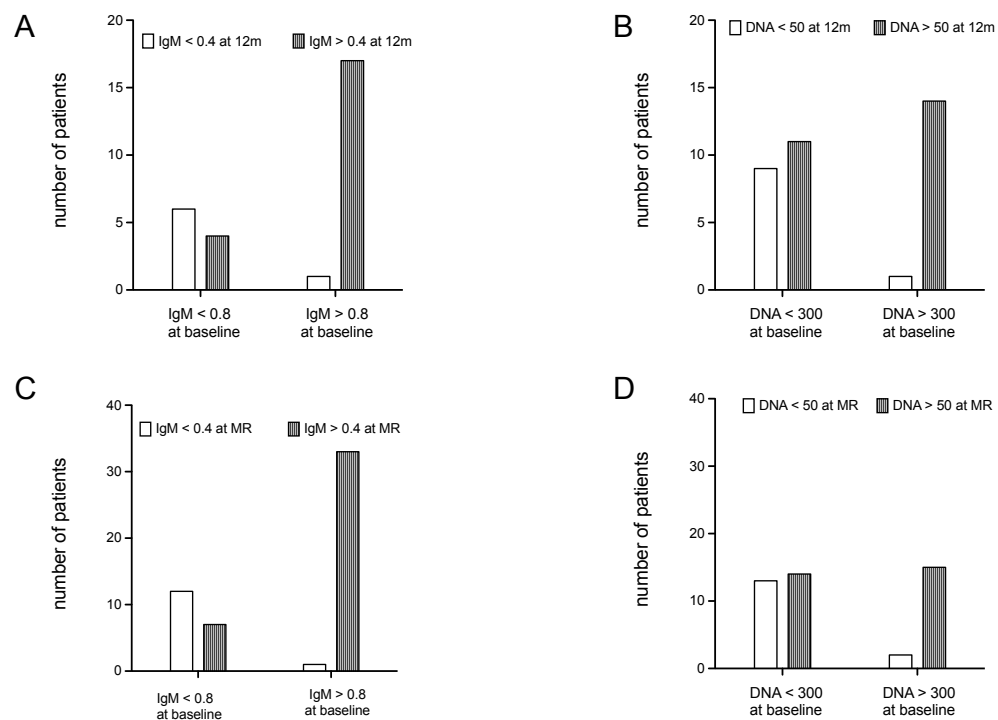

Figure 3.11 IgM hypogammaglobulinemia and normalization of anti-dsDNA antibodies after rituximab: relationship with baseline levels.

Fisher's exact test with respect to threshold levels based on ROC analysis at 12 months and most recent follow-up after rituximab showing: A) serum IgM < 0.4 g/L at 12months; B) anti-dsDNA < 50 IU/mL at 12 months; C) serum IgM < 0.4 g/L at most recent follow-up; and D) anti-dsDNA < 50 IU/mL at most recent follow-up.

In the previous section, ROC analysis suggested that a threshold serum IgM level of 0.8 g/L was associated with a likelihood ratio of > 4 of subsequent low serum IgM levels < 0.4 g/L both at 12 months after rituximab and, also at most recent follow-up after treatment with rituximab. Based on these results, patients were categorised into two subgroups according to whether the baseline serum IgM levels were lower or

higher than 0.8 g/L. Data about serum IgM levels was available from 28 patients at 12 months time point and 53 patients at most recent follow-up.

Seven of 10 patients with baseline serum IgM levels < 0.8 g/L had low serum IgM levels at 12 months after rituximab whereas only one of 18 patients with baseline serum IgM levels > 0.8 g/L had low serum IgM levels at 12 months after rituximab. Fisher's exact test indicated that serum IgM levels < 0.8 g/L were associated with a likelihood ratio of 4.5 of developing low serum IgM levels at 12 months after rituximab (Figure 3.11A). Extended analysis at most recent follow-up revealed that 12 of 19 patients with baseline serum IgM levels < 0.8 g/L had low serum IgM levels (Figure 3.11C). In contrast, only one of 34 patients with baseline serum IgM levels > 0.8 g/L had low serum IgM levels at most recent follow-up (Figure 3.11C). Fisher's exact test indicated that baseline serum IgM levels < 0.8 g/L were associated with a likelihood ratio of 5.2 for developing low serum IgM levels at most recent follow-up after treatment with rituximab. Thus, baseline serum IgM levels were associated with serum IgM levels at most recent follow-up after treatment with rituximab and sequential immunotherapies.

The results from ROC analysis described in the previous section also suggested that a threshold baseline anti-dsDNA antibody level of 300 IU/mL was associated with a likelihood ratio of > 2 for subsequent anti-dsDNA antibody levels < 50 IU/mL, at both 12 months after rituximab and also at most recent follow-up. Based on these results, patients were categorised into two subgroups according to whether the baseline serum anti-dsDNA antibody levels were lower or higher than < 300IU/mL. Data about serum anti-dsDNA antibodies was available from: 35 patients at 12 months after rituximab; and 44 patients at most recent follow-up.

The number of patients who had anti-dsDNA antibody levels < 50 IU/mL at 12 months after rituximab and also at most recent follow-up in the two subgroups were analysed by the Fishers exact test. At 12 months after rituximab, 9 of 20 patients with baseline anti-dsDNA antibody level < 300 IU/mL had serum anti-dsDNA levels < 50 IU/mL (Figure 3.11B). In contrast, one of 15 patients with baseline serum anti-dsDNA antibody levels > 300IU/mL had anti-dsDNA antibody levels in the normal range of < 50 IU/mL. Fisher's exact test suggested that a baseline anti-dsDNA antibody level of 300 IU/mL was associated with a likelihood ratio of 2.2 for achieving subsequent anti-dsDNA antibody levels < 50 IU/mL at 12 months after rituximab (Figure 3.11B).

At most recent follow-up, 13 of 27 patients with baseline anti-dsDNA antibody level < 300 IU/mL had serum anti-dsDNA levels < 50 IU/mL whereas only two of 17 patients

with baseline serum anti-dsDNA antibody levels > 300IU/mL had anti-dsDNA antibody levels in the reference range of < 50 IU/mL. Further, Fisher's exact test analysis suggested that a baseline anti-dsDNA antibody level of < 300 IU/mL was associated with a likelihood ratio of 2.2 for achieving subsequent anti-dsDNA antibody levels < 50 IU/mL at most recent follow-up after rituximab (Figure 3.11D).

Effect of sequential therapy with immunosuppressants

The results from extended data at most recent follow up showed that 13 of 53 patients developed low IgM and 6 of 13 patients were treated with MMF at least 6 months after rituximab and 2 months before the time of analysis. In contrast, only 7 of 43 patients treated with other immunosuppressants including AZT, MTX and CYC and/or only low dose corticosteroids (6 patients) or HCQ (2 patients) or not on any medications (developed low IgM (Figure 3.11). The odds ratio for the analysis was 6.8 (CI, 1.66-27.77).

As discussed in Chapter one, Introduction, treatment with immunosuppressants, particularly MMF, may also contribute to the development of hypogammaglobulinemia. Therefore, patients were categorized into two groups based on whether they received treatment with MMF or alternative conventional therapies including AZT, HCQ, MTX and corticosteroids. The number of patients with persistent low serum IgM levels in both groups was compared by Fisher's exact test.

At 12 months after treatment with rituximab, three of 11 patients treated with MMF and four of 15 patients treated with treated with other conventional therapies had low serum IgM levels (Figure 3.12A). There was no significant difference between the two groups with an odds ratio of one. At most recent follow-up after treatment with rituximab, one patient was not on any treatment and therefore excluded from analysis. Of the remaining 52 patients, six of 11 patients treated with MMF and seven of 41 patients treated with other conventional therapies had low serum IgM levels (Figure 3.12B). The difference between the two groups was significant by Fisher's exact test with an odds ratio of 5.8 (95% confidence interval (CI) 1.3-24.58; p = 0.02). Collectively, these results suggest that sequential therapy with MMF is associated with low serum IgM levels during the long-term follow-up after treatment with rituximab.
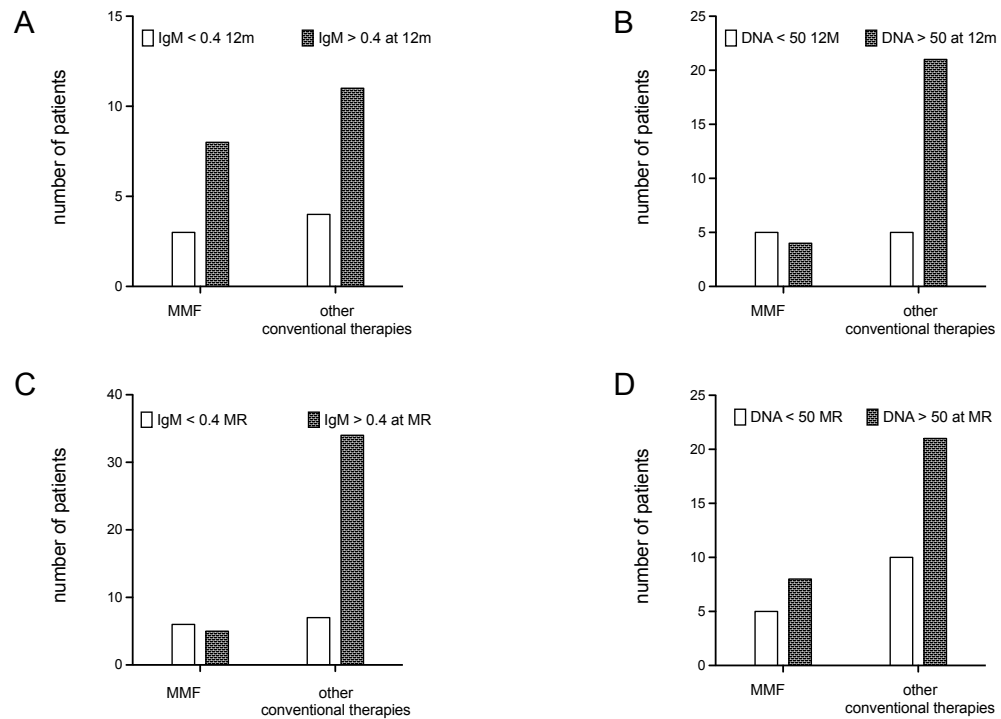
Figure 3.12 The effect of rituximab and/or sequential immunosuppressants on IgM hypogammaglobulinemia and normalising of anti-dsDNA levels after treatment with rituximab in patients with SLE.

DNA, anti-dsDNA antibodies; MMF, mycophenolate mofetil; MR, most recent follow-up.

Changes in serum anti-dsDNA antibody levels after treatment with rituximab suggested that the levels had normalised in a significant number of patients. Fisher's exact test was used to assess the effect of sequential therapies, after treatment with rituximab, on serum anti-dsDNA antibody levels by comparing the number of patients who had serum anti-dsDNA antibody levels < 50 IU/mL between the two groups of patients treated with either MMF or other conventional therapies.

At 12 months after treatment with rituximab, five of nine patients treated with MMF and five of 26 patients treated with other conventional therapies had serum anti-dsDNA antibody levels < 50 IU/mL (Figure 3.12B). There was no significant difference between the two groups by Fisher's exact test. At most recent follow-up after treatment with rituximab, five of 13 patients treated with MMF and 10 of 31 patients treated with other conventional therapies had serum anti-dsDNA antibody levels < 50 IU/mL (Figure 3.12D). The difference between the two groups was not significant by Fisher's exact test. These results suggest that sequential conventional therapies is not associated with normalization of serum anti-dsDNA antibody levels during the long-term follow-up after treatment with rituximab.

B-cell phenotypes in patients with low serum IgM levels

As discussed in Chapter 1, Introduction, the composition of the frequency of B-cell subpopulations may be altered after treatment with rituximab and subsequent immunosuppressants. Therefore, the frequency of different B-cell subpopulations after rituximab therapy was compared between patient groups who had either normal or low serum IgM levels at most recent follow-up.

Data on B-cell subpopulations was available from nine patients with low serum IgM levels and eight with normal serum IgM levels at most recent follow-up after rituximab therapy. The frequency of unswitched (also referred to synonymously as pre-switched memory B cells (IgD+CD27+), but not other phenotypes, was significantly lower in patients who developed low IgM after rituximab (n=9) when compared with those who did not (n=8) (p < 0.05) although a trend for higher frequency of double negative (DN) (IgD-CD27-) B cell subpopulation was also apparent (Figure 3.13). There was no significant difference between the two groups in sex distribution or age or median times since last rituximab treatment (24 months in the low IgM group, 15 months in those with normal IgM), or in percentage of B cells, levels of serum C3, cumulative dose of rituximab or anti-dsDNA levels between the groups (Table 3.1).
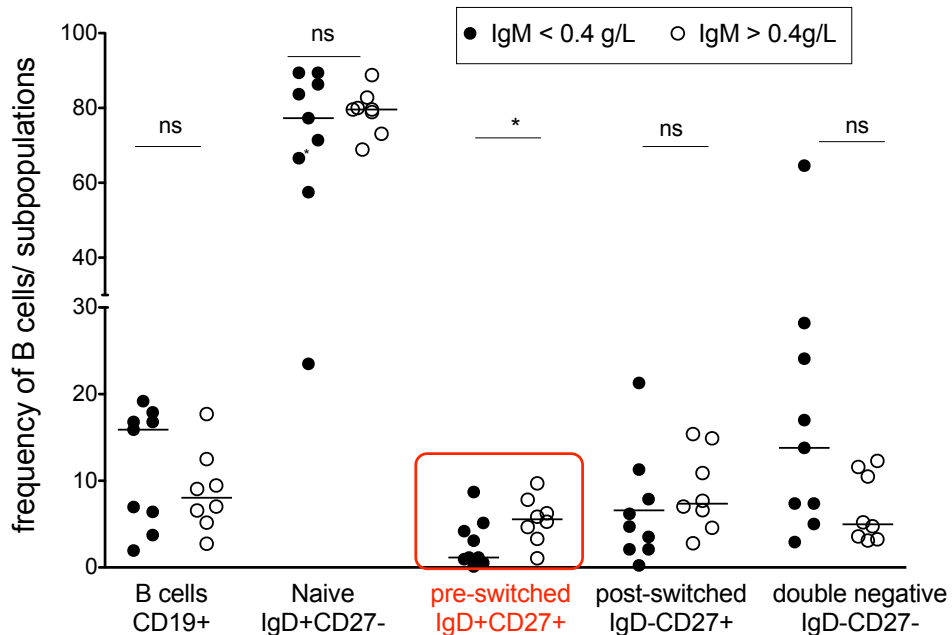
Figure 3.13 The frequency of B-cell subpopulations in patients with SLE who developed low IgM after rituximab.

Horizontal lines represent the median. *, p<0.05.

B-cell depletion and relationship with serum rituximab levels in RA and SLE

As discussed in Chapter 1, Introduction, CD19 + B cell counts do not appear to correlate with serum rituximab levels in both RA and SLE. However, there are no data that directly compare CD19 + B cell counts and serum rituximab levels in RA and SLE. Further, the discrepancy in the detection of CD19+ B cells in some patients as early as at 1 month after rituximab and the absence of CD19+ B cells in peripheral circulation at the same time point in the majority of patients may be due to: 1) altered pharmacokinetics resulting in rapid clearance of rituximab; or alternatively, 2) B cells in some patients may evade rituximab cytotoxicity; or 3) the development of human anti-chimeric antibodies (HACAs) antagonizing rituximab.

Therefore, CD19+ B cell counts were collected and serum rituximab levels were determined retrospectively in a total of 23 patients with RA and 15 with SLE before, and at one and three months after the first-cycle of rituximab. There were no significant differences between CD19+ B cell counts before, or at one and three months after rituximab between patients with RA and SLE (Figure 2.13). Patients were deemed to have achieved an incomplete depletion or a good depletion based on CD19+ B cell counts of $\geq 0.005 \times 10^9$ CD19+Bcells/L or $< 0.005 \times 10^9$ CD19+Bcells/L, respectively. All patients had been treated with two doses of 1g rituximab. Two patients with SLE with vital organ involvement also received a single dose of 750mg of cyclophosphamide. At one month after the second dose of 1g rituximab, 6 patients in each disease category had incomplete B-cell depletion and the others had depleted well.
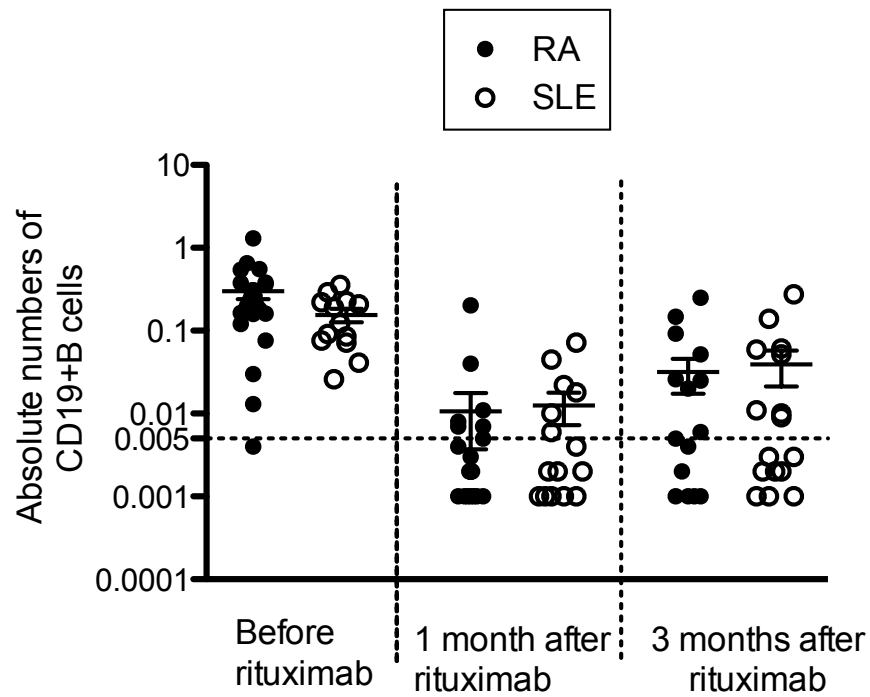
Figure 3.14 CD19+ B cell counts before and after treatment with two doses of 1g Rituximab.

CD19+ B cell counts before and at 1 & 3 months after rituximab in patients with RA (n= 23) and SLE (n= 15). Horizontal lines represent the median and each symbol represents the result of an individual patient.

All but one patient with RA had a CD19+ count of > 0.005 x 10^9 CD19+Bcells/L prior to treatment with rituximab (Figure 3.14 and Table 3.2 and 3.3). A significant variability in CD19+ counts was noted at both one and three months after rituximab, in patients with RA and SLE. The degree of variability in CD19+ B cell counts in both RA and SLE was similar to the larger cohort described earlier. Further, the variability in CD19+ B cell counts allowed for investigation into whether serum rituximab levels were also variable in these patients.
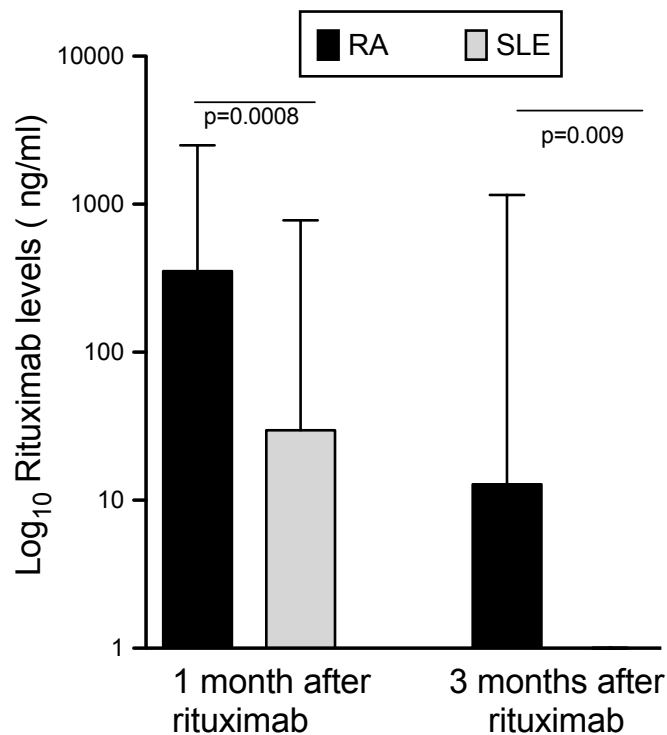
Figure 3.15 Serum rituximab levels at 1 and 3 months after rituximab in patients with RA and SLE.

Serum rituximab levels at 1 and 3 months after rituximab in patients with RA (n= 23) and SLE (n= 15). The bars represent the median and the error bars the range.

Serum rituximab levels were significantly lower in patients with SLE compared to RA at both one and three months after rituximab with a median rituximab level at one month for SLE at 43.07 ng/mL (range 0 - 777) and for RA at 391.9 ng/mL (range 1.3 - 2500) (p=0.0008) (Table 3.2 and 3.3). At three months after rituximab, patients with SLE had significantly lower median serum rituximab levels of 0 ng/mL (range 0 - 54) compared to patients with RA at 2.6 ng/mL (range 0 - 1153) (p = 0.009) (Figure 3.15).

A

CD19+ $\times$ 10^9/L

$r^2 = -0.63$ (p = 0.004)

log$_2$ rituximab levels ng/mL

Rheumatoid Arthritis

B

CD19+ $\times$ 10^9/L

$r^2 = -0.51$ (p = 0.04)

log$_2$ rituximab levels ng/mL

Systemic Lupus Erythematosus
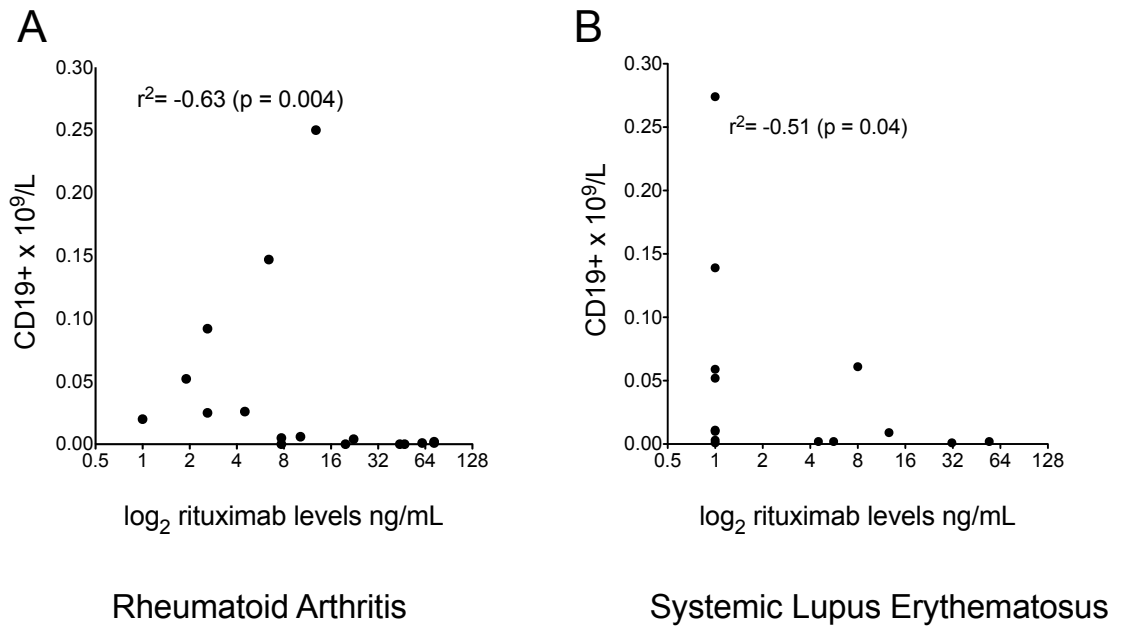
Figure 3.16 Relationship between serum rituximab levels and CD19+ B cell counts in patients with RA and SLE.

Correlation between serum rituximab levels and CD19+ counts at 3 months after 2 doses of 1g rituximab in patients with RA (n= 23) and SLE.

In patients with RA, there was no correlation between serum rituximab levels and CD19+ cell counts at one month after rituximab. However, a significant negative correlation was noted between serum rituximab levels and CD19+ B cell counts at three months after rituximab in patients with RA (r^2 = - 0.63, p = 0.004) (Figure 3.16A).

In patients with SLE also, at 1 month after rituximab, there was no correlation between serum rituximab levels and CD19+ cell counts. However, a significant negative correlation was noted between serum rituximab levels and CD19+ B cell counts at three months after rituximab in patients with SLE (r^2 = - 0.51, p = 0.04) (Figure 3.16B).

Thus, in both RA and SLE, serum rituximab levels correlated with CD19+ B cell counts only at three months, but not at one month, after rituximab treatment.

Table 3.2 CD19+ B cell counts and serum rituximab levels of patients with RA who had depleted well or had incomplete depletion at 1 month and 3 months after rituximab.

	well depleted				
Patient number	CD19x109/L at 0 months	serum rituximab (ng/mL) at 1 month	CD19x109/L at 1 month	serum rituximab (ng/mL) at 3 months	CD19x109/L at 3 months
1	0.454	2500	0.001	61.2	0.001
2	0.153	93	0.002		0.002
3	0.257	461.1	0	7.7	0
4	0.376	1239	0.001		0.001
5	0.236	2500	0.005		0.001
6	0.26	977.6	0.002	47.2	0
7	0.18	2500	0	44	0
8	0.12	884.9	0.001	1153.5	0.001
9	0.004	1270	0	264.7	
10	0.38	347.1	0		
11	0.308	2500	0		0
12	0.03	391.9	0.001	73	0.001
13	0.076	141	0.001	19.8	0
14	0.306	251.8	0.002	73	0.002
15	0.205	208.8	0.202		0
16	0.013	461.1	0.002	2.6	
17	0.164	357.7	0.001	22.3	0.004

	Incomplete depletion				
		2500	0.007		
1	0.039	2500	0.008	220.3	
2	0.206	1.3	0.011		0.008
3	0.16	56.8	0.007	2.6	0.025
4	0.545	70.4	0.04	2.6	0.092
5	0.652	160.8	0.002	12.8	0.25
6	0.199	246	0.002	7.7	0.005
7	1.303	150.6	0.003	1.9	0.052
8	0.279		0.001	4.5	0.026
9	0.556	24.2	0.004	6.4	0.147
10	0.365	47.2	0.002	10.2	0.006
11	0.161	30.6	0.002	0	0.02

Table 3.3 CD19+ B cell counts and serum rituximab levels of patients with SLE who had depleted well or had incomplete depletion at 1 month and 3 months after rituximab.

	well depleted				
Patient number	CD19x109/L at 0 months	serum rituximab (ng/mL) at 1 month	CD19x109/L at 1 month	serum rituximab (ng/mL) at 3 months	CD19x109/L at 3 months
1	0.123	12.857	0.002	0	0.01
2	0.228	32.248	0.001	0	0.003
3		0	0.001	0	0.059
4	0.192	777.449	0.001	0	0.052
5	0.358	24.558	0.002	5.622	0.002
6	0.221	29.643	0.001	0	0.001
7		34.588	0.004	4.506	0.002
8	0.091	6.532	0.002		
9	0.072	43.654	0.001	31.545	0.001
	Incomplete depletion				
1	0.076	0	0.018	0	0.011
2		268.123	0.072	54.384	0.002
3	0.291	373.373	0.006	0	0.139
4	0.209	43.075	0.01	12.61	0.009
5	0.041	13.602	0.022	0	0.274
6	0.085	10.866	0.045	7.997	0.061

Serum rituximab levels were compared between the groups of patients with RA and SLE who had, or not, depleted well at one and three months after rituximab. In well-depleted patients, rituximab levels were significantly lower in patients with SLE compared to RA, at both one (p = 0.003) and three months (p = 0.008) after rituximab (Figure 3.17). There were no significant differences in serum rituximab levels between patients with RA and SLE who had not depleted well. Serum rituximab levels were also higher in patients with RA, who had depleted well compared to those with poor depletion, at both one and three months after rituximab. However, no significant differences in serum rituximab levels were noted between patients with SLE who had, or not, depleted well, at either one or three months after rituximab.
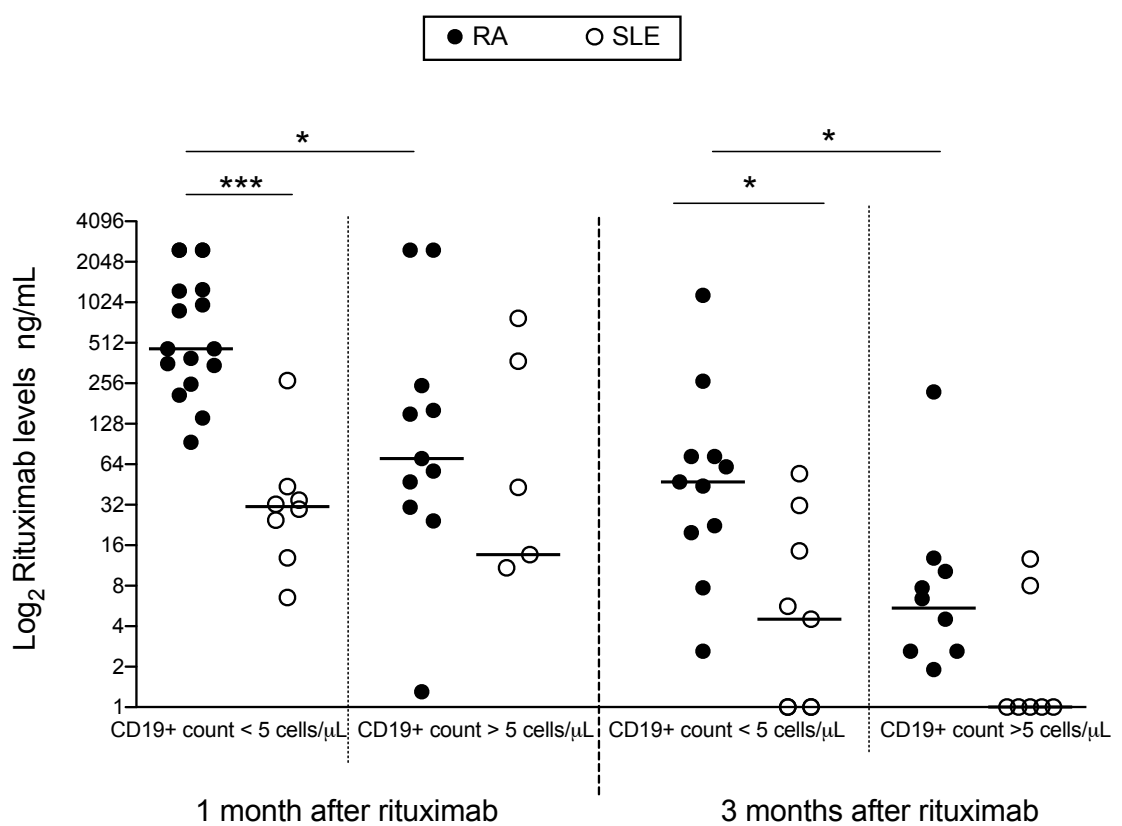
Figure 3.17 Serum rituximab levels and CD19+ cell counts in patients with RA and SLE.

Serum rituximab levels and CD19+ cell counts in patients with RA and SLE, grouped according to CD19+ counts of < / > 5 cells/μL (0.005 x 10^9 CD19+ cells/L) at one and three months after rituximab.

B-cell expression of CD20 and FcγRIIb in RA and SLE

As discussed in Chapter 1, Introduction, malignant B-cell expression of CD20 and the inhibitory FcγRIIb were associated with clinical response to rituximab. Given, the remarkable variability in the efficiency of rituximab at inducing B-cell depletion, in vivo, in RA and SLE, the expression of these two cell surface proteins on B cells from patients with RA (n=44) and SLE (n=81) was compared.

The median (inter-quartile range, IQR) frequency of CD20+ B cells was significantly greater in patients with RA compared to those with SLE, with 9.6 % (7.3 – 12.1 %) and 6 % (3.9 – 10.8 %), respectively (Figure 3.18A). In contrast, B-cell expression of CD20 (MFI) was significantly lower in patients with RA compared with those with SLE with median (IQR) 2292 (1787 – 3654) and 6261 (2334 – 13446), respectively (Figure 3.18B). However, the MFI of CD20 varied remarkably particularly in patients with SLE.

B-cell expression of FcγRIIb (CD32) (MFI) was also significantly lower in patients with RA compared with those with SLE with median (IQR) 280 (181 – 555) and 2414 (1378 – 5707), respectively. The MFI of FcγRIIb varied remarkably between patients with RA and SLE (Figure 3.18C). Further, there was no difference in the relative B-cell expression of CD20 and FcγRIIb (ratio of MFI of CD20 and MFI of FcγRIIb) between patients with RA and SLE (Figure 3.18D).
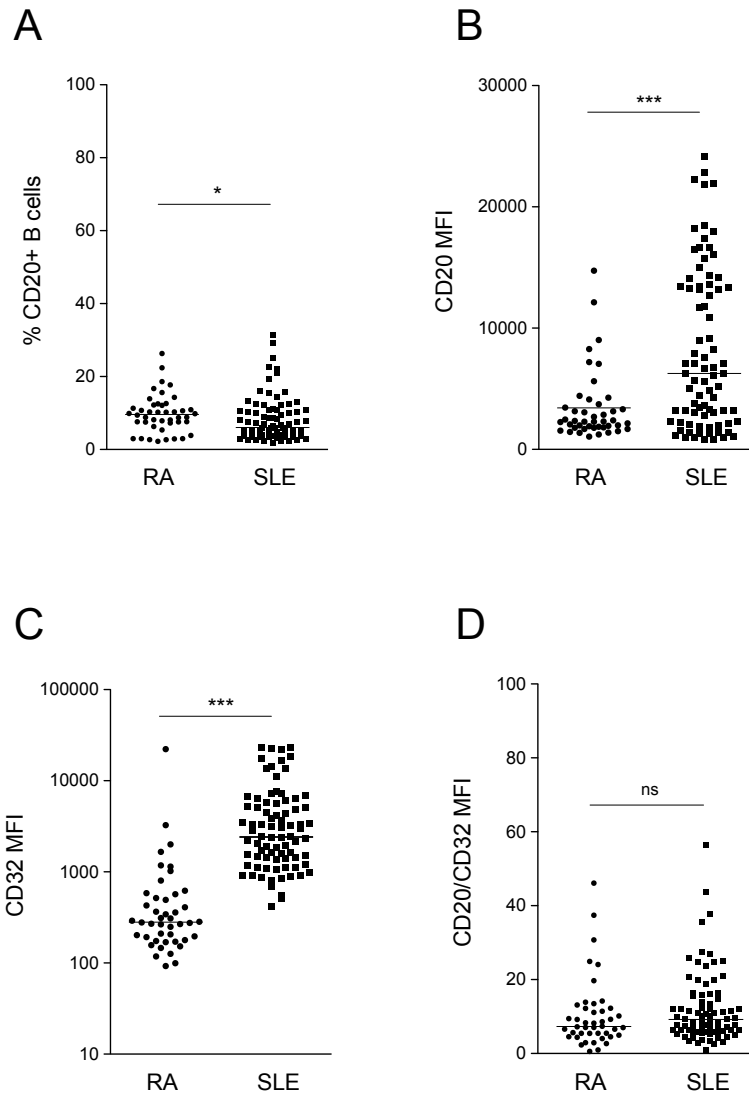
Figure 3.18 The mean fluorescence intensity of CD20 and FcγRIIb on B cells from patients with RA and SLE.

A) The frequency of CD20+ B cells in patients with RA and SLE; B) the mean fluorescence intensity of CD20 in patients with RA and SLE; C) the mean fluorescence intensity of FcγRIIb in patients with RA and SLE; and D) the ratio of MFI of CD20 and MFI of CD32 of CD20+ B cells from patients with RA and SLE. *, p < 0.05; ***, p< 0.0001.

Discussion

B-cell depletion in vivo in RA and SLE

The results of CD19+ B cell counts were remarkably variable between patients with RA and SLE before treatment with rituximab. The analysis of in vivo B-cell depletion, up to 6 months after treatment with rituximab, as assessed by CD19+ B cells in peripheral circulation revealed that, despite treatment with the same dose of rituximab, the duration of B-cell depletion was remarkably variable between individual patients with RA and SLE. Before treatment with rituximab, patients with RA had higher CD19+ B cell counts compared to patients with SLE. However, the observations that CD19+ B cells were detectable in peripheral circulation at an earlier time point after treatment with rituximab in patients with SLE compared to RA suggests that B-cell depletion was less pronounced and/or that B cell repopulation occurred sooner in patients with SLE compared to those with RA. The results also suggest that B-cell depletion appears to be unpredictable in some patients with SLE when compared with RA using current therapeutic regimens. Although absence of CD19+ B cell counts in peripheral blood may not accurately reflect B-cell depletion in lymphoid tissues, the detection of CD19+ B cells in peripheral blood likely suggests the presence of B cells in lymphoid tissues. Currently, there are no directly comparative data about the efficiency of rituximab induced B-cell depletion and/or the duration of B-cell depletion between patients with RA and SLE. These data showing the differences in the extent and duration of B-cell depletion between RA and SLE may, at least in part, explain the disparity in clinical response reported in clinical trials involving patients with RA and SLE.

Effects of rituximab on serum immunoglobulins

As discussed in the Introduction, the long-term effects of rituximab on B-cell depletion and differentiation would also be important to consider particularly in patients with SLE who may be treated with conventional immunosuppressants and/or immunomodulatory agents, sequentially. Because B cells terminally differentiate into plasma cells, which secrete immunoglobulins (Igs), study of the kinetics of Igs may inform about the long-term effects of rituximab on B-cell depletion, repopulation and/or differentiation to plasma cells.

There was a disparity in the dynamics of fluctuations between isotypes of serum Igs after rituximab. At 12 months after the first cycle of rituximab, there was a hierarchy in percentage reduction of Ig isotypes: IgM > IgG > IgA. Hypogammaglobulinemia after

rituximab was mostly restricted to the IgM isotype with seven of 47 (15%) patients with normal IgM levels before rituximab developing low serum IgM levels at most recent follow-up whereas six others with low serum IgM levels before rituximab had persistently low levels at most recent follow-up. In contrast, the incidence of hypogammaglobulinemia with respect to serum IgG and IgA was low at four of 53 (7%) and three of 53 (5%), respectively. Thus, the incidence of hypogammaglobulinemia in this cohort of SLE patients was mostly restricted to the IgM isotype.

In contrast to IgM, serum IgA, after an initial decrease in median levels, started recovering as early as 2 months after rituximab, approaching baseline levels by 6 months. Early recovery in serum IgA levels suggests that the IgA plasma cell pool was rapidly replenished. It has previously been reported that circulating IgA+ plasmablasts can remain detectable early after rituximab, suggesting resistance to depletion of switched IgA+ precursor-B cells, likely in the mucosal microenvironment and/or early regeneration (Mei, Frolich et al. 2010). Recovery of serum IgG levels, despite showing a longer 'lag' when compared with the recovery of serum IgA levels, was apparently also sustainable, attaining pre-treatment levels in most patients by 12 months after rituximab. Indeed, rituximab treatment resulted in correction of hypergammaglobulinemia in most patients in this cohort. At long-term follow up, very few, three of 53 (5% of patients), had serum IgG levels below the lower limit of the normal range.

Serum IgM is derived from both (short-lived) newly generated perifollicular B cells (CD27-) and from CD27+ (un-switched) marginal zone B cells (Weill, Weller et al. 2009). Serum levels of IgA and IgG are largely maintained by long-lived (CD20-) plasma cells, predominantly in the bone marrow. These are therefore not directly targeted by rituximab, and protective immunity is largely maintained in RA patients for instance (Cambridge, Leandro et al. 2003, De La Torre, Leandro et al. 2012, Cambridge, Perry et al. 2014). It is however difficult to differentiate the direct effects of rituximab preventing formation of new plasma cells from indirect effects through disease control.

The proportion of patients achieving a reduction in serum Ig levels also contrasted between IgM vs IgG and IgA isotypes. There were differences between Ig isotypes in the number of patients in the two subgroups, grouped based on whether the immunoglobulin levels decreased or increased from baseline after treatment with rituximab. The majority of patients 47 versus six had lower serum IgM levels at 12 month after rituximab compared to baseline levels whereas for IgG and IgA there were equal numbers of patients in the two subgroups. Similarly, at most recent follow-up

also there were disproportionately large group of patients, 47 versus six, in whom serum IgM levels reduced from baseline whereas for IgG and IgA there were relatively less disproportionate number of patients in the two groups at 36 versus 17 and 32 versus 21, respectively. Furthermore, baseline serum IgG and IgA levels, but not IgM levels, were predictive of further reductions at most recent follow-up after treatment with rituximab. Remarkably, baseline serum IgG and IgA levels, but not IgM levels, were higher in the subgroup of patients who achieved a reduction in serum Ig levels after rituximab.

In the UCLH cohort of patients with RA the incidence of low IgM increased from 9.2% - 38.8% and IgG from 11.8% - 22.2% of patients, after one and 5 cycles respectively (De La Torre, Leandro et al. 2012). Therefore, the incidence of hypogammaglobulinemia in this cohort of patients with SLE was considerably lower compared to the UCH cohort of patients with RA described by De La Torre et al. A potential explanation for the difference in the incidence of hypogammaglobulinemia in these two cohorts of patients may be because the majority of patients with SLE in this cohort received relatively fewer cycles (median 2) of treatment with rituximab. However, another potentially important factor to consider though is that all but one patient in this cohort of patients with SLE also received sequential treatment with immunosuppressants and/or immunomodulatory agents, which may have also increased the risk of the development of hypogammaglobulinemia.

In patients with ANCA-associated vasculitis (AAV) and thrombotic thrombocytopenic purpura (TTP), co-therapies such as cyclophosphamide and plasmapheresis make it difficult to dissect the role of rituximab per se in the development of low serum Igs. None of the patients received plasmapheresis but some patients with vital organ involvement such as the kidney received a single dose of 750mg of cyclophosphamide, substantially lower than that used in AAV. Comparison between patient groups was also confounded due to lower pre-rituximab serum IgG levels in patients with AAV (Marco, Smith et al. 2014). Of direct clinical relevance, rituximab treatment did not result in significant reductions in serum IgG levels in those with low baseline IgG levels of < 6 g/L (Marco, Smith et al. 2014). Collectively, allowing for the differences in the pragmatic management between the conditions, these results suggest that patients with SLE might have relatively lower risk of developing hypogammaglobulinemia, which was mostly restricted to the IgM isotype, compared to RA and AAV.

Differential effects on Ig classes have also been described in patients with multiple myeloma treated with autologous haematopoietic stem cell transplant (HSCT) and rituximab maintenance therapy (Lim, Zhang et al. 2004). Both groups of patients tend

to develop low IgM but not IgG and IgA. In contrast, some patients with refractory follicular lymphoma treated with rituximab and HSCT developed persistently low IgA and IgG with recovery of IgM levels (Hicks, Woods et al. 2009). Underlying disease therefore seems to influence the development of isotype specific hypogammaglobulinemia.

Effects of rituximab on serum anti-dsDNA antibodies

Serum anti-dsDNA antibody levels were significantly lower at one month after rituximab, but not at other time points during the 12 months follow-up after the first cycle of rituximab. Up to 12 months after rituximab, the percentage reduction in serum anti-dsDNA antibodies was also less marked compared to the percentage reduction in serum IgM. Less marked effects on serum anti-dsDNA antibodies may be either due to less efficient depletion of specific clones of B cells and/or due to early recovery of B cells that give rise to DNA antibody secreting plasma cells. Further, a majority of patients achieved a reduction in serum anti-dsDNA antibodies from baseline, in some anti-dsDNA antibodies normalised, compared to the number of patients achieved an increase in anti-dsDNA antibodies, at 26 versus nine, respectively. Similarly, at most recent follow-up 29 patients had lower anti-dsDNA antibody levels compared to baseline levels whereas in 11 patients anti-dsDNA antibody levels increased at most recent follow-up after rituximab. Importantly, serum anti-dsDNA antibody levels normalised in 15 patients whereas only two patients with normal levels at baseline developed high anti-dsDNA antibody levels at most recent follow-up.

Interestingly, patients who had normal anti-dsDNA antibody levels at most recent follow-up also had significantly lower baseline levels compared to a group of patients who had persistently high anti-dsDNA antibody levels at most recent follow-up. These results suggested that baseline levels might be useful in identifying a threshold anti-dsDNA level that would be predictive of the likelihood of normalising levels after rituximab and/or of the likelihood of persistently high anti-dsDNA antibody levels after rituximab. The receiver operating curve (ROC) analysis based on serum anti-dsDNA antibody level of < 50 IU/mL identified a threshold level of 300 IU/mL associated with persistently high levels at both 12 months and long-term follow-up after treatment with rituximab. Furthermore, analysis using the Fisher's exact test also supported that a threshold serum anti-dsDNA antibody level of 300 IU/mL was predictive of the likelihood of normalising serum anti-dsDNA antibody levels at both 12-months and long-term follow-up after rituximab. However, there was no significant difference in the likelihood of normalisation of anti-dsDNA antibody levels based on sequential

conventional therapies MMF versus alternative conventional therapies including AZT, corticosteroids, MTX and HCQ.

Because IgM functions as a scavenger receptor and facilitates the removal of apoptotic cells, it has been suggested that low serum IgM levels might increase the prospects of the development of IgG autoantibodies such as anti-dsDNA antibodies (Ehrenstein, Cook et al. 2000, Ehrenstein and Notley 2010). However, the results of the analysis identified no significant relationships between serum anti-dsDNA antibody levels and serum IgM levels either before rituximab, or at 12 months and most recent follow-up after treatment with rituximab. Therefore, these data are of clinical importance.

Analysis of paired data on changes in serum IgG levels and anti-dsDNA antibody levels revealed disparate changes. Intriguingly, serum IgG levels were not significantly different between baseline and at 12 months after rituximab in either of the two subgroups of patients grouped according to whether anti-dsDNA antibody levels at 12 months after rituximab were lower or higher compared to baseline. In contrast, hypergammaglobulinemia was normalised. A potential explanation for the disparate changes in serum IgG and anti-dsDNA antibody levels might be that anti-dsDNA secreting plasma cell pool may be dependent on continuous feed-in from B-cell differentiation and therefore perturbed by rituximab whereas only a relatively small proportion of serum IgG was constantly formed, hence, remained comparatively stable after treatment with rituximab. Alternatively, rituximab has differential effect on depletion and/or differentiation of B cells toward anti-dsDNA antibody secreting plasma cells whereas IgG secreting plasma cell pool was not significantly affected.

Thus, differences in the patterns of fluctuations in anti-dsDNA antibodies between patients implied a variable contribution from anti-dsDNA committed B cell clones (CD20+) sensitive to B cell depletion and also from long-lived (IgG) plasma cells (CD20-) (Cambridge, Isenberg et al. 2008). Autoantibody-committed B cells may be removed by rituximab, preferentially, as has also been proposed in patients with RA (Teng, Wheater et al. 2012) (Cambridge, Perry et al. 2014). A significant proportion of patients lost seropositivity to anti-dsDNA antibodies at long-term follow-up, however, there was little overall decrease in anti-dsDNA antibodies in those patients with the highest baseline levels, suggesting the presence of a more entrenched autoreactive plasma cell pool (Alexander, Sarfert et al. 2015).

Factors associated with IgM hypogammaglobulinaemia

Given that hypogammaglobulinemia after rituximab was mostly limited to serum IgM, the factors associated with low IgM were explored. Segregating patients based on whether they developed low IgM after rituximab identified two subgroups of patients. Those who developed low IgM had 2 fold-lower median levels of serum IgM before rituximab compared to the group of patients who had normal serum IgM levels at both 12-months and long-term follow-up after rituximab. These findings suggested that baseline levels might be useful in predicting the risk of developing low IgM after rituximab.

The ROC analysis based on the serum IgM of less than 0.4 g/L, identified that a threshold level of 0.8 g/L might be useful for predicting the likelihood of the development of IgM hypogammaglobulinemia at both 12 months and long-term follow-up. Therefore, baseline serum IgM levels may guide monitoring of patients after treatment with rituximab.

As discussed in Chapter 1, Introduction, conventional immunosuppressants such as MMF may also contribute to the development of hypogammaglobulinemia. The results by Fisher's exact test detected an odds ration of > 5 for the association of low IgM levels with sequential MMF treatment compared to alternative conventional therapies including AZT, corticosteroids, MTX and HCQ. These results suggested a strong association between sequential treatment with MMF after rituximab and low serum IgM. MMF preferentially targets type II inosine monophosphate dehydrogenase (IMPDH), which is up regulated in activated lymphocytes (both B and T lymphocytes) (Lee, Pawlak et al. 1985).

The frequency of unswitched memory B-cell subpopulations after rituximab in patients who developed low IgM levels was significantly lower compared to those with normal IgM levels at most recent follow-up after rituximab. There was no difference between time since last rituximab infusion, or in cumulative rituximab dose in the small subgroup of patients studied for B-cell phenotype. In SLE, MMF, but not AZT or HCQ, treatment has been associated with reduced frequency of switched memory B cells and modest decreases in levels of serum Igs and of anti-dsDNA antibodies (Eickenberg, Mickholz et al. 2012, Mino, Naito et al. 2012). The composition of B-cell subpopulations may vary between individuals with SLE and after rituximab repopulation appears to recapitulate ontogeny perhaps further influenced by antigen stimulation (van Zelm, Szczepanski et al. 2007). Also, the frequency of unswitched (IgD+CD27+) memory B cells was significantly lower in patients who developed low IgM after rituximab when

compared with those who did not. Further, unswitched memory B cells may also have a reduced threshold for survival in SLE (Rodriguez-Bayona, Ramos-Amaya et al. 2010). Therefore, both inherent and acquired mechanisms contribute to lower frequency of unswitched memory B cells in some patients with SLE, at risk of developing IgM hypogammaglobulinemia after rituximab.

Co-therapy with MMF has been associated with higher rate of infections in clinical trials using Ocrelizumab (Mysler, Spindler et al. 2013) and low immunoglobulins were noted in patients treated with a combination of MMF and Atacicept (Ginzler, Wax et al. 2012). In the latter study, low IgM levels in SLE patients treated with MMF alone in the placebo arm did not recover over the course of the study. Taken together, these data suggest that the use of sequential immunosuppressants after rituximab warrants careful monitoring and judicious dosing regimen.

The limitations of the methods and data analysis were that the findings are observational and from a single center, and the data were not complete for all time points. However, clinical and laboratory results were available for the majority of patients at most recent follow-up; complemented by prospective analysis of peripheral blood immunophenotyping of most patients who developed low serum IgM.

Serum rituximab levels in RA and SLE

Serum rituximab levels were remarkably variable between patients and between the groups of patients with RA and SLE. All patients had received their first cycle of rituximab treatment, thereby, although not absolutely, minimising the confounding effect of human anti-chimeric antibodies. Intriguingly, patients with SLE had markedly (> 9 fold, at one month) lower serum levels of rituximab than patients with RA, at both one and three months, regardless of the level of B-cell depletion, as assessed by CD19+ B cell counts in peripheral circulation. An important factor influencing serum Ig levels is the balance between synthetic and catabolic rate of different Ig isotypes. IgG catabolism is greater in patients with SLE compared to RA whereas IgM catabolism is greater in RA compared to patients with SLE (Levy, Barnett et al. 1970). Serum half-life of IgG in SLE is remarkably lower at a mean of eight days compared to a mean serum half-life of 14 days in RA (Levy, Barnett et al. 1970) with a catabolic rate of 10% vs 6% in SLE and RA, respectively. Therefore, disease-associated Ig isotype metabolism might further contribute to increased clearance of serum rituximab in SLE compared to RA.

Serum rituximab levels were significantly higher in patients with RA who had depleted well compared to those who did not, at both one and three months after rituximab, suggesting that higher serum rituximab levels may enhance B-cell depletion at least in some patients with RA who do not deplete well. However, there were no significant differences in serum rituximab levels between patients with SLE grouped according to > or < 0.005×10^9 CD19+Bcells/L. No direct correlations between serum rituximab levels and CD19+ cell counts were detected in either RA or SLE at both one and three months after rituximab. The lack of correlation between serum rituximab levels and CD19+ B cell counts at one month after rituximab may be due to either the detected CD19+ B cells were able to evade cytotoxic effects of rituximab and/or due to low number of patients, who had CD19+ B cell counts. Alternatively, the absence of an inverse correlation between serum rituximab levels and CD19+ B-cell counts, at one month, might suggest an intrinsic resistance of B-cells to rituximab-induced depletion.

The study was not designed to investigate whether proteinuria in patients with lupus nephritis influenced serum rituximab levels. However, a larger study in a clinical trial setting may be more appropriate to address whether proteinuria in itself reduces the efficiency of rituximab by mediating increased clearance.

B-cell expression of CD20 and FcγRIIb in RA and SLE

As discussed in Chapter one, introduction, B-cell expression of CD20 and FcγRIIb appear to influence clinical response to rituximab in B-cell malignancies. B-cell expression of CD20 and FcγRIIb varied remarkably between patients RA and SLE. The frequency of CD20+ B cells was higher in patients with RA compared to SLE whereas B-cell expression of CD20 was lower in RA patients compared to SLE. A higher expression of CD20 would be expected to result in more efficient B-cell depletion in patients with SLE. However, the data about the duration of B-cell depletion suggested otherwise raising the possibility of other factors influencing B-cell depletion.

As discussed in Chapter one, malignant B-cell expression of FcγRIIb promotes internalisation of rituximab impairing its efficiency (Lim, Vaughan et al. 2011, Lee, Ashton-Key et al. 2015). In this context, B-cell expression of FcγRIIb was also significantly lower in RA patients compared to SLE, hence, the efficiency of rituximab at depleting B cells in SLE may be impaired. To understand whether a higher expression of FcγRIIb may have offset the effect of higher expression of CD20 in SLE the relative expression of CD20 and FcγRIIb was investigated. However, the relative expression of CD20 and FcγRIIb on B cells was not significantly different between patient with RA

and SLE. Therefore, B-cell expression of CD20 and FcγRIIb do not explain the shorter duration of B-cell depletion in patients with SLE compared to RA. As discussed in Chapter one, B-cell extrinsic factors that are involved in the effector mechanisms of anti-CD20 mAbs may also influence the efficiency of B-cell depletion.

Conclusions

These data showed that the duration of B-cell depletion was variable between patients with RA and SLE, who were treated with the same dosing regimen of rituximab and, that B-cell depletion is less efficient in patients with SLE compared to those with RA. Hypogammaglobulinemia after rituximab in patients with SLE was largely restricted to the IgM isotype, and was associated with low baseline levels and a lower frequency of un-switched B cells. The development of IgM hypogammaglobulinemia was also associated with sequential therapy with MMF. Reassuringly, serum IgM levels after rituximab were not associated with levels of anti-dsDNA antibodies. Thus, these data suggest that due consideration of factors beyond B-cell depletion with rituximab may inform judicious management and monitoring to help minimise the risk of hypogammaglobulinemia.

 Serum rituximab levels, at one and three months after treatment, varied remarkably in this cohort of patients with RA and SLE. Although IgG metabolism may explain the differences in serum half-life of rituximab between patients with RA and SLE (Levy, Barnett et al. 1970), the variability in serum rituximab levels noted between patients with SLE was suggest that alternative mechanisms may be involved. Further, B cell counts did not correlate with serum rituximab levels at one and three months after treatment suggesting that some B cells might evade depletion with rituximab at the levels noted. In this context, as discussed in the Introduction, B cell subpopulations have also been proposed to be differentially sensitive to rituximab-cytotoxicity (Leandro 2013). However, the underlying reasons for this apparent resistance to rituximab-induced B-cell depletion remain elusive and furthermore, whether alternative anti-CD20 mAbs are more efficient at inducing B-cell depletion in patients with RA and SLE is not known. A potential mechanism of B-cell resistance to B-cell depletion with rituximab, as discussed in Chapter one, Introduction, may lie in their ability to internalise rituximab. This will be explored in the next chapter.

Chapter 4   Results: Internalisation of Anti-CD20 mAbs and Efficiency of B-cell Depletion in Vitro

Results

In this chapter, to test the hypothesis that *"Type I anti-CD20 monoclonal antibodies are less efficient than type II anti-CD20 monoclonal antibodies at inducing B cell cytotoxicity in vitro in samples from patients with rheumatoid arthritis and systemic lupus erythematosus"* the following questions were investigated:

1) Are type II anti-CD20 mAbs more efficient than type I anti-CD20 mAbs at inducing B-cell depletion in vitro?
2) What is the effect of B-cell expression of CD20 and FcγRIIb and excess BAFF on the efficiency of anti-CD20 mAbs in whole blood B-cell depletion assay?
3) Is the composition of B-cell subpopulations altered in the presence of anti-CD20 mAbs in whole blood assays?
4) Does internalisation of mAbs influence the efficiency of B-cell depletion, in vitro?
5) What factors determine internalisation of mAbs, in vitro?

Efficiency of Type I and II anti-CD20mAbs

The efficiency of type I and II anti-CD20 mAbs at deleting B cells from patients with RA and SLE was compared in whole blood B-cell depletion assays, as described in Methods. Whole blood B-cell depletion assays were performed using freshly drawn heparinized whole blood samples from a total of 26 patients with RA and 50 with SLE. The characteristics of the patients are described in Tables 4.1 and 4.2. All patients satisfied the criteria for diagnosis based on appropriate diagnostic and/or classification criteria, described in the Methods. The main exclusion criterion was previous treatment with rituximab. The efficiency of rituximab (type I), and GA101$_{gly}$ (type II) at deleting B cells in whole blood assays was compared.

Type II mAbs are twice as efficient at deleting B cells than Type I mAbs

Dose-response experiments

As SLE patients are often lymphopenic, making extensive assays difficult, the optimal concentration of mAbs (0.01, 0.1, 1 and 10 μg/mL) required for the assay was determined using blood from normal healthy controls. In these assays non-glycomodified versions of GA101 (GA101$_{gly}$) were used to assess the effects of type I

versus type II directly without the influence of afucosylation. Independent experiments were performed in whole blood samples from four healthy controls (HC). The mean percentage cell death was used to assess the cytotoxicity of mAbs and determine the optimal dose. GA101gly was significantly more efficient at lysing B cells than rituximab in all four samples at all four concentrations tested (Figure 4.1). As discussed in Chapter three, serum rituximab levels were less than 10 μg/mL, at one month after treatment with rituximab in both RA and SLE. Therefore, all mAbs were used at 1 μg/mL for subsequent experiments.
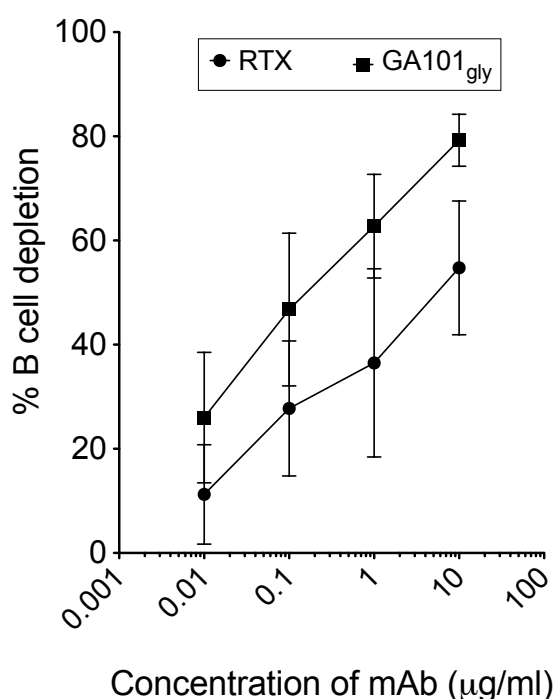
Figure 4.1 Dose-response experiments in whole blood B-cell depletion assay.

Symbols represent the mean and the error bars the range.

Dose-response experiments The optimal concentration of mAbs (0.01, 0.1, 1 and 10 μg/mL) was determined in four independent experiments using freshly drawn heparinized blood from normal healthy controls and using rituximab and non-glycomodified versions of obinutuzumab formerly known as GA101 (GA101gly) to assess the effects of type I versus II anti-CD20 mAbs directly without the influence of afucosylation. Whole blood samples were incubated with or without rituximab or GA101gly at 0.01, 0.1, 1 and 10 μg/mL and percentage B-cell death measured by flow

cytometric analysis after 24 hours and, mean of triplicate wells was used. Cytotoxicity of rituximab and GA101$_{gly}$ were compared in healthy controls (n = 4). Rituximab lysed B cells less efficiently than GA101$_{gly}$ in all four samples at all four concentrations tested (Figure 4.1).

Whole blood B-cell depletion

Whole blood B-cell depletion assays were performed in samples from HC (n=9), patients with RA (n=26) and SLE (n=50) as described in methods. Whole blood samples were incubated with or without RTX or GA101$_{gly}$ at 1μg/mL. Percentage B cell death was measured by flow cytometric analysis after 24 hours. Mean of triplicate wells was used for each individual sample. The cytotoxicity index (CTI) in the autologous whole blood depletion assay was calculated using the formula described in methods. Data were analysed as described in the Methods.

The cytotoxicity of RTX and GA101$_{gly}$ within and between all three cohorts was compared. GA101$_{gly}$ (type II mAb) was found to be significantly more efficient than rituximab (type I mAb) at lysing B cells, in vitro, in all groups. The mean ± SD CTI of GA101$_{gly}$ vs. RTX in HC was 63±11 vs. 36±18 ($p < 0.0001$); in RA, 54±16 vs. 27±16 ($p < 0.0001$); and in SLE, 38±15 vs. 17±12 ($p < 0.0001$). There was no significant difference between CTI of RTX in HC and RA whereas the CTI of rituximab was significantly lower in SLE when compared with HC ($P < 0.005$) and RA ($p < 0.05$). Similarly, there was no significant difference in the CTI of GA101$_{gly}$ between HC and RA whereas the CTI of GA101$_{gly}$ was significantly lower in SLE when compared with HC ($p < 0.0005$) and RA ($p < 0.0001$) (Figure 4.2). The median ratio of CTI of GA101$_{gly}$: rituximab was 1.5, 1.7 and 2.5, for HC, RA and SLE, respectively. Thus, rituximab lyses B cells less efficiently than GA101$_{gly}$ in all three groups. The percentage B-cell depletion achieved by both rituximab and GA101$_{gly}$ was significantly lower in SLE when compared with both healthy controls and RA.
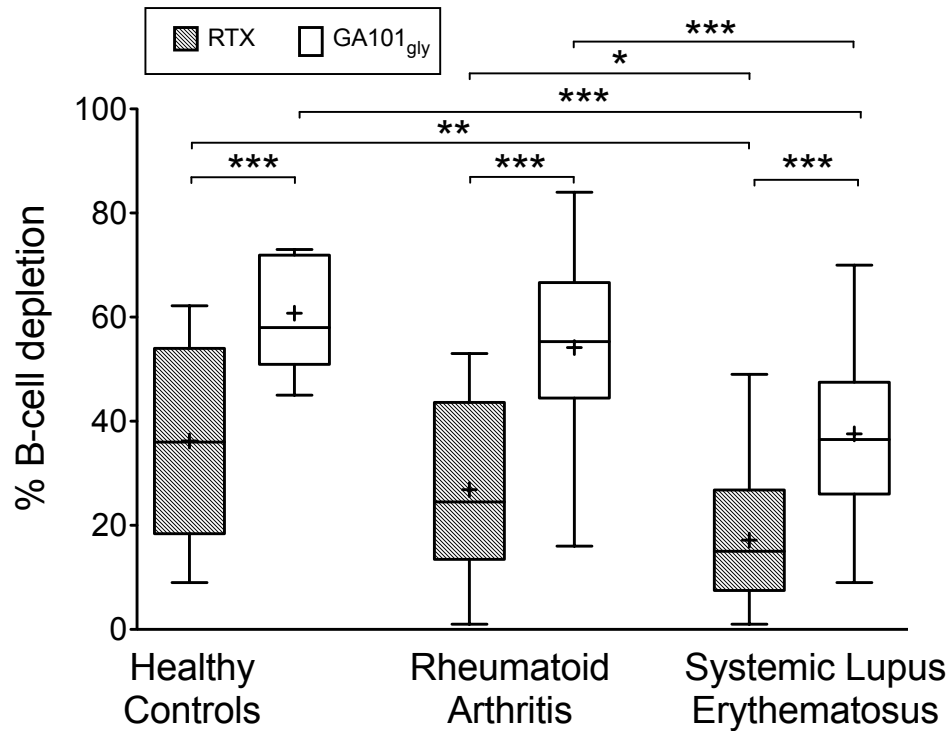
Figure 4.2 B-cell depletion in whole blood assays with rituximab and GA101$_{gly}$ in RA and SLE patient samples.

Horizontal line represents the median; +, the mean; the box, the inter-quartile range; and the whiskers, the range. RTX, rituximab; **p < 0.001, ***p < 0.0001.

Taken together, these results suggested that 1) type II mAbs are more effective at depleting B cells in each group in the whole blood assay; 2) B cells from patients with SLE are less susceptible to lysis by both rituximab and GA101$_{gly}$, suggesting an inherent resistance mechanism.

Table 4.1 Demographics of patients with Rheumatoid Arthritis

Patient number	RTX-CTI	$GA101_{gly}$-CTI	CTI/ RTX-CTI	$GA101_{gly}$- RTX-CTI	Age (years)	Disease duration (years)	Disease activity score DAS28-ESR	Medications
1	12	16	1	54	14	5.2	CS, MTX, HCQ
2	39	51	1	53	12	6.2	CS, MTX, HCQ, LFN
3	15	47	3	42	11	5.99	MTX, Ada
4	43	60	1	68	16	4	MTX
5	15	21	1	62	9	4.65	MTX
6	20	74	4	35	2	5.35	CS, MTX, HCQ, SSZ
7	7	71	11	33	8	5.3	CS, MTX, HCQ
8	48	71	1	45	6	4	SSZ, HCQ
9	17	65	4	49	14	3.3	MTX, Etanercept
10	42	71	2	51	2	3.5	HCQ
11	48	66	1	65	11	4.2	MTX, HCQ, SSZ
12	37	64	2	51	10	3	CS, MTX, Ada

13	47	70	1	56	10	3.5	MTX, Toc
14	10	44	4	46	11	6.3	MTX, SSZ
15	1	37	37	75	14	5.77	CS, MTX
16	6	51	9	33	15	5.9	HCQ, Etanercept
17	13	45	3	66	44	5.68	nil
18	53	84	2	56	3	5.5	CS, MTX, SSZ, HCQ
19	14	57	4	31	8	5	MTX
20	46	53	1	67	25	2.7	nil
21	14	34	2	54	8	6.38	CS, AZA
22	45	58	1	42	20	4.5	SSZ, HCQ
23	30	50	2	59	8	5.12	CS, SSZ, HCQ
24	29	46	2	79	1	5.3	AZA
25	30	60	2	50	14	4	SSZ, MTX
26	16	43	3	24	6	4.3	MTX, SSZ

RTX, rituximab; GA101$_{gly}$, glycosylated GA101; CTI, cytotoxicity index; Ada, Adalimumab (anti-TNF agent); AZA, azathioprine; CS, corticosteroids; HCQ, Hydroxychloroquine; MTX, methotrexate; SSZ, sulfasalazine; Toc, Tocilizumab (anti-IL-6 receptor monoclonal antibody); Etanercept, anti-TNF agent

Table Error! No text of specified style in document..2 **Demographics of patients with Systemic Lupus Erythematosus**

Patient number	RTX-CTI	GA101$_{gly}$-CTI	GA101$_{gly}$-CTI/ RTX-CTI	Age (years)	Ethn-icity	Disease duration (years)	Clinical manifest-ations	C3	Disease activity	BILAG score	Medications
1	39	44	1.15	41	AC	7	LN	1.03	inactive	2	AZA, HCQ
2	18	46	2.52	39	C	23	non-renal	0.86	inactive	2	CS, HCQ
3	8	27	3.21	39	C	26	LN	1.03	Inactive	2	CS, MMF
4	10	47	4.71	41	C	23	non-renal	0.70	inactive	2	CS, HCQ, MMF
5	7	28	4.00	27	AC	14	LN	0.58	active	18	CS, HCQ
6	8	21	2.72	35	C	10	LN	1.00	inactive	0	CS, HCQ, MMF
7	15	32	2.20	34	AC	5	non-renal	0.63	inactive	10	AZA
8	29	52	1.80	24	C	11	non-renal	1.56	inactive	0	AZA, HCQ
9	18	49	2.72	42	A	27	non-renal	1.40	inactive	1	CS
10	28	27	0.96	53	AC	8	non-renal	0.94	inactive	1	CS, HCQ
11	15	49	3.27	61	C	12	non-renal	1.28	active	8	CS
12	5	56	12.31	39	C	19	LN	0.59	active	10	CS, MMF

13	20	26	1.31	22	AC	10	non-renal	0.86	inactive		HCQ, MMF
14	6	15	2.50	52	C	8	non-renal	0.81	inactive	1	CS, AZA, HCQ
15	15	20	1.36	21	AC	4	non-renal	1.13	inactive	1	MTX
16	24	30	1.25	23	AC	8	non-renal	0.93	inactive	0	HCQ, MTX
17	22	46	2.06	32	C	2	non-renal	0.96	inactive	0	HCQ
18	1	24	23.84	39	C	7	LN	0.79	inactive	2	CS, HCQ
19	42	70	1.67	24	AC	16	LN	0.82	inactive	1	CS, HCQ
20	32	9	0.27	31	AC	3	LN	0.75	active	10	CS, MMF
21	36	41	1.14	76	C	19	LN	1.19	inactive	2	CS
22	30	58	1.95	48	C	16	non-renal	1.08	inactive	0	HCQ
23	1	29	47.20	39	C	21	renal	0.99	inactive	0	CS, HCQ, MMF
24	1	26	26.44	56	C	2	non-renal	0.80	inactive	1	CS, AZA, HCQ
25	17	36	2.12	41	C	24	non-renal	0.70	inactive	2	CS, HCQ, MMF
26	1	16	16.00	40	AC	7	LN	1.53	inactive	0	CS
27	11	14	1.31	29	A	29	LN	1.27	active	13	CS, AZA

28	49	64	1.33	24	A	6	LN	1.30	active	9	CS
29	35	69	2.01	26	AC	6	LN	0.91	inactive	2	CS, HCQ, MMF
30	37	58	1.57	51	C	17	non-renal	1.42	inactive	0	Nil
31	18	47	2.61	55	C	27	LN	0.60	inactive	0	HCQ
32	15	17	1.13	44	C	20	LN	1.10	inactive	0	MTX
33	5	41	7.55	46	C	13	non-renal	1.42	active	13	CS, AZA,
34	15	56	3.83	36	AC	6	LN	0.73	active	10	CS, HCQ, MMF
35	29	22	0.74	34	AC	18	LN	1.05	inactive	0	CS, MMF
36	30	49	1.65	32	C	8	non-renal	1.17	inactive	0	HCQ
37	16	66	4.10	29	AC	3	non-renal	1.14	active	8	CS, HCQ
38	12	36	3.00	53	C	4	non-renal	1.07	inactive	0	HCQ
39	17	47	2.76	23	A	14	LN	1.25	inactive	1	HCQ, MMF
40	18	23	1.26	49	AC	15	LN	0.80	inactive	2	CS, AZA, HCQ
41	6	14	2.51	39	C	13	non-renal	0.61	inactive	1	CS, HCQ
42	4	33	7.27	54	A	32	non-renal	1.10	inactive	1	CS, HCQ

43	13	38	2.97	33	C	18	non-renal	1.31	inactive	16	CS, HCQ
44	8	43	5.60	31	CH	15	LN	0.86	inactive	1	CS, HCQ, MTX
45	9	34	3.94	33	AC	20	non-renal	1.06	active	17	CS, HCQ, MTX
46	11	41	3.73	45	C	25	non-renal	1.08	inactive	1	Nil
47	18	37	2.11	39	A	14	non-renal	1.0	inactive	0	HCQ
48	3	36	11.13	38	C	1	non-renal	1.06	inactive	1	CS, HCQ
49	26	38	1.44	59	C	20	LN	1.03	inactive	0	CS, HCQ
50	6	32	5.19	46	C	24	LN	0.94	inactive	1	CS, HCQ, MMF

RTX, rituximab; GA101$_{gly}$, glycosylated GA101; CTI, cytotoxicity index; C3, serum complement 3; LN, lupus nephritis; non-renal, lupus manifestations not including kidney; A, Asian; AC, Afro-Caribbean; C, Caucasian; Ch, Chinese; AZA, azathioprine; CS, corticosteroids; HCQ, Hydroxychloroquine; MTX, methotrexate; MMF, mycophenolate mofetil

Relationships between the efficiency of Type I and II anti-CD20 mAbs and patient demographics

The efficiency of anti-CD20 mAbs at inducing B-cell cytotoxicity may be influenced by patient characteristics including age, disease activity and concurrent medications. Although, the study was not primarily designed to evaluate the effect of each parameter, relationships between the efficiency of mAbs and age of patients, the disease activity score (DAS-28 ESR score for RA and BILAG disease activity score for SLE) and also serum complement levels in patients with SLE were explored. The effect of concurrent therapy on the efficiency of rituximab was evaluated by comparing the co-therapy in patients in whose samples rituximab efficiency varied remarkably. Patient characteristics are described in Table 4.1 and Table 4.2.

Therefore, the relationship between the CTI of rituximab and/or CTI of $GA101_{gly}$ and patient's age (years) and serum C3 levels was next investigated. There was no significant correlation between CTI of rituximab and serum C3 levels. Patient characteristics including serum C3 levels and the CTI of rituximab and CTI of $GA101_{gly}$ are shown in Table 4.2.

B-cell depletion in vitro by rituximab and serum C3 levels

There were no significant relationships between the percentages of B-cell depletion achieved by rituximab in the whole blood B-cell depletion assay in samples from patients with SLE and serum C3 levels, spearman r of -0.17 (p =0.37) Figure 4.3.
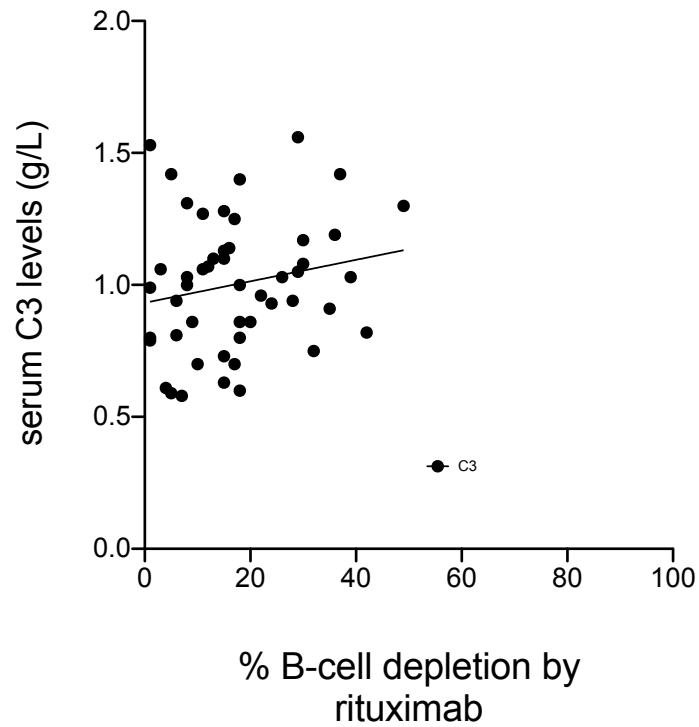
Figure 4.3 Relationships between B-cell depletion in vitro by rituximab and serum C3 levels in SLE.

B-cell depletion in vitro by rituximab and the age of the individual and the duration of disease
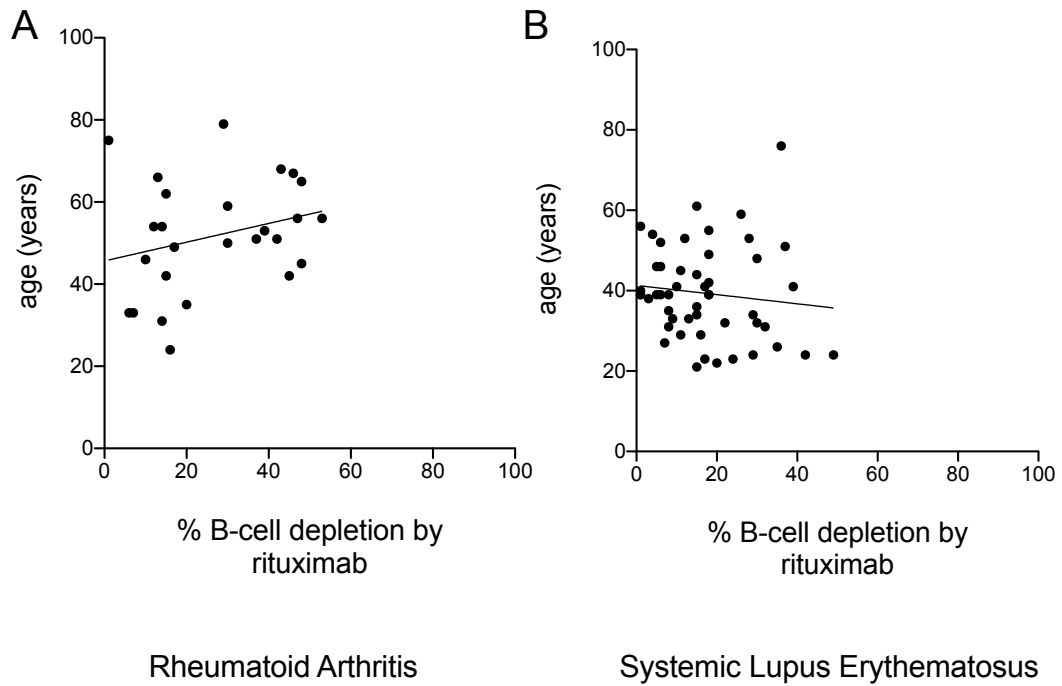
Figure 4.4 Relationship between B-cell depletion in vitro by rituximab and the age of the individual (years) in RA and SLE

There were no significant relationships between the percentages of B-cell depletion by rituximab and the age (years) of patients with RA and SLE shown in Figure 4.4A and B, respectively, with spearman r of 0.07 (p > 0.1) and 0.01 (p > 0.4), respectively.
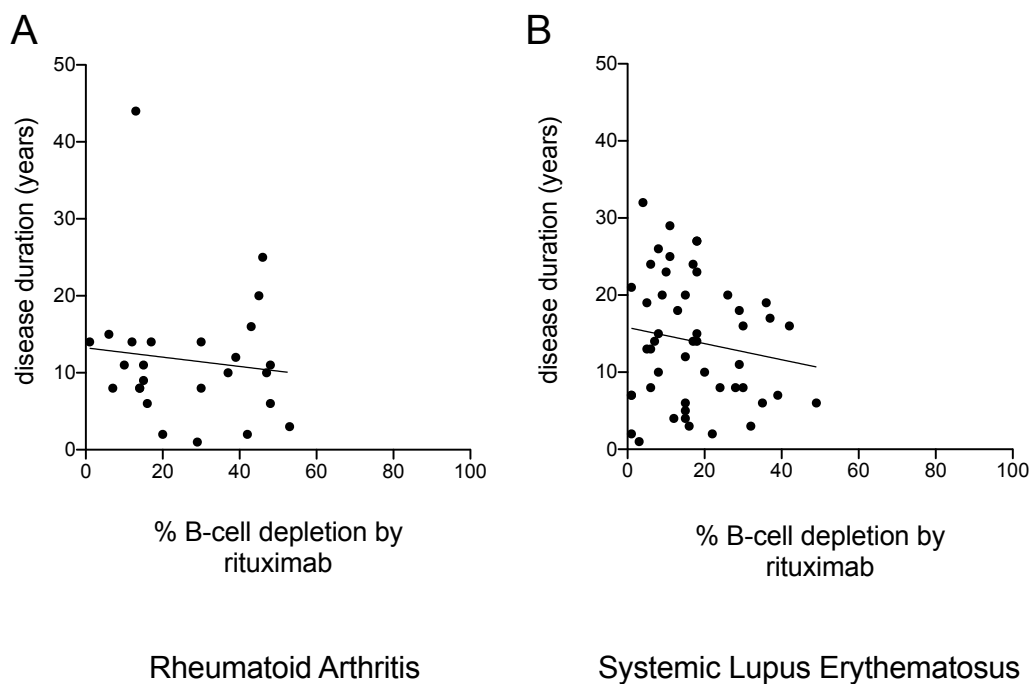
Figure 4.5 Relationship between B-cell depletion in vitro by rituximab and disease duration (years) in RA and SLE

There were no significant relationships between the percentages of B-cell depletion by rituximab in in vitro whole blood B-cell depletion assay and the disease duration in patients with RA and SLE shown in Figure 4.5A and B, respectively, with spearman r of 0.01 (p > 0.5) and 0.02 (p > 0.2), respectively.

Thus, considering these data, the age of the individual and the disease duration did not impact on the percentage B-cell depletion achieved by rituximab in in vitro whole blood B-cell depletion assay.

B-cell depletion in vitro with type I and II anti-CD20mAbs: relationship with B-cell expression of CD20

Both B-cell intrinsic and extrinsic factors may account for the apparent resistance of SLE B cells to depletion. Malignant B-cell expression of CD20 (Golay, Lazzari et al. 2001) and FcγRIIb (Lim, Vaughan et al. 2011) correlated with susceptibility to deletion by anti-CD20 mAbs, Therefore, the relationship between the efficiency of mAbs at inducing B-cell depletion in the whole blood assay and B-cell expression of

the target antigen, CD20 and the inhibitory receptor, FcγRIIb, represented by the MFI in RA and SLE was analysed.
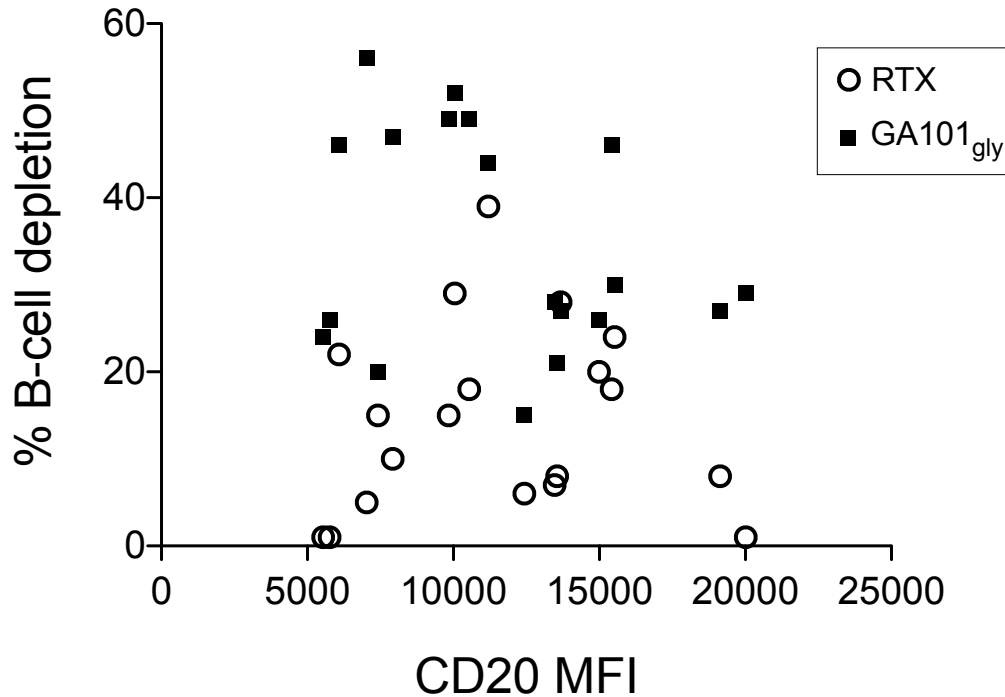
Figure 4.6 Relationship between CD20 MFI and efficiency of B-cell depletion by mAbs.

The MFI of CD20 on B cells and the % B-cell depletion achieved by mAbs in the whole blood B-cell depletion assay in patients with RA (n=7) and SLE (n=12).

CD20 expression on B cells varied remarkably between patients with RA and SLE (Figure 4.6) with a mean±SD of 11555 ±4354. However, there were no significant relationships between B cell expression of the target antigen, CD20 and the CTI of rituximab or GA101$_{gly}$ in the whole blood assay with Spearman r correlation values of -0.11 (p = 0.63) and 0.19 (p =0.42), respectively.

B-cell depletion in vitro with type I and II anti-CD20mAbs: relationship with B-cell expression of FcγRIIb

Whether B-cell expression of FcγRIIb influenced depletion by mAbs was next analysed.
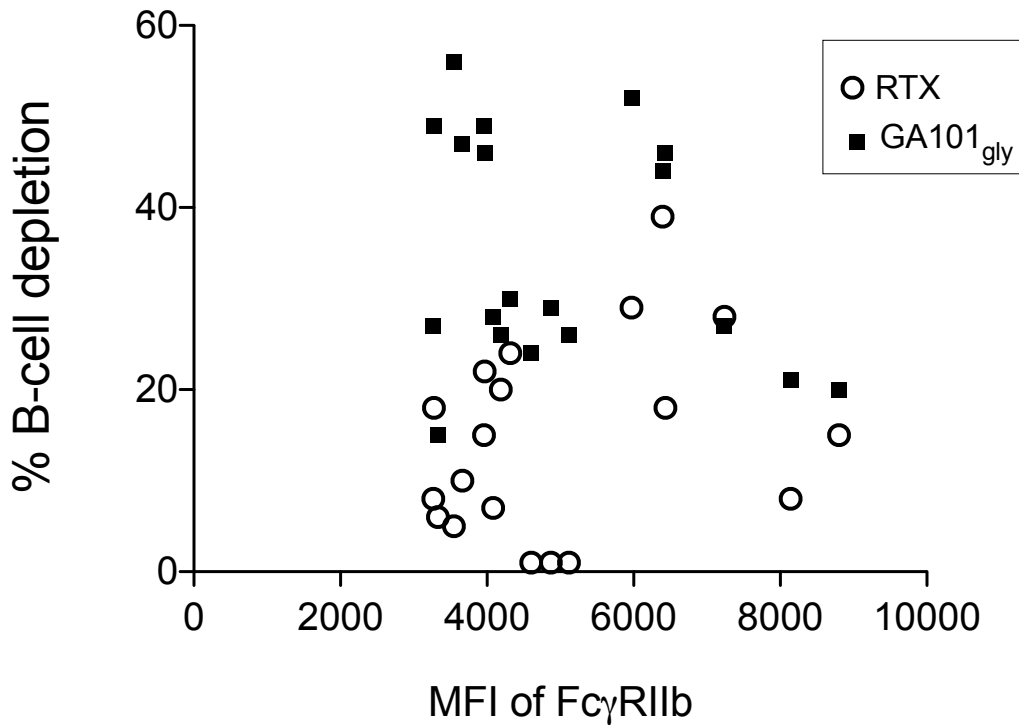
Figure 4.7 Relationship between MFI of FcγRIIb and efficiency of B-cell depletion by mAbs.

The MFI of FcγRIIb on B cells and the % B-cell depletion achieved by mAbs in the whole blood B-cell depletion assay in patients with RA (n=7) and SLE (n=12).

B-cell expression of FcγRIIb was less variable than that of CD20 (Figure 4.6) and Figure 4.7) with a mean±SD of 5008±1587. No significant correlations were found between the expression of CD20, FcγRIIb (Figure 4.4) or their relative expression (ratio of MFI of CD20/ FcγRIIb) to the CTI of rituximab or GA101$_{gly}$ in patients with RA or SLE with Spearman r correlation values of -0.31 (p=0.19) and 0.25 (p=0.29), respectively.

B-cell depletion in vitro with type I and II anti-CD20mAbs: relationship with the composition of B-cell subpopulations

As discussed in Chapter one, B-cell subpopulations may differ in their susceptibility to deletion by mAbs (Leandro 2013). Therefore, the distribution of B-cell subpopulations in samples incubated in the presence of anti-CD20 mAbs for 24 hours in the whole blood B-cell depletion assay was analysed.

There were no differences between B-cell subpopulations in samples incubated with the two mAbs except for the frequency of double negative cells which was higher in samples incubated with rituximab compared to samples incubated without mAbs and those incubated with GA101$_{gly}$ (Figure 4.8). However, these results should be interpreted with caution with regards to attributing changes in the composition to resistance or sensitivity to depletion with rituximab as alterations in one B cell compartment would alter the frequency of other B-cell subpopulations. Allowing for this limitation, it would seem from these findings that DN cells might resist depletion with rituximab, in whole blood B-cell depletion assays, in vitro.
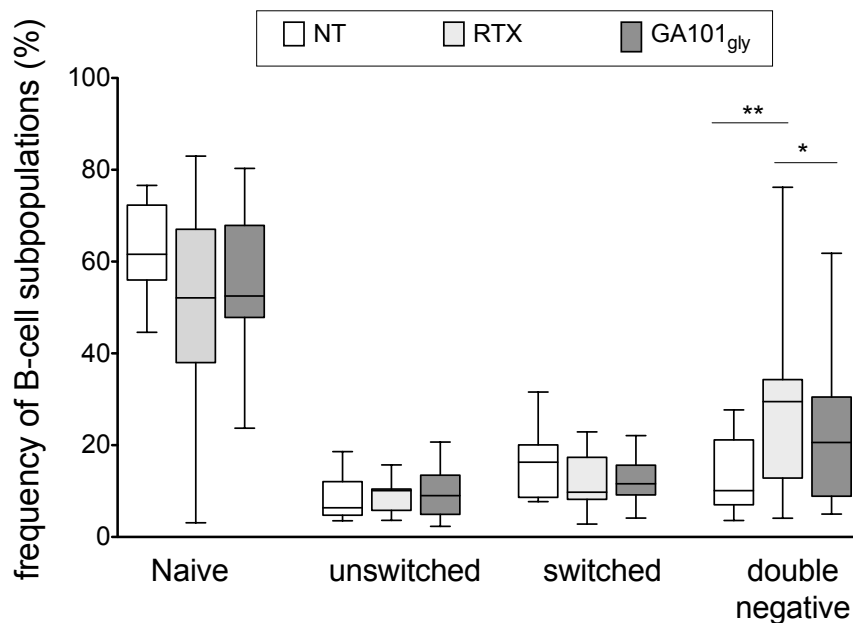
Figure 4.8 Composition of B cell subpopulations by mAbs in the whole blood B-cell depletion assay.

The frequency of B cell subpopulations in samples incubated with or without mAbs in samples from patients with RA (n=9). NT, not treated; rituximab, rituximab; and GA101$_{gly}$, glycosylated GA101. *, p<0.05 and **, p<0.001.

Further, the composition of B cell subpopulations may also vary between individuals with RA and SLE. Therefore, the relationship between the distribution of B-cell subpopulations and CTI of mAbs was investigated.
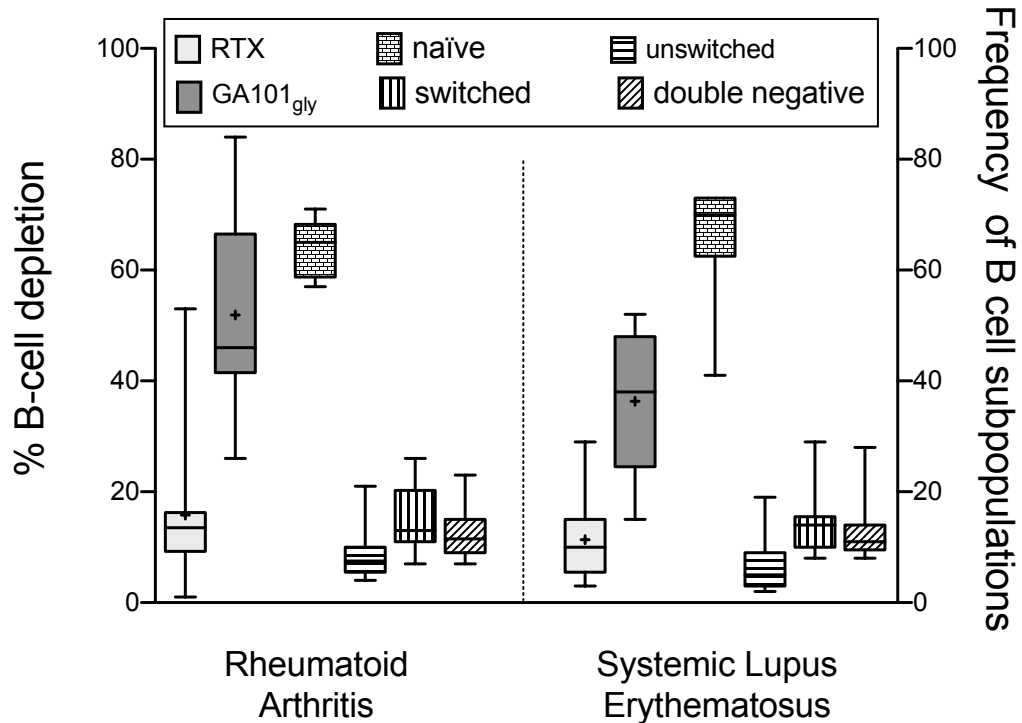
Figure 4.9 CTI of mAbs in whole blood B-cell depletion assay and the distribution of B-cell subpopulations in RA and SLE in peripheral blood.

The CTI of mAbs in the whole blood B-cell depletion assay and frequency of B cell subpopulations and in RA (n=10) and SLE (n=9). RTX, rituximab.

The CTI of rituximab and GA101$_{gly}$, as assessed by whole blood B-cell depletion assay, and the distribution of B-cell subpopulations were analysed in samples from ten patients with RA and nine with SLE. The frequencies of B-cell subpopulations and CTI of rituximab and GA101$_{gly}$, as assessed by the whole blood B-cell depletion assay in samples from these patients are shown in Figure 4.9. As previously noted, the CTI of GA101$_{gly}$ was significantly greater than the CTI of rituximab in both RA and SLE. However, there were no significant differences in the frequencies of B-cell subpopulations between in this cohort of patients with RA and SLE (Figure 4.9).

Taken together, the results shown in Figure 4.8 and 4.9, do not suggest that the composition of B-cell subpopulations impacts on the efficiency of mAbs as assessed by the whole blood B-cell depletion assay.

Efficiency of type I and II anti-CD20 mAbs and the effect of excess BAFF

As discussed in Chapter 1, Introduction, BAFF, B cell activation factor, may promote B cell survival through enhanced expression of apoptotic proteins (Mackay and Browning 2002) particularly in inflammatory tissues (Thaunat, Patey et al. 2008). Given the apparent relative resistance in SLE samples to depletion by rituximab, whether the CTI of mAbs is compromised in the presence of excess BAFF was explored.

The effect of BAFF on the efficiency of anti-CD20 mAbs was investigated in the whole blood assay by incubating whole blood samples from patients with RA (n = 3) and SLE (n = 4). As discussed in Chapter one, patients with SLE may have high BAFF levels detectable in the serum at less than 100 ng/mL and frequently less than 10 ng/mL (Cheema, Roschke et al. 2001). Therefore, the effect of BAFF on the efficiency of anti-CD20 mAbs at inducing B-cell cytotoxicity was assessed by comparing the CTI of anti-CD20 mAbs in samples incubated in the presence or absence of excess BAFF 100 ng/mL.

There were no consistent effects of excess BAFF on the efficiency of mAbs in the whole blood B-cell depletion assay (Figure 4.10). The median (range) CTI of RTX in the absence of excess BAFF was 18 (1 - 29) and in the presence of excess BAFF the median (range) CTI was 34 (14 - 63). There was no significant difference between the CTI of RTX in the absence or the presence of excess BAFF in the whole blood assay (p=0.1875). The median (range) CTI of GA101$_{gly}$ in the absence of excess BAFF was 45 (26 - 52) and in the presence of excess BAFF the median (range) CTI was 50 (12 - 75). There was no significant difference between the CTI of GA101gly in the absence or the presence of excess BAFF in the whole blood assay (p > 0.05). However, even in this small number of samples the CTI of GA101$_{gly}$ was significantly greater than RTX in the absence, but not in the presence, of excess BAFF, p < 0.05. Surprisingly, in some samples, B-cell depletion by mAbs was greater in the presence of excess BAFF. Regardless, even in this small number of samples the CTI of rituximab was significantly lower than that of GA101$_{gly}$ both in the presence and absence of excess BAFF.
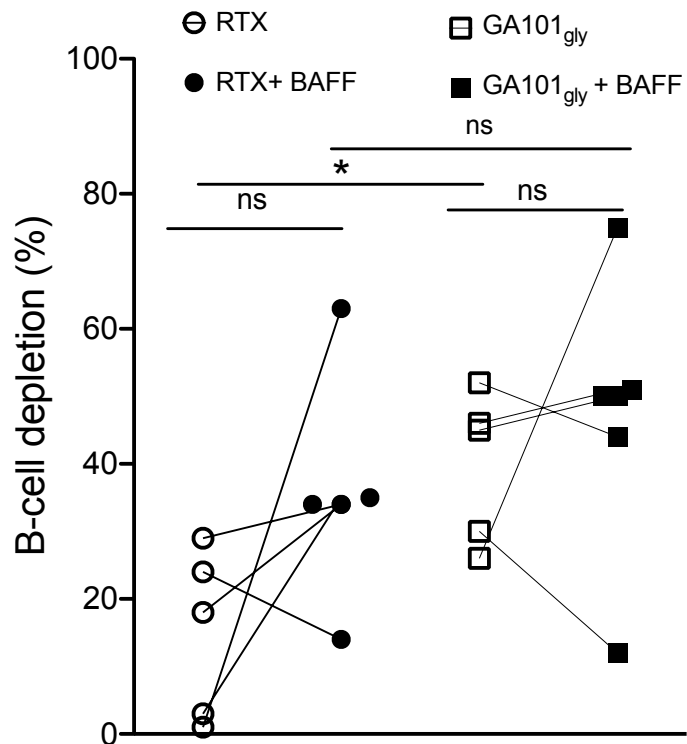
Figure 4.10 The effect of excess BAFF on the efficiency of B-cell depletion by mAbs.

Each symbol represents individual sample and the unfilled symbols represent samples incubated in the absence of excess BAFF and the filled symbols represent samples incubated in the presence of excess BAFF. *, p < 0.05.

The % B-cell depletion induced by mAbs in the whole blood assay in the presence or absence of excess (100 ng/mL) BAFF, B cell activation factor in samples from patients with RA (n = 3) and SLE (n = 3).

Efficiency of alternative type I and II anti-CD20 mAbs

Whether the difference in B cell lysing potential of rituximab and GA101$_{gly}$ was also applicable to additional type I and II mAbs was then investigated. The CTI of two other mAbs: ofatumumab (2F2) and tositumomab (B1) representing type I and II mAbs respectively, were compared. Again, type II mAbs were significantly more efficient than type I at lysing B cells in all samples from patients with RA (n = 3) and SLE (n = 10) (Figure 4.11).
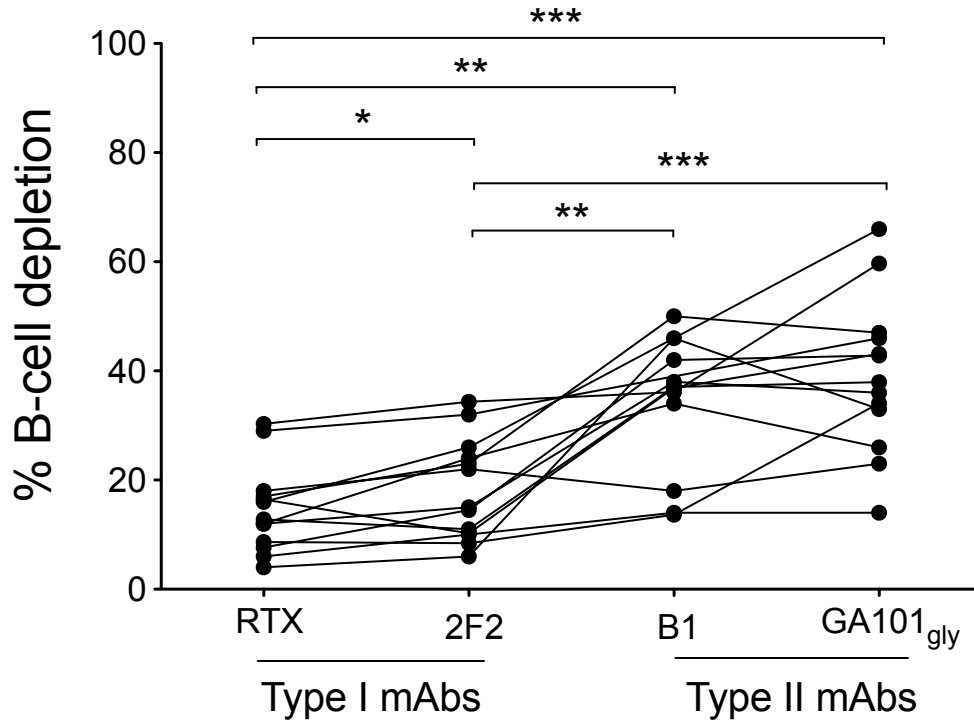
Figure 4.11 Type I anti-CD20 mAbs lyse B cells less efficiently than type II mAbs and anti-CD22mAbs in the whole blood assay.

RTX, rituximab (IgG1, rituximab), 2F2, ofatumumab (human IgG1, 2F2), B1, tositumomab (mouse IgG2a, B1) and GA101$_{gly}$ (IgG1). Each symbol represents an individual sample. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

Whole blood B-cell depletion assays were performed to compare the efficiency of B-cell depletion of alternative type I and type II anti-CD20 mAbs including RTX, rituximab (IgG1, rituximab), 2F2, ofatumumab (human IgG1, 2F2), B1, tositumomab (mouse IgG2a, B1) and GA101$_{gly}$ (IgG1). All mAbs were used at 1 μg/mL. Cytotoxicity of type I and II mAbs were compared in patients with RA (n=3) and SLE (n=10).

Type I mAbs lyse B cells less efficiently than type II mAbs. Cytotoxicity of rituximab < 2F2 < B1 < GA101$_{gly}$. There was a remarkable hierarchy in the efficiency of mAb-CTI: GA101$_{gly}$ > B1 > 2F2 > rituximab with a greater than two fold difference for GA101gly vs. 2F2 (Figure 4.11).

As anti-CD22 mAbs have also been reported to deplete B cells, albeit weakly (Carnahan, Stein et al. 2007) their activity in the whole blood assay was also examined.
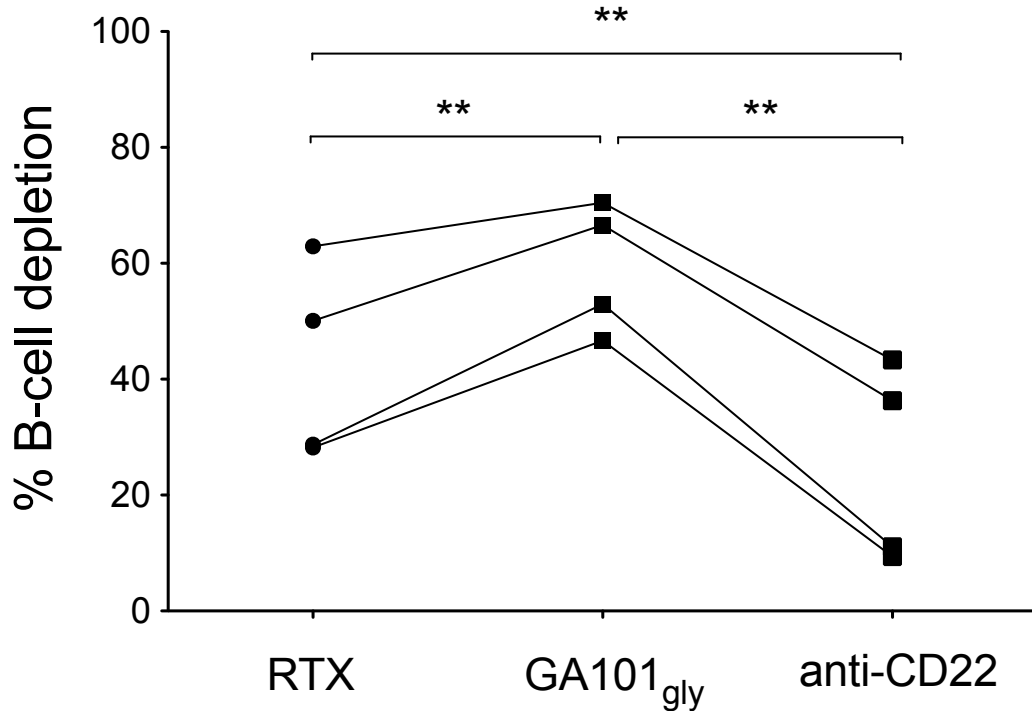
Figure 4.12 Anti-CD22mAbs lyse B cells less efficiently than type I and II anti-CD20 mAbs.

The cytotoxicity index of anti-CD22mAb was significantly lower than that of rituximab and GA101$_{gly}$ in SLE (n=4). *p < 0.05, **p < 0.001, ***p < 0.0001. RTX, rituximab.

The CTI of type I anti-CD20 mAb, rituximab, was compared with that of type II mAb, GA101$_{gly}$, and with the CTI of the anti-CD22 mAb in the whole blood assay in four samples from patients with SLE. In all four samples anti-CD22 mAb induced significantly lower % B-cell depletion compared to type I and II anti-CD20 mAbs. As noted previously, type I anti-CD20 mAbs induced significantly lower % B-cell depletion than type II anti-CD20 mAbs. Thus, the CTI of anti-CD22 mAb was found to be significantly lower than that of anti-CD20 mAbs with a notable hierarchy: anti-CD22 < rituximab < GA101$_{gly}$ (n=4) (Figure 4.12). This variation may, at least in part, be due to the differences in internalisation of mAbs as noted previously for B cell malignancies (Vaughan, Iriyama et al. 2014).

Internalisation of anti-CD20 mAbs and efficiency of B-cell depletion

Type I, but not type II, anti-CD20 mAbs internalise to a greater extent

Given the large variability in depletion afforded by rituximab in SLE and a superior efficiency of type II mAb in the whole blood depletion assays, whether internalisation of mAbs might explain the greater resistance of SLE B cells to depletion was explored. Internalisation was assessed using the surface fluorescence-quenching assay using isolated B cells from five HC, 16 patients with RA and 22 with SLE.

In all groups, a significantly greater % of GA101gly was accessible on the cell surface when compared with rituximab. In the surface fluorescence assay, isolated B cells were incubated with anti-CD20mAbs at a concentration of $5\mu g/mL$ for six hours when optimal internalisation would be expected to have occurred, as described previously (Beers, Chan et al. 2008, Lim, Vaughan et al. 2011). The median % of surface accessible mAbs after 6 hours of incubation for $GA101_{gly}$ vs. rituximab was 67 vs. 57, 69 vs. 55 ($p < 0.005$) and 74 vs. 47 ($p < 0.005$), in HC, RA and SLE, respectively (Figure 4.13). B cells from patients with RA and SLE internalised rituximab at remarkably variable rates, whereas the rate of internalisation of $GA101_{gly}$ was consistently lower than that of rituximab, in all samples tested in both patients with RA and SLE. Also, even in this relatively small sample size, the percentage of surface accessible rituximab, but not $GA101_{gly}$, was lower than 40% in some samples with SLE, but not RA, suggesting that B cells from some patients with SLE internalised rituximab rapidly and possibly to a larger extent. Thus, internalisation of mAbs was a notable feature of B cells from HC and from patients with RA and SLE.
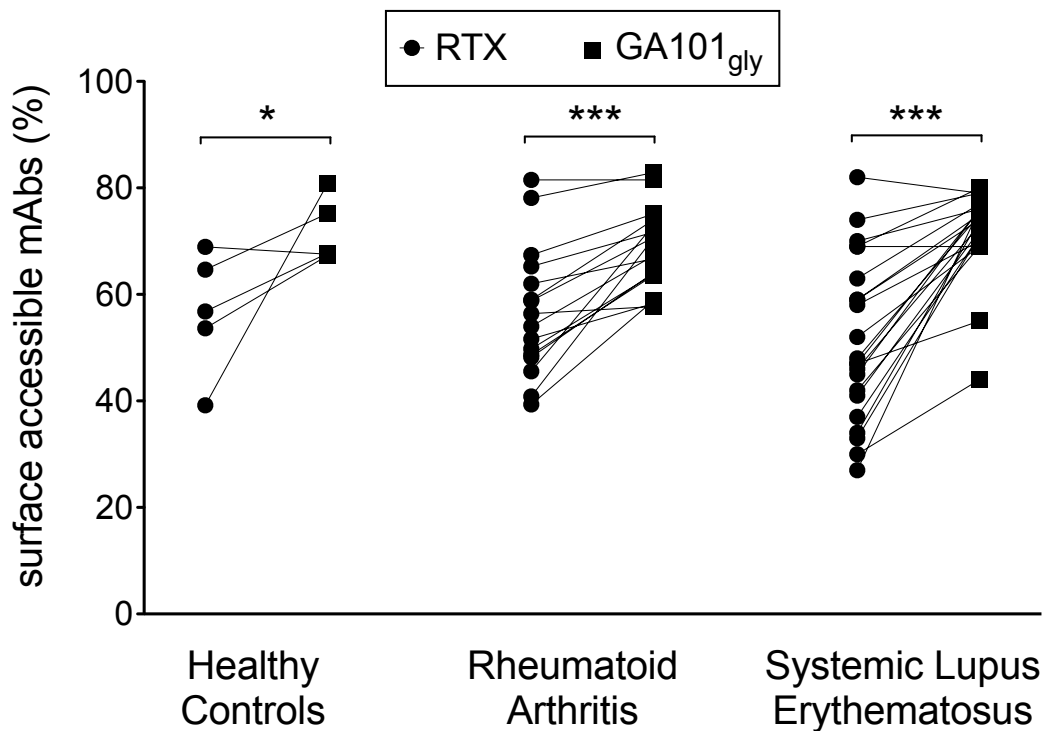
Figure 4.13 Rituximab (RTX) internalised to a highly variable extent and to a significantly greater extent than GA101$_{gly}$.

The frequency of surface accessible mAbs assessed by flow cytometry in the surface fluorescence-quenching assay. *p < 0.05, **p < 0.001, ***p < 0.0001. RTX, rituximab.

Internalisation is associated with lower efficiency of rituximab

The consequences of internalisation on the efficiency of anti-CD20 mAbs was investigated by comparing the frequency of surface accessible anti-CD20 mAbs on isolated B cells in the surface fluorescence-quenching assay, as described in the Methods and, the CTI of anti-CD20 mAbs was assessed in the whole blood B-cell depletion assay in paired patient samples. The relationship between internalisation of mAbs and the efficiency of anti-CD20 mAbs was compared using Spearman rank correlation statistics and also by analyzing the relationship between percentage of surface accessible mAbs and the relative efficiency of type I and II anti-CD20 mAbs using linear regression correlation statistics.
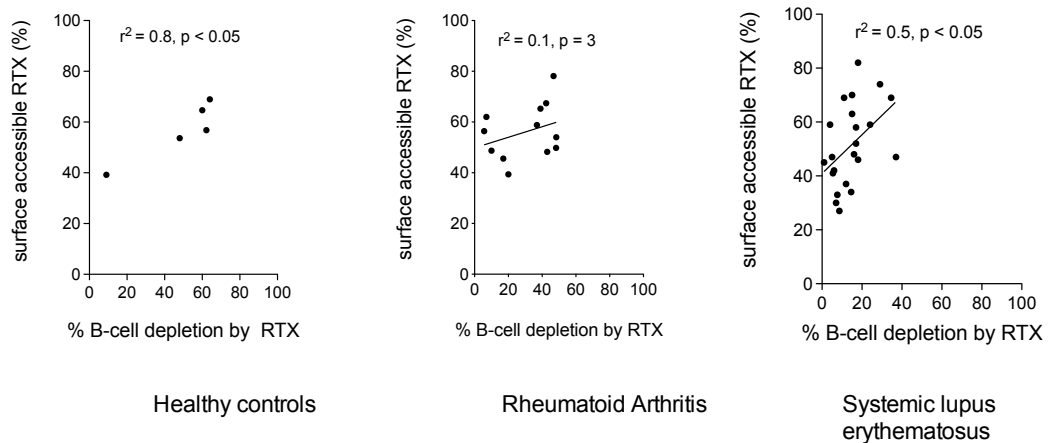
Figure 4.14 The relationship between internalisation and efficiency of rituximab.

CTI, cytotoxicity index

The relationship between the frequency of surface accessible mAbs, assessed in the surface fluorescence-quenching assay, and % B-cell depletion induced by rituximab and GA101$_{gly}$ as assessed by the whole blood depletion assay, was investigated in samples from five HCs, 12 patients with RA and 22 with SLE. There were no significant correlations noted in samples patients with RA ($r^2 = 0.1$, $p > 0.05$). However, significant correlations, between surface accessible rituximab and CTI of rituximab, were noted in samples from HCs ($r^2 = 0.84$, $p < 0.05$) and in samples from SLE ($r^2 = 0.5$, $p < 0.05$) (Figure 4.14). The median and IQR of percentage surface accessible rituximab was 55 % and 48% - 65 % in patients with RA and 47 % and 40 % - 65 % in patients with SLE. Interestingly, samples from patients with SLE that had < 40 % surface accessible rituximab, the percentage B-cell depletion by rituximab was less than 20.
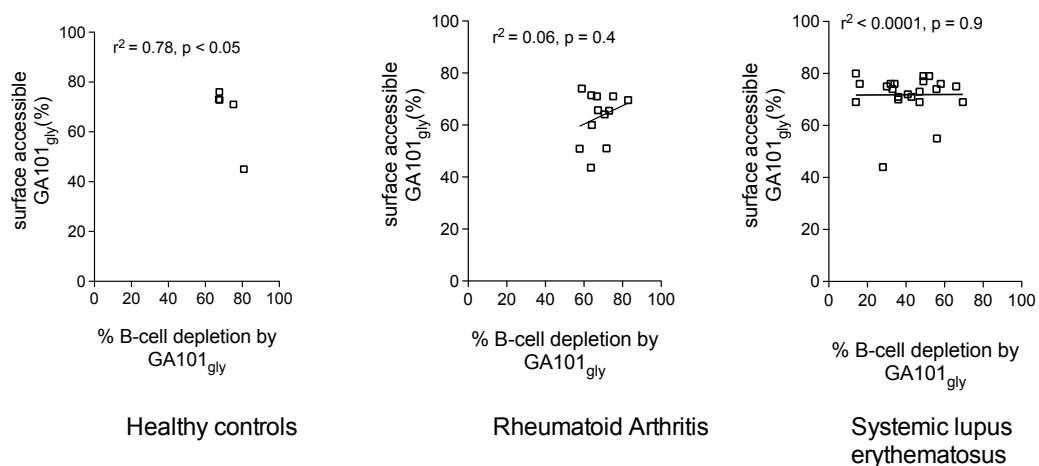
Figure 4.15 The relationship between internalisation and efficiency of GA101$_{gly}$.

CTI, cytotoxicity index

The relationship between internalisation and efficiency of B-cell depletion by GA101$_{gly}$ was next analysed. Similar to rituximab, there was a significant relationship between internalisation and efficiency of B-cell depletion by GA101$_{gly}$, in samples from five HCs ($r^2 = 0.78$, $p < 0.05$), but not in 12 samples from patients with RA ($r^2 = 0.06$, $p > 0.05$) or in 22 samples from patients with SLE ($r^2 < 0.0001$, $p > 0.05$) (Figure 4.15). Thus, there were no significant correlations between surface accessible GA101$_{gly}$ and percentage B-cell depletion in samples from patients with RA and SLE.

There were no significant correlations between the relative CTI of GA101$_{gly}$ and CTI of rituximab and percentage surface accessible rituximab or GA101$_{gly}$ in patients with RA $r^2 = 0.21$, $p > 0.05$ and $r^2 = 0.12$, $p > 0.05$, respectively and in SLE, $r^2 = 0.05$, $p > 0.05$ and $r^2 = 0.09$, $p > 0.05$, respectively (Figure 4.16). There was notable variability in the percentage surface accessible rituximab, in patients with RA, with median and range of 55 % and 39 % – 81 %, respectively, whereas the median and range of percentage surface accessible GA101$_{gly}$ was 69 % and 58 % – 83 %. In patients with SLE a remarkable variability in percentage surface accessible rituximab with median and range of 47 % and 27 % – 82 %, respectively, whereas

the median and range of percentage surface accessible GA101$_{gly}$ was 74 % and 44 % – 80 % .
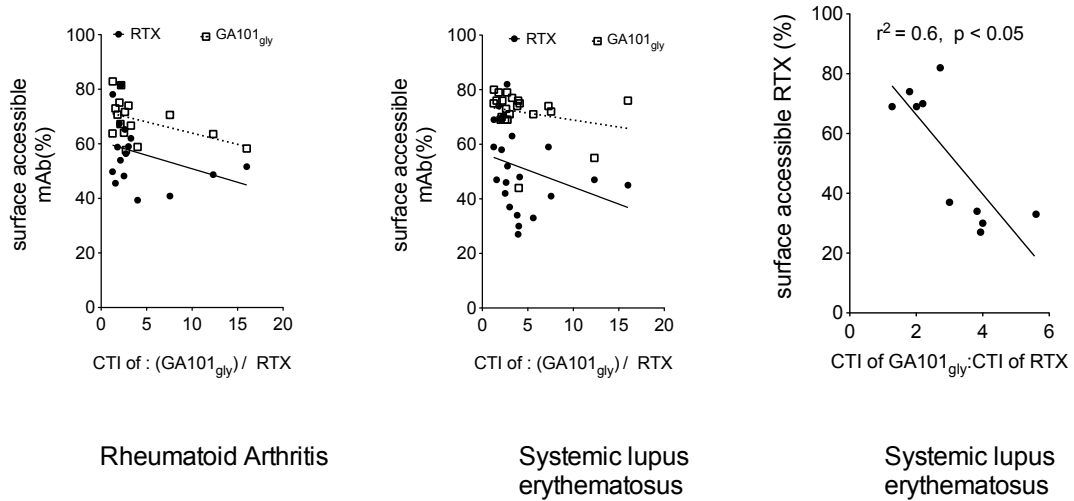
Figure 4.16 Internalisation and the relative efficiency of rituximab and GA101$_{gly}$.

CTI, cytotoxicity index.

Further, the IQR of percentage surface accessible rituximab in patients with SLE was 40 % - 65 % whereas that of GA101gly was 70 % - 76%. Therefore, a subgroup analysis based on the IQR of percentage surface accessible rituximab of 40 % and 65%, was performed, to explore the relationship between the relative CTI of GA101$_{gly}$: CTI of rituximab and surface accessible rituximab. In patients with SLE who had either < 40% or > 65% surface accessible rituximab, there was a significant negative correlation between percentage surface accessible rituximab and the relative CTI of GA101$_{gly}$: CTI of rituximab (r^2 =0.5, p < 0.05) (Figure 4.16) and a significant difference in the relative cytotoxicity, CTI of GA101$_{gly}$: CTI of rituximab between the two groups of patients with two-fold and four-fold, respectively, by Mann Whitney test (p =0.008).

Taken together, these results therefore suggest that internalisation of rituximab contributes to its inferior efficiency of B-cell depletion, as assessed by whole blood assays, in SLE patient samples.

Therefore, the potential mechanisms and/or factors regulating internalisation of rituximab were next explored.

Internalisation of anti-CD20 mAbs and B-cell expression of CD20 and FcγRIIb

The relationship between B-cell expression of CD20 and FcγRIIb and internalisation of mAbs was next examined in paired samples under the same experimental conditions. B-cell expression of CD20 and FcγRIIb were analysed, under the same experimental conditions as the surface fluorescence-quenching assay i.e., after incubation in the absence of anti-CD20 mAbs at 5% CO_2 and 37°C were examined.
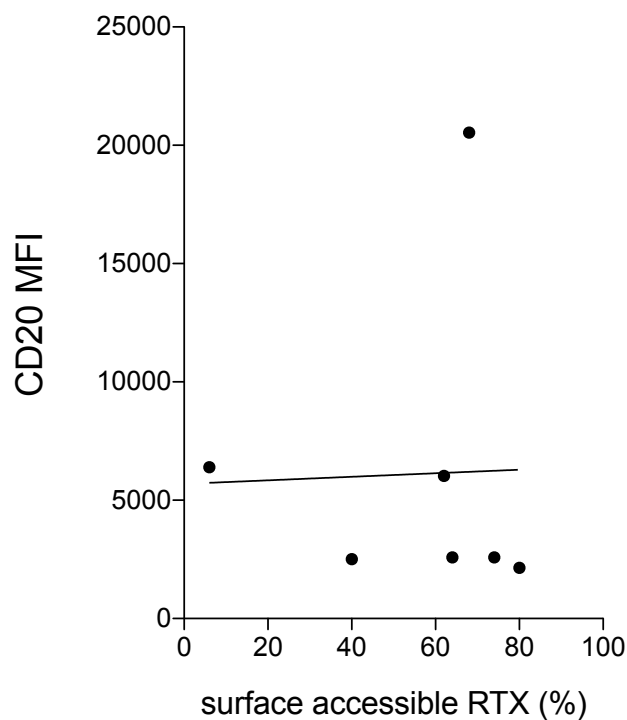
Figure 4.17 Relationship between internalisation of rituximab and B-cell expression of CD20.

First, whether B-cell expression of CD20 was related to surface accessible rituximab was investigated in two samples from HCs and five samples from patients with SLE. In this cohort, there was little variability in CD20 expression on isolated B cells after 6 hours of incubation in the absence of mAbs. There was no significant correlation between the MFI of CD20 and internalisation of rituximab, as assessed by the surface fluorescence-quenching assay (Figure 4.17).

FcγRIIb regulates internalisation of anti-CD20 mAbs

Studies in malignant B cells showed that rituximab internalises as part of a tripartite complex with CD20 and FcγRIIb (Lim, Vaughan et al. 2011). Antagonizing FcγRIIb with an anti-FcγRII mAb, AT10, at least partly, inhibits the internalisation of anti-CD20 mAbs by malignant B cells (Greenman, Tutt et al. 1991). However, B-cell expression of FcγRIIb may be altered in SLE (Su, Yang et al. 2007). Therefore, the potential role of FcγRIIb in the internalisation of rituximab was assessed.
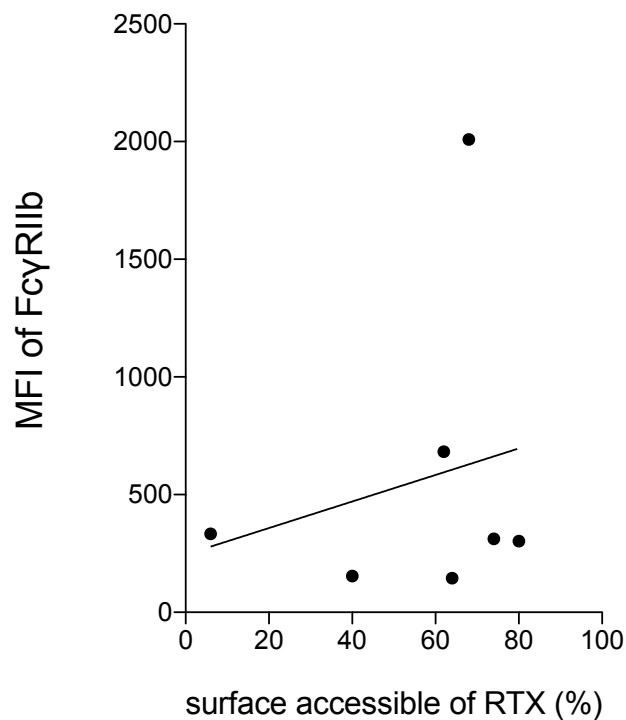
Figure 4.18 Relationship between internalisation of rituximab and B-cell expression FcγRIIb.

In this cohort, there was little variability in B-cell expression of FcγRIIb in isolated B cells incubated for six hours in the absence of anti-CD20mAbs and there was no correlation with % surface accessible rituximab (Figure 4.18). Collectively, the data revealed no correlation between internalisation of rituximab and B-cell expression of CD20 and FcγRIIb.

Whether the inhibitory FcγRIIb regulated internalisation of anti-CD20 mAbs was explored by comparing the percentage of surface accessible mAbs in samples incubated in the presence or absence of an anti- FcγRII blocking antibody, AT10.
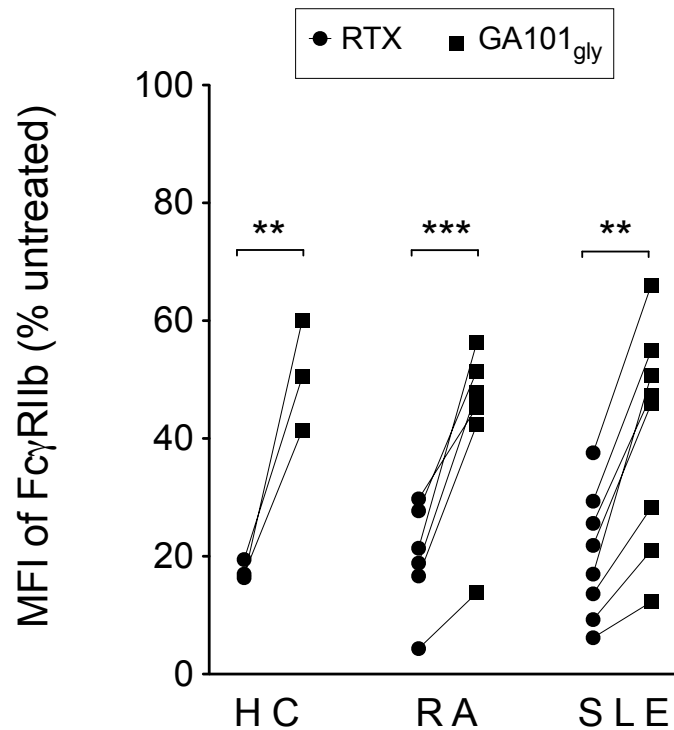
Figure 4.19 FcγRIIb is internalised with Rituximab (rituximab).

**p < 0.001, ***, p < 0.0001.

Whether FcγRIIb also regulated internalisation of mAbs from RA and SLE samples was examined and also whether FcγRIIb internalised to a greater extent with rituximab than GA101$_{gly}$ was examined. If FcγRIIb, as shown in malignant B cells, internalised with rituximab, then the MFI of FcγRIIb would be lower in samples incubated with rituximab compared with samples incubated with GA101$_{gly}$. Based on this principle, the MFI of FcγRIIb in isolated B cells from three HC, six patients with RA and nine with SLE incubated with or without mAbs at 5 µg/mL for 6 hours were assessed. The conditions were similar to that used in the surface fluorescence-quenching assay.

There was a significant difference in the MFI of FcγRIIb ($p < 0.005$ for all) in all 3 groups (Figure 4.19) with the greatest decrease in the MFI of FcγRIIb in samples incubated with rituximab. The decrease in MFI was also noted in all individual

samples tested. Thus, these findings are consistent with the notion that, in B cells from patients with RA and SLE, FcγRIIb internalised with rituximab.

Therefore, whether blocking FcγRIIb inhibited internalisation was also investigated.
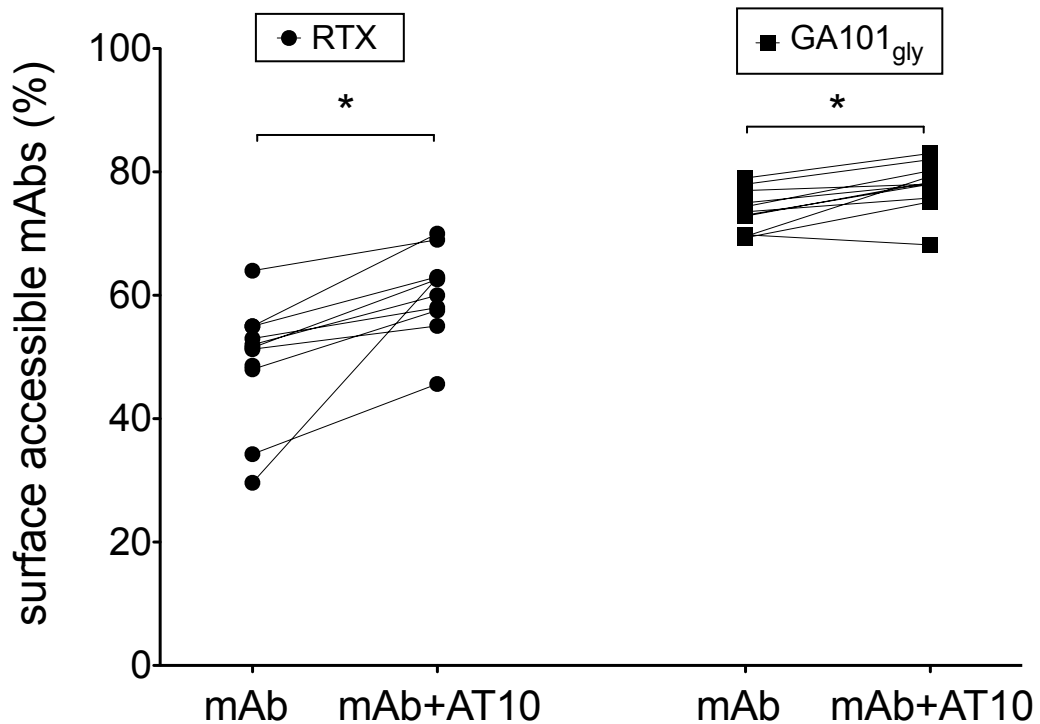
Figure 4.20 FcγRIIb regulates internalisation of rituximab.

The percentage of surface accessible mAbs in samples incubated with anti-CD20 mAbs, RTX (rituximab) and GA101$_{gly}$, with or without prior incubation with the anti-FcγRII mAb, AT10, in samples from patients with SLE (n=11). *p < 0.05.

A surface fluorescence-quenching assay was performed in samples from 11 patients with SLE. Internalisation of both rituximab and GA101$_{gly}$ was inhibited by AT10. However, accessible rituximab was greater in samples incubated with AT10 compared with samples incubated in the absence of AT10 with median 51% vs. 61%, respectively, a mean difference of 11%, whereas this difference was only modest for GA101$_{gly}$ (median 74% vs. 78%, a mean difference of 4%) (Figure 4.20). Intriguingly, despite blocking FcγRIIb, the median surface accessible rituximab was lower than that of GA101$_{gly}$ (median 61% vs. 74%). These findings suggested that rituximab internalised to a greater extent than GA101$_{gly}$, and that FcγRIIb, at least in part, regulated internalisation of anti-CD20 mAbs.

Although there was no direct correlation between the degree of inhibition of internalisation with AT10; or the MFI of FcγRIIb; and the fold difference between CTI of rituximab and CTI of GA101$_{gly}$, it was noted that in the two samples with greatest inhibition of internalisation of mAbs, the CTI of GA101$_{gly}$ was > four-fold higher than the CTI of rituximab whereas the mean for the cohort was a two-fold difference in CTI between the two mAbs. Thus, FcγRIIb facilitated internalisation of Type I CD20 mAbs reduced the efficiency of deletion.

These findings collectively suggest that, at least partly, FcγRIIb-mediated internalisation of anti-CD20 mAbs influenced the efficiency of their B-cell deletion in the whole blood assay. Given the disparity in internalisation between the two types of anti-CD20 mAbs, whether mAbs targeting other B-cell antigens also internalised and whether FcγRIIb regulated internalisation was explored.

Disparity in internalisation of mAbs targeting B-cell antigens

In addition to CD20, mAbs targeting other B cell surface antigens are being explored for use in SLE, including CD19 (Herbst, Wang et al. 2010) and CD22 (Wallace, Kalunian et al. 2014). Therefore, whether the differences in CTI of type I and II anti-CD20 mAbs and anti-CD22 mAb (Figure 4.12), was due to a disparity in internalisation and whether FcγRIIb regulated their internalisation was examined. Vaughan et al. reported that, in malignant B cells, some antigens such as CD22 were rapidly internalised independently of FcγRIIb, whereas others like CD19 and CD38 displayed elements of FcγRIIb-dependency, albeit less than CD20 (Vaughan, Iriyama et al. 2014).

Therefore, the effect of target antigen on the rate of internalisation of mAbs was investigated. Surface fluorescence-quenching assays were performed to assess whether mAbs targeting the following B cell antigens: CD19, CD20, CD22 and CD38. In the case of CD20, internalisation of type I and II anti-CD20 mAbs was compared. These targets were chosen because mAbs targeting these antigens are currently being explored for use in autoimmune conditions such as RA and SLE.
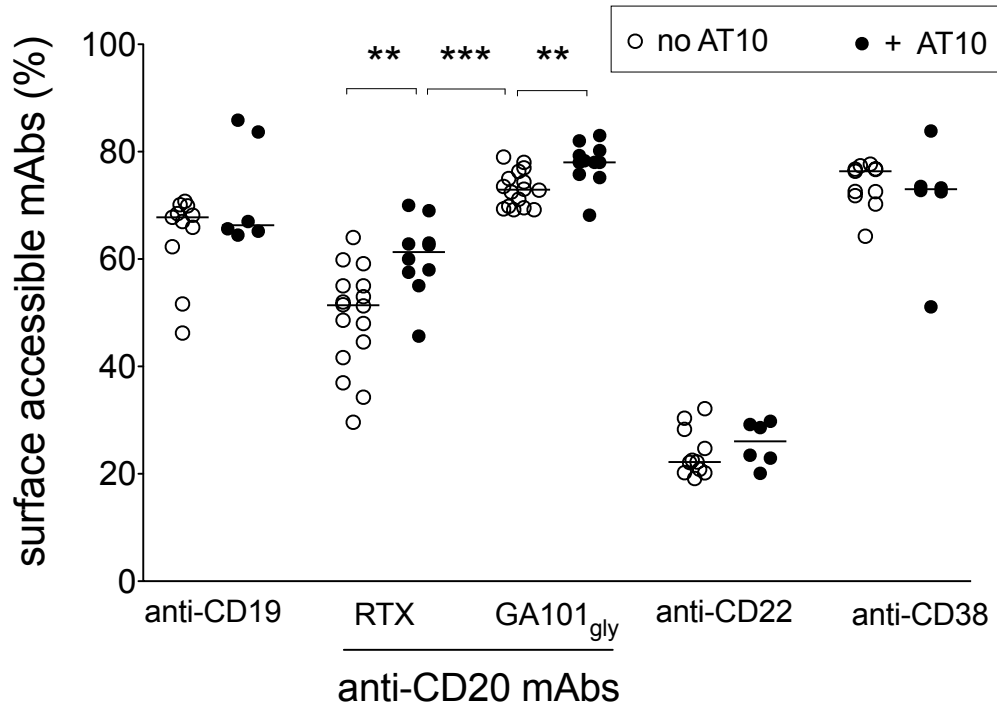
Figure 4.21 Disparity in internalisation of monoclonal antibodies (mAbs) and inhibition by FcγRIIb.

The horizontal line represents the median. *p < 0.05, **p < 0.001, ***p < 0.0001.

A clear disparity in rates of internalisation was noted for the mAbs. As with malignant B cells, SLE B cells displayed a remarkable degree of internalisation of anti-CD22 mAbs, greater than that seen with rituximab, whereas other mAbs (anti-CD19, anti-CD38 and GA101$_{gly}$) internalised to a lesser degree (Figure 4.21) However, even in this small number of samples tested, there was a notable degree of variability in internalisation of rituximab whereas there was little variability in internalisation of GA101$_{gly}$, anti-CD22 mAb and anti-CD38 mAb. Further, internalisation of anti-CD20 mAbs, but not other mAbs, was consistently inhibited by AT10 and was therefore FcγRIIb-dependent.

 In contrast to the hierarchy of depletion with mAbs: anti-CD22 < rituximab < GA101$_{gly}$ (Figure 4.12); a reverse hierarchy of the extent of internalisation: anti-CD22 > rituximab > GA101$_{gly}$ was noted (Figure 4.21).

In summary, these results showed that B cells from patients with RA and SLE internalise anti-CD20 mAbs to a variable degree. Type I anti-CD20 mAbs internalise to a greater extent than type II anti-CD20 mAbs and that FcγRIIb, at least, partly

regulated internalisation of anti-CD20 mAbs. Further, internalisation impaired the efficiency of rituximab in the whole blood B-cell depletion assays. Although, there were no clear differences in the composition of B cell subpopulations, in vivo or in the whole blood assay, between samples with either low or high percentage of B-cell depletion with anti-CD20 mAbs available evidence in vivo suggests some disparity in susceptibility to rituximab-cytotoxicity between B cell subpopulations (Leandro 2013). Therefore, the question of whether there were any differences in internalisation of anti-CD20 mAbs between B cell subpopulations was next investigated.

Internalisation of Type I mAbs is also influenced by IgD and B cell Activation

The effect of B cell related factors including the expression of B cell receptor, IgD, B cell expression of FcγRIIb and B cell activation on internalisation of rituximab was also investigated by comparing the percentage of surface accessible rituximab in B-cell subpopulations based on the expression of IgD, CD27 and CD38. In case of CD38, as most B cells express CD38, the frequency of surface accessible rituximab in B cell subpopulations that expressed either low or high levels of CD38 was compared. Further, surface accessible rituximab and anti-CD22 mAb in B cells incubated with or without 25 μg/mL of anti-IgM F(ab')^2 for 0.5 or 6 hours was compared to assess the effect of B-cell activation on internalisation of rituximab.

B-cell subpopulations were characterized based on the relative expression of IgD and CD27= naïve = IgD+CD27-; unswitched memory cells = IgD+CD27+; switched (switched or switched memory cells) = IgD-CD27+; and double negative cells = IgD-CD27-.

B-cell subpopulations displayed clear differences in internalisation of anti-CD20 mAbs. In all samples from 5 patients with SLE, switched memory cells internalised rituximab significantly less than naïve, unswitched memory B cells and double negative cells (p < 0.05 for all) (Figure 4.22) regardless of prior incubation with AT10 that antagonized the effects of FcγRIIb. Whereas for GA101$_{gly}$ a significant difference was noted between switched cells and naïve and double negative cells before blocking with AT10 and with only naïve cells after blocking with AT10.
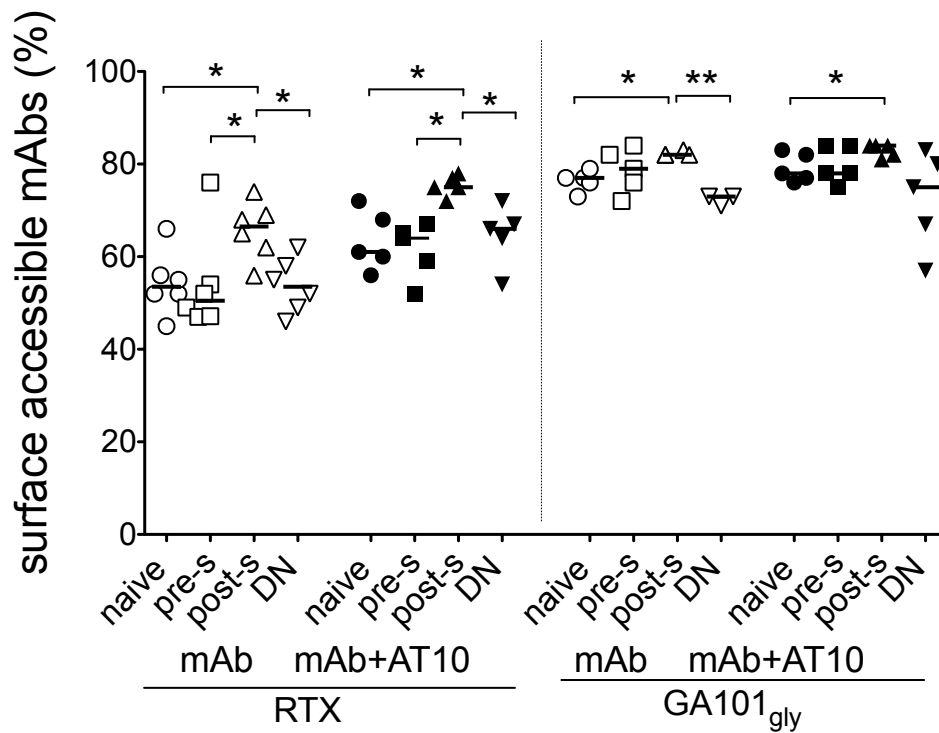
Figure 4.22 Internalisation of anti-CD20 monoclonal antibodies (mAbs) in B cell subpopulations and regulation by FcγRIIb.

The horizontal line represents the median. AT10, anti-FcγRII mAb. *p < 0.05.

Again, as noted in preceding experiments, there was little variability in internalisation of GA101$_{gly}$ within and between B-cell subpopulations whereas internalisation of rituximab varied to a greater extent than GA101$_{gly}$ in different B-cell subpopulations. Rituximab was internalised to a significantly greater extent by switched memory cells (MCs) when compared with other B-cell subpopulations. A greater percentage of rituximab was accessible for quenching on the surface of switched cells when compared with other B-cell subpopulations both before and after blocking with AT10. A significantly greater percentage of GA101$_{gly}$ was accessible on switched cells compared to naïve and double negative cells before blocking with AT10 and only naïve cells after blocking with AT10. Further, the difference in percentage surface accessible mAbs between unswitched and switched cells highlighted the difference in internalisation even within different memory B-cell subpopulations; however, the underlying reasons for this disparity remained unclear.

It has previously been reported that the expression of FcγRIIb differs between B-cell subpopulations in SLE (Su, Yang et al. 2007) and may therefore account for the disparity in internalisation. FcγRIIb expression in B-cell subpopulations was therefore examined.
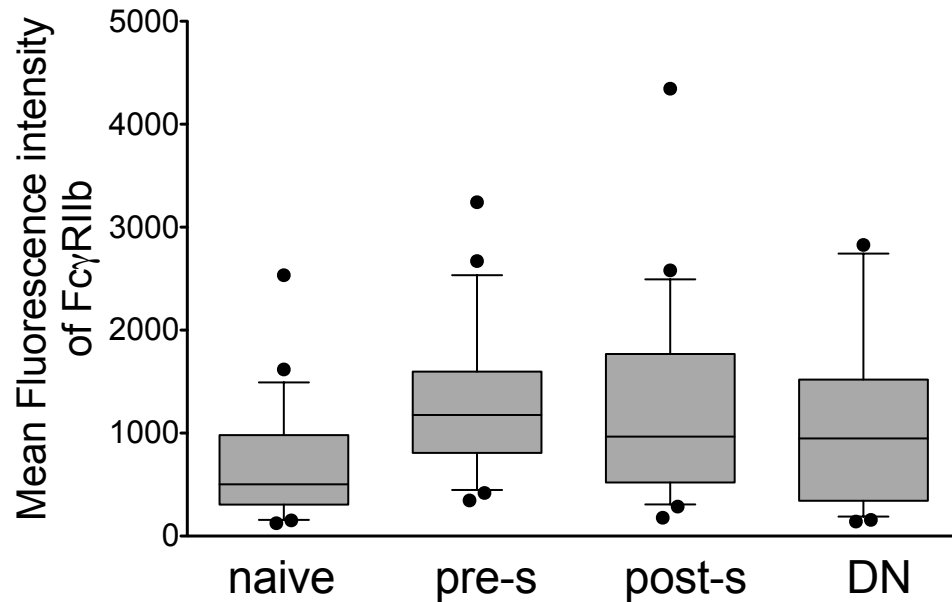
Figure 4.23 IgD and B cell activation influence internalisation of rituximab.

The horizontal line represents the median; the box, IQR; the whiskers, 10-90th percentile; and the dots represent outliers. Pre-s, unswitched memory B cells, post-s, switched memory B cells; and DN, double negative cells.

Similar to a previous report (Mackay, Stanevsky et al. 2006), the MFI of FcγRIIb varied between B cell subpopulations in SLE (Figure 4.23). Naïve cells expressed significantly lower levels when compared with other B-cell subpopulations. Switched memory cells (MCs) expressed FcγRIIb to a similar level as unswitched memory B cells and double negative cells. Naïve cells expressed significantly lower levels when compared with other B-cell subpopulations with a hierarchy of expression: naïve < double negative < switched < unswitched cells. Switched memory cells (MCs) expressed FcγRIIb to a similar level as unswitched MCs and double negative cells. Also, there was a trend toward greater expression of FcγRIIb in unswitched memory cells compared with switched memory cells although this was not statistically significant, perhaps owing to the variability between samples in FcγRIIb

expression. Nonetheless, this pattern is consistent with the reverse trend noted for internalisation of mAbs between the two memory B-cell subpopulations such that unswitched memory B cells with greater levels of FcγRIIb expression also internalised rituximab to a greater extent compared with switched memory B cells with relatively lower levels of FcγRIIb expression. However, there was no difference in internalisation of rituximab between naïve and unswitched memory B cells despite unswitched memory B cells showing significantly greater levels of FcγRIIb expression. Therefore, given the lack of an unequivocal relationship between FcγRIIb expression and internalisation of mAbs between B-cell subpopulations the potential for other factors to influence internalisation of rituximab was investigated.

The role of BCR in internalisation of rituximab in B cells from patients with RA and SLE was investigated. First, the rates of internalisation between B cell subpopulations based on the expression of IgD (IgD+ or IgD-), CD27 (CD27+ or CD27-) and CD38 (CD38 low or CD38 ++) were analysed.

Surface accessible mAbs in B cell subpopulations, grouped based on the expression of IgD with or without prior incubation with anti-FcγRII mAb, AT10. Results from surface fluorescence quenching assays performed using enriched B cells from patients with systemic lupus erythematosus (SLE) (n=6) are shown in Figure 4.24.

IgD+ B cells internalised Rituximab (rituximab) to a significantly greater extent than IgD- B cells (Figure 4.24) with a greater percentage of rituximab remaining accessible on surface for quenching on IgD- B cells when compared with that on IgD+ B cells. Internalisation was also inhibited in samples incubated with AT10 in both IgD+ and IgD- B cell subpopulations. No such differences were noted for GA101$_{gly,}$ which internalised at a consistently low rate in both IgD+ and IgD- B cell subpopulations.
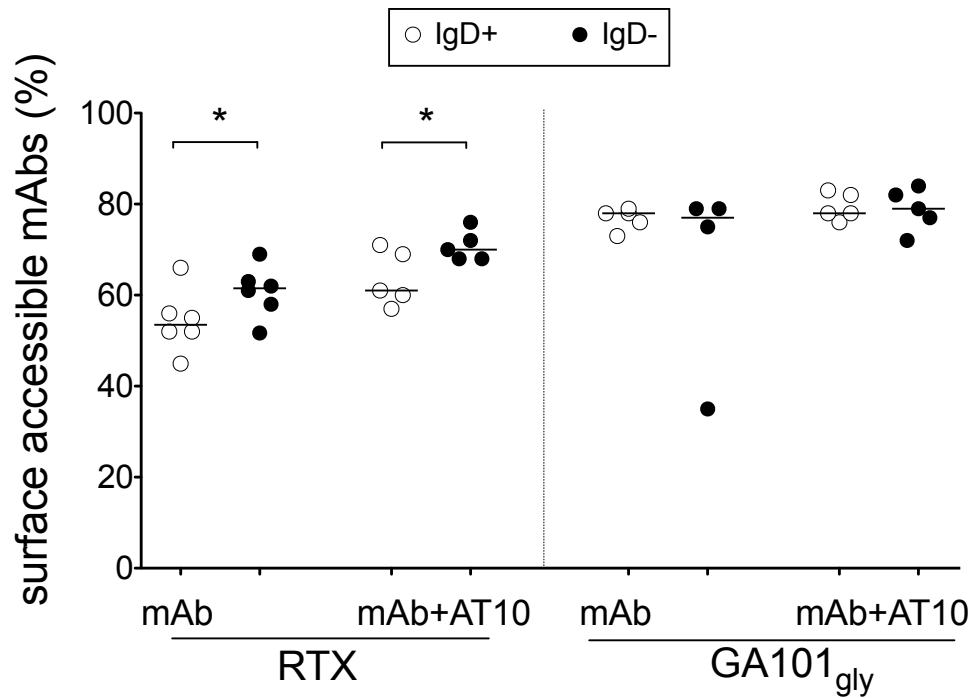
Figure 4.24 Internalisation of Type I mAbs is also influenced by IgD.

The horizontal line represents the median. RTX, rituximab; AT10, anti-FcγRII mAb. *p < 0.05.

The clear difference in internalisation of rituximab between IgD+ and IgD- B-cell subpopulations, therefore, suggested a role for IgD in internalisation of rituximab. Despite different levels of FcγRIIb, there was no clear difference in the rates of internalisation of rituximab between naïve and unswitched memory B cell subpopulations (Figure 4.22). However, both naïve and unswitched memory B cells also express IgD and therefore, it was hypothesized that differences in IgD expression between naïve and unswitched memory B cells may underlie the unexpected lack of difference between the two B-cell subpopulations.
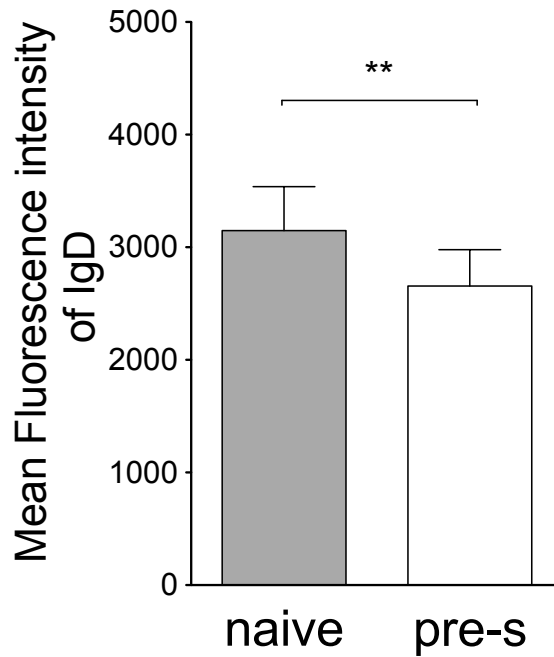
Figure 4.25 Differential expression of IgD and FcγRIIb in B-cell subpopulations.

The mean fluorescence intensity of IgD and FcγRIIb in IgD+CD27- naïve B cells and IgD+CD27+ unswitched memory B cells. The results represent the mean and SD. Pre-s, unswitched memory B cells.

In contrast to the expression of FcγRIIb, the expression of IgD on naïve cells was significantly greater than in unswitched memory B cells (Figure 4.25). This finding was especially of interest when taken in conjunction with the finding that internalisation of mAbs was greatest in IgD+ B cell subpopulation compared with IgD- B cell subpopulation.
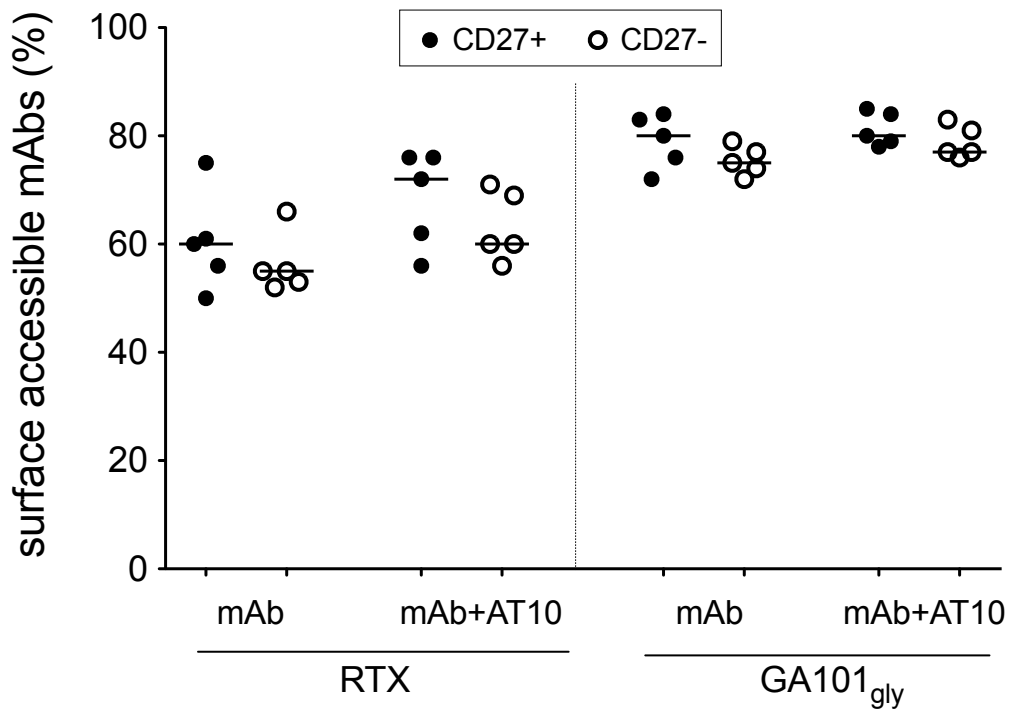
Figure 4.26 Internalisation of Type I mAbs is not influenced by CD27.

The horizontal line represents the median. RTX, rituximab; AT10, anti-FcγRII mAb.

Surface accessible mAbs in B-cell subpopulations, grouped based on the expression of CD27: CD27+ or CD27-; with or without prior incubation with anti-FcγRII mAb, AT10. Results from surface fluorescence quenching assay performed using enriched B cells from patients with systemic lupus erythematosus (SLE) (n=5) are shown in Figure 4.26. There was no significant difference in percentage surface accessible mAbs between CD19+CD27+ and CD19+CD27- B cells (Figure 4.26). Thus, in contrast to what was noted for IgD, B cell expression of CD27 does not appear to influence internalisation of either mAbs.
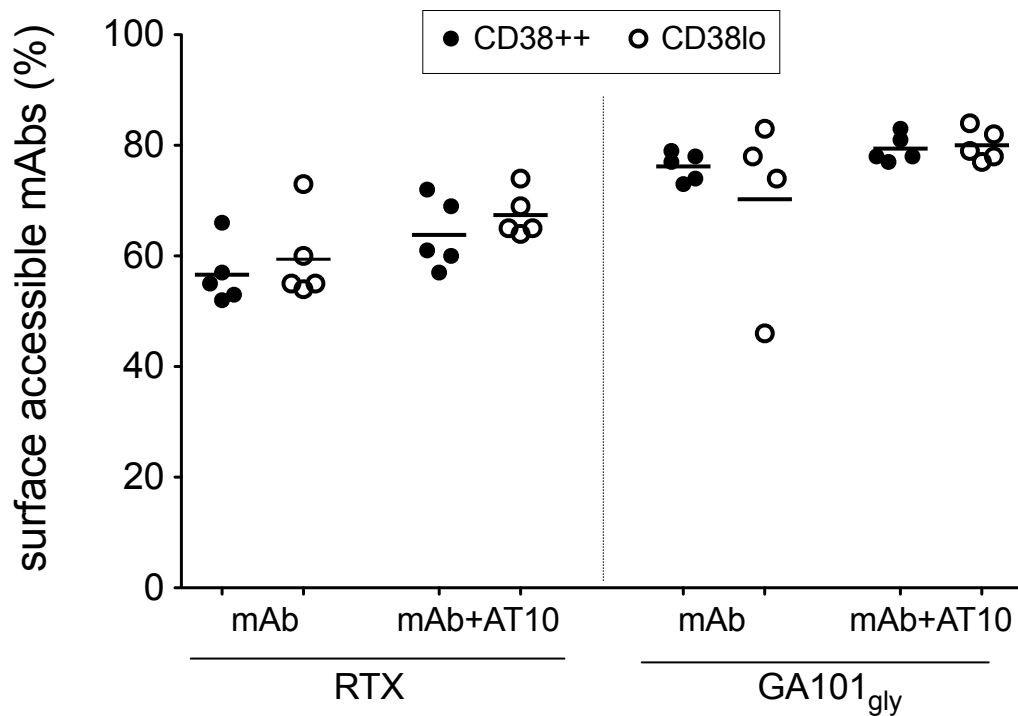
Figure 4.27 Internalisation of Type I mAbs is not influenced by CD38.

Surface accessible mAbs in B-cell subpopulations, grouped based on the expression of IgD with or without prior incubation with anti-FcγRII mAb, AT10. The horizontal line represents the median. RTX, rituximab; AT10, anti-FcγRII mAb.

B cells express CD38 to a variable extent, hence, as such B cell subpopulations are not easily distinguished as positive or negative for CD38. However, a gradient of expression is noted based on which the effect of CD38 on mAb internalisation in two B cell subpopulations grouped as CD38++ with high levels of expression of CD38 or CD38lo with low levels of CD38 expression was analysed.

Results from surface fluorescence-quenching assay performed using enriched B cells from patients with systemic lupus erythematosus (SLE) (n=5) are shown in Figure 4.27. In contrast to what was noted for IgD and similar to CD27 expression, B cell expression of CD38 does not appear to influence internalisation of either mAbs.

Although internalisation of mAbs was higher in the IgD+ B cells suggesting a role for IgD in internalisation of rituximab, IgD itself may be subject to internalisation upon engagement with antigen, as B cells are potent antigen presenting cells. Antigen

engagement of BCR may also trigger BCR signaling leading toward B-cell activation. However, the outcome of BCR-engagement leading to either signaling or internalisation has previously been shown to be mutually exclusive and depends on the phosphorylation of tyrosine-based motifs (Hou, Araujo et al. 2006). CD22, another B-cell antigen, undergoes rapid internalisation constitutively (O'Reilly, Tian et al. 2011) and therefore, whether B cell activation inhibited internalisation of rituximab or anti-CD22 mAb was investigated.
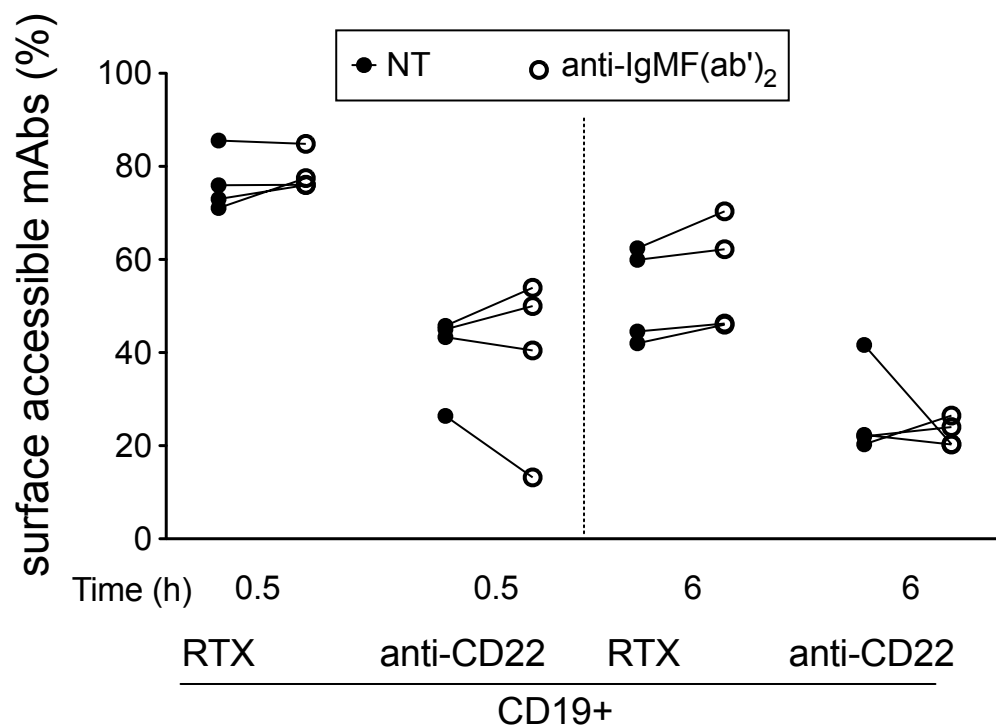
Figure 4.28 Internalisation of Type I mAbs is also influenced by B cell activation.

NT = no treatment. RTX, rituximab.

The frequency of surface accessible mAbs in the surface fluorescence-quenching assay with isolated B cells from patients with SLE (n=4) after incubation with or without 25 μg/mL of anti-IgM F(ab')2 for 0.5 or 6 hours are shown in Figure 4.28. Internalisation of mAbs was assessed at both time points and the percentage surface accessible mAbs was compared in CD19+ B cells as a whole and also in IgD+ and IgD- B cell subpopulations.

No significant differences were noted between samples incubated with or without anti-IgM F(ab')2 (Figure 4.28). Therefore, whether the effect of B cell activation on internalisation was more pronounced in IgD+ cells, which would be activated by anti-IgM F(ab')2 unlike IgD- cells, which do not co-express IgM, was investigated although a small proportion of IgM+IgD- memory B cells (Seifert, Przekopowitz et al. 2015) may also respond to stimulation anti-IgM F(ab')2.

A greater percentage of rituximab was accessible on the surface for quenching in samples incubated with rituximab and anti-IgM F(ab')2 at 6 hours, but not at 0.5 hours. This result suggested that at 0.5 hours, internalisation was not optimal whereas by 6 hours internalisation was partially inhibited by B cell activation with anti-IgM F(ab')2. These findings suggested that activated B cells internalise rituximab to a lesser extent compared with B cells that are not activated.

The frequency of surface accessible mAbs in the surface fluorescence-quenching assay with isolated B cells from patients with SLE (n=4) after incubation with or without 25 μg/mL of anti-IgM F(ab')2 for 6 hours. At 6 hours, a greater percentage of rituximab was accessible on surface for quenching in samples incubated with rituximab and anti-IgM F(ab')2 compared to samples incubated with rituximab alone without anti-IgM F(ab')2 in IgD+ B cells, but not in IgD-B cells (Figure 4.29). No such difference was noted for anti-CD22mAb. Thus, internalisation of rituximab, but not anti-CD22 mAb, was inhibited by B cell activation with anti-IgM F(ab')2 probably because internalisation was inhibited only in IgD+ ($p < 0.05$), but not IgD- B cells, which would not be activated with anti-IgM F(ab')2 stimulation albeit a small proportion of IgD- cells consists of IgM only memory B cells(Seifert, Przekopowitz et al. 2015), which may also be responsive to stimulation with anti-IgM F(ab')2. Thus, internalisation of Type I mAbs, but not Type II was significantly lower in post-switched cells and IgD- B cells as a whole.
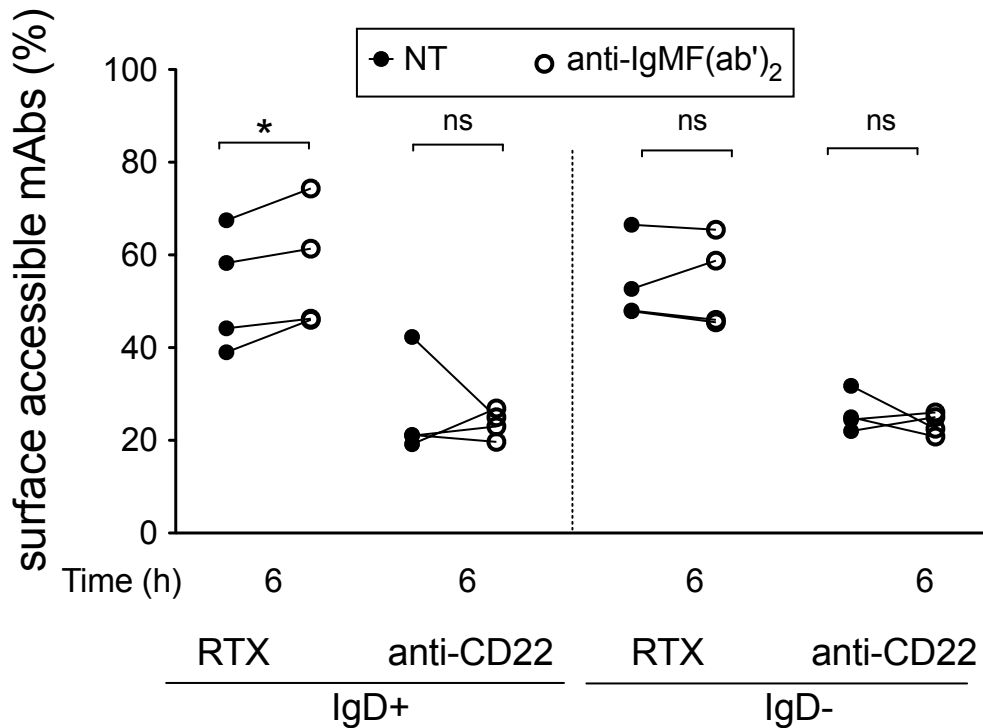
Figure 4.29 Internalisation of Type I mAbs is also influenced by B cell activation.

NT: no treatment; RTX, rituximab; ns: not significant, $*p < 0.05$, $**p < 0.001$.

These results therefore suggest independent roles for $Fc\gamma RIIb$ and the BCR in regulating the internalisation of rituximab, but not $GA101_{gly}$ or anti-CD22 mAbs.

Discussion

In this chapter, the results of a series of experiments comparing the efficiency of type I anti-CD20 mAb, rituximab, and type II anti-CD20 mAbs, and the relationship between the efficiency of type I and II anti-CD20mAbs and internalisation of mAbs are described. The key results are that: 1) type II anti-CD20 mAbs are significantly more efficient at inducing B-cell cytotoxicity in the whole blood assay regardless of the effect of excess BAFF and the composition of B-cell subpopulations; 2) type I anti-CD20 mAbs internalise to a greater extent than type II anti-CD20 mAbs; 3) internalisation influences the efficiency of rituximab; 4) internalisation of anti-CD20 mAbs is significantly variable between B-cell subpopulations with IgD-CD27+ switched memory B cells demonstrating significantly low levels of internalisation;

and that 5) internalisation of anti-CD20 mAbs is influenced by the inhibitory FcγRIIb, IgD and B cell activation.

Efficiency of Type I and II anti-CD20mAbs

First, the efficiency type I mAb, rituximab, and type II mAb, GA101$_{gly}$, in the whole blood B-cell depletion assay showed that rituximab demonstrated inferior B-cell cytotoxicity to GA101$_{gly}$ in both RA and SLE. The difference in the efficiency of rituximab and GA101$_{gly}$, with similar glycosylation of Fc as rituximab, is attributable to the type I and II nature of the mAbs, respectively. Further, comparing the efficiency of type I mAbs rituximab and ofatumumab with type II mAbs tositumomab (B1) and GA101gly revealed that other type II mAbs were also significantly more effective than type I mAbs including 2F2 (Ofatumumab) at depleting B cells from patients with RA and SLE in whole blood depletion assays. Thus, type II mAbs demonstrated superior B-cell cytotoxicity to type I mAbs in RA and SLE samples.

Anti-CD20 mAbs demonstrated superior B-cell cytotoxicity in RA compared to SLE samples suggesting resistance to depletion. Alterations of the immune system in patients with SLE such as defective phagocytosis (Munoz, Lauber et al. 2010) and NK cell function (Sibbitt, Mathews et al. 1983) may further explain why even type II anti-CD20 mAbs failed to achieve comparable depletion to that of RA patients and HCs. Further, SLE-related phagocytic defects and defects in NK cell function may also contribute to the relative resistance to rituximab-induced B-cell depletion and to some extent the variability in B-cell depletion evident between SLE patient samples.

Type I CD20 mAbs (rituximab) induce CDC whereas Type II mAbs are poor inducers of CDC (Glennie, French et al. 2007), therefore, the efficiency of Type I CD20 mAbs may be compromised in conditions with defects in complement function such as SLE (Walport 2002). However, there was no correlation between serum complement (C3) levels and the efficiency of rituximab at inducing B-cell cytotoxicity in whole blood B-cell depletion assay.

Although, not the focus of this study, the results described here clearly demonstrate that anti-CD22 mAb also induces modest levels of B-cell depletion compared with anti-CD20 mAbs. Phase III studies investigating the safety and efficacy of anti-CD22 mAb reported negative results in SLE (Iowse MEB 2015). These results are first to demonstrate the in vitro activity of anti-CD22 mAb at inducing B-cell cytotoxicity and internalisation in samples from patients with RA and SLE.

Relationships between the efficiency of Type I and II anti-CD20 mAbs and patient demographics

Regardless of the patient's age, their disease activity and / or the use of medications, Type II anti-CD20 mAbs were more efficient than Type I anti-CD20 mAbs at inducing B-cell depletion in vitro in whole blood assays in all individual samples tested.

The relationship between the efficiency of anti-CD20 mAbs and B-cell expression of CD20 and FcγRIIb and the composition of B-cell subpopulations

B-cell expression of CD20 and/or FcγRIIb did not directly correlate with the in vitro efficiency of mAbs in both RA and SLE. These results contrast with what has been described in some B-cell malignancies where malignant B-cell expression of FcγRIIb was associated with clinical response to rituximab (Lim, Vaughan et al. 2011, Lee, Ashton-Key et al. 2015) whereas CD20 expression on B cells was shown to correlate with in vitro activity of rituximab, particularly, complement-dependent cellular cytotoxicity induced by rituximab in samples from patients with chronic lymphocytic leukemia (Golay, Zaffaroni et al. 2000, Golay, Lazzari et al. 2001, van Meerten, van Rijn et al. 2006, Horvat, Kloboves Prevodnik et al. 2010). Thus, although B-cell expression of CD20 and/or FcγRIIb was variable between individuals and also in B-cell subpopulations, the findings described here suggest that the thresholds of B-cell expression of CD20 and FcγRIIb and/or baseline composition of B-cell subpopulations do not seem to influence the efficiency of anti-CD20mAbs at inducing B-cell depletion in RA and SLE patient samples in vitro. As yet, there are no comparative studies to which these findings can be related.

Efficiency of anti-CD20 mAbs and the effect of excess BAFF

It has been proposed that BAFF inhibits apoptosis in B cells by down regulating the expression of BCR-induced pro-apoptotic factor, Bim, mediated through phosphorylation of ERK (Craxton, Draves et al. 2005) and in malignant B cells by up regulating anti-apoptotic factors Bcl-2 and Bcl-XL (He, Chadburn et al. 2004). However, there were no consistent effects of BAFF on the efficiency of mAbs in this small number of samples and the efficiency of mAbs was not reduced in the presence of excess BAFF. Whether B cells acquire resistance to anti-CD20 mAb

induced cytotoxicity in the presence of BAFF was not comprehensively explored here, but such data would be useful for clinical application.

Internalisation of anti-CD20 mAbs and efficiency of B-cell depletion in vitro

These results show, for the first time that RA and SLE B cells also internalise mAbs and that internalisation of mAbs clearly distinguishes the two types of anti-CD20 mAbs. Whilst the rates of internalisation seen here are comparable to those described in malignant B cells for type II mAbs such as Tositumomab and GA101$_{gly}$ the internalisation rates noted here for rituximab are not directly comparable because of the wide variability in internalisation of rituximab seen in different B cell malignancies (Lim, Vaughan et al. 2011). A potential explanation for the lack of such clear difference between RA and SLE in terms of internalisation may be due to the relatively small difference in B cell expression of FcγRIIb in RA and SLE.

The results described here demonstrated a clear relationship between internalisation of rituximab and the efficiency of B-cell depletion by rituximab in SLE, but not in RA patient samples. The difference between RA and SLE with respect to correlation between internalisation and efficiency of rituximab may, at least in part, be due to the relatively low number of patient samples. Although a remarkable degree of internalisation was noted, as for example, in the case of SLE and to a lesser extent in RA. A wide variability in the efficiency of depletion was observed in the case of rituximab and SLE, which correlated with the level of internalisation, particularly with rituximab. This suggested that internalisation of rituximab is a probable 'resistance mechanism' in some patients with SLE and may, at least in part, explain the variability in B-cell depletion noted in some patients with SLE (Albert, Dunham et al. 2008).

Although there were no clear correlations between B-cell expression of FcγRIIb and efficiency of mAbs, the role of FcγRIIb in regulating internalisation of mAbs was clearly revealed in internalisation experiments using AT10, where internalisation of mAbs, particularly rituximab, was significantly inhibited by antagonising FcγRIIb. These results are similar to studies in B cell malignancies where FcγRIIb was shown to regulate internalisation of rituximab, albeit with a remarkable degree of variability between different B cell malignancies (Lim, Vaughan et al. 2011). However, antagonizing FcγRIIb only had a partial effect on internalisation of rituximab, the

levels of which were greater compared to internalisation of GA101$_{gly}$. These findings indicating a lack of clear and complete regulatory effect of FcγRIIb on internalisation, suggest that other factors may also operate to influence internalisation of mAbs in RA and SLE.

Disparity in internalisation of mAbs targeting B-cell antigens

mAbs targeting B cell surface proteins other than CD20 are also being explored for use in SLE including anti-CD19 (Herbst, Wang et al. 2010) and anti-CD22 mAbs (Wallace, Gordon et al. 2013) aimed at depleting and/or modulating B cell function. The results described here revealed a disparity in internalisation of these mAbs by B cells from patients with SLE, with rapid internalisation of anti-CD22 mAb occurring unaffected by FcγRIIb and variable internalisation and regulation by FcγRIIb for anti-CD19 mAb. Internalisation of mAbs results in lower amounts of mAb on the target cell surface being accessible to immune-effector cells, (Beers, French et al. 2010) thereby compromising their cytotoxicity, particularly in SLE, which showed rapid internalisation of rituximab.

A possible explanation for the disparity in internalisation of mAbs targeting different B cell surface antigens may relate to the constitutive endocytosis of these antigens. As an example, CD22 is known to undergo rapid endocytosis in B cells (Shan and Press 1995, O'Reilly, Tian et al. 2011) with some alterations in anti-CD22 mAb triggered endocytosis upon surface IgM engagement (Zhang and Varki 2004). CD38-anti-CD38mAb complexes have been shown to internalise slowly both in healthy (Funaro, Reinis et al. 1998) and in malignant B cells independent of FcγRIIb (Vaughan, Iriyama et al. 2014), albeit to a relatively small extent. Similarly, B cells from patients with RA and SLE internalised anti-CD19 mAbs to a relatively lower extent, albeit with some variability, as described previously (Vaughan, Iriyama et al. 2014). CD20 is not considered to shed or internalise from the surface of B cells (Stashenko, Nadler et al. 1980), however, internalisation of CD20 may be induced to a notable degree by binding of type I anti-CD20 mAbs that modulate CD20, but not type II mAbs, which seem to be relatively less potent at modulating CD20, consequently, less likely to be internalised regardless of FcγRIIb signaling (Lim, Vaughan et al. 2011, Vaughan, Iriyama et al. 2014). These results are also consistent with studies in malignant B cells that described different rates of internalisation of mAbs targeting various B cell surface antigens (Press, Farr et al. 1989). The prototype type II anti-CD20 mAb (B1) was shown to internalise less

rapidly than type I ati-CD20 mAb, rituximab (Press, Howell-Clark et al. 1994). Thus, internalisation of mAbs relates to both constitutive endocytosis of target antigen and differentially induced by mAbs.

Upon antigen engagement B cell receptors (BCR) use at least two distinct pathways of endocytosis: clathrin-coated-pit mediated and plasma membrane lipid raft mediated (Putnam, Moquin et al. 2003). Whereas CD20 exists in distinct clusters with variable association with lipid rafts (Li, Ayer et al. 2004), following ligation with anti-CD20 mAbs, CD20 has been shown to co-localize with the BCR (Petrie and Deans 2002). Therefore, a plausible alternative mechanistic explanation for the disparity in internalisation may relate to the requirement for CD20 to associate with lipid rafts, either constitutively or after binding by mAbs (Li, Ayer et al. 2004) to facilitate internalisation (Petrie and Deans 2002). Indeed early studies reported that type I mAb, 1F5, redistributed CD20 into detergent insoluble membrane compartment five-fold more efficiently than type II mAb, B1 (Deans, Robbins et al. 1998). Anti-CD20 mAb-induced in vitro calcium flux studies revealed that type I, but not type II, anti-CD20 mAbs utilize the BCR signaling pathway in an FcγRIIb dependent manner (Walshe, Beers et al. 2008). Studies in B cell malignancies have also shown that internalisation of mAbs targeting different antigens related to their association with lipid rafts (Cragg, Walshe et al. 2005). Taken together, these studies suggest that type I, but not type II anti-CD20 mAbs, promote co-localization of CD20 with the BCR, consequently, internalisation of type I mAbs is more efficient.

Other factors influencing internalisation of rituximab

Intriguingly, the experiments evaluating the role of FcγRIIb in internalisation of mAbs showing that internalisation is only partially inhibited by blocking FcγRIIb suggested that other factors are involved in internalisation of mAbs in RA and SLE. Further, internalisation of rituximab, but not anti-CD22 mAb, was independently inhibited, both by blocking of FcγRIIb and by B cell activation in IgD+ B cells. Internalisation of rituximab, but not anti-CD22 mAb, was variable across B cell subpopulations; being low in switched (IgD-CD27+) memory cells and IgD- cells compared with IgD+CD27+ unswitched memory cells and IgD+ cells as a whole. These differences between B-cell subpopulations in the rates of internalisation of anti-CD20 mAbs suggested that the B-cell receptor, IgD, also influenced internalisation, accordingly the rate of internalisation of rituximab was greater in IgD+ B cells compared with that in IgD- B cells. As discussed earlier, the co-localization of CD20 and BCR (IgD) may

explain why internalisation was significantly higher in IgD+ B cells compared with IgD- B cells (Petrie and Deans 2002). A majority of B cells that express IgM also co-express IgD and both molecules are capable of signaling (Brink, Goodnow et al. 1995). Some B cells express IgM only (Seifert, Przekopowitz et al. 2015), and the specific roles, if at all, of IgM and IgD on internalisation of anti-CD20mAbs remains to be explored in this context. Type I, but not type II, anti-CD20 mAb-induced in vitro calcium flux engaging the BCR signaling pathway (Walshe, Beers et al. 2008) and excessive calcium flux is a potential mechanism by which anti-CD20 mAb mediate apoptosis of B cells. In this regard, type I anti-CD20 mAbs were shown to be more efficient at inducing apoptosis compared to type II anti-CD20 mAbs, in vitro (Deans, Li et al. 2002).

The BCR has at least two key functions: to transduce signals of B cell activation and/or to facilitate internalisation of the antigen for processing in the endosomes prior to antigen presentation. However, both roles are mutually exclusive in that at an individual receptor level, following antigen engagement, the BCR either triggers signal transduction or undergoes internalisation to mediate antigen presentation (Hou, Araujo et al. 2006). Exploiting this principle, the results presented here show that B cell activation inhibits internalisation of IgD and thereby also internalisation of rituximab. This may be advantageous in a clinical setting where rituximab may be more efficient at lysing activated B cells with lower rates of internalisation because B cell activation reduces internalisation, consequently, more rituximab is available on cell surface to evoke effector mechanisms. This is a novel finding in that the role of IgD in partly regulating internalisation of mAbs has not been described before.

Taken together, these results suggest that FcγRIIb and BCR activation influence internalisation of type I anti-CD20 mAbs, but not anti-CD22 mAbs. Thus, distinct mechanisms operate to facilitate internalisation of different mAbs and the differences in internalisation between antigen-specific mAbs may relate to the constitutive endocytosis of the target antigen as for CD22 (Shan and Press 1995) and/or induced association with specific protein complexes after redistribution of CD20 into lipid rafts following incubation with rituximab (Beers, French et al. 2010).

Conclusions

These data suggest that internalisation of rituximab is a potential intrinsic mechanism of resistance by which some B cells from patients with RA and SLE may

evade rituximab-induced cytotoxicity. Internalisation of rituximab appears to be complex, predominantly influenced by FcγRIIb and to some extent by IgD and B cell activation. Therefore, the prospects for targeting the mechanisms influencing internalisation of rituximab in RA and SLE are limited. In contrast, less rapidly internalizing type II anti-CD20 mAbs may be considered as alternative B-cell depleting agents in RA and SLE. Further, factors beyond internalisation might be important to consider in the quest toward more efficient B-cell depleting agents in RA and SLE.

As discussed in the Introduction, both inherent and/or acquired immune defects associated with autoimmune diseases, such as SLE, including complement system, hypergammaglobulinemia, NK cell function and phagocytosis may impact on the effector mechanisms evoked by anti-CD20 mAbs. Therefore, it would be important to understand the differences between type I anti-CD20 mAb, rituximab, with that of the commercially available type II anti-CD20 mAb, obinutuzumab, at evoking the four different effector mechanisms in RA and SLE.

Chapter 5   Results: Efficiency of B-cell Depletion and Effector Mechanisms: Rituximab versus Obinutuzumab

Results

In this chapter, the hypothesis that *"Obinutuzumab induces superior B cell cytotoxicity to rituximab in samples from patients with rheumatoid arthritis and systemic lupus erythematosus in vitro through FcγR-dependent and –independent effector mechanisms"* was tested.

The following questions were investigated in this chapter:

1) Is obinutuzumab superior to rituximab at inducing B-cell cytotoxicity in RA and SLE patient samples, in vitro?
2) Is rituximab more potent than obinutuzumab at activating complement-mediated cellular cytotoxicity?
3) Is obinutuzumab more efficient than rituximab at evoking FcγR-mediated effector mechanisms, in vitro?
4) Is obinutuzumab more competent at inducing FcγR-independent direct cell death?
5) Is obinutuzumab more efficient at inducing direct cell death of B-cell subpopulations?

Efficiency of Rituximab and Obinutuzumab in vitro

Obinutuzumab is more efficient than rituximab at inducing B-cell cytotoxicity in RA and SLE patient samples

In the previous Chapter, the efficiency of Type I and II mAbs on B cell cytotoxicity in RA and SLE samples, whole blood B-cell depletion assays were described. The efficiency of rituximab, a type I anti-CD20mAb and $GA101_{gly}$, a type II anti-CD20mAb engineered in house with an identical Fc portion as rituximab, at inducing B cell cytotoxicity in RA and SLE patient samples was compared. Therefore, the differences noted in the efficiency of B-cell depletion between the two mAbs would be attributable to the type I and II nature whereas the commercially available type II mAb, obinutuzumab (OBZ) that is FDA approved for use in some B cell malignancies bears an afucosylated Fc portion, which may enhance its potential at evoking FcγR-mediated effector mechanisms.

Therefore, the efficiency of rituximab, obinutuzumab and OBZ$_{Gly}$ (obinutuzumab with glycosylated Fc portion similar to rituximab) at inducing B cell cytotoxicity in RA and SLE patient samples, as assessed by the whole blood B-cell depletion assay, was compared. Whole blood B-cell depletion assay was performed in samples from patients with RA (n=31) and SLE (n=34). Obinutuzumab was > two-fold more efficient than rituximab at deleting B-cells from patients with RA and SLE with both OBZ$_{Gly}$ and obinutuzumab more efficient than rituximab in all samples tested (Figure 5.1). Demographics of patients with RA and SLE are shown in Table 5.1 and 5.2, respectively.
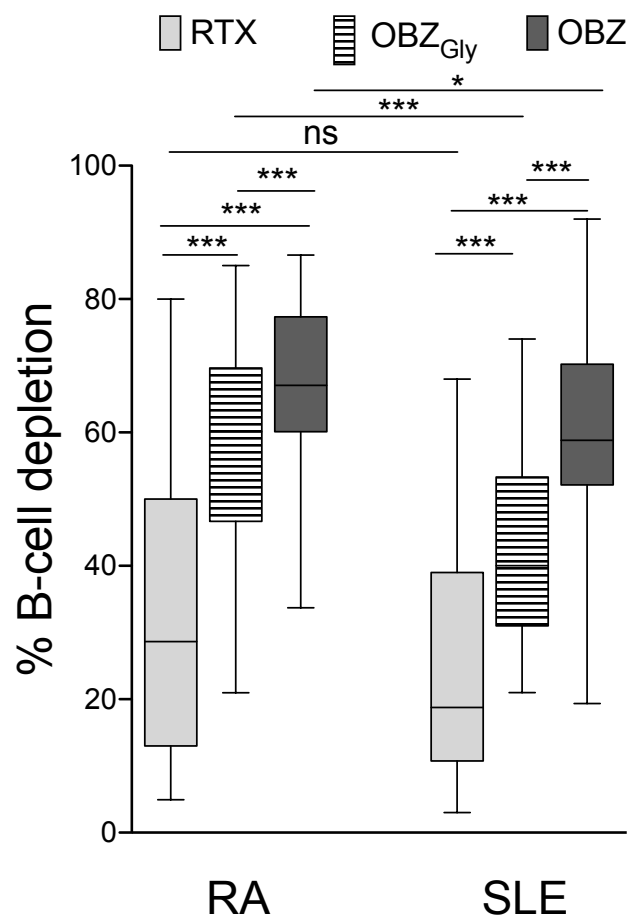
Figure 5.1 Efficiency of rituximab, OBZ$_{Gly}$ and obinutuzumab in whole blood B-cell depletion assay.

Values are the mean of triplicate wells. The horizontal line in the box represents the median, the box represents the IQR range and the whiskers represent the range. * $p<0.05$; **, $p<0.001$; ***, $p<0.0001$.

In both RA and SLE, the median CTI of obinutuzumab was significantly greater than the CTI of OBZ_{Gly} and the CTI of rituximab and, the CTI of OBZ_{Gly} was significantly higher than the CTI of rituximab in both RA and SLE. In RA, the median (IQR) CTI of rituximab, OBZ_{Gly} and obinutuzumab was 29 (13 - 50), 60 (47 - 70) and 67 (60 - 77), respectively and, in SLE, 19 (11 - 39), 40 (31 - 53) and 59 (52 - 70), respectively. Thus, in both RA and SLE, a hierarchy in the efficiency of mAbs: rituximab < OBZ_{Gly} < obinutuzumab was noted. The superior efficiency of OBZ_{Gly} with similar Fc glycosylation to rituximab suggests that type II nature alone probably account for the difference between the two types of mAbs in the efficiency of B-cell depletion in the whole blood assay, whereas superior efficiency of obinutuzumab when compared to OBZ_{Gly} is likely attributable to afucosylation of the Fc portion.

Rituximab displayed significantly lower efficiency of B-cell depletion in the whole blood assay compared to OBZ_{Gly} and the commercially available afucosylated obinutuzumab, consistent with the observations from previous experiments comparing the two types of mAbs in previous cohort of patients with RA and SLE, by Wilcoxon matched-pairs signed rank test, $p < 0.0001$ for all (Figure 5.1). Data about the efficiency of mAbs in RA and SLE are shown in Table 5.3. A significant difference was also noted between the efficiency of B-cell depletion achieved by rituximab and both type II mAbs, OBZ_{Gly} with unmodified Fc, and the afucosylated obinutuzumab, in both RA and SLE, in all individual samples, albeit with a degree of variability between individual samples.

Unlike previous results, no significant difference was noted between the efficiency of rituximab in RA and SLE samples, perhaps due to the lower number of patients with SLE here compared to the previous experiments that may have limited the detection of the variability between patient samples in B-cell depletion afforded by the mAbs. However, similar to the results of previous set of experiments the efficiency of OBZ_{Gly} and obinutuzumab was the significantly lower in SLE samples compared to RA (Figure 5.1) suggesting that B-cell depletion by anti-CD20 mAbs was less efficient in SLE compared to RA.

Given the clear superiority of type II mAbs, regardless of afucosylation, compared to rituximab in the whole blood B-cell depletion assay, the next objective was to investigate whether the characteristics of type I and type II mAbs, particularly their ability to internalise and evoke CDC demonstrated in malignant cells, also applied to non-malignant B cells of RA and SLE. Differences in internalisation between rituximab and OBZ_{Gly} by B cells from RA and SLE were apparent in previous

experiments. Therefore, the focus, here, was on assessing the internalisation dynamics of rituximab and obinutuzumab.

Internalisation of Anti-CD20 mAbs

Surface fluorescence-quenching assays were used to determine internalisation of mAbs, as described previously and detailed in the Methods (Chapter 2), in samples from five patients with RA and eight with SLE. The effect of FcγRIIb on B cell internalisation of anti-CD20 mAbs was also assessed as described previously and detailed in the Methods.

B cells internalise rituximab to a greater extent than obinutuzumab

Here, the internalisation dynamics of rituximab and obinutuzumab in the surface fluorescence-quenching assay were compared. The frequency of surface accessible mAbs was assessed by flow cytometry after six hours of incubation with isolated B cells from patients with RA (n=5) and SLE (n=8) with or without prior incubation with anti- FcγRII mAb, AT10. As noted in the previous set of surface fluorescence-quenching experiments, after 6 hours of incubation, rituximab internalised rapidly, to a greater extent than obinutuzumab in all samples, with a median (range) percentage of surface accessible rituximab vs obinutuzumab of: 55 (51 - 57) versus 83 (81 - 84), respectively in RA; and 60 (49 – 77) versus 76 (70 – 80), respectively in SLE, by Wilcoxon matched-pairs signed rank test, $p < 0.05$ for all, (Figure 5.2).

FcγRIIb regulates internalisation of rituximab and obinutuzumab

AT10, an inhibitor of FcγRII, was again used to show that antagonizing the effects of FcγRIIb partially inhibited internalisation of rituximab, and to a smaller extent, obinutuzumab (Figure 5.2), similar to the results described in the previous chapter using OBZ$_{Gly}$ (GA101$_{gly}$). Further, the variability in internalisation was lowest in samples incubated with obinutuzumab with prior blocking of FcγRIIb with AT10. Rituximab, but not obinutuzumab, internalised to a greater extent in this cohort of RA compared to SLE samples. Thus, B cells from patients with RA and SLE internalise rituximab more than obinutuzumab.

Table 5.1 Baseline characteristics of patients with Rheumatoid Arthritis

patient number	Age (years)	Ethnicity	Disease duration (years)	Disease activity DAS-28-ESR	Medications
1	45	AC	20	5.68	CS, MTX, SSZ
2	52	A	8	8.39	SSZ, HCQ
3	58	C	5	5.4	HCQ, MTX, SSZ
4	86	C	13	5.9	IFX
5	79	A	5	3.6	MTX, SSZ
6	43	C	15	2.72	CS, HCQ
7	70	C	12	5.12	CS, HCQ
8	36	C	10	5.7	SSZ, MTX, Adalimumab
9	55	C	7	4.2	MTX, Adalimumab
10	60	C	18	3.8	IFX
11	86	C	2	7.97	MTX, SSZ
12	67	C	3	4.3	HCQ, Adalimumab
13	56	F	35	3.8	IFX
14	79	C	39	5.12	MTX
15	25	C	8	5.4	MTX, Golimumab
16	30	C	14	4.3	Enbrel
17	54	C	8	5.07	IFX
18	34	AC	1	3.1	MTX
19	27	C	2	5.5	MTX,SSZ, Adalimumab
20	69	C	11	1.9	MTX, SSZ,

					Etanercept
21	63	A	1	5.5	CS, HCQ, MTX
22	59	C	11	5.4	CS
23	49	AC	9	5.4	CS, HCQ, LFN
24	70	C	14	2.45	MTX
25	70	C	18	2.72	RTX
26	56	C	4	7.4	MTX, SSZ
27	55	C	10	3.8	CS, AZA
28	82	C	3	5.2	CS
29	72	C	14	5.6	HCQ, MTX, SSZ
30	34	AC	11	4.17	CS, MTX
31	58	C	3	3.22	HCQ, MTX, SSZ

DAS-28-ESR, disease activity score-28- erythrocyte sedimentation rate; AZA, azathioprine; CS, corticosteroids; HCQ, Hydroxychloroquine; MTX, methotrexate; SSZ, sulfasalazine; LFN, leflunomide; IFX, infliximab; Adalimumab, anti-TNF agent; Etanercept, anti-TNF agent; Golimumab, anti-TNF agent

Table 5.2 Baseline characteristics of patients with Systemic Lupus Erythematosus

Patient number	Age (years)	Ethn -icity	Disease duration (years)	Clinical manifest-ations	C3	Disease activity	BILAG score	Medications
1	36	AC	8	LN	0.73	active	21	CS, HCQ, MMF
2	52	C	26	non-renal	1.31	active	13	CS
3	56	C	31	non-renal	0.87	inactive	0	CS, HCQ
4	57	AC	7	non-renal	1.22	active	9	CS, HCQ
5	34	AC	6	non-renal	1.44	active	8	CS, HCQ
6	48	C	30	non-renal	0.94	inactive	2	HCQ
7	35	C	16	non-renal	1.13	inactive	0	HCQ
8	35	AC	18	LN	0.82	active	8	CS, MMF
9	60	C	44	non-renal	1.04	active	13	CS, AZA, HCQ
10	40	AC	15	non-renal	1.17	active	10	CS, AZA
11	72	C	32	non-renal	0.82	inactive	1	nil
12	30	AC	10	LN	0.82	active	13	CS
13	43	A	13	LN	1.01	inactive	2	CS, HCQ
14	26	C	15	non-renal	1.07	inactive	0	HCQ, MMF
15	50	C	26	LN	1.1	inactive	1	CS, HCQ
16	45	C	34	LN	0.82	inactive	3	HCQ, MMF
17	44	Ch	7	non-renal	1.01	inactive	2	HCQ
18	38	C	12	LN	1.1	inactive	0	HCQ, MTX
19	47	C	12	non-renal	0.97	active	13	CS, AZA
20	48	C	16	non-renal	1.08	inactive	0	nil

21	60	C	15	LN	1.07	inactive	1	CS, HCQ
22	47	C	25	LN	0.9	inactive	1	CS, HCQ, MMF
23	46	C	26	LN	0.86	active	8	HCQ
24	51	AC	21	non-renal	1.12	active	9	CS
25	24	A	13	LN	0.91	active	17	HCQ
26	33	C	13	LN	1.05	active	8	CS
27	43	C	23	LN	0.68	inactive	2	CS, AZA, HCQ
28	37	AC	24	LN	0.67	inactive	2	CS, HCQ
29	42	A	22	non-renal	1.04	active	9	HCQ
30	52	AC	8	non-renal	1.2	inactive	1	CS, HCQ, MMF
31	33	A	9	non-renal	0.82	inactive	3	HCQ, AZA
32	41	C	24	non-renal	0.7	inactive	2	CS, HCQ, MMF
33	40	AC	7	LN	1.53	inactive	0	CS
34	29	A	29	LN	1.27	active	13	CS, AZA
35	24	A	6	LN	1.3	active	9	CS
36	26	AC	6	LN	0.91	inactive	2	CS, HCQ, MMF
37	51	C	17	non-renal	1.42	inactive	0	NIL
38	55	C	27	LN	0.6	inactive	0	HCQ
39	44	C	20	LN	1.1	inactive	0	MTX
40	46	C	13	non-renal	1.42	active	13	CS, AZA,
41	36	AC	6	LN	0.73	active	10	CS, HCQ, MMF
42	34	AC	18	LN	1.05	inactive	0	CS, MMF
43	32	C	8	non-	1.17	inactive	0	HCQ

A, Asian; AC, Afro-Caribbean; C, Caucasian; Ch, Chinese; AZA, azathioprine; CS, corticosteroids; HCQ, Hydroxychloroquine; MTX, methotrexate; MMF, mycophenolate mofetil. LN, lupus nephritis

Table 5.3 Flow cytometry data from the whole blood B-cell depletion assay

patient number	Rheumatoid arthritis		Systemic lupus erythematosus	
	RTX-CTI	OBZ-CTI	RTX-CTI	OBZ-CTI
1	13	77	8	58
2	9	53	41	77
3	17	40	21	53
4	12	37	11	49
5	11	76	18	58
6	11	64	3	48
7	13	64	26	68
8	5	41	6	61
9	29	77	54	71
10	35	64	67	70
11	59	77	15	47
12	39	75	37	63
13	18	67	24	53
14	13	66	39	57
15	34	63	10	59
16	18	50	39	47
17	34	85	17	64
18	15	76	14	55
19	50	77	62	82
20	30	87	10	19
21	25	75	4	57
22	68	82	49	79

23	55	60	32	61
24	28	65	3	74
25	56	66	39	80
26	70	81	9	53
27	44	86	19	69
28	67	34	16	43
29	13	57	28	51
30	80	85	17	27
31	34	82	68	79
32			14	64
33			29	59
34			19	92

RTX, rituximab; OBZ, obinutuzumab; CTI, cytotoxicity index

Given the superior efficiency of type II mAbs at inducing B-cell cytotoxicity in the whole blood assay with significantly lower rates of internalisation, the next objective was to investigate the ability of mAbs to elicit CDC.
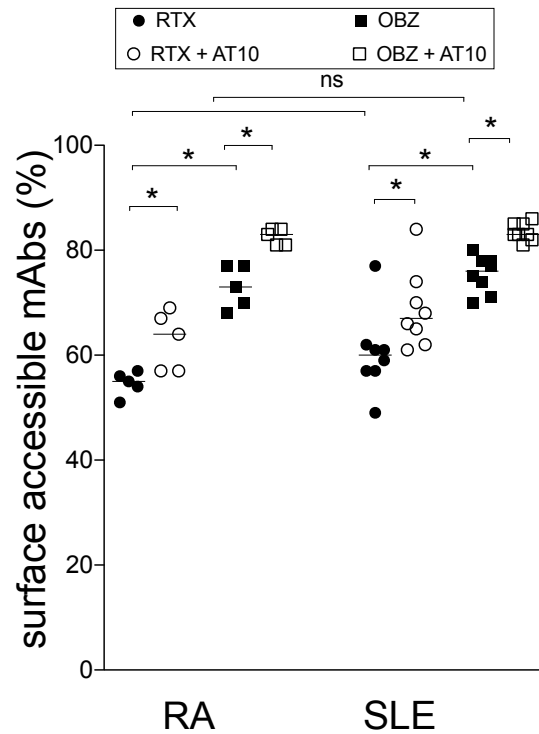
Figure 5.2 Internalisation of mAbs and regulation by FcγRIIb.

The horizontal line represents the median. * p<0.05.

Rituximab is more efficient than obinutuzumab at inducing complement-dependent cellular cytotoxicity

The efficiency of mAbs at recruiting complement was compared in in vitro CDC assays performed using isolated B cells from two healthy controls, two patients with RA and three with SLE. Freshly isolated sera from healthy controls were used as the source of complement in all experiments. Anti-CD20 mAbs were used at a concentration of 10 μg/mL. The frequency of CD19+AnV+PI+ cells in samples incubated with normal healthy serum (NHS) was compared with the frequency of CD19+AnV+PI+ in samples incubated with heat-inactivated serum (HIS) to assess the efficiency of anti-CD20 mAbs at recruiting complement to induce B-cell

cytotoxicity. The fold increase in the frequency of CD19+AnV+PI+ cells in samples incubated with anti-CD20 mAbs in the presence of NHS vs HIS was also analysed to evaluate the efficiency of anti-CD20 mAbs at recruiting complement to induce B-cell cytotoxicity.
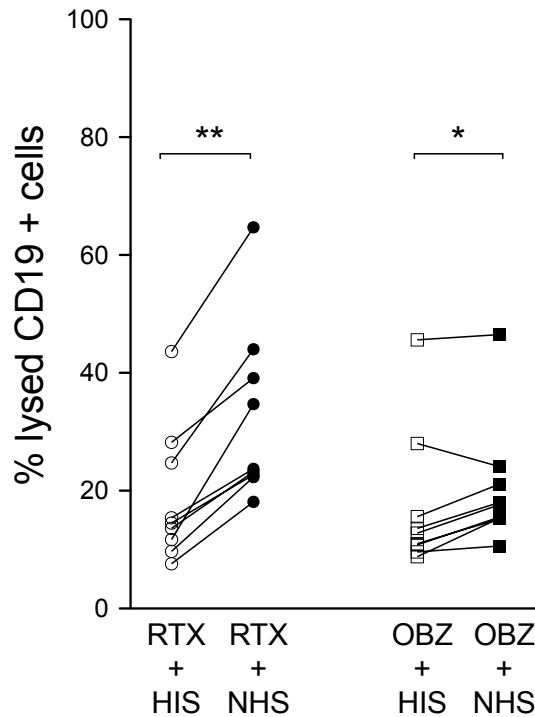
Figure 5.3 Complement-dependent cellular cytotoxicity elicited by Rituximab and obinutuzumab.

Each symbol represents a sample. NHS, normal healthy serum; HIS, heat inactivated serum. * p<0.05; **, p<0.005.

CDC assays were performed as described in the Methods. Isolated B cells (HC, n = 2; RA, n = 2 and SLE, n = 3) were incubated with rituximab or obinutuzumab for 30 minutes with NHS or HIS before analyzing for the frequency of CD19+Av+PI+ B cells. In these assays, the frequency of lysed B cells (CD19+Av+PI+) was significantly greater in samples incubated with rituximab in the presence of NHS compared to HIS with a median (range) difference of 10.9% (8.1 - 21) whereas the difference for obinutuzumab was 4.8% (0.9 - 6.5) (Figure 5.3). These results suggested that rituximab was more efficient than obinutuzumab at inducing

complement activation to lyse CD19+ B cells. The relative efficiency of rituximab and obinutuzumab at inducing CDC was next compared.
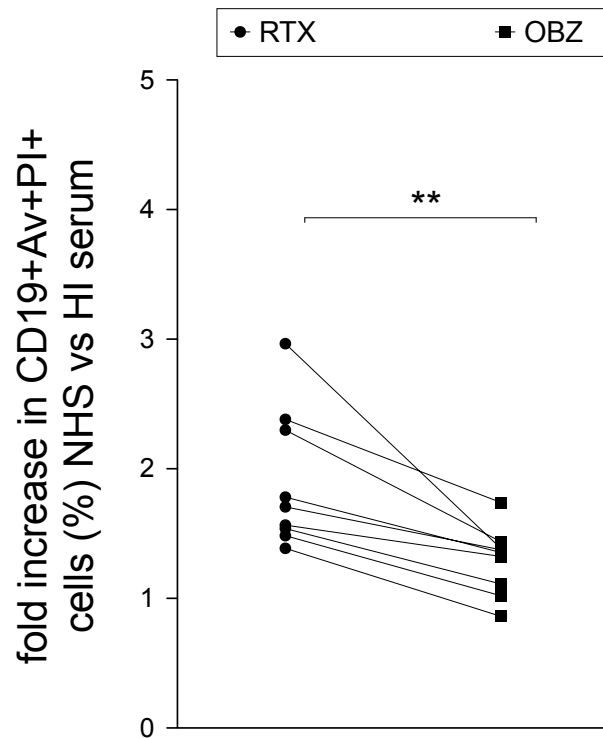
Figure 5.4 Complement-dependent cellular cytotoxicity elicited by Rituximab and obinutuzumab.

Each symbol represents the result of an experiment of complement-dependent cellular cytotoxicity assay. **, $p < 0.005$.

The ratio of the frequency of CD19+Av+ cells in samples incubated with HIS compared to the samples incubated with NHS represented the fold increase in CDC induced by mAbs. The mean±SD fold increase in lysed cells in samples incubated with NHS vs HIS was 1.9±0.5 and 1.2±0.2 for rituximab and obinutuzumab, respectively (Figure 5.4). Thus, rituximab was superior to obinutuzumab at evoking CDC.

The results of CDC were at odds with the superior efficiency of type II mAbs in the whole blood assay, but consistent with type I and II nature of mAbs. Therefore, these observations suggest that type II mAbs are likely to evoke other effector mechanisms more efficiently than type I mAbs in RA and SLE patient samples. So,

the next objective was to investigate the ability of mAbs to evoke other effector mechanisms.

FcγR-mediated effector mechanisms: NK cell mediated Antibody-dependent Cellular Cytotoxicity (ADCC)

As discussed in Introduction, ADCC is a clinically relevant mechanism of B-cell depletion induced by anti-CD20 mAbs in both RA (Kastbom, Coster et al. 2012) and SLE (Anolik, Campbell et al. 2003) and NK cells, which express FcγRIIIA, act as key mediators of ADCC. However, it has been suggested that complement deposition on anti-CD20 mAbs bound to B cells may hinder the interaction between FcγRIIIA bearing NK cells and anti-CD20 mAbs bound to B cells (Wang, Racila et al. 2008, Wang, Veeramani et al. 2009). Therefore, the ability of mAbs to activate NK cells was assessed in whole blood assays (in the presence of complement) in samples from 18 patients with RA and 23 with SLE, which also allowed for analyzing correlations between the efficiency of anti-CD20 mAbs and their ability to activate NK cells in whole blood assays.

The ability of mAbs to activate NK cells in the whole blood B-cell depletion assay was assessed. NK cell activation was assessed on flow cytometry by increases in the expression of CD107a and decreases in the expression of CD16, as discussed in the Methods.

Obinutuzumab is more efficient than Rituximab at activating NK cells

Flow cytometry gating to assess NK cell activation by anti-CD20 mAbs is indicated in Figure 2.7. This gating strategy allowed assessment of NK cell degranulation relative to their expression of CD16. Alternative flow cytometry gating strategy was employed to assess whether activated NK cells may be characterised based on the relative expression of CD56 and CD16, as shown in Figure 5.5.
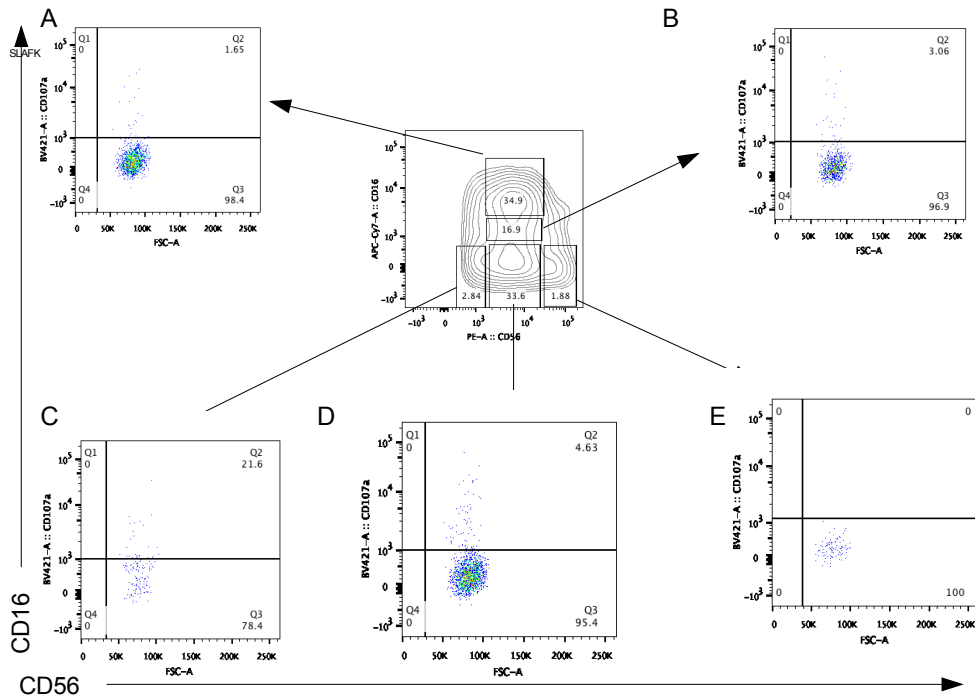
Figure 5.5 NK cell degranulation in subpopulation of NK cells grouped based on the relative expression of CD56 and CD16.

An example of NK cell activation induced by type II anti-CD20 mAb, OBZ, in the whole blood assay. NK cells (CD3-CD56+) categorized based on the relative expression of CD56 and CD16 revealed five subpopulations such that the proportion of CD107a+ activated NK cells in: A) CD56++CD16++ NK cells was 1.5 %; B) CD56++CD16+ NK cells was 3 %; C) CD56+CD16-NK cells was 25%; D) CD56++CD16- NK cells was 4.5 %; and E) CD56+++CD16- NK cells was 0%.

In the previous gating strategy shown in Figure 2.7, NK cells were categorized based on the degree of CD16 expression revealing at least 3 subpopulations: CD56+CD16++, CD56+CD16+ and CD56+CD16- that contained inverse proportions of CD107a+ activated NK cells suggesting that CD16 expression is reduced in activated NK cells. In the current example shown in Figure 5.5, NK cells were alternatively categorized based on the relative expression of CD16 and CD56 to show that CD56+CD16- and CD56+++CD16- contained the highest and lowest proportion of CD107a+ activated NK cells, respectively. These results suggested that activated NK cells expressed lower levels of CD56 or alternatively NK cells with lower expression of CD56 were more frequent in samples incubated with anti-CD20 mAbs.

Having established these parameters, next, equivalent assays comparing rituximab and obinutuzumab were performed.
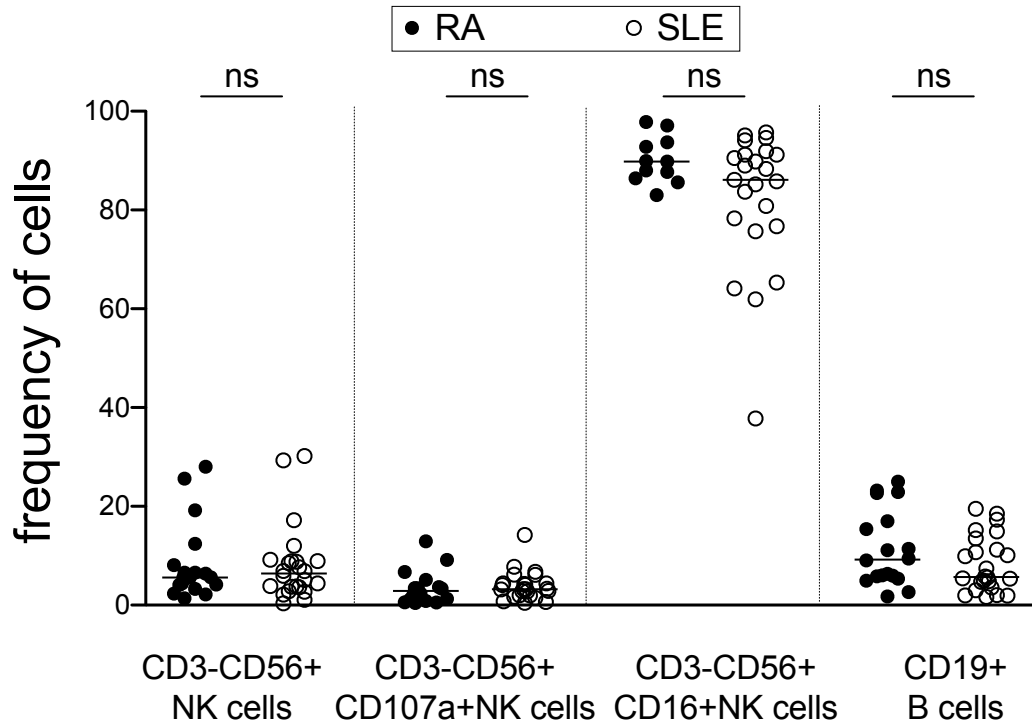
Figure 5.6 The frequency of NK cells and B cells in RA and SLE.

Horizontal lines represent the median. RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; rituximab, rituximab; OBZ, obinutuzumab. **, $p < 0.005$.

There were no significant differences in the frequency of CD3-CD56+ NK cells in the lymphocyte gate, CD3-CD56+CD107a+ NK cells and CD3-CD56-CD16+ NK cells in total NK cells or CD19+ cells in lymphocyte gate in samples from patients with RA (n = 18) and SLE (n = 23) incubated for 24 hours at 37°C and 5% CO_2 in the absence of mAbs (Figure 5.6).

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However, in both RA and SLE patient samples, the median (range) frequency of CD3-CD56+CD107a+ activated NK cells was significantly higher, by Wilcoxon matched-pairs signed rank test, in samples incubated with obinutuzumab compared to rituximab 5.1% (1.9% - 22%) vs 2.8% (0.3% - 14%) (p < 0.0001) and 5.5% (0.6% - 12%) vs 4.3% (1.2% - 8.9%) (p < 0.0001), respectively. There was also a significantly higher fold-increase in the frequency of CD3-CD56+CD107a+ NK cells, by Wilcoxon matched-pairs signed rank test, in samples incubated without mAbs to that in samples incubated with obinutuzumab compared to those incubated with rituximab in RA with a median (inter-quartile range) of 1.2 (1-2.4) vs 2.3 (1.6- 3.5) (p < 0.0001) and in SLE with a median (inter-quartile range) of 1.1 (1-1.6) vs 1.4 (1-2.4) (p < 0.05) (Figure 5.7).
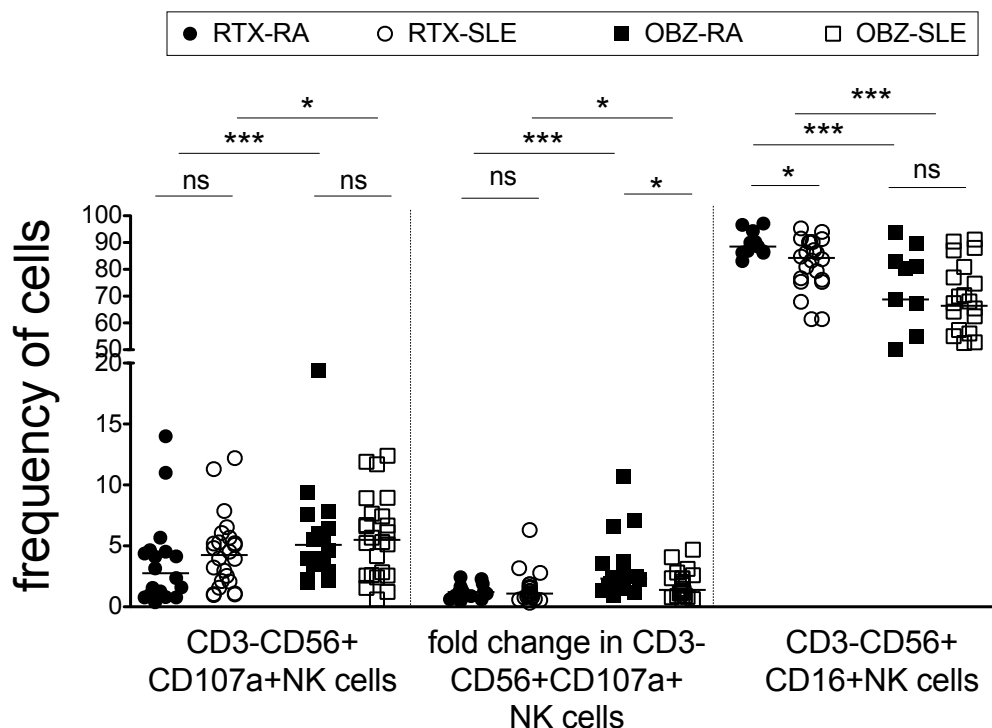
Figure 5.7 mAbs induced NK cell activation in RA and SLE.

Horizontal lines represent the median. RTX, rituximab; OBZ, obinutuzumab. * p < 0.05; **, p < 0.001; ***, p < 0.0001.

There was also a significantly lower median (range) frequency of CD16+ NK cells in in both RA and SLE patient samples incubated with obinutuzumab compared to rituximab, by Wilcoxon matched-pairs signed rank test, 69% (36% - 94%) vs 89%

(83% – 97%) (p < 0.0001) and 66% (42% – 91%) vs 84% (61% – 95%) (p < 0.0001), respectively (Figure 5.7). Furthermore, NK cell activation evoked by rituximab, as assessed by loss of CD16, was lower in SLE samples compared to RA samples (Wilcoxon matched-pairs signed rank test, p < 0.05) (Figure 5.7) whereas the fold increase in the frequency of CD3-CD56+CD107a+ NK cells in samples incubated with obinutuzumab, was greater in RA compared to SLE (Wilcoxon matched-pairs signed rank test, p < 0.05) (Figure 5.7). Thus, these results demonstrated a remarkable variability in activation of NK cells in samples incubated in the absence of mAbs as well as after incubation in the presence of mAbs.

To understand whether the differences in mAb-induced NK cell activation related to the frequency of activated NK cells in samples incubated in the absence of mAbs, the relationship between the frequencies of CD3-CD56+CD107a+ NK cells in samples incubated with mAbs compared to that in samples incubated without mAbs was analysed.

The relationship between the frequency of CD3-CD56+CD107a+ NK cells in samples incubated in the presence or absence of rituximab and obinutuzumab was assessed in samples from patients with RA (n=18). NK cell activation, as assessed by the frequency of CD3-CD56+CD107a+ NK cells by rituximab and obinutuzumab, correlated significantly with that in samples incubated without mAbs with r^2 = 0.89, p < 0.05; r^2 = 0.78, p < 0.05, respectively, in RA (Figure 5.8A) suggesting that baseline activation of NK cells may influence their response to activation with anti-CD20 mAbs.

NK cell activation, as assessed by the frequency of CD3-CD56+CD107a+ NK cells by rituximab and obinutuzumab, correlated significantly with that in samples incubated without mAbs with r^2 = 0.52, p < 0.05; r^2 = 0.36, p < 0.05, respectively, in SLE (n = 23) (Figure 5.8B). However, correlations were stronger in RA compared to SLE for rituximab at r^2 of 0.89, 0.52, respectively; and for obinutuzumab at r^2 of 0.78, 0.36, respectively (Figure 5.8A and Figure 5.8B).

Taken together, these data raise the possibility of SLE-associated defects in the activation of NK cells contributing to inefficient B-cell depletion noted in whole blood B-cell depletion assays (Figure 5.1) and that baseline activation status of NK cells may influence response to activation by mAbs in both RA and SLE (Figure 5.8).
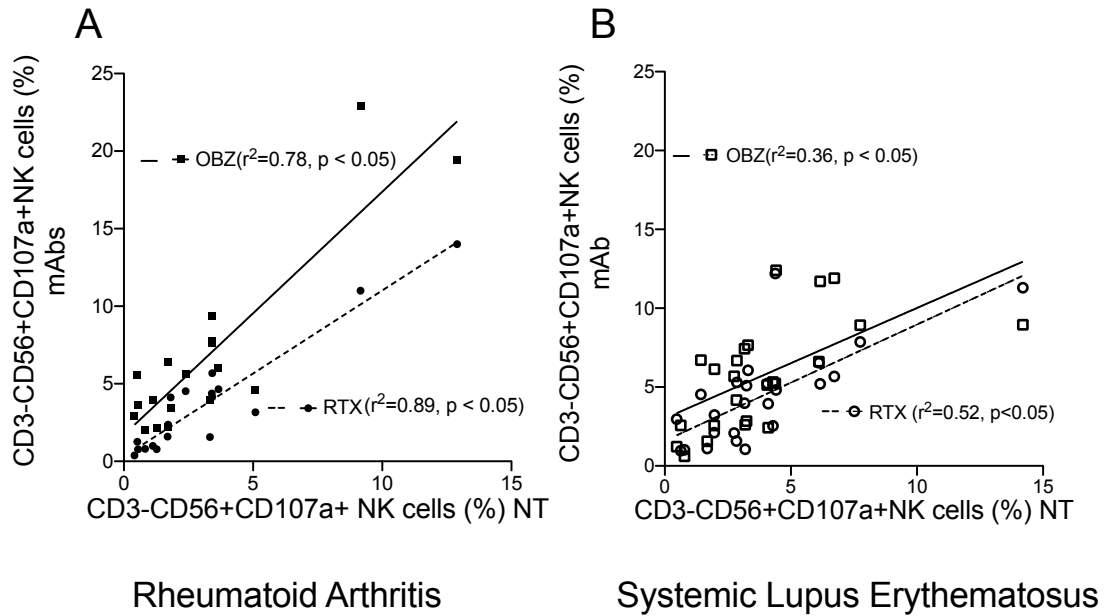
Figure 5.8 Relationships between mAb-induced activation of NK cells to activation of NK cells in test conditions in RA and SLE.

Each symbol represents the result from a patient sample. The dotted lines represent the correlation by linear regression and the correlation coefficient, r^2 was considered significant when p was at least < 0.05.

Although, there were clear differences in NK cell activation between rituximab and obinutuzumab, to what extent the type I and II nature of mAbs and/or afucosylation of Fc accounted for the difference between the mAbs remained unclear. Therefore, the next set of experiments investigated whether differential activation of NK cells by rituximab and obinutuzumab is due to type I and type II characteristics and/or due to the effect of Fc glycosylation.

ADCC and the effect of Fc glycosylation

As discussed in the Introduction chapter, Fc glycosylation influences the binding of mAbs to FcγRIII (Umana, Jean-Mairet et al. 1999, Ferrara, Stuart et al. 2006, Iida, Misaka et al. 2006, Ferrara, Grau et al. 2011). To explore the effect of Fc glycosylation on ADCC, the efficiency of four different anti-CD20 mAbs at activating NK cells: rituximab (type I); obinutuzumab (commercially available, afucosylated type II mAb); glycosylated OBZ (OBZ$_{Gly}$); and OBZ-LALA, OBZ with engineered Fc lacking engagement with FcγRIII was compared.

Whole blood B-cell depletion assays were performed in samples from patients with RA (n = 18) and SLE (n = 23) comparing the efficiency of B-cell depletion and NK cell activation with rituximab, OBZ$_{Gly}$ and obinutuzumab and also with OBZ-LALA in six samples from patients with RA and five with SLE. Again, as noted in the previous set of experiments shown in Figure 5.1, Obinutuzumab was more efficient than OBZ$_{Gly}$ and rituximab at depleting B cells in the whole blood assay in both RA and SLE, by Wilcoxon matched-pairs signed rank test with p < 0.0001, shown in Figure 5.10. There was no significant difference between the % B-cell depletion achieved by rituximab and OBZ-LALA, by Wilcoxon matched-pairs signed rank test (Figure 5.9).

Figure 5.9 Obinutuzumab is more efficient than Rituximab at evoking NK Cell-mediated cellular cytotoxicity in RA and SLE.

Each symbol represents %B-cell depletion result of whole blood B-cell depletion in a sample. ***, p<0.0001; and ns, not significant.

There were no significant differences in the frequency of CD3-CD56+CD107a+ NK cells in samples incubated without mAbs compared to samples incubated with rituximab in both RA (n = 18) and SLE (n = 23) (Figure 5.10). The frequency of CD3-CD56+CD107a+ NK cells in samples incubated without mAbs was significantly lower compared to samples incubated with OBZ-LALA, by Wilcoxon matched-pairs

signed rank test, in SLE (n = 12) albeit a small difference with a median (IQR range) 3.2% (2.0 % - 4.4%) and 3.1% (1.5%-4.9%), respectively, (p < 0.05), but not RA (n=6) (Figure 5.10).

Figure 5.10 Obinutuzumab is more efficient than Rituximab at evoking NK Cell-mediated cellular cytotoxicity in RA and SLE.

Bars represent the median and the error bars the IQR. * p < 0.05; **, p < 0.001; ***, p < 0.0001.

However, the frequency of CD3-CD56+CD107a+ NK cells in samples incubated with rituximab was significantly higher, by Wilcoxon matched-pairs signed rank test, compared to samples incubated with and OBZ-LALA in SLE (n = 12) (p < 0.005) with median (IQR) 4.2% (2.1 % - 5.6%) and 3.1% (1.5%-4.9%), respectively, but not in RA (n=6) (Figure 5.10).

In RA, an increasing hierarchy was noted in the frequency of CD3-CD56+CD107a+ NK cells such that the frequency was lowest in samples incubated with no mAbs < rituximab < OBZ$_{Gly}$ < obinutuzumab with median (IQR) of 2.1% (1.0 % - 3.5 %), 2.8 % (1.0 % - 4.5 %), 3.0% (1.3 % - 5.6 %) and 5.1% (3.3 % - 7.7 %), respectively (Figure 5.10). Similarly, in SLE also, there was an increasing hierarchy was noted in the frequency of CD3-CD56+CD107a+ NK cells such that the frequency was lowest

in samples incubated with no mAbs < rituximab < OBZ$_{Gly}$ < obinutuzumab with median (IQR) of 3.2% (1.9 % - 4.4 %), 4.2 % (2.1 % - 5.5 %), 3.7% (2.4 % - 5.6 %)
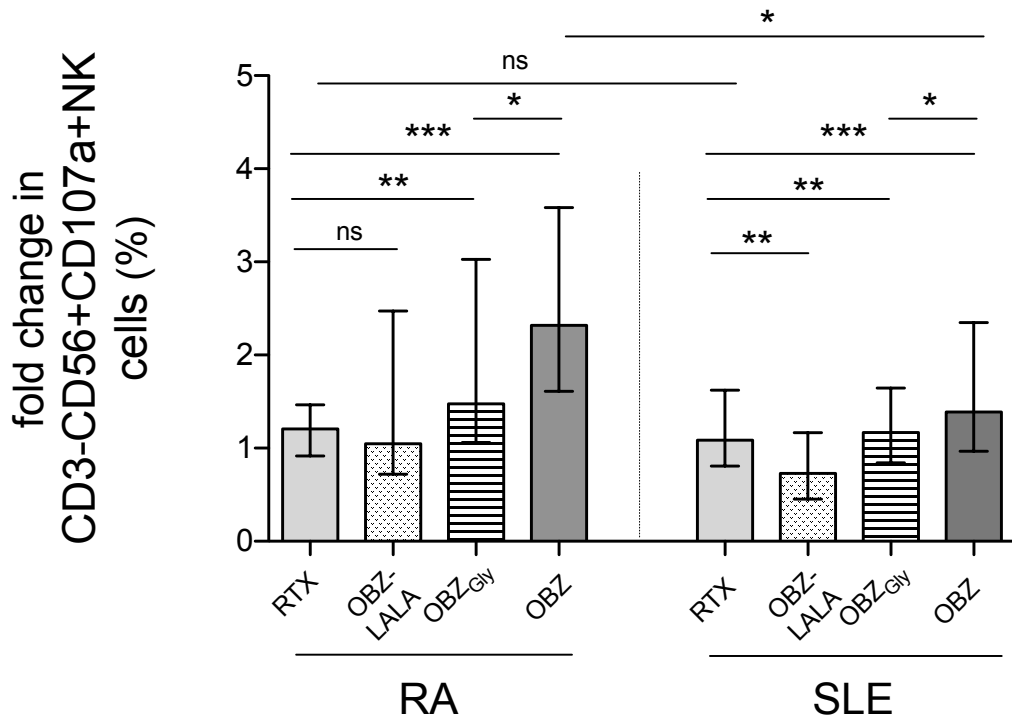
and 5.5% (2.6 % - 7.6 %), respectively (Figure 5.10).

Figure 5.11 Obinutuzumab is more efficient than Rituximab at evoking NK Cell-mediated cellular cytotoxicity in RA and SLE.

Bars represent the median and the error bars the IQR. * p<0.05; **, p < 0.001; ***, p < 0.0001.

In RA, an increasing hierarchy was noted in the fold-increase in CD3-CD56+CD107a+ NK cells such that the frequency was lowest in samples incubated with rituximab < OBZ$_{Gly}$ < obinutuzumab with median (IQR) of 1.2 % (1.0 % - 1.5 %), 1.5 % (1.1 % - 3.0 %) and 2.3 % (1.6 % - 3.6 %) respectively (Figure 5.11). In SLE also, an increasing hierarchy was noted in the fold-increase in CD3-CD56+CD107a+ NK cells such that the frequency was lowest in samples incubated with rituximab < OBZ$_{Gly}$ < obinutuzumab with median (IQR) of 1.1 % (0.8 % - 1.6 %), 1.2 % (0.8 % - 1.6 %) and 1.4 % (1.0 % - 2.3 %) respectively (Figure 5.11). Further, the fold-change in the frequency of CD3-CD56+CD107a+ NK cells was significantly higher in RA samples incubated with obinutuzumab, but not rituximab, compared to

SLE samples (Figure 5.11). Collectively, these results suggested that although obinutuzumab was more efficient than rituximab at activating NK cells in both RA and SLE, obinutuzumab was only partially able to overcome SLE-associated immune defects.
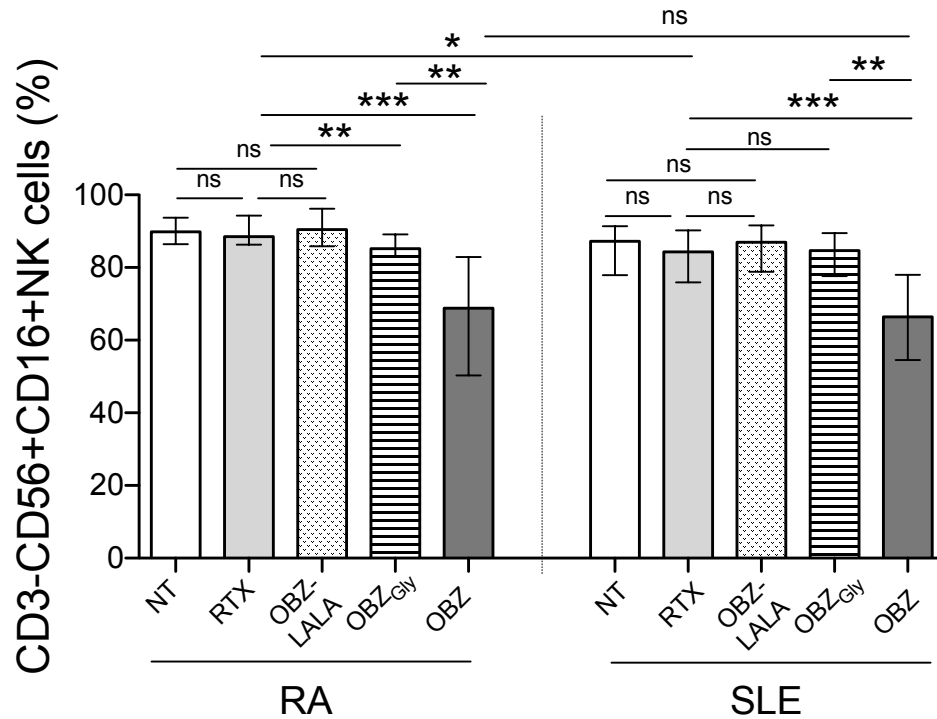
Figure 5.12 Obinutuzumab is more efficient than Rituximab at evoking NK Cell-mediated cellular cytotoxicity in RA and SLE.

Bars represent the median and the error bars the IQR. * $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

There were no significant differences in the frequency of CD3-CD56+CD16+ NK cells in samples incubated without mAbs compared to samples incubated with OBZ-LALA in both RA (n = 6) and SLE (n = 12) (Figure 5.12).

In RA, a decreasing hierarchy was noted in the frequency of CD3-CD56+CD16+ NK cells such that the frequency was highest in samples incubated with rituximab > OBZ$_{Gly}$ > obinutuzumab with median (IQR) of 88 % (86 % - 94 %), 85 % (83 % - 89 %) and 69 % (50 % - 83 %) respectively (Figure 5.12). In SLE, the frequency of CD3-CD56+CD16+NK cells was significantly lower in samples incubated with obinutuzumab compared to OBZ$_{Gly}$ and rituximab with a median (IQR) of 66 % (54

% - 78 %), 85 % (78 % - 89 %) and 84 % (76 % - 90 %), respectively (Figure 5.13). The frequency of CD3-CD56+CD16+NK cells was also lower in samples incubated with OBZ_{Gly} compared to rituximab in RA, but not SLE (Figure 5.12).

The ability of mAbs to up-regulate the expression of CD107a on CD3-CD56+ NK cells was greater in RA compared to SLE, such that the median fold difference in samples incubated with rituximab, OBZ_{Gly} and obinutuzumab compared to samples incubated without mAbs was 1.2, 1.5, and 2.3, respectively, in RA whereas the median fold difference was 1.1, 1.2 and 1.4, respectively, in SLE (Figure 5.11). Thus, the pattern of B-cell depletion achieved by mAbs in RA and SLE (Figure 5.9) was similar to the pattern of NK cell activation by mAbs (Figure 5.11).
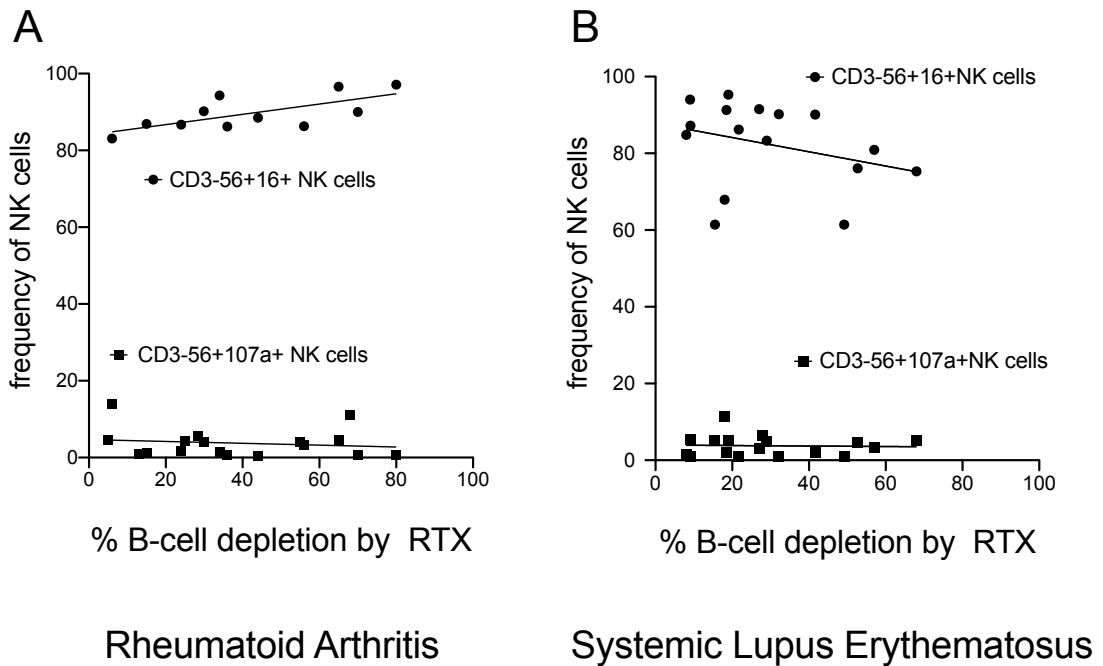
Figure 5.13 Obinutuzumab is more efficient than Rituximab at evoking NK Cell-mediated cellular cytotoxicity in RA and SLE.

Each symbol represents the result from a patient sample. The lines represent the correlation by linear regression and the correlation coefficient, r^2 was considered significant when p was at least < 0.05.

In RA, the percentage B-cell depletion by rituximab correlated with the frequency of CD3-CD56+CD16+ NK cells ($r^2 = 0.47$, p < 0.05), but not CD3-CD56+CD107a+ NK cells (Figure 5.13A and B). In SLE, there were no significant correlations between

the percentage B-cell depletion induced by rituximab and the frequency of CD3-CD56+CD16+ NK cells and CD3-CD56+CD107a+ NK cells.
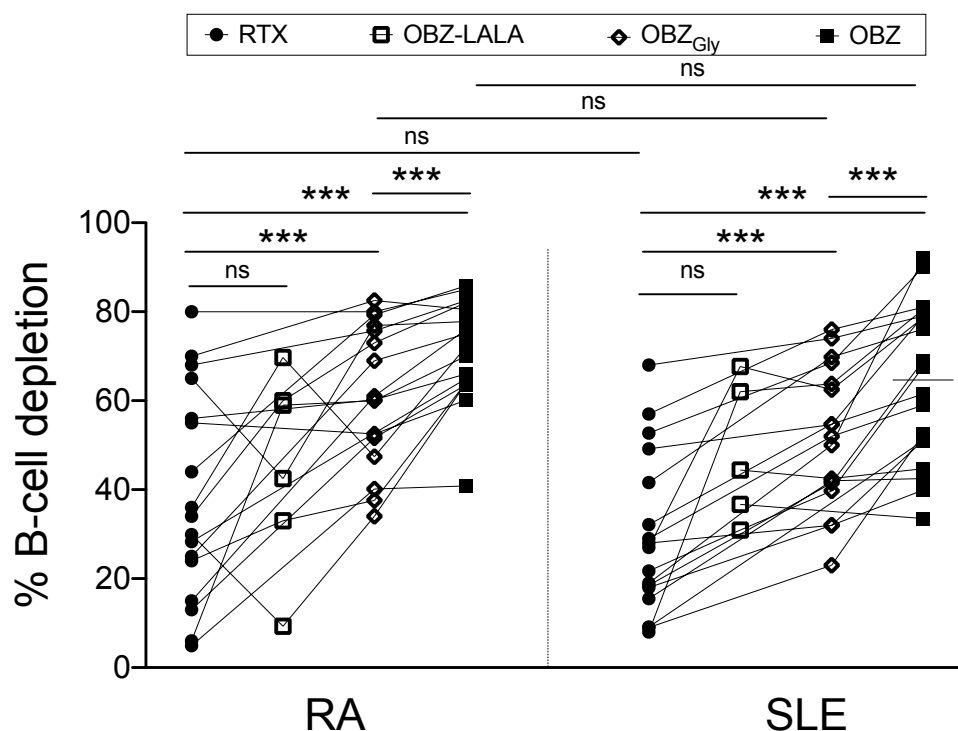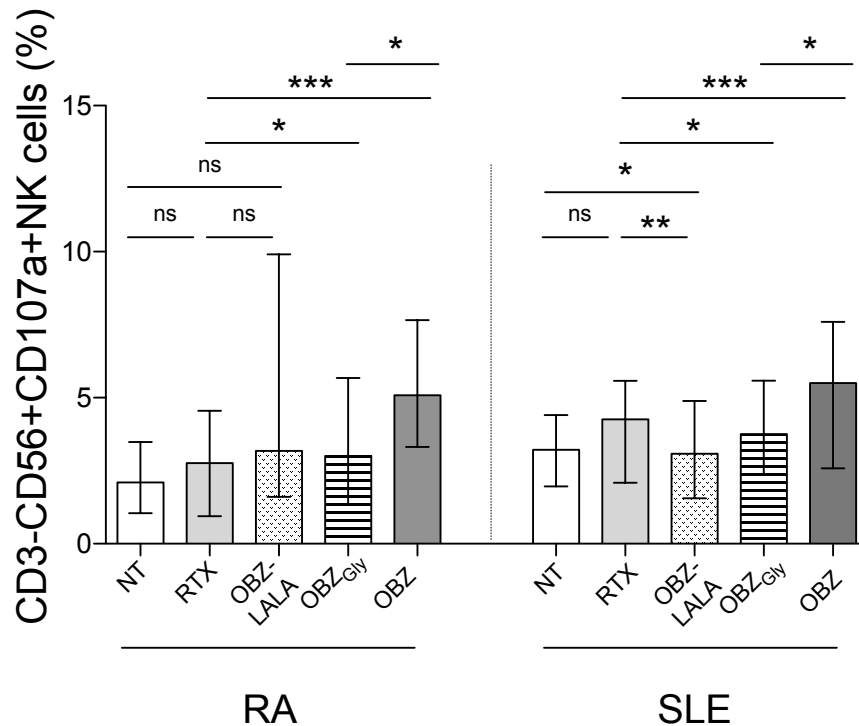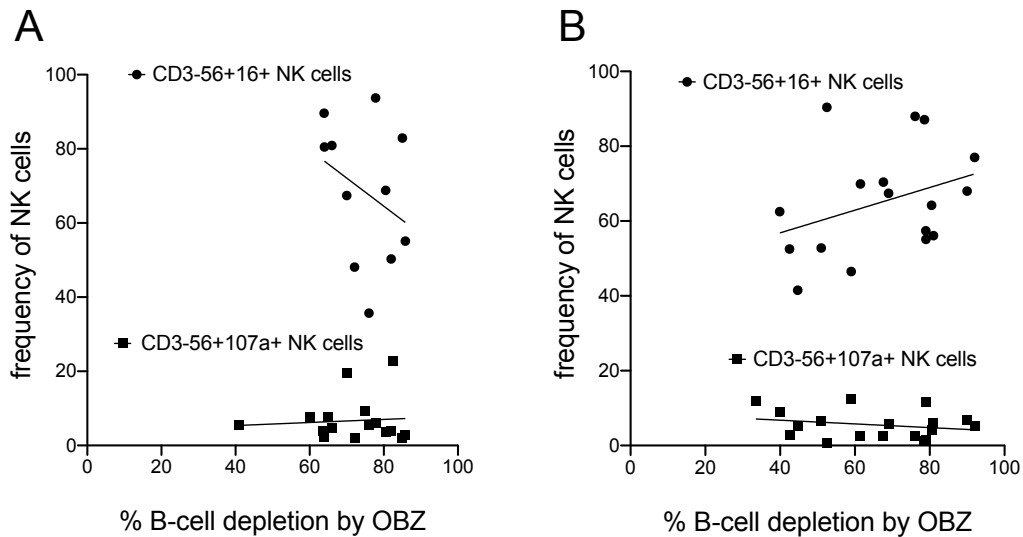

Figure 5.14 Obinutuzumab is more efficient than Rituximab at evoking NK Cell-mediated cellular cytotoxicity in RA and SLE.

Each symbol represents the result from a patient sample. The lines represent the correlation by linear regression and the correlation coefficient, r^2 was considered significant when p was at least < 0.05.

There were no significant correlations between the percentages of B-cell depletion induced by obinutuzumab and the frequency of CD3-CD56+CD16+ NK cells and CD3-CD56+CD107a+ NK cells in both RA and SLE (Figure 5.14A and B).

FcγR-mediated effector mechanisms: Neutrophil mediated Antibody-dependent Cell Phagocytosis (ADCP)

Neutrophils are the most abundant white blood cells in peripheral blood and have been proposed to be effector cells for B-cell depletion by anti-CD20 mAbs (Golay, Da Roit et al. 2013). Neutrophils express FcγRIIIb, which facilitates phagocytosis of anti-CD20 mAb opsonized B cells. Assessment of neutrophil activation by mAbs in whole blood environment probably accounts for the potential adverse effects of

complement interfering with interaction between anti-CD20 mAbs bound to B cells and FcγRIIIb bearing neutrophils. Therefore, as for ADCC experiments, the ability of anti-CD20 mAbs to evoke neutrophil activation in whole blood assays was assessed in samples from ten patients with RA and 22 with SLE.

Obinutuzumab is more efficient than rituximab at activating neutrophils

The ability of mAbs to induce neutrophil activation was assessed by measuring the expression of CD11b and CD62L, as described previously (Wittmann, Rothe et al. 2004) according to flow cytometry gating shown in the Methods. CD11b forms part of the βintegrin (Mac-1) complex expressed on neutrophils. Several genetic variants of this complex have been associated with lupus-related phagocytic defects (Zhou, Wu et al. 2013). Upon neutrophil activation the surface expression of CD11b is up regulated whereas the expression of the adhesion molecule CD62L is down regulated (Wittmann, Rothe et al. 2004, Golay, Da Roit et al. 2013).

After 24-hour incubation of whole blood samples with mAbs at 1μg/mL in the whole blood assay, the MFI of CD11b on CD15+ neutrophils in samples incubated in the presence or absence of mAbs in samples from patients with RA and SLE. In these assays, the MFI of CD11b in samples incubated with obinutuzumab, but not other mAbs, was significantly higher in RA (n = 10) (Figure 5.15) compared to samples incubated without mAbs ($p < 0.005$). In RA, the MFI of CD11b in samples incubated with obinutuzumab was also significantly higher compared to samples incubated with rituximab ($p < 0.005$) and OBZ$_{Gly}$ ($p < 0.005$) (Figure 5.15).

In SLE (n = 22), compared to samples incubated in the absence of mAbs the MFI of CD11b was higher in samples incubated with rituximab ($p < 0.05$), OBZ$_{Gly}$ ($p < 0.005$), and obinutuzumab ($p < 0.0005$), respectively. The MFI of CD11b was also significantly higher in samples incubated with obinutuzumab compared to rituximab ($p < 0.005$) and OBZ$_{Gly}$ ($p < 0.0005$) (Figure 5.15). However, there were no significant differences in the MFI of CD11b in samples incubated without mAbs compared to samples incubated with OBZ-LALA in both RA (n = 6) and SLE (n = 12).
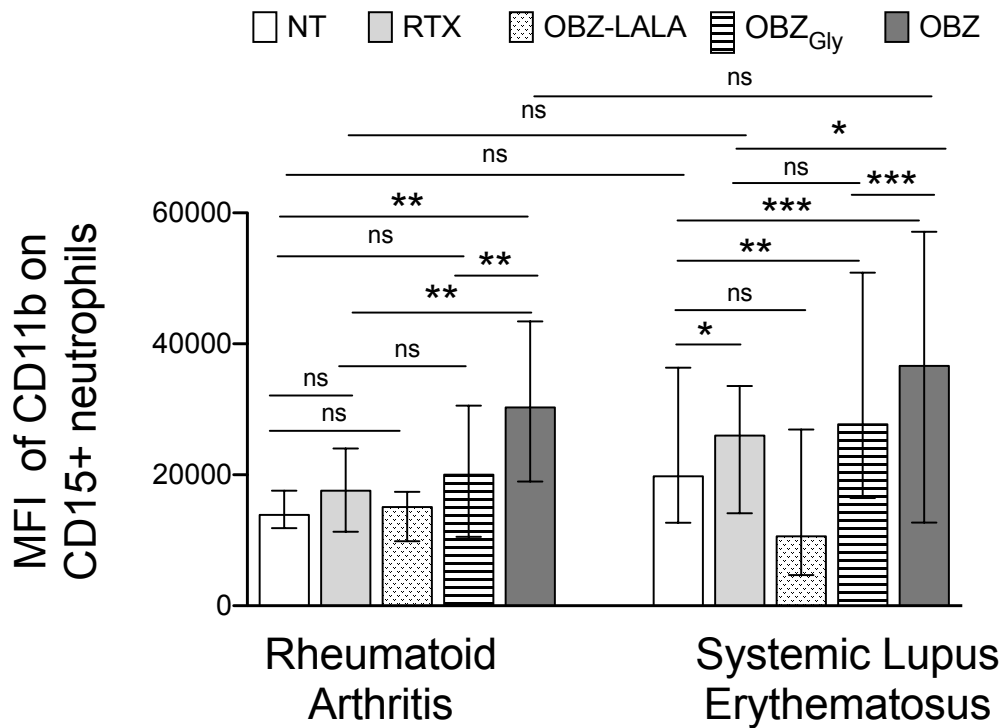
Figure 5.15 Obinutuzumab is more efficient than Rituximab at Activating Neutrophils in Rheumatoid Arthritis and Systemic Lupus Erythematosus.

The Median and IQR ranges are represented by the error bars. * p < 0.05.

The MFI of CD11b was lower in samples incubated with RTX < obinutuzumab revealing significant differences in the ability of mAbs to up-regulate CD11b.

Variants of CD11b are not only associated with SLE, but may also influence phagocytosis (Rhodes, Furnrohr et al. 2012, Zhou, Wu et al. 2013), therefore, whether baseline expression of CD11b influenced the ability of anti-CD20mAbs to induce up-regulation of MFI of CD11b was investigated.
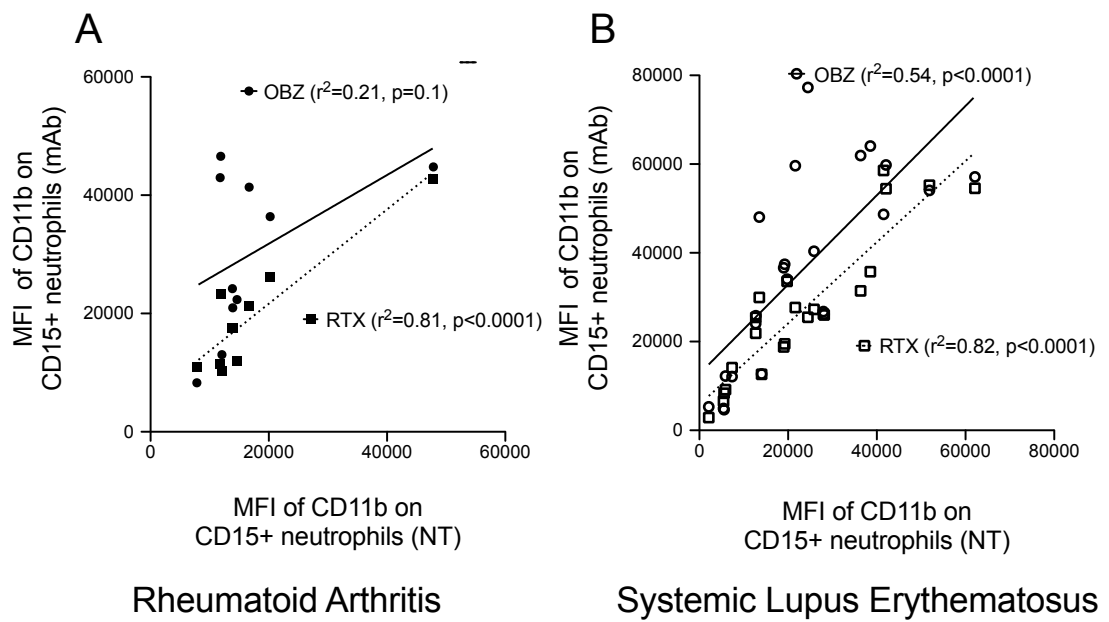
Figure 5.16 Obinutuzumab is more efficient than Rituximab at Activating Neutrophils in Rheumatoid Arthritis and Systemic Lupus Erythematosus.

Each symbol represents the result from a patient sample. The lines represent the correlation by linear regression and the correlation coefficient, r^2 was considered significant when p was at least < 0.05.

In both RA and SLE, significant correlations were noted between the MFI of CD11b in samples incubated in the absence of mAbs and in samples incubated with rituximab (r^2 = 0.81, 0.82, respectively) whereas such significant correlation for obinutuzumab was noted in SLE (r^2 = 0.81), but not RA (Figure 5.16A and B).
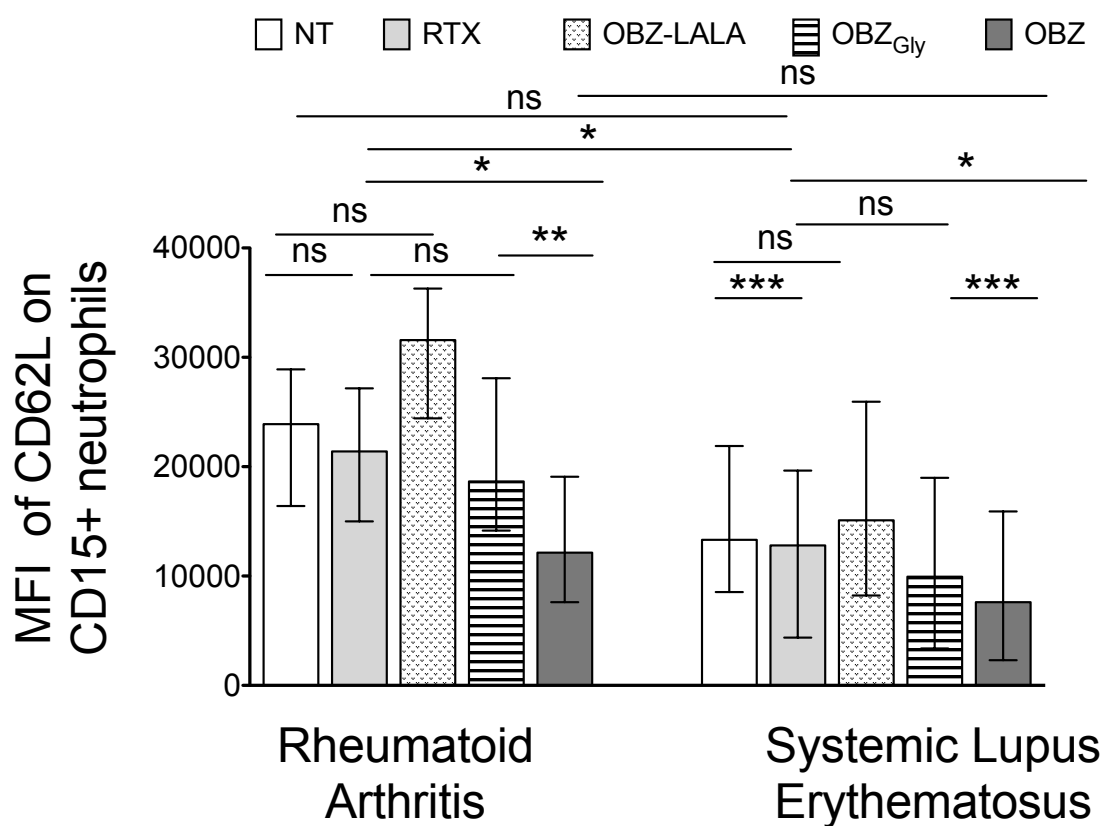
Figure 5.17 Obinutuzumab is more efficient than Rituximab at Activating Neutrophils in Rheumatoid Arthritis and Systemic Lupus Erythematosus.

The Median and IQR are represented by the error bars. * p < 0.05. * p < 0.05; **, p < 0.001; ***, p < 0.0001.

There were no significant differences in the MFI of CD62L in samples incubated without mAbs compared to samples incubated with rituximab and OBZ-LALA in RA (n = 6). However, in SLE, a small but significant difference in the MFI of CD62L was noted in samples incubated without mAbs compared to samples incubated with rituximab (p < 0.0001) (n=23), but not OBZ-LALA (n= 12). In both RA and SLE, the MFI of CD62L was greater in samples incubated with: rituximab > obinutuzumab, but not OBZ$_{Gly}$, by Wilcoxon matched-pairs signed test, p < 0.05 for both (Figure 5.17). However, in both RA and SLE, the MFI of CD62L was significantly lower in samples incubated with obinutuzumab compared to samples incubated with OBZ$_{Gly}$, by Wilcoxon matched-pairs signed test, p < 0.05 and p < 0.0001, respectively (Figure 5.17).
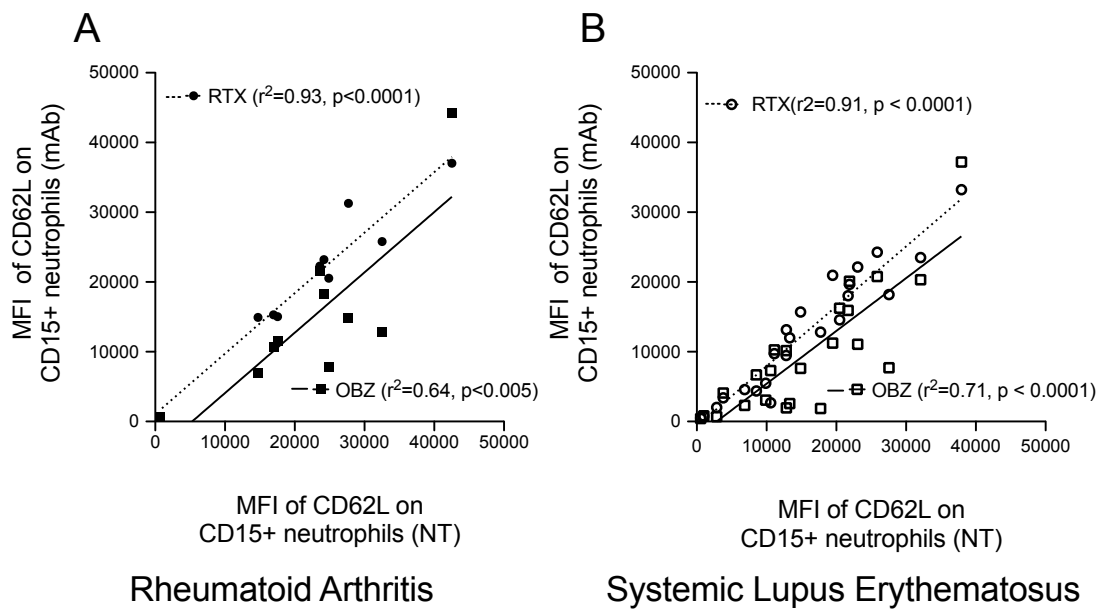
Figure 5.18 Obinutuzumab is more efficient than Rituximab at Activating Neutrophils in Rheumatoid Arthritis and Systemic Lupus Erythematosus.

Each symbol represents the result from a patient sample. The lines represent the correlation by linear regression and the correlation coefficient, r2 was considered significant when p was at least < 0.05.

In RA, significant correlations were found between the MFI of CD62L in samples incubated in the absence of mAbs and that in samples incubated with rituximab (r^2 = 0.93, 0.91, respectively) and obinutuzumab (r^2 = 0.64, 0.71, respectively) (Figure 5.18A). In SLE, also significant correlations were found between the MFI of CD62L in samples incubated in the absence of mAbs and that in samples incubated with rituximab (r^2 = 0.93, 0.91, respectively) and obinutuzumab (r^2 = 0.64, 0.71, respectively) (Figure 5.18B).

Taken together, these data indicated that obinutuzumab was superior to rituximab in activating neutrophils in the whole blood assay in both RA and SLE samples.

Direct Cell Death by anti-CD20 mAbs

Finally, the fourth effector mechanism by which anti-CD20 mAbs induce B-cell depletion is by direct cell death (DCD). Whilst anti-CD20 mAbs activate all four mechanisms, in vivo, the predominant mechanism may potentially differ based on the location of B cells, as access to effector cells may be limited by circulatory dynamics (Gong, Ou et al. 2005). As discussed in the Introduction, B cells opsonized with anti-CD20 mAbs were detectable in lymph nodes and spleen of patients four weeks after treatment with a single low (500mg) dose of rituximab (Kamburova, Koenen et al. 2013, Wallin, Jolly et al. 2014). These findings suggest that at least DCD by anti-CD20 mAbs was not effective.

Obinutuzumab is more efficient than rituximab at inducing direct cell death

The ability of mAbs to induce direct cell death was next assessed. Previous in vitro data on the efficiency of mAbs at inducing direct cell death was mostly derived from studies using malignant B cell lines in experiments performed with hyper-cross linking of mAbs. However, whether hyper-cross linking of mAbs occurs in vivo is not known and therefore, here, the DCD assays were performed without hyper-cross linking of mAbs, using B cells isolated from patients with RA and SLE. The frequency of Annexin V + cells represented cell death.

Spontaneous cell death

Isolated B cells incubated in RPMI supplemented with 10% foetal calf serum for 6 hours at 37°c and 5% CO_2 were analyzed by flow cytometry. The frequency of Annexin V + cells represented direct cell death in CD19+ cells as a whole and also in B-cell subpopulations categorized into naïve (IgD+CD27-), unswitched memory cells (IgD+CD27+), switched memory cells (IgD-CD27+) and double negative cells (IgD-CD27-).

A remarkable disparity was noted between B-cell subpopulations in susceptibility to spontaneous cell death. IgD+CD27+ unswitched memory cells were significantly more susceptible to spontaneous cell death compared to IgD+CD27- naïve cells and IgD-CD27+ switched memory cells (Figure 5.19). Although there was a trend toward greater frequency of Av+ cells in IgD+CD27+ unswitched memory cells compared to Av+ IgD-CD27- cells, perhaps owing to a remarkable variability in samples, this was not significant.
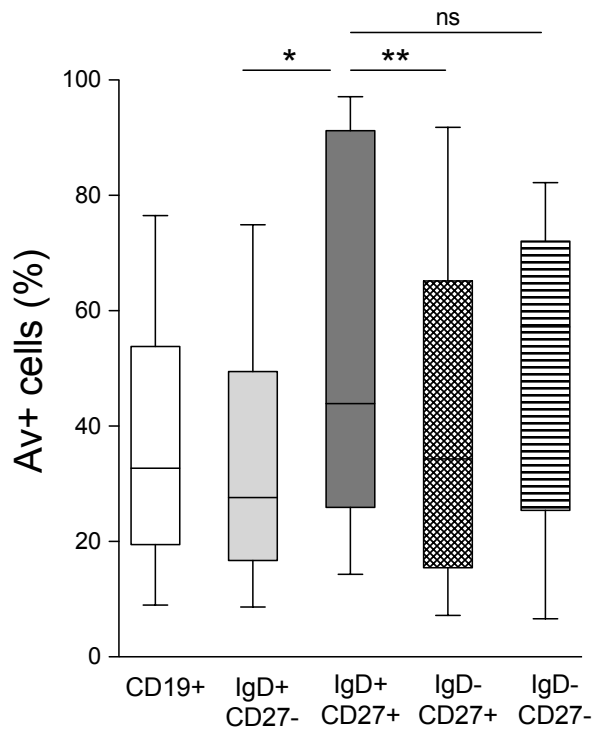
Figure 5.19 Susceptibility to spontaneous cell death in B-cell subpopulations.

Av, Annexin V; * p < 0.05; ***, p < 0.0001; ns, not significant.

Having established this, the ability of type I and II anti-CD20 mAbs to induce direct cell death in isolated B cells from five patients with RA and four with SLE was compared. As described in Chapter 2, Methods, isolated B cells were incubated for 6 hours, with anti-CD20 mAbs, used at a concentration of 10 μg/mL before analysing by flow cytometry. The frequency of CD19+AnV+ cells represented non-viable B cells. Further, the ability of mAbs to induce cytotoxicity in different B-cell subpopulations categorized based on the relative expression of IgD and CD27 was investigated.

Obinutuzumab induced greater direct cell death than rituximab in both CD19+ cells as a whole and in B-cell subpopulations including IgD+CD27- naïve cells and IgD-CD27+ switched memory cells (Figure 5.20).
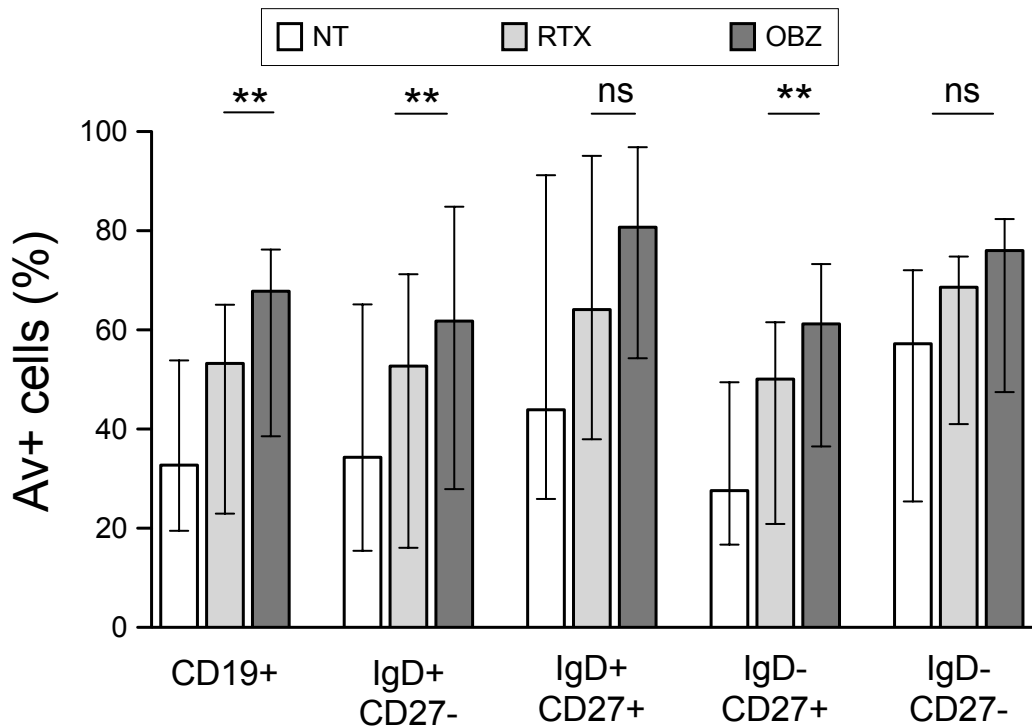
Figure 5.20 Disparity among B-cell subpopulations in anti-CD20 mAb-induced direct cell death.

The error bars represent the median and IQR. **, p < 0.001.

The frequency of Annexin V+ cells in CD19+ cells as a whole and in B-cell subpopulations based on the relative expression of IgD and CD27: IgD+CD27- naïve cells; IgD+CD27+ unswitched memory cells; IgD-CD27+ switched memory cells; and IgD-CD27- double negative cells; in samples from patients with RA (n = 5) and SLE (n = 4) incubated with or without mAbs. Obinutuzumab induced significantly greater direct cell death than rituximab in isolated CD19+ B cells in this assay.
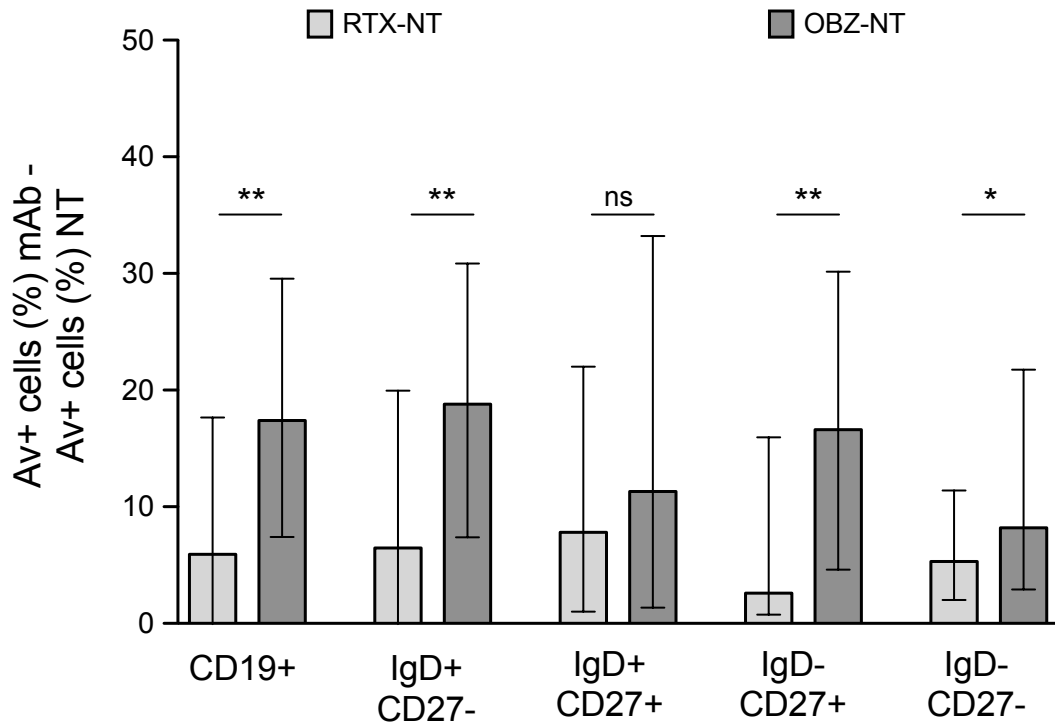
Figure 5.21 Anti-CD20 mAb-induced direct cell death in B-cell subpopulations

NT, not treated; Av, Annexin V; *, p<0.05; **, p<0.005.

After allowing for background cell death noted in samples incubated without mAbs, mAb-induced DCD was significantly lower in samples incubated with rituximab compared to obinutuzumab with a median (IQR) CD19+Av+ cells of 5.9% (1.5 %-17.65%) and 17.4%(7.4 – 29.5%), respectively (Figure 5.21). There was a significant difference in the median (IQR) frequency of Av+ B-cell subpopulations in samples incubated with rituximab and obinutuzumab compared to samples incubated with mAbs with 6.4 % (-2.2% - 19.9%) and 18.8% (7.3% - 30.8 %) for IgD+CD27- naïve; 7.8% (1%- 22%); 2.6% (0.7%- 15.9%) and 16.6% (4.6%-30.1%) for IgD-CD27+ switched memory cells; 5.3% (2%-11.4%) and 8.2% (2.9%-21.7%) for IgD-CD27- B cells, respectively (Figure 5.21). However there was no such difference noted for IgD+CD27+ unswitched memory cells (Figure 5.21). Thus, obinutuzumab induced greater DCD of CD19+ B cells as a whole and also in all but IgD+CD27+ B-cell subpopulations, compared to rituximab in this assay, after allowing for spontaneous cell death in test conditions.
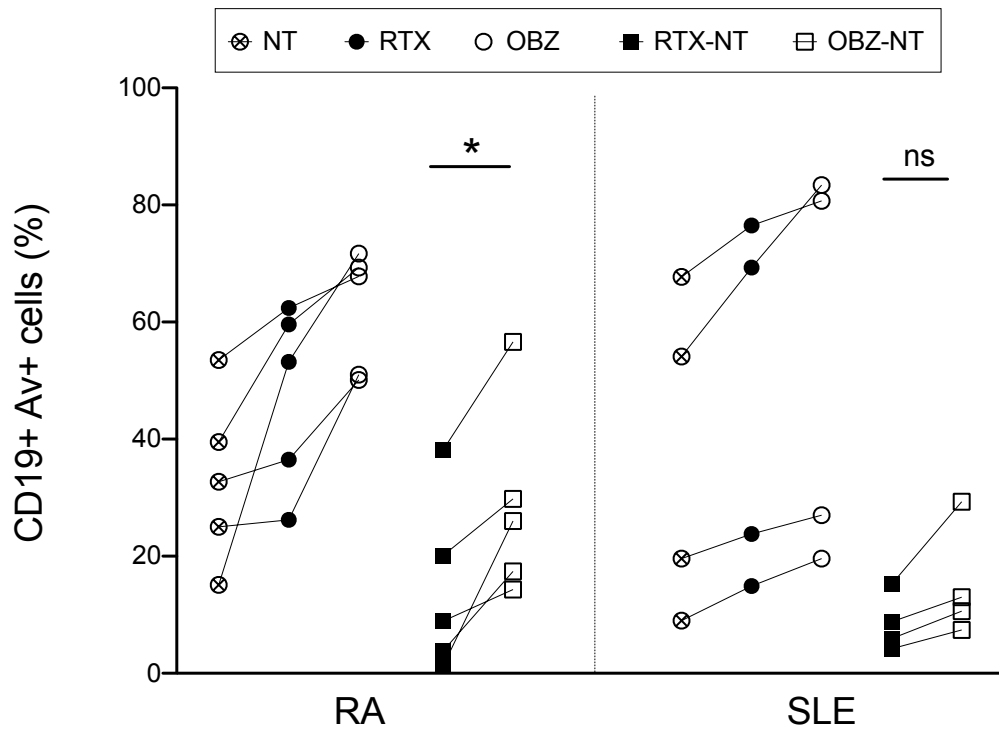
Figure 5.22 mAb-induced direct cell death in RA and SLE.

NT, not treated; Av, Annexin V; * p<0.05; ns, not significant.

The results were also analysed separately for samples from patients with RA and SLE. In the subgroup analysis of DCD in RA and SLE samples, obinutuzumab induced greater DCD in RA, but not in SLE samples, compared to rituximab in the DCD assay (Figure 5.22). Thus mAb induced DCD was relatively weak in this assay, particularly with rituximab. However, a larger sample size would be required to draw any firm conclusions.

Therefore, next, whether differences between B-cell subpopulations in the expression of CD20, FcγRIIb and/or their ability to internalise mAbs provided explanations for resistance to mAb-induced DCD was explored.

Factors influencing DCD induced by anti-CD20 mAbs

The series of experiments in the previous chapter highlighted that B cell surface expression of the inhibitory FcγRIIb and B-cell activation influenced internalisation of rituximab to a greater extent than GA101$_{gly}$. Based on these observations, the objective, here, was to investigate whether B cell expression of CD20 and FcγRIIb also influenced internalisation of rituximab and obinutuzumab in the experiments described earlier in the this Chapter (Figure 5.2) using paired patient samples for

both internalisation assays and for B-cell expression of CD20 and FcγRIIb. The mean fluorescence intensity of CD20 and FcγRIIb was used to represent surface expression as described in the Methods. Samples from five patients with RA and four with SLE were studied.

Internalisation of mAbs in B-cell subpopulations
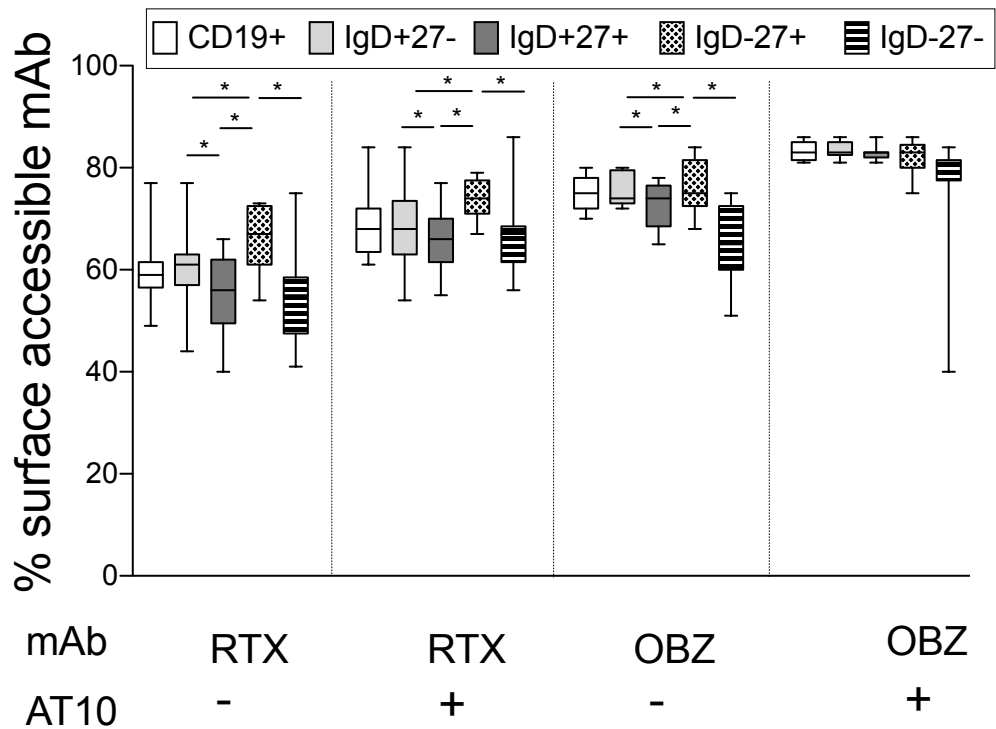
Figure 5.23 Internalisation of type I and II anti-CD20 mAbs in B-cell subpopulations.

*, $p < 0.05$. AT10, anti-FcγRII mAb.

As noted in previous chapter, B-cell subpopulations displayed varying ability to internalise mAbs such that IgD-CD27+ switched memory cells internalised mAbs to a significantly lesser extent than other B-cell subpopulations whereas IgD+CD27+ unswitched memory cells internalised mAbs to a greater extent than other B-cell subpopulations, however, antagonizing the effects of FcγRIIb on B cells significantly reduced internalisation of mAbs in both cases. The least variability in internalisation in CD19+ cells was noted in samples incubated with obinutuzumab after blocking FcγRIIb with AT10 in contrast to samples incubated with rituximab alone, where a

remarkable variability was noted (Figure 5.23). Variability in internalisation was also noted in B cell subpopulations, IgD-CD27- DN cells in particular.

CD20 expression in B-cell subpopulations

B-cell subpopulations express variable levels of CD20. As discussed earlier, CD20 engagement with anti-CD20 mAbs, in vitro, was shown to trigger BCR signaling, consequently, cell death. Given, the variability in susceptibility of B-cell subpopulations to DCD by mAbs, whether CD20 expression on B-cell subpopulations may underlie the disparity noted in DCD by mAbs was investigated.

IgD+CD27+ unswitched memory cells had significantly greater expression of CD20 than other B-cell subpopulations (Figure 5.24). However, there was no significant difference in CD20 expression between naïve and IgD-CD27+ switched memory cells whereas IgD-CD27- DN cells displayed variable degrees of CD20 expression. Thus, there were some similarities in the pattern of CD20 expression in B cell subpopulations and the pattern of spontaneous cell death and DCD by mAbs.
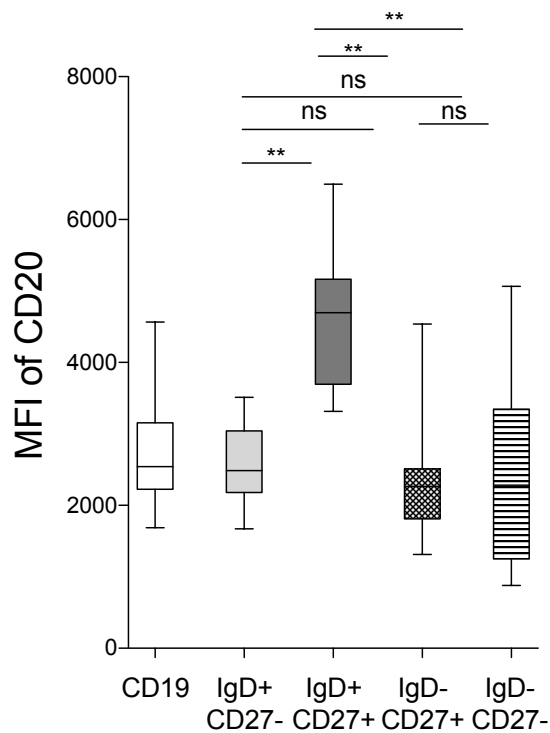
Figure 5.24 CD20 expression in B-cell subpopulations.

Horizontal bar in the box represents the median; the box, the inter-quartile range; and the error bars, the range. **, $p < 0.001$.

FcγRIIb expression in B-cell subpopulations

Previous experiments showed that FcγRIIb, at least in part, regulates internalisation of mAbs and that B-cell subpopulations vary in their ability to internalise mAbs. In SLE, B-cell subpopulations express different levels of FcγRIIb and selective down-regulation of FcγRIIb in CD27+ memory cells was shown to increase B cell hyperactivity (Mackay, Stanevsky et al. 2006). Therefore, the relationship between the pattern of FcγRIIb expression on B-cell subpopulations and the disparity between B-cell subpopulations in their ability to internalise mAbs was explored.
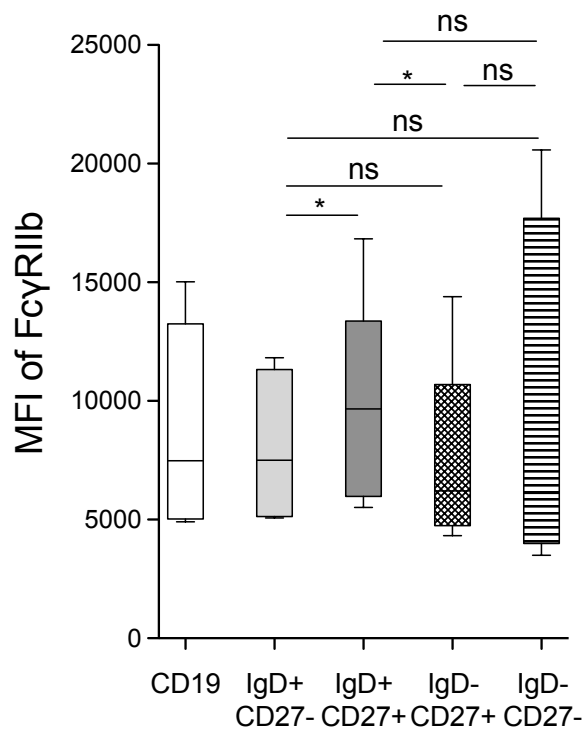
Figure 5.25 FcγRIIb expression in B-cell subpopulations.

Horizontal bar in the box represents the median; the box, the inter-quartile range; and the error bars, the range. **, $p<0.005$; and ns, not significant.

Similar to the results described in the previous chapter, IgD+CD27+ unswitched memory cells had significantly greater expression of FcγRIIb compared to IgD+CD27- naïve, IgD-CD27+ switched memory cells, but not IgD-CD27- DN cells, which displayed remarkable variability in FcγRIIb expression. In this regard, to some extent, the pattern of FcγRIIb expression contrasts with the pattern of internalisation by mAbs (Figure 5.23 and Figure 5.25).

Taken together, when compared to naïve and IgD-CD27+ switched memory cells, IgD+CD27+ unswitched memory cells had significantly greater expression of CD20 and FcγRIIb and displayed significantly greater ability to internalise mAbs whereas naïve and IgD-CD27+ switched memory cells had significantly lower expression of CD20 and FcγRIIb and displayed significantly lower levels of internalisation. IgD-CD27- DN cells had remarkably variable levels of expression of CD20 and FcγRIIb, but internalised rituximab to a significantly greater extent than IgD-CD27+ switched memory cells. B cells from both RA and SLE samples consistently displayed low ability to internalise obinutuzumab. Therefore, the ability to internalise mAbs and B cell expression of CD20 and FcγRIIb likely influence the susceptibility of B-cell subpopulations to mAb-induced DCD.

Discussion

Given that anti-CD20 mAbs evoke four distinct effector mechanisms to delete target B cells: antibody-dependent cellular cytotoxicity (ADCC); antibody-dependent cell phagocytosis (ADCP); complement-dependent cellular cytotoxicity (CDC); and direct cell death (DCD) (Glennie, French et al. 2007), the efficiency of the two types of anti-CD20 mAbs at evoking different effector mechanisms were compared in the whole blood B-cell depletion assay.

Type II anti-CD20 mAb, OBZ_{Gly}, ($GA101_{gly}$) bearing a Fc portion similar to that of rituximab demonstrated a clear superiority in the efficiency of B-cell depletion in whole blood assays compared to the type I anti-CD20 mAb, rituximab. This result suggested that type II nature alone is sufficient to increase the efficiency of anti-CD20 mAbs, in vitro, because the fundamental difference between the two anti-CD20 mAbs lies in the fine epitope specificities, which determines the rate of internalisation (Klein, Lammens et al. 2013). As discussed in the Introduction, the differences between the two types of anti-CD20 mAbs with regards to the predominant mechanisms they evoke are also relevant in RA and SLE, in particular. However, whether type I and II anti-CD20 mAbs differ in their ability to recruit different effector mechanisms as shown in malignant B cells (Cragg and Glennie 2004) in RA and SLE remains elusive.

Assessment of the efficiency of anti-CD20 mAbs in in vitro whole blood assays would be close to the physiological environment in which the anti-CD20 mAbs evoke various effector mechanisms in vivo. However, dissecting the extent to which

individual effector mechanisms are activated is not feasible in whole blood assays. Therefore, complement-dependent cellular cytotoxicity (CDC) and direct cell death (DCD) mechanisms were analysed in respective assays as described in the Methods (Chapter 2). Since both complement (Wang, Racila et al. 2008, Wang, Veeramani et al. 2009) and immunoglobulins (Bologna, Gotti et al. 2011) may interfere with the ability of the mAbs to interact with FcγR-bearing effector cells, the potency of anti-CD20 mAbs at evoking FcγR-mediated effector mechanisms, ADCC and ADCP, were assessed in whole blood assays, simultaneously, which allowed for direct comparison of the efficiency of the two types of anti-CD20 mAbs t o evoke these mechanisms in a physiological environment not devoid of complement and/or immunoglobulins.

The commercially available humanised type II anti-CD20 mAb, obinutuzumab, was glycoengineered to bear an afucosylated Fc portion with enhanced affinity for FcγRIII (CD16). Given, the previous series of experiments demonstrating superior efficacy of OBZ_{Gly}, a type II anti-CD20 mAb with glycosylated Fc similar to rituximab, compared to rituximab, here, the efficiency of rituximab, OBZ_{Gly}, and obinutuzumab at inducing B-cell cytotoxicity was compared in, in vitro, whole blood B-cell depletion assays. The efficiency of the two types of anti-CD20 mAbs at recruiting the four effector mechanisms was also explored.

Efficiency of type I and II mAbs in whole blood B-cell depletion assay

The results from the experiments described in the previous chapter, also revealed that B cells from patients with RA and SLE internalise rituximab influencing its efficiency of depletion, in vitro. Both type II mAbs, OBZ_{Gly} and obinutuzumab demonstrate superior efficiency over type I mAb, rituximab, at inducing B-cell death in whole blood assays in both RA and SLE patient samples. Obinutuzumab was also superior to OBZ_{Gly} at inducing B-cell depletion in whole blood assays. The superior efficiency of obinutuzumab in whole blood assays was noted in all individual samples. Taken together, these results indicated that type II nature and afucosylation of Fc had independent effects at enhancing the efficiency of obinutuzumab at inducing B-cell cytotoxicity.

Complement-dependent cellular cytotoxicity

Consistent with previous data in malignant B-cells and/or cell lines (Mossner, Brunker et al. 2010, Bologna, Gotti et al. 2011, Tipton, Roghanian et al. 2015),

rituximab recruited complement more efficiently than obinutuzumab to induce B-cell cytotoxicity, in in vitro CDC assays. The effect of CD20 expression on CDC was not explored here, but would be important particularly in the context of variable expression of CD20 in B-cell subpopulations. Given, that complement defects are characteristic of SLE (Walport 2002), the efficiency of rituximab may be compromised in SLE, where type II characteristics of obinutuzumab may provide a mechanistic advantage over rituximab in SLE patients with complement defects.

FcγR-dependent cell cytotoxicity: NK cell mediated ADCC

The superior efficiency of obinutuzumab in the whole blood assay despite inferior ability to evoke CDC suggests that the predominant mode of action of obinutuzumab is through FcγR-mediated effector mechanisms and/or direct cell death. Whilst there was no difference between patients with RA and SLE in the frequency of activated NK cells that lacked CD16 expression and/or expressed CD107a, NK cells from patients with both RA and SLE responded less well to stimulation with rituximab compared to obinutuzumab. Activation of NK cells by anti-CD20 mAbs is also associated with loss of cell surface expression of CD16 revealing remarkable differences in activation of NK-cell subpopulations based on relative expression of CD16 and up-regulation of the degranulation marker, CD107a. Whereas rituximab was less efficient at activating NK cells in both RA and SLE, obinutuzumab induced a greater fold-increase in activating NK cells in samples from patients with RA compared to SLE, suggesting that SLE-associated NK cell defects such as impaired up-regulation of CD107a may also contribute to poor depletion with rituximab (Katz, Zaytoun et al. 1982, Neighbour, Grayzel et al. 1982, Hervier, Beziat et al. 2011, Henriques, Teixeira et al. 2013).

The highest proportion of CD107a+ NK (CD3-CD56+) cells was seen in the CD56+CD16- fraction and lowest proportion of in the CD56+CD16++ fraction suggesting that degranulating NK cells had down regulated CD16 as previously reported (Grzywacz, Kataria et al. 2007). NK cell subpopulations may also differ in the levels of expression of CD56. CD3-CD56+CD16- subpopulation of NK cells are considered mature and mostly resident in lymphoid tissues with a small variable frequency of approximately 10% detectable in peripheral circulation whereas CD3-CD56++CD16+ NK cells form the majority subpopulation in the periphery (Moretta, Marcenaro et al. 2008). Therefore, CD3-CD56+CD16- NK cells are less likely to be activated by anti-CD20 mAbs.

The relative inefficiency of rituximab at evoking ADCC in vitro may, at least partly, be due to internalisation of mAbs leading to reduced surface accessibility of mAbs for engagement with FcγR-receptor bearing effector cells, as shown previously (Cragg and Glennie 2004, Tipton, Roghanian et al. 2015). In vitro studies in malignant B-cell-lines suggest that HLA expression and the interaction between HLA and inhibitory killer immunoglobulin receptors (KIR) may also diminish rituximab-induced cytotoxicity by NK cells (Borgerding, Hasenkamp et al. 2010). Other factors such as complement (Wang, Racila et al. 2008) and immunoglobulins (Bologna, Gotti et al. 2011) purportedly inhibit mAb-induced ADCC whereas afucosylation of Fc increases the affinity of IgG1 to FcγRIIIa with little effect on binding to complement (Shields, Lai et al. 2002), which may explain the superior efficiency of obinutuzumab at activating NK cells in the whole blood assay even in the presence of complement (Kern, James et al. 2013) and immunoglobulins (Iida, Misaka et al. 2006). However, NK cell mediated cytotoxicity is particularly important for B cells in peripheral circulation, but probably less likely so for depletion in tissues, as tissue resident NK cells do not express CD16 (Moretta, Marcenaro et al. 2008). Taken together, the superior efficiency of obinutuzumab at activating NK cells is attributable to a greater surface accessibility owing to type II nature and a greater affinity for CD16 conferred by afucosylation of Fc.

FcγR-dependent cell cytotoxicity: neutrophil mediated ADCP

These results indicated that obinutuzumab is superior to rituximab at activating neutrophils. The findings of superior neutrophil activation by obinutuzumab compared to rituximab in RA and SLE samples are consistent with studies in malignant B-cells (Golay, Da Roit et al. 2013). Polymorphisms of CD16b may at least partially account for the variability in mAb-induced activation, most notable for rituximab whereas afucosylation may have reduced this variability, as described previously (Golay, Da Roit et al. 2013). A number of polymorphisms of CD11b associated with SLE may have contributed to the variability between patients in neutrophil activation (Zhou, Wu et al. 2013), regardless; glycoengineered obinutuzumab was more efficient than wild type obinutuzumab and rituximab at inducing neutrophil activation.

Direct cell death

Obinutuzumab induced greater DCD, in vitro, in CD19+ cells and IgD-CD27+ switched memory cells from patients with RA and SLE, compared to rituximab.

These findings are similar to those in malignant B cells (Mossner, Brunker et al. 2010). As discussed chapter one, Introduction, several lines of enquiry in malignant B cells suggested that B-cell expression of the target antigen CD20 influences sensitivity to rituximab, particularly in relation to induction of CDC. A tantalizing possibility is that lower levels of CD20 expression of IgD-CD27+ switched memory cells and IgD-CD27- DN cells may, at least in part, aid evasion of rituximab-cytotoxicity, as proposed for chronic lymphocytic leukemia (Horvat, Kloboves Prevodnik et al. 2010), where in combination with chemotherapy obinutuzumab was superior to rituximab in improving clinical response (De Vita, Quartuccio et al. 2014). Also B cell expression of IgD and the activation state of B cells may also influence internalisation of mAbs compromising their cytotoxicity.

Patterns of susceptibility to direct cell death by anti-CD20 mAbs, expression of CD20 and FcγRIIb and internalisation of mAbs in B-cell subpopulations

As discussed in Chapter 1, IgD-CD27+ switched memory B cells appear to evade cytotoxic effects of rituximab both in peripheral blood and in lymph nodes suggesting that sensitivity to rituximab-induced depletion is dependent on both B-cell subpopulations and also on their location (Kamburova, Koenen et al. 2013, Wallin, Jolly et al. 2014). These findings are also clinically relevant in RA and SLE because, following rituximab, IgD-CD27+ switched memory cells and IgD-CD27- DN cells are detectable in peripheral blood before IgD+CD27- naïve and IgD+CD27+ unswitched memory cells in patients with RA and SLE (Leandro, Cambridge et al. 2006, Leandro 2013, Adlowitz, Barnard et al. 2015). Although IgD-CD27+ switched memory cells and IgD-CD27- DN cells are likely not depleted by rituximab, it is also plausible that a proportion may have been newly formed from a small frequency of non-depleted IgD+CD27- naïve cells that are detectable in lymph nodes as shown in patients treated with low-dose (500mg) rituximab as part of pre-conditioning for kidney transplantation (Kamburova, Koenen et al. 2013, Wallin, Jolly et al. 2014). Non-depleted B cells from lymph nodes appear to be capable of activation and proliferation, in vitro, even in the presence of low-concentration of rituximab (Kamburova, Koenen et al. 2013). Taken together, achieving a better depletion of IgD-CD27+ memory B cells is important, but the underlying mechanisms for the apparent resistance to rituximab are poorly understood.

Conclusions

Thus, the results described in this chapter show that obinutuzumab, a type II anti-CD20 mAb with afucosylated Fc portion demonstrated at least 2-fold greater potency at inducing cytotoxicity in B-cells from patients with both RA and SLE, effected predominantly through Fc gamma receptor (FcγR)-mediated effector mechanisms and FcγR-independent DCD compared to rapidly internalizing rituximab, a type I mAb, which recruited complement more efficiently, but was significantly less efficient at evoking FcγR-mediated effector mechanisms and DCD. The findings also suggest that the apparent resistance of IgD-CD27+ switched memory cells and IgD-CD27- DN cells to removal by rituximab may, at least partly, be due to the relatively lower levels of CD20 expression compared to IgD+CD27+ unswitched memory cells.

Taken together, these results showing superior efficiency of obinutuzumab over Rituximab, noted in the whole blood assay system is most likely to be due to FcγR-mediated effector mechanisms and FcγR-independent direct cell death. These results therefore provide compelling mechanistic basis for considering the use of obinutuzumab as an alternative B-cell depleting agent in RA and SLE, in particular.

Chapter 6   General Discussion of Results

Improving B-cell depletion in Rheumatoid Arthritis and Systemic Lupus Erythematosus: Resistance to Rituximab and the Potential of Obinutuzumab

Several mechanisms of resistance to rituximab potentially influence the clinical response to rituximab in RA and SLE. Whilst a single resistance mechanism is unlikely to explain poor response noted in a significant number of patients, a critical evaluation of the currently available evidence suggests that a failure to achieve effective B-cell depletion with rituximab may be an important resistance mechanism. Therefore, a better understanding of the resistance mechanisms that underlie incomplete B-cell depletion would therefore help our efforts to improve B-cell depletion and enhance clinical response with B-cell depletion therapy in RA and SLE. The availability of the glycoengineered afucosylated type II anti-CD20 mAb, obinutuzumab, which has been shown to be superior to rituximab in treating patients with CLL with coexisting conditions (Goede, Fischer et al. 2014), and also demonstrated to be useful in some patients with rituximab-refractory follicular lymphoma (Salles, Morschhauser et al. 2013), provided the impetus to investigate its potential in delivering more efficient B-cell depletion in RA and SLE.

Duration of B-cell depletion in vivo in RA and SLE

The observed associations between incomplete B-cell depletion and poor and/or partial clinical response in RA (Vital, Rawstron et al. 2011) and SLE (Vital, Dass et al. 2011); and the association between prolonged duration of B-cell depletion and better clinical response in SLE (Dias, Rodriguez-Garcia et al. 2015) suggest the clinical relevance of achieving complete and/or durable B-cell depletion. Further, the improvement in clinical response in those with partial response following an additional dose of rituximab in RA (Vital, Dass et al. 2015) and also in a small number of patients with SLE (Isenberg, unpublished observations) also suggest that incomplete or shorter duration of B-cell depletion may not only explain, at least partly, the variability in clinical response noted in patients with RA and SLE, in particular, but also support the notion that improving B-cell depletion may enhance clinical response in both RA and SLE.

During the early studies at UCLH investigating the use of B-cell depletion therapy in patients with refractory RA and SLE, a remarkable variability was observed in B-cell depletion achieved between patients. It was also noted that a majority of patients

with RA achieved predictable and durable duration of B-cell depletion whereas the extent and duration of B-cell depletion achieved in some patients with SLE was limited compared to patients with RA although no directly comparative data was published. The data presented here demonstrated that the duration of peripheral B-cell depletion was shorter in patients with SLE compared to RA. Some patients with SLE had detectable CD19+ B cells in peripheral circulation as early as one month after the first dose of rituximab with the proportion of patients with detectable CD19+B cells in peripheral circulation increasing at three and six months after the first dose of rituximab. These observations, given the same therapeutic regimen of rituximab employed in both RA and SLE, allowing for the differences in co-therapies, may serve to speculate that the relatively shorter duration of B-cell depletion noted in patients with SLE may be due to inherent disease-associated mechanisms of resistance to rituximab.

In this context, the use of varied and arbitrary thresholds for defining B-cell depletion (typically 10,000 – 20,000) in clinical studies thus far (Leandro, Edwards et al. 2002, Leandro, Edwards et al. 2002, Leandro, Cambridge et al. 2005, Smith, Jones et al. 2006, Dias, Rodriguez-Garcia et al. 2015) and the EXPLORER and LUNAR randomised clinical trials (Merrill, Neuwelt et al. 2010, Rovin, Furie et al. 2012), has constrained any efforts at identifying a threshold of B-cell depletion that is predictive of clinical response. Therefore, it would be clearly important to define a standard threshold for defining B-cell depletion that may be tested in future clinical trials. Furthermore, using highly sensitive flow cytometry in clinical trials to evaluate B-cell depletion may help define thresholds of B-cell subpopulations associated with clinical response, which otherwise are less likely to be identified using a lower threshold of CD19+ B cell counts (Vital, Dass et al. 2011).

Although riddled with practical limitations, the evaluation of B-cell depletion at the sites of inflammation such as the kidney, shown to be important in understanding rejection of transplanted organ where rituximab was used prophylactically to prevent organ rejection (Thaunat, Patey et al. 2008); and in lymphoid tissues may also be vital to understand the mechanisms of resistance to B-cell depletion therapy. Minimally invasive techniques with acceptable adverse risk profile such as fine needle aspiration cytology of peripheral lymph nodes may be one such tool worth considering. Synovial studies in RA revealed the presence of plasma cells in those with poor response to rituximab and therefore represent an important mechanism of resistance, as plasma cells are not directly targeted by rituximab. The identification

of long-lived plasma cells in the spleen of rituximab-refractory patients with ITP, but not in those not treated with rituximab, suggests that persistence of plasma cells in lymphoid tissues as a mechanism of resistance to rituximab (Mahevas, Patin et al. 2013). Thus far, evidence about B-cell depletion and/or in the plasma cell persistence in lymphoid tissues in RA and SLE after rituximab remains elusive.

Long-term effects of rituximab on serum immunoglobulins and anti-dsDNA antibodies

Immunoglobulins are continuously generated by terminally differentiated plasma cells, which are only indirectly targeted by rituximab through transient B-cell depletion whereas removal of pathogenic antibodies such as anti-dsDNA antibodies would be beneficial for clinical response. Whilst data about the long-term effects of rituximab on immunoglobulins in RA are well described, such data in SLE are lacking, but clearly needed. Given, the pragmatic use of repeat cycles of rituximab-based B-cell depletion therapy concurrently with immunosuppressants in SLE, the long-term effects on changes in serum Ig levels and anti-dsDNA antibodies was explored here.

In patients with SLE, rituximab had disparate effect on serum levels of different immunoglobulin isotypes and anti-dsDNA antibodies. Hypogammaglobulinemia was restricted to the IgM isotype. Patients with lower baseline serum IgM levels and those who received sequential therapy with MMF either developed or had persistently low IgM levels in the long-term after rituximab therapy. The data about IgM is consistent with previous studies investigating the use of the combination of MMF (3 g/day) and rituximab (Mysler, Spindler et al. 2013) or atacicept (Ginzler, Wax et al. 2012), where patients developed low serum immunoglobulins and/or infections associated with this combination of therapies. However, the data presented here is not directly comparable to these studies because of the pragmatic nature of the use of MMF, often used at a lower dose < 3g/day, following rituximab and the long-term follow-up in contrast to the two studies with pre-defined therapeutic regimen and fixed duration of follow-up although both studies were discontinued prematurely due to the adverse events. Therefore, the data about the long-term effects of rituximab on serum immunoglobulins may be useful in stratifying patients at risk of developing low serum IgM levels in routine clinical practice.

Serum IgA and IgG levels on the other hand remained within the normal range at long-term follow-up after rituximab therapy and concurrent immunosuppressive

therapy. Allowing for the differences in therapeutic regimens including the cumulative dose of rituximab, the choice of and the duration of concurrent immunosuppressive therapy, the data about serum IgG levels contrasts with that reported in patients with ANCA-associated vasculitis (Marco, Smith et al. 2014, Roberts, Jones et al. 2015) and immune thrombocytopenia (Cooper, Davies et al. 2009), where some patients required IVIG replacement to treat hypogammaglobulinemia associated complications. Nonetheless, in SLE patients treated with rituximab, judicious use of MMF and monitoring of serum Ig levels is essential to minimise the risk of hypogammaglobulinemia.

The effect of rituximab on serum anti-dsDNA antibodies varied between patients. Anti-dsDNA antibodies normalised in patients with lower baseline levels, whereas serum anti-dsDNA antibody levels were persistently high in a significant number of patients, particularly in those with higher baseline levels. A plausible consideration for the latter finding may be that elevated serum BAFF levels following B-cell depletion therapy in patients with SLE (Cambridge, Stohl et al. 2006) may promote the survival of autoreactive B cells and/or plasma cells (Benson, Dillon et al. 2008) facilitating increased secretion of anti-dsDNA antibodies (Carter, Isenberg et al. 2013). However, the analysis of the data here revealed that baseline anti-dsDNA antibody levels, regardless of serum BAFF levels, were predictive of persistently high levels following rituximab therapy. Hence, persistently elevated anti-dsDNA antibody levels in those with high baseline levels may represent a potential resistance mechanism to rituximab-based B-cell depletion therapy. It would be interesting to explore whether plasma cell persistence in the kidneys may account for rituximab-refractory lupus nephritis. Collectively, these findings may be useful in stratifying patients who are likely to require additional therapies targeting excess BAFF with Belimumab and/or plasma cells with proteasome inhibitors such as bortezomib (Alexander, Sarfert et al. 2015).

Thus, understanding the variability in biological response as assessed by changes in serum immunoglobulins and anti-dsDNA antibody levels may help in the management of the risk of hypogammaglobulinemia and predict the long-term outcomes about anti-dsDNA antibody levels in patients with SLE following treatment with rituximab and concurrent immunosuppressant therapy.

B-cell depletion and serum rituximab levels

The relationship between serum rituximab levels and the duration of B-cell depletion in RA (Breedveld, Agarwal et al. 2007) and SLE (Looney, Anolik et al. 2004) has been explored in previous studies raising several unanswered questions. For example, what would be a desired therapeutic level of rituximab to achieve effective B-cell depletion? Of particular interest, would be to understand whether the simultaneous detection of CD19+B cells and serum rituximab in peripheral circulation represents resistance to rituximab. Do the 'non-depleted' CD19+ B cells represent truly CD19+CD20- B cells such as plasma blasts / plasma cells or alternatively the 'non-depleted' CD19+Bcells also express CD20, which is simply masked by rituximab and therefore undetectable by flow cytometry. Evidence for the latter interpretation has been demonstrated in nonmalignant B-cell depletion in the lymphoid tissues using fluorescent-tagged anti-rituximab mAbs (Kamburova, Koenen et al. 2013). Thus, at least, in the lymphoid tissues CD19+ B cells evade depletion despite opsonisation with rituximab.

What is the relevance of HACAs to rituximab resistance? Intriguingly, the occurrence of HACAs is higher in SLE than RA. HACAs may inhibit rituximab-induced B-cell depletion by simply neutralising rituximab. However, the clinical response to rituximab was not significantly different in patients with SLE who developed HACAs compared to those with no HACAs in the EXPLORER study (Merrill, Neuwelt et al. 2010). Moreover, in RA, humanised anti-CD20 mAbs do not appear to improve clinical response compared to that achieved by rituximab although there are no directly comparable data. However, humanised mAbs may provide mechanistic advantages in that serum sickness like reactions noted with rituximab would be expected to be lower and therefore better tolerated (Thornton, Ambrose et al. 2015).

Factors other than HACAs such as IgG metabolism may also influence serum rituximab levels. Lower serum rituximab levels noted here in some patients with RA and SLE, may impair the efficiency of anti-CD20 mAbs, particularly in SLE. A more rapid clearance of rituximab in SLE may also be influenced by lower serum half-life of IgG due to altered metabolism in SLE compared to RA, independent of proteinuria (Levy, Barnett et al. 1970). Therefore, potentially IgG-based mAbs would achieve shorter half-life in SLE. Although internalisation of rituximab by B cells may potentially affect its clearance to what extent this is relevant is not known. Thus, a

number of mechanisms relevant to RA and SLE may influence IgG-based mAb metabolism.

A potential solution to counterbalance rapid clearance of rituximab may be to consider unconventional therapeutic regimens to achieve a steady state level of rituximab for the desired duration, for example, by delivering smaller doses more frequently administered either intravenously or by using subcutaneous preparations. Alternatively, mAbs engineered with a mutated Fc to increase FcRn recycling may be expected to have a longer serum half-life and therefore less susceptible to rapid clearance of mAbs, however, it is pertinent to ensure such modifications do not adversely affect the effector functions.

B-cell expression of CD20 and FcγRIIb

Studies in B cell malignancies revealed differences in the levels of expression of CD20 based on histological type with relatively higher levels in MCL compared to CLL (Ginaldi, De Martinis et al. 1998) and also on location of B cells in CLL, with a decreasing hierarchy of expression in peripheral blood, bone marrow and the lymphoid tissues (Huh, Keating et al. 2001). For example, poor clinical response to rituximab occurs in both MCL and CLL. Further, differences in the target cell expression of FcγRIIb was also shown to be variable between patients with different histological type of B cell malignancy with inverse relationship with clinical response to rituximab (Lim, Vaughan et al. 2011). However, whether there are differences in CD20 and FcγRIIb expression between B cells in peripheral blood, lymphoid tissues and inflammatory sites in RA and SLE are not clearly understood.

The data presented here revealed clear differences in B-cell expression of CD20 and FcγRIIb between patients with RA and SLE. However, the relative expression of CD20 and FcγRIIb, analysed to allow for the combined effect of CD20 expression and internalisation of rituximab mediated by FcγRIIb, was not significantly different between patients with RA and SLE. Therefore, in contrast to studies in B cell malignancies that demonstrated the potential of target cell expression of CD20 and FcγRIIb as biomarkers of clinical response to rituximab, non-malignant B-cell expression of CD20 and FcγRIIb in patients with RA and SLE do not appear to predict response to rituximab. However, the expression of CD20 and FcγRIIb in B-cell subpopulations in patients with RA and SLE provided new insights in that the

relatively lower expression of CD20 by IgD-CD27+ B cells may suggest, at least in part, an inherent ability to evade the cytotoxic effects of rituximab.

Utility of whole blood B-cell depletion assay in comparing the efficiency of mAbs at inducing B-cell depletion

Whilst studies revealing FcγRIIIa genotype associations with clinical response to rituximab have served to inform us about the importance of the effector mechanisms in vivo, there remains a need for an assessment tool that has the potential to compare the efficiency of different anti-CD20mAbs in in vitro assays. Whole blood B-cell depletion assays have been used to compare the efficiency of anti-CD20mAbs in deleting malignant B cells in vitro (Mossner, Brunker et al. 2010, Bologna, Gotti et al. 2013). Given the significant differences in the target cell load and immune effector mechanisms between B cell malignancies and autoimmune disease such as RA and SLE, the utility of whole blood B-cell depletion assay to compare the efficiency of anti-CD20 mAbs at inducing cytotoxicity of B cells from patients with RA and SLE in vitro was explored.

The results described here clearly demonstrate the utility of the autologous whole blood B-cell depletion assay in comparing the efficiency of different anti-CD20mAbs in close-to physiological conditions that could be employed on freshly drawn whole blood samples. Further, the whole blood B-cell depletion assay also accounts for the previously reported complex interactions between complement and excess immunoglobulins and/or immune complexes on FcγR-mediated effector functions. Therefore the results of the whole blood B-cell depletion assay are relevant for the individual patient and also allow for comparison between the two disease cohorts RA and SLE with significant differences in disease-specific immune abnormalities such as defects in complement, excess immunoglobulins and inefficient FcγR-mediated effector mechanisms (discussed later).

As a number of factors, discussed in Chapter one, potentially influence the efficiency of anti-CD20mAbs at inducing B-cell depletion, whether, the efficiency of rituximab as measured by the whole blood B-cell depletion assay is predictive of the duration of B-cell depletion in vivo would also be clearly important to evaluate.

Internalisation of rituximab and the efficiency of B-cell depletion

CD20 was targeted to deplete B cells as malignant B cells express it and because it was not considered to shed or internalise from B-cell surface (Beers, Chan et al. 2010). However, subsequent studies investigating the mechanisms of resistance to rituximab revealed that CD20 became internalised in complex with anti-CD20mAbs to a greater extent after engagement with type I anti-CD20mAbs compared to type II anti-CD20mAbs limiting their efficiency of B-cell depletion (Beers, Chan et al. 2008, Lim, Vaughan et al. 2011, Tipton, Roghanian et al. 2015). Efforts to improve B-cell depletion in RA and SLE with humanised type I anti-CD20mAbs such as ocrelizumab and ofatumumab delivered comparable clinical responses to rituximab (Taylor, Quattrocchi et al. 2011, Rigby, Tony et al. 2012, Mysler, Spindler et al. 2013, Reddy, Jayne et al. 2013) suggesting that factors other than HACAs may potentially impair the efficiency of rituximab in RA and SLE (Reddy, Dahal et al. 2016).

The results of the surface fluorescence-quenching assays described here suggest that B cells from patients with RA and SLE also internalise type I anti-CD20mAb, rituximab, to a greater extent than type II anti-CD20mAb, obinutuzumab, and thereby impair its efficiency of B-cell depletion in SLE patient samples in the in vitro whole blood B-cell depletion assay. The lack of significant correlations between internalisation in RA samples and also in subsequent experiments using obinutuzumab may be due to the differences in the levels of internalisation of rituximab in different cohorts. For example, in the latter cohort of RA and SLE samples, the rates of internalisation were lower with surface accessible rituximab > 45% in all samples tested whereas in the earlier cohort there was a greater degree of variability in internalisation with < 45 % surface accessible rituximab in a significant number of patient samples which influenced correlations with B-cell depletion achieved by rituximab in vitro. These seemingly discordant results between the two cohorts are not surprising because a majority of the patients with RA and SLE achieve complete B-cell depletion with rituximab in vivo.

Intriguingly, internalisation of both type I and II anti-CD20mAbs was significantly different between B-cell subpopulations. Internalisation in nonmalignant B-cell subpopulations has not been reported before, but the results of the two cohorts of patient samples described here consistently showed similar patterns in internalisation of anti-CD20mAbs such that IgD-CD27+ B cells internalised anti-CD20mAbs to a significantly lower extent compared to other B-cell subpopulations

whereas IgD+CD27+ B cells internalised to a significantly greater extent than other B-cell subpopulations. Lower levels of internalisation of anti-CD20mAbs would be desirable for B-cell depletion and in that respect IgD-CD27+ B cells would be expected to be more susceptible to B-cell depletion. Further, the results also revealed that B-cell activation also seemed to inhibit internalisation, thus potentially increasing the susceptibility of activated B cells to depletion by rituximab. This could be advantageous as a higher frequency of IgD-CD27+ B cells is associated with poor clinical response to rituximab.

However, factors beyond internalisation including the expression of CD20 and FcγRIIb were shown to influence malignant B-cell depletion. In this context, the results described here highlighted differences between B-cell subpopulations in the expression of CD20 and FcγRIIb. Data about depletion in vivo suggested that IgD-CD27+ B cells are less susceptible to depletion, as assessed by the detection of an increased frequency of IgD-CD27+ B cells in the lymph nodes of patients treated with a single 500mg dose of rituximab (Wallin, Jolly et al. 2014). A plausible interpretation may be that whilst IgD-CD27+ B cells show low levels of internalisation of CD20 probably owing to lower levels of FcγRIIb expression, their baseline CD20 expression was also lower. Thus, the cumulative effect of B-cell intrinsic factors including the expression of CD20, FcγRIIb and B-cell activation and B-cell extrinsic factors such as the microenvironment and circulatory dynamics, as discussed in Chapter one, influence the susceptibility of B-cell subpopulations to depletion in vivo.

Thus, the importance of internalisation as an important rituximab resistance mechanism is underscored by two observations: i) the correlation between surface accessible rituximab in the surface fluorescence-quenching assays and in vitro whole blood B-cell depletion; and ii) the superior efficiency of the wild type obinutuzumab to rituximab, despite similar Fc glycosylation, at inducing B-cell cytotoxicity in in vitro whole blood B-cell depletion assays in RA and SLE patient samples. Further, support for the inverse relationship between internalisation and the efficiency of B-cell depletion was provided by the opposite orders of hierarchy between internalisation and the efficiency of B-cell depletion between isotype matched anti-CD22mAb, type I and II anti-CD20mAbs.

Although obinutuzumab was superior to rituximab at inducing B-cell cytotoxicity in RA and SLE patient samples, the data revealed that the efficiency of B-cell

depletion by anti-CD20mAbs in RA patient samples was significantly greater than compared to SLE patient samples suggesting that disease-associated defects may also contribute to resistance to depletion by anti-CD20mAbs.

Disease-associated immune defects and efficiency of B-cell depletion

Anti-CD20mAbs mediate cell death by four distinct effector mechanisms (Glennie, French et al. 2007), three of which are dependent on the host immune system including complements for CDC and efficient functioning of FcγR-bearing effector cells for ADCC and ADCP. As discussed in Chapter one, defects in complements and FcγR-bearing effector cells have been reported in SLE and disease-related defects in complement, NK cell function and phagocytosis may also reduce the efficiency of type I anti-CD20 mAbs like rituximab, in SLE. In this regard, here, type II anti-CD20 mAbs were more efficient than rituximab, at inducing B-cell cytotoxicity, in vitro, therefore, may provide mechanistic advantages to type I anti-CD20 mAbs.

Complement dependent cellular cytotoxicity

Complement defects are well described in SLE (Walport 2002). Therefore, the efficiency of type I anti-CD20 mAbs such as rituximab that evoke CDC may be compromised. In this regard, the data described here indicated that rituximab is more potent than obinutuzumab at evoking CDC in vitro. Stronger evidence than that described here is required to support any ideas for improving rituximab-induced CDC by simultaneous complement replacement in vivo. Moreover, it has also been suggested that complement deposition may hinder the interaction between anti-CD20mAb-bound target cells and FcγR-bearing effector cells and thereby interfere with ADCC (Wang, Racila et al. 2008, Wang, Veeramani et al. 2009).

As yet, there are no in vivo data to suggest that anti-CD20mAb induced B-cell depletion is compromised in SLE patients with complement deficiency. Perhaps CDC would be important in patients with compromised FcγR-dependent effector mechanisms. Nonetheless, the efficiency of type II anti-CD20 mAbs such as obinutuzumab may be relatively less affected compared to type I anti-CD20 mAbs rituximab by complement defects noted in SLE. In this regard, obinutuzumab may provide mechanistic advantages for achieving relatively uncompromised B-cell depletion compared to rituximab in patients with SLE-associated complement defects.

Antibody-dependent cell cytotoxicity

The associations between the high affinity 158v FcγRIIIa polymorphism and favorable clinical response in patients with RA and SLE highlight the clinical relevance of ADCC as an anti-CD20mAb effector mechanism. NK cells are predominant effector cells for ADCC and their function may be defective in SLE. Therefore, the efficiency of anti-CD20mAbs at evoking ADCC may be compromised in SLE. The data described here revealed remarkable variability in anti-CD20mAb induced activation of NK cells in vitro, which was compromised in with SLE patient samples compared to RA patient samples providing evidence for the relevance of SLE-associated defects at influencing anti-CD20mAb-induced activation of NK cells. Whilst there were modest differences in NK cell activation induced by rituximab and obinutuzumab-wild type, the glycoengineered afucosylated obinutuzumab was consistently more efficient than rituximab at activating NK cells. In this regard, given that FcγRIIIa polymorphisms contribute to variability in the efficiency of B-cell depletion, consequently, clinical response to glycosylated anti-CD20 mAbs such as rituximab, at least in some patients with RA and SLE, this resistance mechanism may potentially be overcome by afucosylated mAbs such as obinutuzumab with a greater affinity for FcγRIIIa. Thus, obinutuzumab may provide mechanistic advantage compared to rituximab in SLE in improving ADCC.

However, even in samples incubated with obinutuzumab there was remarkable variability in NK cell activation. Intriguingly, the correlation between baseline activation status of NK cells and anti-CD20mAb induced NK cell activation in RA and SLE suggest that rectifying SLE-associated NK cell defective function may enhance the efficiency of anti-CD20mAbs at inducing NK cell activation. For example, a better understanding of whether disease-acquired defects such excessive immunoglobulins and/or immune complexes and the effect of concomitant immunosuppressants including CS, AZT, CYC and MMF on NK cell function and the efficiency of anti-CD20mAb-mediated effector functions in SLE may inform our efforts to optimise B-cell depletion by anti-CD20mAbs.

Therefore, obinutuzumab is likely to engage with FcγRIIIa-bearing effector cells such as NK cells and, although not directly assessed here, macrophages, more efficiently, and enhance their ability to induce cytotoxicity and/or phagocytose mAb-bound B cells, respectively.

Antibody-dependent cell phagocytosis

ADCP mediated by macrophages has been shown to be important in malignant B-cell depletion in vivo. However, as yet there is no direct evidence about the importance of ADCP in anti-CD20mAb induced B-cell depletion in RA and SLE. Neutrophils are the predominant effector cells for mediating ADCP in in vitro whole blood B-cell depletion assay. Alterations in cell surface expression of CD62L and CD11b, important for neutrophil migration and phagocytosis, respectively, were studied. Of particular importance is CD11b, the genetic variants of which are associated with defective FcγR-mediated phagocytosis in SLE. The data revealed a significant variability in anti-CD20mAb induced activation of neutrophils in both RA and SLE patient samples in vitro. Obinutuzumab was significantly more efficient than rituximab and obinutuzumab wild type at activating neutrophils in RA and SLE suggesting that afucosylation of the Fc portion conferred additional gains for activating neutrophils.

Although the effect of FcγRIIIb polymorphisms on the efficiency and clinical response to rituximab in RA and SLE is currently not known, obinutuzumab is likely to deliver better B-cell depletion even in patients with the lower affinity FcγRIIIb polymorphism, thereby providing another mechanistic advantage over rituximab. Further, similar to NK cell activation by anti-CD20mAbs, neutrophil activation correlated significantly with baseline activation status suggesting that rectifying disease-associated phagocytic defects may improve anti-CD20mAb induced B-cell depletion in RA and SLE.

Macrophage mediated ADCP was not assessed here, but it would be very important to compare the efficiency of rituximab and obinutuzumab at inducing macrophage-mediated ADCP in RA and SLE patient samples to understand whether the differences noted here between the two types of anti-CD20mAbs in their ability to induce neutrophil activation are also applicable to macrophages.

Direct cell death

The relative importance of DCD in vivo is not clear. However, the observations that the detection of IgD-CD27+ B cells, but not IgD+CD27- naïve B cells, in peripheral circulation at four weeks after rituximab despite the presence of albeit a small frequency of IgD+CD27- naïve B cells in the lymph nodes suggest that both B-cell intrinsic factors, their microenvironment and circulatory dynamics influence the

susceptibility to anti-CD20mAb cytotoxicity (Wallin, Jolly et al. 2014). Circulatory dynamics may compromise access for effector cells to remove anti-CD20mAb opsonised B cells. However, the consistent demonstration of nondepleted B cells despite opsonisation with rituximab suggests that DCD by anti-CD20mAbs is relatively inefficient although microenvironment such as excess BAFF levels associated with SLE may confer resistance to rituximab-induced DCD by apoptosis whereas obinutuzumab induces non-apoptotic lysosome-mediated DCD, and therefore, the potency of obinutuzumab to induce DCD is less likely to be hindered by BAFF-mediated anti-apoptotic effects. Further, whether internalisation influences susceptibility to rituximab cytotoxicity in B-cell subpopulations in vivo and whether type II anti-CD20mAbs such as obinutuzumab, with greater ability to induced DCD in vitro compared to rituximab, may prove advantageous in this regard in vivo remains to be determined.

Although the effect of BAFF on anti-CD20mAb efficiency in whole blood B-cell depletion assay was assessed here, the effects on DCD specifically were not addressed, but would be important to understand. The effect of B-cell expression of anti-apoptotic proteins such as Bcl-2 and the effect of concomitant immunosuppressant drugs on the efficiency of anti-CD20mAbs to induce DCD in B-cell subpopulations was not studied here, but would be important to address. Thus, a better understanding about the signaling pathways of DCD induction by anti-CD20mAbs would inform our efforts to optimise anti-CD20mAb-induced DCD.

Conclusions

In summary, the duration of B-cell depletion is shorter and serum rituximab levels lower in SLE compared to RA whilst hypogammaglobulinemia was limited to the IgM isotype, particularly with sequential mycophenolate therapy. These observations may be useful for judicious monitoring of patients treated with rituximab and improve the therapeutic index of B-cell depletion therapy.

Finally, obinutuzumab, a humanised, type II anti-CD20 mAb with afucosylated Fc portion is not only two-fold more efficient than rituximab at inducing B-cell depletion, in RA and SLE patient samples, in vitro, but also provides multiple mechanistic advantages relevant to autoimmune disease such as SLE including: i) lower risk of HACAs; ii) less reliability on complement for inducing B-cell cytotoxicity; iii) improved FcγR-dependent effector mechanisms through enhanced interaction with FcγRIII on

effector cells; and iv) FcγR-independent lysosome mediated direct cell death that is less likely to be influenced by the effects of excess BAFF, to improve B-cell depletion, compared to rituximab. Therefore, these data provide a compelling mechanistic basis for expecting better outcomes with obinutuzumab as an alternative B-cell depleting agent in RA and SLE, in particular.

Chapter 7   Future perspectives

What next for optimising B-cell depletion strategies in autoimmune diseases?

The available data clearly demonstrate that rituximab-based B-cell depletion therapy improves outcomes for the majority of patients with autoimmune diseases such as RA and SLE. However, a significant proportion of patients respond poorly, likely due to various rituximab-resistance mechanisms. Although, humanised, anti-CD20 mAbs have now entered clinical trials for use in RA and SLE, some demonstrating efficacy in rituximab-naïve RA, it is not yet clear whether they will be more effective as currently there is no data comparing these mAbs in head-to-head trials with rituximab and, furthermore whether they will also be effective in patients with incomplete B-cell depletion with rituximab is not known. Presumably this will depend upon the reason for resistance in the first place. If development of HACA or anti-idiotype responses is responsible, then alternative humanised mAbs should be effective. Alternatively, if a patient-intrinsic mechanistic defect is responsible, then unless this is overcome with the new treatment no improvement in response would be expected. In interpreting the head-to-head trials it is important to note that the doses used may not be directly comparable to those for rituximab in routine clinical practice (either two doses of 1g given 1-2 weeks apart or four doses of 375-mg/m^2 given weekly). This point is particularly pertinent given that rituximab resistance may, at least in part, be overcome by using an extra dose of rituximab as shown in the case of RA (Vital, Dass et al. 2015). Another important factor needing careful consideration is to distinguish the biological activity of these anti-CD20 mAbs from their clinical activity. For example, in some patients efficient depletion may not always lead to good clinical response, conversely, good clinical response may occur despite inefficient B-cell depletion. Therefore, it would be important to monitor these activities separately and where possible perform mechanistic studies involving patients in clinical trial settings to provide clinically meaningful information and provide insights into how best to optimise B-cell depletion therapy further. Given its enhanced ability to delete B cells in other diseases and the capacity to elicit alternative effector functions, the type II anti-CD20 mAb obinutuzumab may provide a useful alternative B-cell depleting agent for at least some patients.

A significant minority of patients with RA and SLE respond less well to B-cell depletion therapy despite complete B-cell depletion, as assessed by peripheral blood CD19+ B cell counts. In a proportion of these patients poor response to B-cell

depletion therapy may relate to higher BAFF levels and/or type I IFNs, which may promote B-cell differentiation and/or plasma cell activity. Therefore, sequential anti-BAFF and anti-IFN agents after B-cell depletion therapy may target these potential alternative resistance mechanisms. Some patients with SLE who have persistently high anti-dsDNA antibodies despite B-cell depletion therapy may respond poorly owing to high plasma cell activity. Direct targeting of plasma cells using proteasome inhibitors may benefit this subgroup of patients.

Thus, optimising B-cell depletion therapy with agents that potentially overcome disease-related immune defects and, targeting pathways of resistance that operate beyond B-cell depletion would be important to explore in clinical trials, if we are to improve overall clinical response to B-cell targeted therapies, particularly, in SLE.

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Publications resulting from the work

Articles

Reddy, V., S. Croca, D. Gerona, I. De La Torre, D. Isenberg, V. McDonald, M. Leandro and G. Cambridge (2013). "Serum rituximab levels and efficiency of B cell depletion: differences between patients with rheumatoid arthritis and systemic lupus erythematosus." Rheumatology (Oxford) 52(5): 951-952.

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Review articles

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