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Mechanisms Of Relapse In Rheumatoid Arthritis Following B-Lymphocyte Depletion

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Thesis submitted for the degree of
Doctor of Philosophy

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ABSTRACT

An open label trial of B-lymphocyte depletion therapy (BLDT) based on rituximab in 5 patients with rheumatoid arthritis, initiated in 1998, had shown that this therapy was associated with major clinical improvement. B-lymphocyte repopulation had occurred before relapse in all patients and, interestingly, it had not been always associated with relapse. This thesis starts with an extension of the initial open label trial to 22 patients with results suggesting a dose response to rituximab. Further follow up of these and an additional 18 patients revealed that, although all patients eventually relapsed, as in the previous small study, relapse could occur either at the time of B-lymphocyte repopulation of the peripheral blood or at a variable time after this time point, up to almost 3 years. Re-treatment with BLDT was found to be well tolerated and effective. Upon re-treatment, patients usually repeated the same pattern of relapse.

Disease-associated autoantibodies, rheumatoid factor of immunoglobulin A, G and M classes and antibodies to cyclic citrullinated peptides, fell in all patients following BLDT but only decreased significantly in those who responded to treatment. Comparison with changes observed in total immunoglobulin levels and anti-microbial antibodies, suggested a specific effect on autoantibodies. Relapse was almost always preceded or associated with a rise in autoantibody levels, particularly rheumatoid factor of immunoglobulin M isotype, and was more closely associated with a rise in autoantibody levels than with the presence of B lymphocytes per se. B-lymphocyte stimulator (BLyS) serum levels increased following BLDT. Levels decreased following B-lymphocyte repopulation but tended to be lower at the time of repopulation in the group of patients who relapsed at this time point.

Immunophenotyping of peripheral blood B lymphocytes showed that depletion was major but not complete and that it involved all B-lymphocyte subpopulations. It suggested that a quantitative threshold of depletion needed to be reached for patients to respond to therapy. B-lymphocyte repopulation occurred mainly from naïve cells with immature B lymphocytes present in increased numbers and frequency. Patients who relapsed at the time of B-lymphocyte repopulation tended to show increased frequency

and increased numbers of circulating memory B lymphocytes at the time of repopulation.

Immunophenotyping studies of bone marrow aspirates in 6 patients, 3 months after treatment with BLDT, showed that the frequency of cells of B-lymphocyte lineage at this time point varied between patients. The variable relationships between the frequencies of more immature and more mature B-lymphocyte precursors in the samples suggested different degrees of depletion. Samples from patients who relapsed the earliest showed a pattern suggesting less efficient depletion.

In conclusion, the results presented here suggest that relapse following BLDT in patients with rheumatoid arthritis can be attributed to incomplete depletion of pathogenic B-lymphocyte clones, persistence of long-lived plasma cells producing pathogenic autoantibodies or to a combination of both. This will contribute to hypothesis-based development of B-lymphocyte targeting therapies.

To Jo Edwards and Jo Cambridge who made this thesis possible.

To Paulo for his constant support and encouragement.

To my Mother for her capacity to enjoy life and her courage, when faced with a certain early death.

> "But, as Bacon has well pointed out, truth is more likely to come out of error, if this is clear and definite, than out of confusion, and my experience teaches me that it is better to hold a well–understood and intelligible opinion, even if it should turn out to be wrong, than to be content with a muddle-headed mixture of conflicting views, sometimes miscalled impartiality, and often no better than no opinion at all."
>
> William Maddock Bayliss,
>
> Principles of General Physiology, 1915, X.

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DECLARATION

The work described in this thesis was carried out by the author unless otherwise stated.

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LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
ADCC	Antibody-dependent cell-mediated cytotoxicity
ANA	Antinuclear antibodies
APC	Antigen-presenting cell
APC	Allophycocyanin
BAFF	B-cell activator factor
BCMA	B-cell maturation antigen
BCR	B-cell receptor
BLDT	B-lymphocyte depletion therapy
BLyS	B-lymphocyte stimulator
BMT	Bone marrow transplantation
BR3	BLyS receptor 3
CCL	Chemokine from the CC group
CCR	Receptor for chemokine of the CC group
CD	Cluster of differentiation antigen
CDC	Complement-dependent cytotoxicity
CDR	Complementarity determining region
CHOP	Cyclophosphamide, doxorubicin, vincristine and prednisone
CLL	Chronic lymphocytic leukaemia
CR	Complement receptor
CRP	C reactive protein
CTLA4	Cytotoxic T-lymphocyte associated antigen 4
CXCL	Chemokine from the CXC group
CXCR	Recptor for chemokine of the CXC group
DAF	Complement decay accelerating factor
DMARDs	Disease modifying anti-rheumatic drugs
DNP	Dinitrobenzene
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate

Fc	Fragment crystallisable
FcγR	Fc receptor for IgG
FcεR	Fc receptor for IgE
FITC	Fluorescein isothiocyanate
GPI	Glucose-6-phosphate isomerase
GVHD	Chronic graft versus host disease
HACA	Human anti-chimaeric antibodies
HAMA	Human anti-mouse antibodies
HLA	Human leucocyte antigen
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgA-RhF	Rheumatoid factor of IgA class
IgG-RhF	Rheumatoid factor of IgG class
IgM-RhF	Rheumatoid factor of IgM class
IL	Interleukin
IFN-γ	Interferon γ
MHC	Major histocompatibility complex
NHL	Non-Hodgkin's lymphoma
NK	Natural killer
NSAIDs	Nonsteroidal anti-inflammatory drugs
PBS	Phosphate-buffered saline
PCP	Pneumococcal capsular polysaccharides
PCR	Polymerase-chain reaction
PE	Phycoerythrin
PE-Cy5	Phycoerythrin-indodicarbocyanine, also called Cy-chrome
PerCP	Peridinin-chlorophyll *a* complex protein
PE-TR	Phycoerythrin-Texas Red
RA	Rheumatoid arthritis
RAG-1	Recombinant gene 1 protein
RAG-2	Recombinant gene 2 protein
RAPA	Rheumatoid arthritis particle agglutination
RhF	Rheumatoid factor

SAA	Serum amyloid A protein
SDF-1	Stromal cell-derived factor 1
SLE	Systemic lupus erythematosus
SS	Sjögren's syndrome
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TD	Thymus-dependent
TdT	Terminal deoxynucleotidyl transferase
TI	Thymus-independent
TLR	Toll-like receptor
TNF	Tumour necrosis factor (previously tumour necrosis factor alfa)
TT	Tetanus toxoid
UCL	University College London
UCLH	University College London Hospitals
VCAM-1	Vascular cell adhesion molecule-1
VH	Variable heavy-chain
VLA-4	Very late antigen 4

CHAPTER 1 INTRODUCTION

1.1 Rheumatoid arthritis: clinical description and natural history

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease involving predominantly diarthrodial joints and synovial tissue surrounding tendons. It characteristically involves the small joints of the hands and feet and the wrists in a symmetrical pattern. Joint symptoms are usually associated with malaise and fatigue and less frequently with weight loss and low-grade fever. In some patients, subcutaneous nodules and chronic inflammation of other tissues, particularly the pericardium, lung and pleura, small blood vessels and sclera is clinically evident.

In Caucasian European and North American populations RA prevalence ranges from 0.2% to 1% in individuals 16 years or older (Seldin et al., 1999, MacGregor and Silman, 1998). Most studies show a female to male excess prevalence of between 2 and 4 times (MacGregor and Silman, 1998). Overall annual incidences vary between different studies. In a UK study in 1990 reported annual incidence of RA was 0.36/1000 person years in females and 0.14/1000 in males (Symmons et al., 1994). Disease incidence and age-specific prevalence rates rise with age (MacGregor and Silman, 1998).

Frequently disease activity is at first intermittent; with the passage of time it becomes more sustained. Approximately 20 percent of patients experience an acute onset of symptoms. Typically disease activity waxes and wanes. In the individual patient flares are frequently attributed to coincident infections or times of physical or emotional stress. Disease severity varies but many patients experience progressive damage of the joints. Spontaneous remission is rare if disease has been continuous for two or more years.

Several pieces of evidence suggest that RA is an autoimmune disease, in particular, its association with the presence of a number of autoantibodies. In approximately 75 to 80% of patients autoantibodies directed to the Fc (fragment crystallisable) portion of immunoglobulin G (IgG), known as rheumatoid factors (RhF), are detected (Williams, 1998). Other autoantibodies can also be detected in these patients including antibodies to citrullinated peptides (anti-CCP antibodies) and to nuclear antigens. RA is frequently

associated with other syndromes in which there is evidence for organ-specific or systemic autoimmunity such as autoimmune thyroiditis or Sjögren's syndrome. Disease activity responds to immunosuppressive or immunomodulatory therapies.

Patients with detectable RhF in serum (so called seropositive patients) have a higher incidence of extra-articular manifestations (De Rycke et al., 2004). In particular, subcutaneous nodules and vasculitis are rare in patients without these autoantibodies (Williams, 1998). The presence of detectable RhF and also of anti-CCP antibodies at the time of disease presentation has been associated with a worse prognosis, being associated with more frequent development of joint erosions (Dixey et al., 2004).

1.1.1 Clinical response to conventional treatment

The treatment of RA includes the use of non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and, in many patients, the use of drugs classified as disease modifying antirheumatic drugs (DMARDs). DMARDs used in RA include azathioprine, cyclophosphamide, cyclosporine, D-penicillamine, oral or parenteral gold, hydroxychloroquine, leflunomide, methotrexate, sulfasalazine and biological agents targeting the cytokine tumour necrosis factor (anti-TNF agents) and interleukin 1 (IL-1). All these drugs are immunomodulatory with known effects on different types of cells from both the innate and adaptive immune systems. However, with the probable exception of anti-TNF agents, the precise mechanism or mechanisms through which these drugs are beneficial in RA are unknown.

With the exception of anti-TNF agents, which can have a clinical effect within 24 to 48 hours, most DMARDs have a slow onset of action that varies between them. For most drugs it is recommended that treatment be continued for at least three months before deciding to withdraw it for lack of efficacy. It is always unpredictable whether a particular patient with RA is going to respond to treatment with a specific DMARD or not.

All DMARDs can induce clinical remission as defined by the absence of clinical symptoms and signs of active disease and normal laboratorial indices of inflammation. However, in the majority of patients treated with the different drugs, either in isolation

or in combination, disease activity is reduced but not abolished. Usually, a sustained response to a drug is dependent on continuation of treatment with recurrence of symptoms occurring within weeks to months if treatment is stopped. Only a small number of patients are able to discontinue all medication and remain in clinical remission. In some patients it is possible to decrease the total dose of the drug administered once disease control is achieved without clinical relapse occurring. Even on stable doses of medication many patients experience flares of their disease.

Altogether these data show that in the majority of patients none of the current DMARDs is able to reverse the disease process or to interfere with it in a way that induces a long-term remission. This fact remains true even after widespread use of anti-TNF agents, whose remarkable efficacy has allowed many patients to achieve significant clinical improvements, including very low disease activity or clinical remission. However, as with previous DMARDs, only rarely is clinical remission sustained after stopping treatment. Establishment of long-term remission that is sustained after stopping therapy must be the ultimate objective in the treatment of a chronic disabling disease such as RA, which current treatment involves the use of drugs with potentially severe side effects.

1.1.2 Summary

RA is a chronic, systemic autoimmune disease that targets in particular the synovial tissue. Its severity varies but frequently it is a chronic, debilitating disease leading to progressive cumulative damage to diarthrodial joints. Spontaneous remission is rare once the disease has been present for two or more years. A sustained response to currently available DMARDs depends largely on continuation of treatment and is not always achievable with flares being frequently observed. In the absence of a cure, long-term remission is the ultimate objective in the treatment of RA and it is this need that provided the basis for the enquiry described in this thesis.

1.2 Pathogenesis of rheumatoid arthritis

The cause of RA remains unknown despite many years of intensive investigation. Perhaps the most concrete clue to pathogenesis that we have is that the cytokines, TNF

and, to a lesser extent, IL-1 and IL-6, derived from activated macrophages, are directly or indirectly responsible for the majority of the manifestations of RA (Firestein, 1998). Clinical efficacy of anti-TNF agents has proved the central role of this cytokine in the disease perpetuation in a significant proportion of patients (Feldmann and Maini, 2001). The stimulus or stimuli that activate macrophages in the rheumatoid synovium and that initiate and perpetuate local and systemic inflammation remain a question of debate.

As described below, various pieces of evidence suggest that the aetiology of RA, probably involves interactions between genetic, environmental and random factors occurring within the immune system.

1.2.1 Risk factors for developing rheumatoid arthritis

The strongest identified risk factors for developing RA are female sex, a family history of RA, presence of a peptide sequence known as the shared epitope on human leucocyte antigen (HLA) DR (also known as Major Histocompatibility Complex (MHC) class II) molecules, smoking and the presence of detectable RhF and anti-CCP in serum.

Women have a higher risk of developing RA with most studies showing an increased prevalence of 2 to 4 times when compared to men (MacGregor and Silman, 1998).

Siblings of patients with RA have an increased risk of developing RA. Concordance for RA in monozygotic twins is probably in the range of 12 to 15% and RA affects between 2 and 4% of siblings of patients with RA (Seldin et al., 1999, MacGregor and Silman, 1998). It has been estimated that approximately one-third to one-half of this total genetic contribution in RA can be attributed to genes in the MHC (Deighton et al., 1989, Jawaheer et al., 2001). An association between RA and HLA was first demonstrated in the 1970s, when the mixed lymphocyte culture type Dw4 was found to be present at increased frequencies among B lymphocytes of patients with RA when compared with control individuals (Stastny, 1978). Since then, a large number of population studies have confirmed the association between RA and HLA-DRB1*0401 and HLA-DRB1*0404 at least in Caucasian populations (Nepom and Nepom, 1998). The vast majority of studies have focused on a direct role for HLA-DRB1 alleles that encode a conserved amino acid sequence designated the "shared epitope" (Ollier and

Thomson, 1992, Nepom and Nepom, 1998). HLA-DRB1*0101, HLA-DRB1*0405 and HLA-DRB1*1402 carry the shared epitope and have also been associated with RA (Nepom and Nepom, 1998). Also several studies have documented a dose-effect of the HLA molecules, with the risk of developing RA being higher in patients homozygous for shared epitope alleles (Nepom and Nepom, 2004). Several studies have shown that patients with the shared epitope are more likely to have seropositive disease and to have more severe disease (Ollier and MacGregor, 1995, Nepom and Nepom, 1998).

Nevertheless, several studies showed that the association between RA and HLA is more complex. Individuals that carry certain combinations of HLA-DRB1 alleles have an especially high risk of developing RA (Hall et al., 1996). It is unclear whether these interactions are mediated by the HLA-DRB1 molecules themselves or reflect the action of other genes on these haplotypes. Several studies have shown that the association of RA with the HLA complex also involves other genes, including possibly the TNF gene (Seldin et al., 1999, Jawaheer et al., 2001, Waldron-Lynch et al., 2001, Etzel et al., 2006).

Smoking is so far the only environmental factor that has been unambiguously demonstrated in several studies to be a risk factor for RA (Vessey et al., 1987, Silman et al., 1996, Stolt et al., 2003). A stronger association with smoking has been found for seropositive cases of RA than for seronegative cases with some studies finding an association with only seropositive cases (Heliovaara et al., 1993, Stolt et al., 2003). In a recent large, population based, case-control study using incident cases in Sweden, smoking has been found to be a risk factor for seropositive rheumatoid arthritis in both sexes but not for seronegative rheumatoid arthritis (Stolt et al., 2003). The increased risk occurred after a long duration (20 or more years), but merely a moderate intensity (6 to 9 cigarettes per day) of exposure and results suggested that it might remain for several years after smoking cessation (remained for up 10 to 19 years). The risk increased with increased cumulative dose of smoking. This dose-response relationship documented in some studies suggests that smoking may exert pro-arthritogenic effects through repeated stimulation over a period of several years.

The presence in serum of detectable RhF of immunoglobulin M (IgM), IgG and immunoglobulin A (IgA) classes (IgM-RhF, IgG-RhF and IgA-RhF) or of anti-CCP

antibodies have been identified as risk factors for developing RA (Thorsteinsson et al., 1975, Halldorsdottir et al., 2000, Rantapaa-Dahlqvist et al., 2003). In a study by Nielen and colleagues, approximately one-half of patients with RA had positive IgM-RhF and/or anti-CCP antibodies on at least one occasion, a median of almost 5 years prior to disease onset (Nielen et al., 2004). The presence of these antibodies in the serum can predate the onset of clinical overt disease by several years (up to 14 years) (Rantapaa-Dahlquist et al., 2003, Nielen et al., 2004,).

Recent studies have provided possible clues for associations between several of the identified risk factors for the development of RA. The combined presence of the shared epitope with anti-CCP antibodies or a RhF of any isotype have been found to confer a higher risk of developing RA with a marked interaction suggested between the two risk factors (presence of autoantibody and shared epitope) (Berglin et al., 2004). The risk was particularly high in individuals who carried the shared epitope and had anti-CCP antibodies. In a mouse model transgenic for HLA-DRB1*0401, conversion of arginine to citrulline at the peptide side-chain position interacting with the SE significantly increases peptide-MHC affinity and leads to activation of CD4 T lymphocytes suggesting that DRB1 alleles with the shared epitope could initiate an autoimmune response to citrullinated self-antigens in RA patients (Hill et al., 2003).

An interaction between smoking and the presence of the shared epitope as risk factors for developing seropositive RA has been described (Padyukov et al., 2004). In this study, individuals carrying double copies of the shared epitope and who were smokers were found to have approximately a 16 times increased risk of developing seropositive RA when compared to individuals who did not smoke and carried no copy of the shared epitope. None of these factors alone or in combination increased the risk for development of seronegative RA. More recently, smoking has been found to increase the risk of developing anti-CCP antibodies only in RA patients carrying HLA-DRB1 shared epitope alleles (Linn-Rasker et al., 2006). The same study found similar effect for IgM-RhF but stratification showed that the predominant association was with anti-CCP antibodies.

The association of seropositive and seronegative RA and, more recently, anti-CCP positive and anti-CCP negative RA with different risk factors suggests that they may be

two different pathogenic processes. As RA is a disease defined by clinical criteria different pathogenic pathways may result in clinical conditions with enough similarities to be classified into the entity we currently call RA.

Although the risk factors described above go some way to explaining lifetime risk for the development of RA, a significant contribution to risk must come from as yet unidentified factors. Moreover, the time of onset of disease is not explained. This suggests that environmental factors or possibly stochastic phenomena within the immune system may also be involved in the development of clinically overt RA and this issue will be returned to later (Edwards and Cambridge, 1998, Edwards et al., 1999, Edwards and Cambridge, 2006).

1.2.2 Histology

Although rheumatoid arthritis has many features of a systemic disease, the synovium is the primary site of inflammation (Gardner and Arnold, 1992, Edwards, 1998). The synovium is a soft connective tissue that lines diarthrodial joints, tendon sheaths and bursae. Normal synovium is composed of a surface layer of cells, the intima, and the underlying tissue, the subintima. The lining of cells is situated in the inner side of the synovium, in direct contact with the intra-articular cavity, and is one to two cell layers deep. It is made of two types of cells, macrophages (historically known as synoviocytes type A), and fibroblast-like cells (synoviocytes type B). In normal synovium, the majority of cells in the intima are fibroblast-like cells. This lining is not a true epithelial surface and can be discontinuous. The cells are not tightly joined and there is no basal membrane separating it from the underlying tissue, although the specialised intercellular matrix includes several molecules associated with basal membranes. The subintima has normally only a relatively small number of cells. It contains blood vessels and scattered fibroblasts, macrophages and mast cells. Small numbers of lymphocytes and interdigitating dendritic antigen-presenting cells can also be present. Capillaries are found immediately below the lining. A plexus of small arterioles and venules is found deeper in the subintima. The subintimal matrix contains different macromolecules including collagen, fibronectin and proteoglycans.

The synovium provides a non-adherent surface that facilitates movement and participates in the nutrition of the articular cartilage. It controls the volume and composition of synovial fluid. Synovial fluid is an ultrafiltrate of plasma to which hyaluronic acid is added. Intimal fibroblasts secrete hyaluronic acid and through the differential expression of adhesion molecules participate in regulating cell trafficking. Intimal fibroblasts are mesenchymal cells thought to arise by local division. Intimal macrophages are involved in the removal of cellular debris from the intra-articular space.

In rheumatoid arthritis the synovium presents major macroscopic and microscopic changes (Gardner and Arnold, 1992). Macroscopically it is oedematous and has multiple redundant folds and villi. Microscopically it shows marked increased number of cells in the synovial intima, increased numbers of blood vessels and infiltration of the subintima with mononuclear cells. The increase in intimal cells is mainly due to an increased number of macrophages which is thought to be due mainly to migration of new cells into the lining rather than *in situ* proliferation (Henderson et al., 1988, Edwards and Wilkinson, 1995). There is marked infiltration of the subintima with mononuclear cells, predominantly T cells, macrophages and plasma cells but also other types of cells such as B cells, mast cells and neutrophils (Gardner and Arnold, 1992, Edwards and Wilkinson, 1995, Tak and Bresnihan, 2000). The cellular infiltrate can be diffuse or form smaller or larger aggregates. When aggregates are present, different regions can be distinguished in the subintima in rheumatoid synovium based on the characteristics of its cellular composition (Firestein, 1998). These include lymphocyte-enriched regions, usually perivenular, containing about 80% lymphocytes, zones rich in plasma cells, containing about 75% plasma cells and regions referred to as transitional zones containing different kind of cells including small lymphocytes, macrophages, plasma cells and antigen-presenting cells (dendritic cells and possibly transformed macrophages). Lymphocytes are not found in the intima (Firestein, 1998, Edwards and Wilkinson, 1995). The majority of lymphocytes seen in the rheumatoid synovium are T lymphocytes, expressing CD4 and CD45RO surface antigens that characterise T helper memory cells (Gardner and Arnold, 1992, Firestein, 1998). Interdigitating dendritic antigen-presenting cells (APC) are probably present both in normal and particularly in diseased synovium. Their separation from macrophages with similar phenotype in the human is still controversial (Edwards, 1998). In some rheumatoid synovium samples,

cells with phenotypes similar to follicular dendritic cells can be found in lymphoid aggregates in association with B lymphocytes (Gardner and Arnold, 1992, Firestein, 1998).

Increased numbers of plasma cells are frequently present in rheumatoid synovium (Gardner and Arnold, 1992, Edwards and Wilkinson, 1995, Tak and Bresnihan, 2000). These cells secrete all three major types of Ig with IgG secreting cells predominating and IgA secreting cells being usually the minority (Freemont and Rutley, 1986) (Gardner and Arnold, 1992). Rheumatoid factor production has been demonstrated in the synovium. In one study, 50-60% of those plasma cells synthesizing IgG produced RhF (Youinou et al., 1984). In comparison, up to 90% of the cells synthesizing IgM secreted rheumatoid factor while only 10% of IgA plasma cells were RhF positive.

Although many plasma cells are present in the rheumatoid synovium there is only a small proportion of B lymphocytes in the majority of samples (Gardner and Arnold, 1992, Edwards and Wilkinson, 1995, Tak and Bresnihan, 2000, Firestein, 1998). However, in about 20% of cases, in larger lymphoid aggregates, B lymphocytes are arranged in structures resembling follicles with germinal centres (Takemura et al., 2001a). Very few B lymphocytes are found in the synovial fluid (Gardner and Arnold, 1992).

At the synovial interface with cartilage and bone the rheumatoid synovium has the propensity to become locally invasive and form what is called "pannus" (Tak and Bresnihan, 2000). The 'pannus" contains primarily fibroblastic cells with macrophages in the subintima but very few lymphocytes (Gardner and Arnold, 1992, Tak and Bresnihan, 2000). The presence of typical "pannus" is characteristic of rheumatoid arthritis but not specific (Tak and Bresnihan, 2000, Edwards and Wilkinson, 1995). Other histologic changes are also not specific although several findings are more characteristic of rheumatoid than other diseases and indices can be developed that can identify rheumatoid synovium with good specificity.

The synovial fluid in RA contains high concentrations of proteins such as IgG, presence of immune complexes, evidence of complement activation and large numbers of neutrophils (Gardner and Arnold, 1992, Firestein, 1998). It appears that neutrophils are

attracted to the synovium but not retained by it and that they migrate to the synovial fluid (Firestein, 1998).

Twenty to 35 percent of patients with RA develop rheumatoid nodules (Harris, 2005). A mature rheumatoid nodule has a central area of necrosis rimmed by a corona of palisading macrophages and fibroblasts that is surrounded in turn by a collageneous capsule with perivascular collections of chronic inflammatory cells (Gardner and Arnold, 1992, Edwards and Wilkinson, 1995). The nodule grows by accumulating cells that expand centrifugally, leaving behind central necrosis. The cells are presumed to derive mainly from a mixture of proliferating fibroblasts and an influx of macrophages from the circulation. The pattern of cytokines produced in a rheumatoid nodule is similar to the one found in rheumatoid synovium, however T and B lymphocytes are absent.

The synovium is targeted in several immunological diseases, although the reasons for this are still not clear. Primary autoimmune responses to synovial antigens might theoretically occur but evidence is lacking in humans (Edwards, 1998). Also, synovium inflammation is usually associated with inflammation in other tissues and targeting of the different tissues needs to be explained. A number of autoantibodies are associated with several autoimmune diseases that target the synovium but they are directed to ubiquitous antigens. Targeting of the synovium in these diseases might therefore reflect the local microenvironment and responsiveness of local resident cells to different immunological stimulus rather than the presence of specific articular antigens (Edwards, 1998).

Certain characteristics of the normal synovium seem to make it a frequent target in inflammation consequent to different immunological processes (Edwards, 1998). Synovial intimal macrophages constitutively express high levels of the Fc receptor for IgG, FcγRIIIa (Edwards et al., 1997, Bhatia et al., 1998). In vitro, if FcγRIIIa is cross-linked it leads to activation of expressing macrophages with production of reactive oxygen species and inflammatory mediators, including cytokines such as TNF (Abrahams et al., 2000). Synovial fibroblasts appear hyper-responsive to certain cytokines, including to TNF, by producing cytokines and metalloproteinases (Edwards et al., 1997). Intimal fibroblasts also constitutively express vascular cell adhesion

molecule-1 (VCAM-1, CD106) unlike many other fibroblasts and complement decay accelerating factor (DAF, CD55) (Edwards, 1998). VCAM-1 may be involved in retaining mononuclear leucocytes, including both macrophages and lymphocytes, in the synovium (Edwards et al., 1997).

1.2.3 Role of macrophages and mediators of inflammation

In rheumatoid arthritis, many studies support the idea that joint manifestations are due to the local production of inflammatory mediators by activated synovial macrophages, particularly intimal macrophages (Tak and Bresnihan, 2000, Firestein, 1998). Extra-articular manifestations, in particular vasculitis, are generally accepted to be due, at least in part, to immune complex formation with consequent activation of complement and effector cells (Harris, 2005).

In rheumatoid synovium, activated macrophages produce large amounts of TNF, IL-1 and smaller amounts of IL-6 (Firestein, 1998). In vitro, synovial fibroblasts are activated by TNF and IL-1 and secrete IL-6 and matrix degrading enzymes, which are principal contributors to synovial inflammation and joint destruction (Firestein, 1998). The importance of macrophage- and fibroblast-derived cytokines in the clinical manifestations of the disease is underlined by the beneficial effect of strategies aimed at targeting TNF, IL-1 and IL-6 (Feldmann and Maini, 2001, Dayer and Bresnihan, 2002, Choy et al., 2002).

Synovial macrophages include intimal and subintimal macrophages. It is thought that both populations derive from monocytes from the peripheral blood and that subintimal macrophages may mature into intimal macrophages (Edwards, 1998). There are, however, phenotypic differences between these two populations. Normal intimal macrophages express constitutively high levels of HLA-DR and FcγRIIIa while subintimal macrophages express lower levels of HLA-DR and low levels or no FcγRIIIa (Edwards, 1998).

In RA, an increased number of macrophages are present in the synovium (Gardner and Arnold, 1992, Firestein, 1998). Macrophages make up a minority of cells in normal intima but in rheumatoid synovium the proportion of macrophages in the intima may

rise to 80 percent (Edwards, 1998). In enzymatically dispersed rheumatoid synovium macrophages make up about 20 percent of all cells (Firestein, 1998). Increased numbers of macrophages have been found in assymptomatic joints in patients with RA (Kraan et al., 1998). A positive correlation has been found between synovial macrophage numbers and expression of TNF and IL-6 and the severity of the synovitis, with no differences being detected when samples from early and long-standing disease were compared (Tak et al., 1997). The number of synovial macrophages appears to correlate with joint destruction in RA (Mulherin et al., 1996). Changes in numbers of synovial subintimal macrophages correlate with clinical improvement independently of the treatment used (Yanni et al., 1994, Dolhain et al., 1998, Kraan et al., 2000, Pettit et al., 2001, Haringman et al., 2005).

Even though synovial macrophage activation is widely considered to be the effector mechanism that leads to rheumatoid synovial inflammation and eventually to joint destruction, what drives macrophage activation is not known and continues to be a question of debate and controversy.

1.2.4 Mechanisms driving activated macrophages

Through the years, a number of hypotheses have been proposed to explain the initiation and perpetuation of synovial inflammation in RA. Most hypotheses have been centred on the possibility of specific autoimmune responses by cells of the adaptive immune system, mainly B lymphocytes and CD4 T lymphocytes, but some have suggested primary roles for synovial fibroblasts or cells of the innate immune system and antigen-independent mechanisms.

Specific adaptive immunity was historically divided into humoral and cell-mediated components. These two components of specific immunity are closely related to each other (Janeway et al., 2001). Briefly, T lymphocytes interact with B lymphocytes in the production of antibody against most antigens and, for example, in secondary immune reactions, specific antibodies facilitate antigen presentation and the recruitment and activation of specific T lymphocytes. Specific antibodies and cell-mediated immunity are induced in all immune reactions to infectious organisms, but the magnitude and quality of the components varies in different infections. In RA, as in other autoimmune

diseases, the main question is not if multiple immune mechanisms occur but which ones are primary and which ones are secondary, and whether different mechanisms are responsible for the initiation of the disease and for its perpetuation.

Early autoimmune hypotheses were formulated after the discovery of RhF. These were based on B lymphocytes as the producers of autoantibodies (mainly RhF) capable of forming immune complexes and causing inflammation by consequent deposition and activation of complement and effector cells such as neutrophils. The existence of seronegative patients, the lack of a clear and close association between RhF measured in serum and disease clinical activity and the fact that complement activation by immune complexes could not explain all the pathology led to doubts about a primary role for B lymphocytes in the pathogenesis of RA. In the 1980s and in the 1990s, the central pathogenic role was attributed to autoreactive CD4 T lymphocytes, based in particular on RA association with DR4 and the predominance of T lymphocytes in rheumatoid synovium. Such T lymphocytes were suggested to recognise autoantigens specific to the joint and to be capable of activating macrophages through the production of cytokines, particularly interferon-γ (IFN-γ).

When the central role of macrophage activation and cytokine production was established, the CD4 T-lymphocyte centred hypothesis became predominant, and a mechanism driven by B lymphocytes themselves or by autoantibodies seemed more unlikely to most researchers. However, in more recent years, the consideration of other potential pathogenic roles for autoantibodies, including activation of macrophages through interaction with Fcγ receptors and participation in aberrant immune interactions between B and T lymphocytes, has led to a new hypothesis based on a specific pathogenic role for certain B lymphocytes and the specific immunoglobulins they produced (Edwards and Cambridge, 1998, Edwards et al., 1999). This hypothesis led to the use of B-lymphocyte depletion therapy (BLDT) based on rituximab as a treatment for RA, which forms the topic of this dissertation (Edwards and Cambridge, 2001).

1.2.4.1 Functions of B lymphocytes

B lymphocytes are thought to have two main functions: the production of antibodies and the presentation of antigen to T lymphocytes, in particular, soluble antigen in secondary

immune reactions (Janeway et al., 2001). In addition, B lymphocytes are now recognised to have an important role in the development of a normal architecture in secondary lymphoid tissues before birth, to be able to produce cytokines and possibly be involved in the differentiation and activation of APC (Lipsky, 2001, Shlomchik and Madaio, 2003, Serra and Santamaria, 2006).

B lymphocytes express the necessary molecules to be able to prime naïve T-lymphocytes (particularly CD80 and CD86) and are able to concentrate antigen with a high efficiency due to the expression of specific surface immunoglobulin (the antigen specific B-cell receptor - BCR). Nevertheless, it is thought that B lymphocytes do not play an important role as APC priming naïve T lymphocytes in primary immune reactions due to the small number of specific naïve B and T lymphocytes and to the low probability of these cells contacting each other when compared to the efficiency of the other professional APC mechanisms (Janeway et al., 2001). Even in secondary immune reactions to soluble antigens, initial antigen presentation to T lymphocytes is thought to be done by other APC, facilitated by the presence of specific circulating antibodies and increased uptake of the antigen in the form of immune complexes by these cells.

1.2.4.2 B lymphocytes as producers of autoantibodies

Rheumatoid arthritis is associated with the presence of autoantibodies both in the serum as well as in the synovium and synovial fluid. In the majority of patients with RA, synovium samples contain very small numbers of B lymphocytes but they frequently contain large numbers of plasma cells that produce large amounts of antibodies, including RhF (Gardner and Arnold, 1992, Firestein, 1998, Edwards and Wilkinson, 1995).

Many of these autoantibodies may arise secondary to joint inflammation and damage with the consequent release of cryptic antigens or modification of antigens leading to specific adaptive immune reactions or they may be a consequence of polyclonal B-lymphocyte stimulation. These autoantibodies include antibodies to antigens found in normal cartilage, such as anti-human cartilage glycoprotein-39 and anti-type II collagen antibodies, antibodies to glucose-6-phosphate-isomerase, antibodies to nuclear proteins

including A2 protein (RA33) and anti-calpastatin antibodies (Blass et al., 1999, van Boekel et al., 2002). They usually have low sensitivities and low specificities for RA.

In contrast, rheumatoid factor and anti-CCP antibodies are significantly associated with RA. They are present in the serum of the majority of patients with RA. The presence of anti-CCP and of high titres of RhF or IgA or IgG-RhF has a high specificity for RA. Also, these autoantibodies can be detected in the serum of individuals several years (up to 14 years) before clinical signs of disease become apparent and their presence in the serum increases the risk of developing RA (Rantapaa-Dahlqvist et al., 2003, Nielen et al., 2004). This suggests that either the autoantibodies or the specific B lymphocytes generating these antibodies play a role in disease pathogenesis (in the initiation of the synovitis).

In the 1960s and 1970s, following the discovery of RhF in the serum and particularly in the synovium (and synovial fluid) of patients with RA, the disease manifestations were seen as a consequence of RhF immune complex formation and consequent complement activation with recruitment of a variety of inflammatory cells that became activated, leading to inflammation and joint damage (Zvaifler, 1973). Subsequently, the existence of patients with a clinical diagnosis of RA but no detectable RhF in serum, the presence of RhF in patients with other diseases and in a proportion of normal individuals and the fact that complement activation by immune complexes could not explain all features of RA, particularly the synovitis, meant that attention was drawn to CD4 T cells (Janossy et al., 1981). As indicated below, the role of classic complement activation in immune complex mediated disease may have been overemphasised in the past and has now been reassessed. Other possible pathogenic roles for immune complexes both in efferent but also in afferent arms of immune responses have been advanced (Roosnek and Lanzavecchia, 1991, Leadbetter et al., 2002, Viglianti et al., 2003, Jiang et al., 2003, Lau et al., 2005, Edwards and Cambridge, 2006).

1.2.4.2.1 Rheumatoid factor

Rheumatoid factors are autoantibodies directed against antigenic determinants on the Fc region of IgG molecules first discovered by Erik Waaler in 1937 (Waaler, 1940). When he added the serum of a patient with RA to sheep erythrocytes sensitised with rabbit

antibodies to sheep red blood cells, agglutination of the cells was observed. He termed the substance responsible for this "agglutination activation factor". Similar observations were made and published by Rose and collaborators from the College of Physicians and Surgeons in New York and provided the impetus for a number of investigations on these antibodies in patients with RA. The term "rheumatoid factor", was suggested by Pike and collaborators (Pike et al., 1949). Kunkel and his colleagues established the molecular nature of RhF (Franklin et al., 1957).

Rheumatoid factors may be of the IgM, IgG, IgA or IgE isotype (Tighe and Carson, 2005). These autoantibodies are considered to be the immunological hallmark of RA and are widely used as a diagnostic marker for this disease (Arnett et al., 1988). Nevertheless, they are also present in a variety of other chronic inflammatory and infectious diseases as well as in normal individuals (Tighe and Carson, 2005). Studies suggest that autoreactivity specific for the Fc fraction of normal human IgG is part of the normal human B-lymphocyte and antibody repertoires. This specificity is present in foetal liver and cord blood B-lymphocyte clones (Lydyard et al., 1990).

Many of the conditions associated with the presence of detectable RhF in serum are also associated with hypergammaglobulinemia, indicative of polyclonal B-lymphocyte activation, or with circulating immune complexes. Transient RhF production can be observed in normal individuals following secondary immunisation (Bonagura et al., 1998). Several studies have focused on differences and similarities of RhF detected in RA patients when compared to RhF detected in other diseases or in normal individuals. The RhFs associated with RA are polyclonal and generally exhibit broad reactivity with both human and heterologous (e.g. rabbit) IgG, are of higher affinity, are present at higher titre and include not only IgM-RhF but also IgG-, IgA- and IgE-RhF isotypes (Tighe and Carson, 2005). In contrast, RhF associated with other diseases tend to react preferentially with human IgG (although exceptions occur), are of lower affinity and of the IgM isotype (Tighe and Carson, 2005). It remains unknown the exact mechanism why in RA there is a sustained and antigen-driven response to the Fc fraction of IgG. In one study, RhF from RA patients were relatively enriched for reactivity with IgG3, particularly in rheumatoid synovium, when compared with those from normal immunised individuals and to monoclonal IgM-RhF from patients with Waldenstrom macroglobulinemia (Artandi et al., 1991). It has been described that RA patients have

significantly fewer galactose residues on their IgG Fc compared to age-matched healthy controls due to reduced B-lymphocyte galactosyltransferase activity (Axford et al., 1987). A lack of terminal galactose residues early in disease has been associated with a worse prognosis (van Zeben et al., 1994).

At a dilution of serum at which 95 percent of the normal population will be RhF negative, 70% of patients with RA (diagnosed by other criteria) will have detectable RhF in serum by latex agglutination (i.e. agglutination of latex particles coated with IgG) (Tighe and Carson, 2005). This number increases to 90% when the more sensitive enzyme-linked immunosorbent assays (ELISA) are used (Tighe and Carson, 2005). Some seronegative patients, on repeated testing, convert to seropositive. The presence of RhF of more than one isotype, particularly in the joint, is considered highly specific for RA and is very rarely found in other rheumatic diseases (Tighe and Carson, 2005). Also, the specificity of RhF for RA increases with the titre. IgG-RhF is abundant in the sera and particularly in the joints of many patients with severe RA.

Several pieces of circumstantial evidence suggest that RhF is involved in the pathogenic processes underlying RA. The existence of seronegative cases of RA is said to argue against RhF being a causative factor in joint disease but it can also reflect the possibility that truly seronegative cases of RA represent a different disease. The presence of detectable RhF on standard agglutination tests (seropositive disease) has been associated with more severe disease and higher incidence of extra-articular manifestations (Bukhari et al., 2002, De Rycke et al., 2004). The presence of IgA-RhF is associated with rapidly progressive, more severe disease and bone erosions (Jonsson et al., 1995). Both IgA- and IgG-RhF are associated with systemic features such as vasculitis (Allen et al., 1981, Scott et al., 1981, Quismorio et al., 1983, Westedt et al., 1985a). Serial measurements of IgM-RhF modestly correlate with disease activity (Cats and Hazevoet, 1970, Westedt et al., 1985b, Robbins et al., 1986). Spontaneous production of IgM-RhF by peripheral blood mononuclear cells in vitro has also been found to correlate with disease activity (Olsen et al., 1982, Olsen et al., 1984). Immune complexes containing IgM-RhF are present in the synovium and in the synovial fluid. These complexes are able to activate complement and may lead to an increase in the influx of inflammatory cells contributing to tissue damage. Seronegative RA patients usually display milder symptoms and seldom develop extra-articular rheumatoid disease

(Harris, 2005). Furthermore, asymptomatic individuals with persistently raised RhF have a significantly increased risk of developing RA, suggesting that dysregulation of RhF production is a predisposing factor in the development of the disease (Thorsteinsson et al., 1975, Halldorsdottir et al., 2000, Rantapaa-Dahlqvist et al., 2003). The presence in the serum of all isotypes of RhF can precede the development of clinically detectable RA for years (Nielen et al., 2004, Rantapaa-Dahlquist et al., 2003). IgA-RhF can precede RA without the presence of IgM-RhF (Berglin et al., 2004).

IgG-RhF are unique amongst autoantibodies in that they are directed against antigenic determinants present on the Fc region of the same molecular type as the antibody itself (Pope et al., 1974). For this reason IgG-RhF can self-associate and form unique immune complexes in the absence of exogenous antigen. Such complexes have been detected in the serum and synovial fluid of patients with RA (Winchester et al., 1970, Brown et al., 1982). Their small size allows them to escape clearance by not activating complement very efficiently in contrast to IgM-RhF based complexes (Brown et al., 1982, Sabharwal et al., 1982). Circulating IgG-RhF has been found to more accurately reflect articular manifestations than other classes of RhF or the presence of immune complexes (McDougal et al., 1982, Lessard et al., 1983).

Traditionally, antibodies were thought to carry their actions mainly by activating complement, facilitating phagocytosis (opsonisation) and leading to antibody-dependent cell-mediated cytotoxicity (ADCC) (Janeway et al., 2001). Over time, other effector functions have been described, including activation of mast cells through the Fc receptor for IgE, FcεRI, activation of macrophages through the interaction with FcγRIIIa (and FcγRIIa) and also inhibitory functions through interaction with FcγRIIb on B lymphocytes (Janeway et al., 2001).

Immune complexes are present in the rheumatoid synovium and in rheumatoid sera. Thus Fc receptors may play an important role in synovial inflammation by triggering macrophage effector mechanisms through immune complexes (Edwards and Cambridge, 1998, Edwards et al., 1999, Abrahams et al., 2000). IgG-RhF self-associating immune complexes had previously been shown to stimulate monocytes (Nardella et al., 1983).

At University College London (UCL), research into the pathogenesis of RA led in the late 1990s to a hypothesis in which B lymphocytes had a central role in the initiation and perpetuation of the inflammation in RA (Edwards and Cambridge, 1998, Edwards et al., 1999). Briefly, it was suggested that certain pathogenic species of disease-associated autoantibodies, in particular IgG-RhF, would be able to form small immune complexes, escape clearance, as they do not fix complement efficiently, and activate macrophages directly through interaction with FcγRIIIa. Significant numbers of macrophages bearing Fcγ receptors are present in rheumatoid synovium and distinctive patterns of expression of the different subtypes of Fcγ receptors suggest differential regulation of these molecules on macrophages in synovium (Broker et al., 1990). FcγRIIIa was found to be expressed preferentially by macrophages located at sites in synovium and other organs involved in RA (Bhatia et al., 1998). Activation of macrophages through FcγRIIIa in a way that simulated binding of small immune complexes through FcγRIIIa, but not other Fcγ receptors, was shown to lead to secretion of TNF by these cells (Abrahams et al., 2000).

It had previously been shown that there was no need for autoreactive T lymphocytes to initiate production of RhF, as RhF-committed B lymphocytes are able to obtain T-lymphocyte help without need for loss of T-lymphocyte tolerance to the Fc fraction of the IgG molecule (Roosnek and Lanzavecchia, 1991). RhF-producing B lymphocytes can take up immune complexes containing IgG attached to foreign antigen and present peptide fragments of the foreign antigen to specific T lymphocytes, thereby receiving help to undergo further development. In addition, the capacity of IgG-RhF to form self-complexes with consequent binding of the complement fragment C3d provides a mechanism for delivering a second survival signal to RhF-committed B lymphocytes (by interacting simultaneously with the B-lymphocyte receptor and complement receptor (CR) 2 (CD21) on the cell surface and consequent self-perpetuation of the RhF-committed B-lymphocyte clones. The initial, published hypothesis was based on IgG-RhF-specific B lymphocytes. It is possible that other RhF (particularly certain species of IgA-RhF or monomeric IgM-RhF) or other autoantibodies can substitute for IgG-RF in this model.

Subsequent to these human studies, arthritis in K/BxN mice was found to be autoantibody mediated. K/BxN mice were created, by Mathis and collaborators, through

introduction of a T-lymphocyte receptor transgene on a NOD background (Kouskoff et al., 1996). These mice spontaneously develop a chronic erosive polyarthritis that shows similarities to human RA. The disease starts with a T-lymphocyte dependent response to glucose-6-phosphate isomerase (GPI) in which autoreactive T lymphocytes recruit anti-GPI B lymphocytes, which then differentiate into plasma cells producing polyclonal anti-GPI antibodies (Matsumoto et al., 1999). The antibodies to this ubiquitously expressed antigen (GPI is a cytosolic enzyme involved in glycolysis) subsequently cause arthritis through a mechanism that is both Fc receptor and complement dependent and mediated by immune complexes (Ji et al., 2002a). Studies in cytokine knockout mice suggest an absolute requirement for Il-1 and a lesser role for TNF in disease development (Ji et al., 2002b). The initiation of synovitis is solely dependent on the autoantibodies, because transfer of sera or purified anti-GPI antibodies from healthy K/B mice into K/BxN mice with no T lymphocytes results in the transient development of arthritis (Korganow et al., 1999, Maccioni et al., 2002). Repeated administration of K/BxN serum or antibody is required to maintain the disease. Interestingly, monoclonal antibodies to GPI were not able to induce disease but were effective when combined in pairs or when in larger pools (Maccioni et al., 2002).

1.2.4.2.2 Antibodies to cyclic citrullinated peptides

Antibodies to citrullinated antigens have the highest specificity for RA yet described. Second generation anti-CCP assays seem to equal RhF assays sensitivity in RA but with higher specificity. Sensitivities of this test in clinically diagnosed RA patients are in the range of 70 percent and specificities as high as 99 percent have been reported (Vincent et al., 2002). Approximately 40% of seronegative RA patients have been described as being anti-CCP positive (Kroot et al., 2000, Vallbracht et al., 2004).

The presence of anti-CCP in the serum is particularly useful for the diagnosis of RA in seronegative inflammatory arthritis and in patients with concomitant diseases where the presence of IgM-RhF is frequent. The presence of anti-CCP, in early undifferentiated arthritis, very strongly suggests a diagnosis of RA and predicts development of erosions (Forslind et al., 2004, Kastbom et al., 2004, van Gaalen et al., 2004, Vittecoq et al., 2004, Ronnelid et al., 2005). However, more recent studies have described positive anti-CCP in 8% of 134 patients with Sjögren's syndrome and in 8% of 192 patients with

psoriatic arthritis (Vander Cruyssen et al., 2005, Gottenberg et al., 2005). Whether these patients are at a higher risk of developing RA is not known. In a study by de Rycke and colleagues, no association was found between anti-CCP and extra-articular manifestations of RA (de Rycke et al., 2004).

Anti-CCP can be found in serum samples up to 14 years before development of clinical disease in RA patients (Nielen et al., 2004, Rantapaa-Dahlqvist et al., 2003). A 5-year follow up study of 160 patients with early (less than 1 year duration) RA showed that anti-CCP status changed very little over time (Ronnelid et al., 2005). At baseline, 57.3% had detectable anti-CCP. Only three patients initially anti-CCP negative became anti-CCP positive during follow up. Only eight of the initially positive patients lost demonstrable anti-CCP at any occasion during follow up and most qualitative changes occurred in samples only marginally positive.

Citrulline is formed by deimination of the amino acid arginine by enzymes known as peptidylarginine deiminases (Vossenaar et al., 2003). Antibodies directed to citrullinated antigens have been found in serum of many patients with RA several decades ago. These were detected by indirect immunofluorescence assays and were known as antiperinuclear factor and antikeratin antibodies. They were later found to be reactive with mature filaggrin and renamed antifilaggrin antibodies. Approximately 50% of RA patients test positive for antifilaggrin antibodies (Zendman et al., 2006). Binding of these antibodies to mature filagrin was later found to be dependent on citrulline residues. Assays to detect antibodies to citrullinated antigens using either naturally occurring citrullinated proteins such as filaggrin or fibrinogen, or in vitro-citrullinated proteins show slightly higher sensitivities up to 60 percent but have numerous technical problems in particular with standardisation (Zendman et al., 2006). The most reliable assays to detect antibodies to citrullinated antigens are based on binding of immunoglobulin to synthetic cyclic citrullinated peptides (cyclization increases sensitivity). First generation anti-CCP tests (CCP1) with a filagrin-based cyclic peptide showed a sensitivity of 68% with a specificity of 98% (Schellekens et al., 2000). As filaggrin is not present in synovium, libraries of citrullinated peptides were screened to find a peptide that allowed higher sensitivity. This led to the second-generation anti-CCP test (CCP2) that shows a sensitivity of up to 80% with similar specificity (Zendman et al., 2006).

The identity of the specific citrullinated antigen driving the production of anti-CCP in RA is not yet known. As previously mentioned, filaggrin is not present in normal or diseased synovium. Protein citrullination occurs in apoptotic cells (Gyorgy et al., 2006, Zendman et al., 2006). Peptidylarginine deiminases and citrullinated proteins (including fibrin) can be detected in inflamed synovia of RA but also non-RA patients and in many cases this is not associated with the presence of anti-CCP antibodies (Vossenaar et al., 2004, Chapuy-Regaud et al., 2005).

Recently, an association has been described between the presence of anti-CCP and the shared epitope (HLA-DRB1*0404/0401). Individuals who carry a shared epitope allele and have detectable anti-CCP antibodies in serum show a very high relative risk of developping RA (odds ratio 66.8, 95% confidence interval 8.3-222.2) (Berglin et al., 2004). The comparison with the odds ratio associated with the presence of each of these risk factors isolated suggests a functional association between the two. Smoking has been found to increase the risk of developing anti-CCP antibodies only in RA patients carrying HLA-DRB1 shared epitope alleles (Klareskog et al., 2006, Linn-Rasker et al., 2006). A study in transgenic mice expressing HLA-DRB1*0401 showed that citrullination at the peptide side-chain position interacting with the shared epitope significantly increased peptide-MHC affinity and led to the activation of CD4 T lymphocytes (Hill et al., 2003). A hypothesis has been advanced that in genetically predisposed individuals, non-specific joint inflammation could eventually cause cell necrosis leading to a T-lymphocyte driven autoantibody response to citrullinated antigens (possibly located on fibrin) with production of immune complexes that would lead to perpetuation of inflammation (Zendman et al., 2006). A role for anti-CCP antibodies as a disease amplifier has also been suggested (Weyand and Goronzy, 2006).

In recent years some studies have looked at changes in anti-CCP antibodies serum levels following treatment, mainly with methotrexate with or without infliximab. Several studies have not shown significant changes in anti-CCP levels following treament (De Rycke et al., 2004, Nissinen et al., 2004, Caramaschi et al., 2005). When a significant decrease was observed it was either not sustained or detected only in patients with disease of shorter duration (Bobbio-Pallavicini et al., 2004, Mikuls et al., 2004, Ronnelid et al., 2005). Only in one study, a decrease in anti-CCP antibodies following

treatment correlated with clinical response (Alessandri et al., 2004). In all the studies that included RhF, IgM-RhF decreased following treatment and was found to correlate with clinical response in some of them (Alessandri et al., 2004, Bobbio-Pallavicini et al., 2004, de Rycke et al., 2004, Mikuls et al., 2004, Nissinen et al., 2004, Caramaschi et al., 2005).

1.2.4.2.3 Other autoantibodies

A number of other autoantibodies have been detected in serum, synovial membrane or synovial fluid of patients with RA but their sensitivity or specificity is lower than that of RhF and anti-CCP. These autoantibodies have been described in the serum, synovial membrane or synovial fluid. These include antibodies to self antigens absent from the joint (Sa protein), to antigens that are ubiquitously expressed (antigens present in nuclear proteins including A2 protein (RA33), heat shock proteins, heavy chain binding protein (p68, BiP), glucose-6-phosphate isomerase, calpastatin) and antigens that are major joint components (human cartilage glycoprotein 39, type II collagen, chondrocyte membrane antigens) (Blass et al., 1999, van Boekel et al., 2002). For some of these antigens, T-lymphocyte autoreactivity has been demonstrated in patients with RA but also in normal controls (Snowden et al., 1997).

1.2.4.3 Other possible roles of B lymphocytes

B lymphocytes are not only the precursors of autoantibody-producing plasma cells but are also efficient APC. Furthermore, B lymphocytes are known to secrete a variety of cytokines and chemokines that can influence the function of other cells in the immune system, and, at least in mice, play an important role in promoting normal follicular dendritic cell formation and normal lymphoid architecture (Fu et al., 1998, Pistoia, 1997, Harris et al., 2000, McDonald et al., 2005, Lipsky, 2001, Shlomchik and Madaio, 2003, Serra and Santamaria, 2006, Gonzalez et al., 1998).

One of the important functions of B lymphocytes as APC is their ability to greatly concentrate antigen by virtue of the specificity of its surface immunoglobulin, the BCR (Watts et al., 1989, Lanzavecchia, 1990, Roosnek and Lanzavecchia, 1991). Antigen-specific B lymphocytes may play an important role in diversifying the immune response, in particular, by presenting sequestered antigens to CD4 T lymphocytes

(Mamula and Janeway, 1993). Interestingly, the specificity of the BCR may direct processing of the protein antigen and therefore which peptides are presented to T lymphocytes (Watts et al., 1989, Jaume et al., 2002). In vitro and in vivo animal studies suggest that activated B lymphocytes can stimulate naïve T lymphocytes and contribute to the loss of T-lymphocyte self-tolerance, particularly by down-regulating presentation of immunodominant epitopes and increasing presentation of cryptic epitopes (Lin et al., 1991, Mamula et al., 1992, Jaume et al., 2002). Whether in human autoimmune diseases, B lymphocytes are able to prime naïve T lymphocytes is not known.

A number of studies have focused on the pathogenic role of B lymphocytes in animal models of autoimmunity. These have suggested that complex interactions between B and T lymphocytes underly disease pathogenesis. In the NOD mouse model of diabetes in which T lymphocytes are known to be the end-stage effector cells, B lymphocytes are important for the development of diabetes as NOD.μMT-/- mice, which do not have B lymphocytes, only rarely develop diabetes (Wong et al., 2004). It has been suggested that B lymphocytes, presumably through their capacity to concentrate and present antigen, may play an essential role in determinant spreading of T-lymphocyte autoimmunity in this model (Tian et al., 2006). However, experiments with chimaeric animals showed that NOD B lymphocytes were not sufficient to disrupt established T-lymphocyte tolerance (Moore et al., 2005). B-lymphocytes are able to activate autoreactive T lymphocytes but only if T-lymphocyte tolerance has not been established (Moore et al., 2005).

Whether secreted autoantibodies play an important pathogenic role in the NOD mice is not known. NOD transgenic mice that express surface IgM but that can not secrete antibodies developed increased islet inflammation and showed an increased incidence of diabetes when compared to NOD B-lymphocyte deficient mice but diabetes was not restored to the level seen in "normal" NOD mice (Wong et al., 2004). Whether this is due to the absence of secreted autoantibodies or to the restricted repertoire that the mIg B lymphocytes have is not known. Transfer of total serum from NOD mice does not precipitate disease but recently one study has shown that maternal autoantibodies are necessary for NOD mice to develop diabetes (Serreze et al., 1998, Greeley et al., 2002). It is possible that these autoantibodies could indirectly promote disease either by

enhancing antigen presentation by APC other than B lymphocytes or by causing tissue damage that releases islet cell antigens.

In animal models of lupus possible pathogenic roles of B lymphocytes independent of secreted autoantibodies have also been suggested. MRL/lpr mice with no B lymphocytes (JhD mutation, targeted deletion of the Jh region which prevents normal B lymphocyte development) had normal kidney histology at an age in which MRL/lpr mice showed severe disease (Shlomchik et al., 1994). These mice did not develop glomerulonephritis, vasculitis or interstitial nephritis. The absence particularly of interstitial nephritis was unexpected as T lymphocytes were thought to play a role in these lesions. In the B-lymphocyte deficient MRL/lpr mice, a 10- to 20-fold reduction in the number of memory T lymphocytes was observed, suggesting a role for B lymphocytes on T-lymphocyte activation (Chan et al., 1999b). In MRL/lpr mice with a transgene that allowed for the expression of surface immunoglobulin but not secretion of antibodies, B-lymphocyte development was restored and T-lymphocyte memory cell numbers and activation was again similar to MRL/lpr mice with normal B lymphocytes (Chan et al., 1999a). These mice developed significant cellular infiltrates in the renal interstitium and around the vessels, composed mainly of T lymphocytes and they showed increased mortality when compared with B-lymphocyte deficient mice. These studies demonstrated a potential pathogenic role of B lymphocytes in T-lymphocyte pathogenicity in this mouse model independent of secreted antibodies. A study in NZW/B F1 mice with a limited B-lymphocyte repertoire showed decreased T-lymphocyte activation (Wellmann et al., 2001).

What these studies suggest is that the reality of human as well as animal model autoimmune diseases is likely to be more complicated than first thought. It is possible that the role of B lymphocytes in the pathogenesis of many autoimmune diseases is dependent on its antigen-presenting capacity as well as on the production of autoantibodies. Studies on the role of B lymphocytes in development of diabetes in the NOD mice are a good example of this. But no doubt there are differences between diseases as far as effector mechanisms are concerned and for RA there is no data strongly suggesting primary loss of T-lymphocyte self-tolerance or that CD4 T lymphocytes are the main effector cells driving macrophage activation in synovium. A study in scid-rheumatoid synovia chimaeras showed that T-lymphocyte activation

seemed to be B lymphocyte dependent but only when T lymphocytes were derived from rheumatoid synovium samples with formation of germinal centres (Takemura et al., 2001b). These infiltrates are only present in about 20% of patients with RA (Takemura et al., 2001a).

1.2.5 Role of T lymphocytes

Hypotheses proposing a primary role for autoreactive T lymphocytes in the pathogenesis of RA are based on observations in humans including: a) the majority of lymphocytes infiltrating the rheumatoid synovium are T lymphocytes; b) RA shows an association with specific HLA-DR genes of the MHC and c) many of the animal models of arthritis are T-lymphocyte dependent.

The majority of lymphocytes seen in the rheumatoid synovium are T lymphocytes, expressing CD4 and CD45RO surface antigens compatible with a memory helper phenotype (Gardner and Arnold, 1992, Firestein, 1998). The activation status and cytokine profile of these cells has been thoroughly studied and suggest that the majority of T lymphocytes in the synovium are in fact not activated and that they have a Th1-like phenotype (Gardner and Arnold, 1992, Firestein, 1998). This attribution of Th1-like phenotype is based on the secretion of small amounts of IFN-γ (despite no secretion of IL-2) and no secretion of IL-4 (Steiner et al., 1999, Firestein et al., 1990).

However, the T-lymphocyte infiltrate in rheumatoid synovium is characteristic of T-lymphocyte infiltrates found in chronically inflamed tissues. It is possible that at least some of the T lymphocytes present in synovium are contributing to local inflammation but there is no strong evidence that these cells are the major effector cells driving macrophage activation. T lymphocytes are rarely seen in contact with intimal synoviocytes (fibroblasts or macrophages) or chondrocytes (Edwards and Wilkinson, 1995). INF-γ has only been detected in small amounts in RA synovium and intimal macrophages do not show the range of gene expression that might be expected in response to this cytokine (Firestein, 1998). Specific T-lymphocyte depletion therapies have not been effective (Moreland et al., 1995, Wendling et al., 1998). However, the efficacy of co-stimulation modulation with CTLA4-Ig (fusion protein consisting of the binding domain of human cytotoxic T-lymphocyte-associated antigen 4 to human IgG,

abatacept) supports a role of T lymphocytes in many patients with RA (Genovese et al., 2005).

As previously discussed, the major identified genetic risk factor for developing RA is the presence of HLA class II alleles that encode for a conserved amino acid sequence designated the "shared epitope". This epitope influences both disease susceptibility and disease severity. These shared epitope residues are located on the α-helical portion of the DRβ chain, in a position where they may influence both peptide binding and T-lymphocyte receptor interaction with the DRB1 molecule. Due to the importance of class II in the presentation of antigen by APC to CD4 T lymphocytes this association has been stated as an argument for the role of T lymphocytes as effector cells in the pathogenesis of RA.

A number of different hypothesis have been advanced to explain the shared epitope association with RA (Nepom and Nepom, 1998). The shared epitope hypothesis postulates that the shared epitope itself is directly involved in the initiation or propagation of RA by allowing the presentation of a peptide antigen, or set of related antigens to "arthritogenic" CD4 T lymphocytes. But the identity of these putative disease-causing peptide antigens has never been identified despite intensive research. Recent studies have provided interesting data on the association of the shared epitope, smoking and the presence of anti-CCP (Linn-Rasker et al., 2006).

A second hypothesis postulates that these risk alleles regulate the formation of the peripheral T-lymphocyte repertoire by acting to select for particular T-lymphocyte receptors during thymic selection. Several studies have tried to find evidence for oligoclonal expansion of T lymphocytes in synovium compatible with specific antigen derived expansion. The results have not been consistent and, to date, there is no evidence that the accumulation of T lymphocytes in rheumatoid synovium is due to a T-lymphocyte antigen-specific response (Paliard et al., 1991, Howell et al., 1991).

Any attempt to explain the association of the shared epitope and RA has to take into account that not all shared epitope alleles carry the same risk and that the strength of the association varies in different populations. The shared epitope itself does not appear to associate with RA in African American and some Hispanic populations. Furthemore,

certain combinations of risk alleles carry higher risks of developing RA than the risk of each one of them added together (Hall et al., 1996). It is not known whether this interaction is due to the HLA-DRB1 molecules themselves or reflect the action of other genes on these haplotypes.

In several animal models of arthritis T lymphocytes are known to play an essential role in disease pathogenesis but there are doubts whether any of these models are good animal models for RA.

Taken together these data support a role for T lymphocytes in immunopathogenic events in RA but it remains unclear whether T lymphocytes are involved in critical initiating events or in the inflammatory effector mechanisms.

1.2.6 Role of resident synovial cells

Resident fibroblastic cells are found at increased numbers at sites of cartilage destruction and clearly play an important role in joint damage by secreting cytokines and metalloproteinases and other proteolytic enzymes that digest matrix proteins such as collagen and proteoglycans (Firestein, 1998). They are also thought to contribute to inflammation by secreting cytokines and to regulate the differentiation of monocytes into osteoclasts (Firestein, 1998). It is thought that synovium inflammation is driven mainly by cytokine-dependent mechanisms but cytokine-independent mechanisms have also been described (Karouzakis et al., 2006, Stanczyk et al., 2006).

A more fundamental role for synovial fibroblasts in rheumatoid synovitis has been proposed by some groups of investigators (Zvaifler and Firestein, 1994, Pap et al., 2005, Firestein and Zvaifler, 2002). Some studies have identified a population of synovial fibroblasts that is present in RA and has characteristics that distinguish them from other synovial fibroblasts (Zvaifler et al., 1997, Pap et al., 2000, Ospelt et al., 2004). These cells express an activated phenotype and secrete metalloproteinases and other proteolytic enzymes. It has also been suggested that they may be able to prolong B- and T-lymphocyte survival by preventing apoptosis. The expression of this "transformed" phenotype can be maintained in immunodeficient mice chimaeras in the absence of

other inflammatory cells (Muller-Ladner et al., 1996). However, it is not clear how specific these changes are and whether they are of pathogenic significance.

1.2.7 Summary

Despite many years of intensive investigation the cause of RA remains unknown. However, its autoimmune nature is widely accepted and significant progress has been made in understanding its pathogenesis. The central role of macrophage activation and cytokine production in causing disease manifestations has been well established and has led to very effective therapeutic approaches. There is clear evidence for B-lymphocyte autoreactivity and circumstantial evidence suggests its involvement in disease pathogenesis, in particular the fact that it often precedes clinically overt disease. There is clear evidence for a T-lymphocyte supportive role but no strong evidence for T-lymphocyte autoreactivity or a primary effector role for T lymphocytes in synovitis. Synovial fibroblasts are involved in joint inflammation and joint damage but there is uncertainty regarding any other pathogenic role.

When investigating the pathogenesis of autoimmune diseases it is important to consider that initiating antigens (or epitopes) might be distinct from perpetuating antigens (or epitopes). Either antigen spread or antigen replacement might be a driving force in established disease. Furthermore, as shown in the K/BxN mouse model of arthritis, responses to ubiquitous antigens may lead to localised joint disease eliminating the need for the inciting stimulus to be based on a joint-associated antigen.

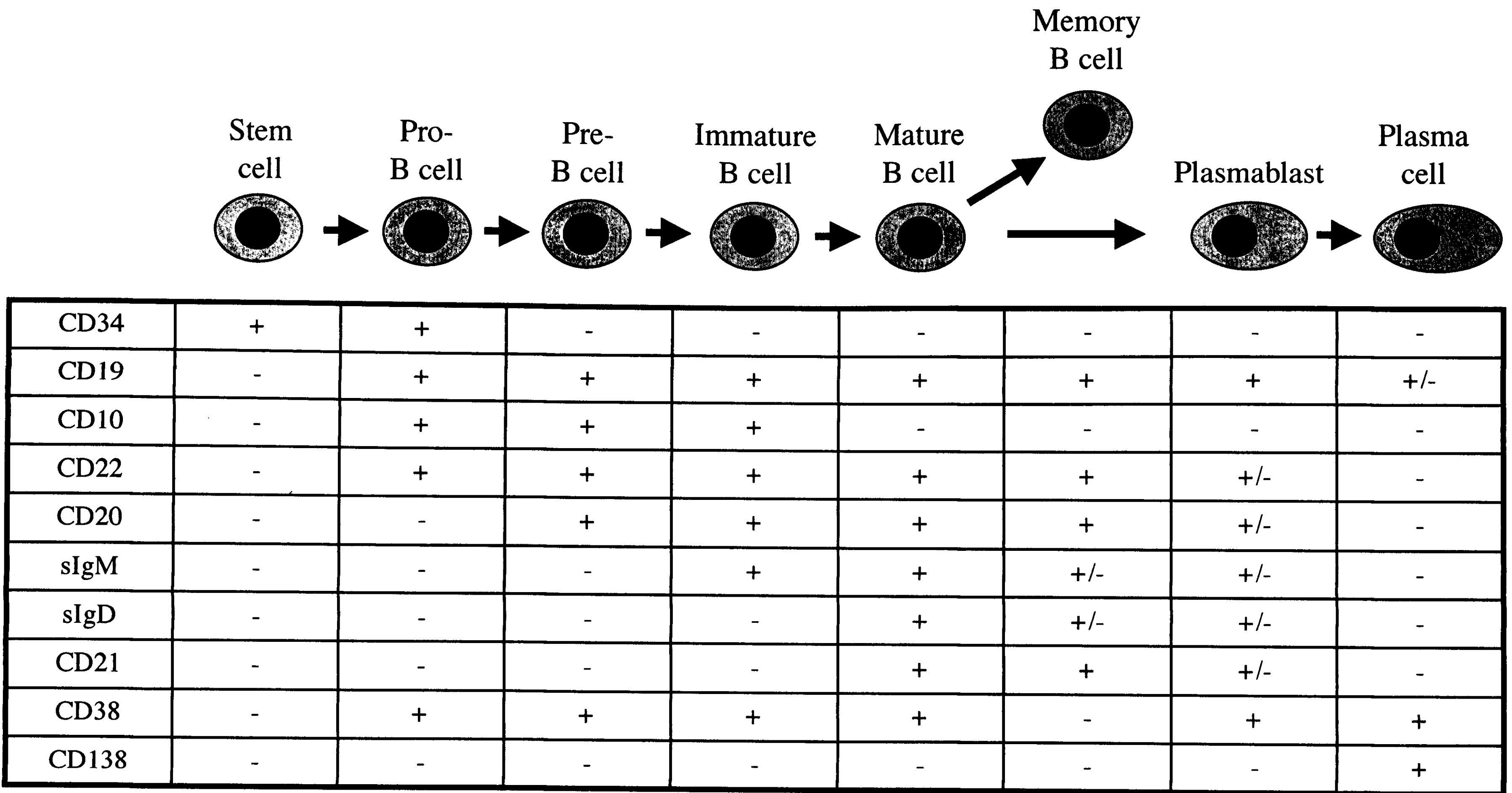
1.3 B-lymphocyte biology

1.3.1 B-lymphocyte development in the bone marrow

During foetal development in the human, B lymphocytes are initially generated in the liver and then in the bone marrow (Gathings et al., 1977, Kamps and Cooper, 1982). After birth, the earlier stages of B-lymphocyte development take place exclusively in the bone marrow. In humans, the rate of B-lymphopoiesis decreases with age but new B lymphocytes are formed continuously throughout life (Nunez et al., 1996, Rossi et al., 2003). B-lymphocyte development is a highly ordered hierarchical process that

involves sequential rearrangement of the immunoglobulin heavy and light chain genes resulting in expression on the cell membrane of a functional IgM molecule (BCR), followed by surface expression of both IgM and IgD (by differential mRNA splicing) that characterizes the mature B-lymphocyte stage. Several studies using multivariable flow cytometry and single-cell polymerase-chain reaction (PCR) techniques have allowed to order B-lymphocyte precursors in the bone marrow in their development pathway, based on their differential surface and intra-cellular expression of different molecules and the rearrangement status of their heavy and light-chain immunoglobulin genes (Loken et al., 1987, Pontvert-Delucq et al., 1993, Ghia et al., 1996, Dworzak et al., 1997, Schneider et al., 1997, Ghia et al., 1998, McKenna et al., 2001, Pellat-Deceunynck and Bataille, 2004) (figure 1.1).

The earliest B-lymphocyte precursors detectable in the bone marrow are pro-B lymphocytes. These cells still express the CD34 marker, characteristic of haematopoetic stem cells, but already express CD19, the characteristic B-lymphocyte marker, and CD10. It is controversial whether there are earlier B-lymphocyte precursors expressing CD10 but not CD19 or CD19 but not CD10 (that is whether one of the markers is expressed earlier on B-lymphocyte ontogeny) (Uckun and Ledbetter, 1988). Pro-B lymphocytes express the recombination activating genes 1 and 2 proteins (RAG-1 and RAG-2), and terminal deoxynucleotidyl transferase (TdT) intracellularly and the components of the surrogate light chain, λ5 and Vpre-B. Rearrangement of the heavy chain immunoglobulin genes takes place in these cells. Once a productive VDJ_H rearrangement has occurred and the cell starts producing μ heavy chain molecules it is known as a pre-B lymphocyte. It is at this stage that CD20 starts being expressed on the cell surface. Production of a μ heavy chain leads to the expression of this molecule on the cell surface in association with the surrogate light chain and Igα and Igβ, as the pre-B-cell receptor. This is believed to lead to cell division and proliferation, and these cells are called 'large' pre-B lymphocytes. They express CD19 and CD10 on their surface but have lost CD34. They do not express RAG-1, -2 or TdT intracellularly. 'Large' pre-B lymphocytes mature to become 'small' pre-B lymphocytes in which rearrangement of the light chain immunoglobulin gene occurs. These 'small' pre-B lymphocytes no longer express the pre-B-cell receptor on their surface. They also lose intracellular expression of the surrogate light chain components. They express RAG-1 and RAG-2 but do not express TdT. When one of the light chains rearranges productively, an IgM



	Stem cell	Pro-B cell	Pre-B cell	Immature B cell	Mature B cell	Memory B cell	Plasmablast	Plasma cell
CD34	+	+	-	-	-	-	-	-
CD19	-	+	+	+	+	+	+	+/-
CD10	-	+	+	+	-	-	-	-
CD22	-	+	+	+	+	+	+/-	-
CD20	-	-	+	+	+	+	+/-	-
sIgM	-	-	-	+	+	+/-	+/-	-
sIgD	-	-	-	-	+	+/-	+/-	-
CD21	-	-	-	-	+	+	+/-	-
CD38	-	+	+	+	+	-	+	+
CD138	-	-	-	-	-	-	-	+

Figure 1.1 Schematic representation of some of the antigens associated with B-lymphocyte differentiation.

molecule is expressed on the cell surface and the cell is named immature B lymphocyte. These cells still express CD10 but do not yet express CD21. The process of immunoglobulin gene rearrangement is tightly controlled; each B lymphocyte expresses only one heavy and one light immunoglobulin chain so that its antigen receptors have only one specificity. At this immature B-lymphocyte stage, central tolerance is induced to antigens present in the bone marrow. Immature B lymphocytes that are stimulated by antigen either die or are inactivated. Further development of B lymphocytes to form naïve mature B lymphocytes involves the expression of both IgM and IgD on the cell surface due to alternative splicing of the mRNA molecule, loss of expression of CD10 and expression of CD21, among other changes.

Despite this orderly sequence of events, within each subset, there is a continuum of maturation. This could account for some of the variability in results on the expression of different markers in different B-lymphocyte precursors, which cannot be explained on the basis of differences in techniques, protocols or reagents used by different investigators.

B-lymphocyte formation in the bone marrow is dependent on cell contact with bone marrow stromal cells and on soluble growth factors and cytokines. These are thought to include IL-7, IL-3 and B-lymphocyte growth factor (Saeland et al., 1992, Moreau et al., 1993).

1.3.2 B-lymphocyte development in the peripheral lymphoid tissues

Mature naïve B lymphocytes enter the peripheral blood to circulate through the secondary lymphoid tissues and can have different fates. Studies in mice have shown that only a small number of the newly made B lymphocytes that are produced daily enter the peripheral pool (Janeway et al., 2001). The majority of them die within a few days presumably due to lack of survival signals (Chung et al., 2003). To survive, B lymphocytes need regular survival signals, for which they have to compete (Rathmell, 2004). These signals are most likely delivered by follicular dendritic cells, when B lymphocytes circulate through the primary follicles in secondary lymphoid tissue. If a new naïve B lymphocyte survives, it will enter the relatively long-lived recirculating

mature naïve B-lymphocyte pool with a half-life between 1 and 2 months (Janeway et al., 2001).

When a naïve B lymphocyte is stimulated by antigen and gets appropriate T-lymphocyte help, it homes to the follicles where it undergoes rapid proliferation. In these secondary follicles, affinity maturation and isotype switching occur. The cells that survive in the germinal center reaction are therefore more likely to be of high affinity and many of them will have undergone class switching to produce immunoglobulins of the IgG, IgA or IgE classes. These cells can now have two different fates. Either they undergo terminal differentiation into plasma cells or they become resting memory cells that will be recruited in the future if a challenge with the same or a similar antigen occurs. The different B-lymphocyte subpopulations can be distinguished by the differential expression of different surface molecules.

The factors that make a B lymphocyte develop into a memory B lymphocyte or differentiate into an antibody-secreting cell are not completely understood.

1.3.3 Memory B lymphocytes

Memory B lymphocytes are descendents of cells that have undergone stimulation by antigen, participated in a germinal centre reaction, acquired somatic mutations in their immunoglobulin variable region genes, with or without isotype switching, and have undergone affinity maturation (Janeway et al., 2001). These cells can be distinguished from naïve B lymphocytes by the presence of somatic mutations on their immunoglobulin genes or by surface expression of the CD27 antigen (Klein et al., 1998, Agematsu et al., 2000).

Memory B lymphocytes are thought to be formed in immune reactions against thymus-dependent (TD) antigens (Janeway et al., 2001). TD antigens include proteins and hapten-protein conjugates for which MHC class II-restricted T-lymphocyte help is needed.

Memory B lymphocytes re-circulate through the peripheral blood and secondary lymphoid tissues in the same way as naïve B lymphocytes (Klein et al., 1998, Janeway

et al., 2001). In the peripheral blood of healthy human adults, memory B lymphocytes represent about 40% of all B lymphocytes (Klein et al., 1998). Human blood memory B lymphocytes form at least four subpopulations (Klein et al., 1998). Two large populations are constituted by "switched" CD27+ cells (cells expressing IgG, IgA or IgE) and by IgD+ IgM+ CD27+ cells, respectively. There is a smaller population of IgD- IgM+ CD27+ cells, called "IgM only" memory B lymphocytes. Finally, a smaller population of IgD+ IgM- CD27+ cells, called "IgD only" memory B lymphocytes, occurs at very low frequency.

Memory B lymphocytes are also found in the marginal zone of the spleen in humans (Zandvoort et al., 2001, Steiniger et al., 2005). In adults, they constitute the larger population of B lymphocytes in this location (Tangye et al., 1998). It is still controversial whether these cells re-circulate or stay within the marginal zone. A recent study has found several similarities between marginal zone B lymphocytes and IgM memory B lymphocytes in the peripheral blood, suggesting that they do re-circulate (Weller et al., 2004). In addition, presence of this memory B lymphocyte population in the peripheral blood seems to dependent on the presence of the spleen (Kruetzmann et al., 2003).

It is still controversial whether memory B lymphocytes themselves can survive for years in a resting state or whether it is memory B-lymphocyte clones who persist for years but individual cells undergo recurrent activation and expansion (Gray and Skarvall, 1988, Maruyama et al., 2000, Fearon et al., 2001, Kelly et al., 2005). Studies on specific memory B lymphocytes suggested that the majority of the cells were in a resting state and do not proliferate but that a small proportion expressed activation markers (Schittek and Rajewsky, 1990) (Janeway et al., 2001). Studies of responses to non-replicating antigens show that germinal centres are present for only 3-4 weeks after initial antigen exposure. Small numbers of B lymphocytes, however, continue to proliferate in the follicles for months. Studies in patients with paroxysmal nocturnal haemoglobinuria have shown that memory B-lymphocyte clones have a long-life span but it is not possible to distinguish between B lymphocytes that have been formed many years before and descendents of these cells (Richards et al., 2000). In a normal adult the total number of B lymphocytes is thought to be kept within certain limits despite the fact that new naïve and new memory B lymphocytes are being formed all the time (Janeway et

al., 2001). It is thought that memory B lymphocytes will have to compete for survival signals, provided by follicular dendritic cells, not only with naïve cells but also between each other. This suggests that at least some state of semi-activation is needed for cells or clones to be able to persist for many years.

If memory B-lymphocyte clones persist for many years due to recurrent activation and expansion, these recurrent cycles of activation could be a consequence of specific antigen stimulation by persistence of specific antigen in the form of immune complexes on follicular dendritic cells or in other forms (e.g. persistence of virus in cells) (Szakal et al., 1989, Gray and Skarvall, 1988, Kosco-Vilbois, 2003). Alternatively, they could be a consequence of activation of these clones by cytokines or other soluble mediators, either constitutively expressed or released during non-cross reactive immune reactions, so called "by-stander activation" (Maruyama et al., 2000, Haberman and Shlomchik, 2003). These antigen-independent mechanisms may include activation by pathogen-pattern signals that interact with Toll-like receptors (TLR) (Peng, 2005). TLR9 has been found to be expressed constitutively on memory B lymphocytes while its expression on naïve B lymphocytes was mainly induced by activation (Bernasconi et al., 2002). These antigen-independent mechanisms could take advantage of the fact that memory B lymphocytes have lower thresholds for activation than naïve B lymphocytes and are also less restrictive in the signals needed for their reactivation (Tangye et al., 2003).

It is known that antigen in the form of immune complexes can remain for a long time on follicular dendritic cells (Gray and Skarvall, 1988, Szakal et al., 1989). Feedback mechanisms have been proposed to regulate antibody serum levels with the antibodies contained in these immune complexes being in equilibrium with the antibodies present in the serum (Gray and Skarvall, 1988, Szakal et al., 1989). It has been hypothesised that when enough plasma cells die or antibody clearance increases and this leads to specific antibody serum levels to decrease below a certain level, antigen epitopes on immune complexes on follicular dendritic cells could be freed and could lead to activation of specific memory B lymphocytes with subsequent activation, expansion and differentiation into plasma cells and increase again in specific serum antibody levels (Bernasconi et al., 2002, Traggiai et al., 2003).

Although most investigators agree that antigen persistence on follicular dendritic cells may have a role on memory B-lymphocyte function not all agree that this interaction is antigen-specific. Some authors argue that the survival signals supplied by these follicular dendritic cells may be non-specific and mediated through complement receptors (Habernan and Shlomchik, 2003). Also, cells may benefit more or less from these signals depending on their state of activation.

1.3.4 Plasma cells

Plasma cells are terminally differentiated B lymphocytes. Their role is to produce and secrete immunoglobulin molecules (antibodies). Antibody-secreting cells can be distinguished as plasmablasts, activated B lymphocytes in the earlier stages of differentiation and still proliferating, and plasma cells, cells that are at the end-stage of differentiation and no longer proliferating. When B lymphocytes activated by antigen undergo differentiation to plasma cells they upregulate expression of CD27 and particularly CD38, start expressing CD138 and lose expression of CD20 and surface immunoglobulin among other changes (Janeway et al., 2001, Medina et al, 2002).

In adaptive immune responses to TD antigens, after encounter with their specific antigen, B lymphocytes enter the T-lymphocyte areas of peripheral lymphoid organs. There they can interact with T lymphocytes specific for peptides which have been precessed from the antigen and are presented on the surface of the B lymphocytes. In these T-lymphocyte areas, primary foci of proliferating B lymphocytes develop. These activated B lymphocytes then migrate either to adjacent follicles or to extra-follicular sites of proliferation located mainly in the medullary cords of the lymph nodes and in those parts of the red pulp of the spleen that are next to the T-lymphocyte zones (Bhan et al., 1981). In these extra-follicular sites, B lymphocytes undergo cell division and differentiation into antibody-producing plasma cells. The B lymphocytes that migrate to the primary follicles form germinal centres, undergo somatic hypermutation, affinity maturation and isotype switching before either becoming memory cells or leaving the germinal centre as plasmablasts and homing either to the bone marrow or to the lamina propria of the gut and other epithelial surfaces (Benner et al., 1981, Dilosa et al., 1991, Tseng, 1981, Smith et al., 1996) (Janeway et al., 2005). Antigen-specific plasmablasts can be detected circulating in the peripheral blood with a peak 6 to 7 days after

immunisation in secondary immune responses (Stevens et al., 1979, Cupps et al., 1984, Medina et al., 2002). Plasmablasts originating in peripheral lymph nodes or spleen migrate to the bone marrow. Those originating in the follicles of Peyer's patches and mesenteric lymph nodes migrate to the lamina propria of the gut and other epithelial surfaces. The majority of these later cells secrete IgA. Antibody-secreting cells in the bone marrow secrete IgM, IgG and monomeric IgA (Hibi and Dosch, 1986).

It is not known what drives plasma cells to have these two different fates. Whether antibody-secreting cells can become long-lived plasma cells may depend on the timing and mode of their generation, as well as whether they are specific for T-dependent or T-independent (TI) antigens. It is possible that plasma cells formed initially in an immune response, stay locally while plasma cells that differentiate from cells that have participated in a germinal centre reaction and have therefore higher affinity antibodies migrate to the bone marrow (Benneer et al., 1981). A detailed comparison between human antibody-secreting cells isolated from tonsils, peripheral blood 6 days after secondary immunisation with tetanus toxoid and bone marrow showed heterogeneity within the plasmablast/plasma cell compartment and suggested a gradient of increased maturity in the direction tonsil → peripheral blood → bone marrow (Medina et al., 2002). Indeed, plasma cell populations in the bone marrow are enriched for cells that synthesise antibody of higher affinity (Ho et al., 1986, Manz et al., 1997, Smith et al., 1997, Slifka et al., 1998). This would make sense from an evolutional point of view, as these are the antibody species most likely to be useful on re-infection. Different chemokines are involved in homing of antibody-secreting cells to the bone marrow (CXCL12 (SDF-1alfa) and its receptor CXCR4) or to the lamina propria of the gut or other epithelia (CCL28 and CCR10, CCL25 and CCR9) (Kunkel and Butcher, 2003).

The bone marrow is thus an important site of production of specific antibodies, not only on secondary immune responses but also on primary responses (Smith et al., 1997). In an established humoral memory response the bone marrow is the principal site of persistent antibody production (McMillan et al., 1972, Benner et al., 1981, Tew et al., 1992). Plasma cells in the bone marrow are thought to provide the majority of circulating immunoglobulins found in the serum and to be responsible for secretion of the antibodies that can last in the blood for years after an initial immune response.

Maintenance of serum antibody levels

The discussion around plasma cell survival is closely related to the discussion about life span and population kinetics of individual memory B lymphocytes and specific memory B-lymphocyte clones and essential to understanding which phenomena are responsible for the persistent detection of specific antibodies in serum for several years following an earlier infection. The half-life of antibody molecules in serum is less than 3 weeks. Therefore, maintenance of specific serum antibody levels requires continuous secretion of antibodies and thus persistence of antibody-secreting cells or their frequent renewal.

Plasma cells can synthesize and secrete several thousand antibody molecules per second (Hibi and Dosch, 1986). Thus, the antibody levels in serum and other body fluids can be maintained by a relatively small population of antibody-secreting cells, which make up only about 0.1% to 1.0% of the cells of secondary lymphoid organs and the bone marrow (Brieva et al., 1991).

In the bone marrow, plasma cells are thought to occupy special niches that allow their survival. The survival of plasma cells in the bone marrow is dependent on soluble factors as well as cell-to-cell contact with bone marrow stromal cells (Cassese et al., 2003). It is thought that the total serum levels of immunoglobulins may be dependent on the number of plasma cells niches available in the body, particularly in the bone marrow. The capacity of the bone marrow for plasma cells seems to be limited as studies showed very similar frequencies of plasma cells in bone marrow samples from different individuals (between 0.2% and 0.5%) (Brieva et al., 1991). A small number of similar niches that allow long-term survival of plasma cells may also be present in the spleen (Sze et al., 2000).

Transfer experiments show that the activity of plasma cells is not dependent on the presence of antigen with increases and decreases in antibody serum levels being due to changes in the numbers of specific antibody-producing cells assuming the clearance of the antibody remains stable (Manz et al., 1998). However, it is not known whether plasma cells change their antibody-secretion rate in response to external stimulus. Several pieces of data from plasmapheresis suggest that the secretion rate of a plasma cell clone is kept relatively constant (Balfour et al., 2005). When plasmapheresis is used alone to remove circulating antibodies produced by plasma cell clones whose numbers

are thought to remain relatively stable for a short period of time, as in lymphoma, there is frequently a fast return of the paraprotein to a total level very similar to the one before treatment. When used to remove antibodies in the context of autoimmune diseases in which an immune reaction is on-going with new plasma cells being formed all the time, rebounds in antibody serum levels following treatment with plasmapheresis are observed. These rebounds are thought to be due to increased generation of new plasma cells presumably by abolition of negative feedback mechanisms exerted by circulating- or follicular dendritic cell- bound immune complexes on specific memory B lymphocytes. These rebounds can be prevented by concomitant use of cyclophosphamide, which is cytotoxic to proliferating cells.

In mice, researchers have shown that long-lived plasma cells can maintain specific serum antibody levels for the lifetime of the animals (Manz et al., 1997, Slifka et al., 1998). It is controversial whether this happens in humans and the life span of individual plasma cells is not yet known. Currently, the most widely accepted view is that the majority of plasma cells found in the induction areas of secondary lymphoid tissues die within a few days but that, in contrast, plasma cells in the bone marrow can survive for several months. The main controversy is whether plasma cells in the bone marrow can live for years or for the lifetime of an individual.

In humans, information on the different life span of plasma cells is mainly circumstantial or based in in vitro studies. Following vaccination or infection, persistent levels of specific antibodies are detectable in human serum for decades even in the apparent absence of the antigen (Slifka and Ahmed, 1996b, Hammarlund et al., 2003). Detectable specific antibodies have been described in patients immunised with vaccinia 25 and more years (up to 75 years) after without any evidence of contact with the virus (Hammarlund et al., 2003). Both mucosal and systemic infections induce long-lasting humoral immunity but antibodies induced by systemic infection persist longer (Slifka and Ahmed, 1996).

Human Ig-forming cells from secondary organs such as tonsil and lymph nodes exhibit short Ig secretion kinetics (3 days) in culture, whereas bone marrow and lamina propria plasma cells secrete Ig in vitro for more prolonged periods (Hibi and Dosch, 1986, Peters et al., 1989, Brieva et al., 1994). Studies suggest that antibody-secreting cells in

secondary lymphoid tissues, peripheral blood and in the bone marrow are at different stages of maturation and this could contribute to their different half-life (Medina et al., 2002). Chimaerism studies, using Gm allotypes, in patients after allogeneic bone marrow transplantation (BMT) have revealed persistence of serum IgG1 and IgG2 of recipient origin more than 1 year (up to 8 years) after BMT in a significant number of patients (van Tol et al., 1996). This suggests that at least a fraction of immunoglobulin-producing cells of recipient origin is not eradicated by the conditioning regimens and can survive for several years.

In patients suffering from autoimmune diseases in which disease-associated antibodies can be detected, autoantibodies frequently persist in serum despite intensive immunosupression (Wahren et al., 1998, Westman et al., 1998, Rosen et al., 2000, Traynor et al., 2000, Izumi et al., 2003). The persistence of autoantibodies in serum despite immunosupression suggests that such autoantibodies are secreted by long-lived plasma cells, refractory to immunosupression and feedback signals from antigen-antibody complexes.

Animal studies

Earlier studies on secondary lymphoid tissues in rodents showed that the majority of plasma cells found in these tissues during an immune reaction died within a few days (Nossal and Makela, 1962). In some of these early studies there was already a suggestion that a small percentage of plasma cells could be long-lived (Miller and Nossal, 1964). Later on, studies showed the importance of the bone marrow as a deposit organ for plasma cells and in the production of specific antibodies at later time points after immunisation (McMillan et al., 1972, Benner et al., 1981) and these plasma cells were shown to live for more than 3 weeks (Ho et al., 1986). More recently, the existence of long-lived, nonproliferating, autoreactive plasma cells has been demonstrated directly in the murine NZB/W model of SLE (Hoyer et al., 2004). Several experiments suggest that plasma cells have the capacity to live for unlimited time if rescued by specific factors provided in a limited number of survival niches in the body (Manz et al., 1997, Slifka et al., 1998, Manz and Radbruch, 2002). The survival of these long-lived plasma cells persisting in the bone marrow following a secondary immune response to ovalbumin was not dependent on the presence of antigen (Manz et al., 1997, Manz et al., 1997). The survival niches are present mostly in the bone marrow but

possibly also in the spleen although at much lower numbers (Slifka and Ahmed, 1996a, Sze et al., 2000, Ellyard et al., 2004). The existence of such survival niches provides an explanation for the progressive decrease seen in many serum antibody responses as competition for survival niches would eventually lead to a replacement of old plasma cells by new ones. This competition for a limited number of niches offers an explanation for the regulation of serum antibody levels (Manz et al., 2002).

The concept of competition between newly generated plasmablasts and old plasma cells for bone marrow survival niches implies that the maintenance of humoral memory for a specific antigen would be mainly regulated by the frequency and strength of subsequent immune responses, that is, the memory for more recent antigens would be established at the expense of the memory for "old" antigens not encountered anymore. Memory for old antigens could be refreshed when such antigens are encountered again and levels of specific antibodies in serum have dropped below protective concentrations. This could lead to progressive replacement of the long-lived plasma cell population to secrete the antibodies that have proved to be the most necessary to deal with the changing exposures through life. In the case of autoimmune reactions where antigen persists and is in excess, the relative contribution to the maintenance of serum autoantibody levels of long-lived versus short-lived antibody-producing cells as well as what is the contribution to the formation of new autoantibody-producing plasma cells of specific memory B lymphocytes or newly formed auto-reactive naïve B lymphocytes is not known.

Plasma cell differentiation

Several factors have been found to interfere either with differentiation of B lymphocytes into plasma cells or directly with plasma cells survival. Differentiation into the plasma cell stage has been associated with loss of expression of the transcription factor BSAP (B cell-specific activating protein), the product of the *pax* 5 gene, and expression of the transcription factor PRDI-BF1 (positive regulatory domain-I-binding factor 1) for which Blimp-1 is the murine homologue) (Calame et al., 2003). It is not known whether there are intrinsic factors to plasma cells that contribute to the differences in their life-span (for example differences in the expression of anti-apoptotic or pro-apoptotic factors or whether plasma cells are intrinsically predisposed to undergo apoptosis within a few days, differences in their life-span being therefore mainly a result of their differential

migration to different microenvironments and the survival signals provided by the different environments (Strasser et al., 1991). Most likely, the numbers of plasma cells originally formed and their migration potential will depend on the amount and form of antigen that triggers the immune response and the quality of help by other cells. Early plasma cells obtained from induction areas in secondary lymphoid organs appear to express molecules that confer propensity to undergo apoptosis, whereas plasma cells that home to the bone marrow seemed to express a non-apoptotic phenotype (Medina et al., 2002). Nevertheless, most data seem to show that survival of plasma cells in the bone marrow is not an intrinsic property of the plasma cells but depends mainly on the ability of the plasma cell to respond to a combination of signals provided by its environment (Minges Wols et al., 2002, Cassese et al., 2003). In very young children antibody responses are shorter than in adults and this is may be due to differences in bone marrow stroma cells and their capacity to support final differentiation of plasmablasts into fully differentiated non-dividing plasma cells (Pihlgren et al., 2006). The persistence of plasma cells in the bone marrow is supported by soluble factors and cell-cell contact. The stromal cells contribute with both soluble survival signals (such as IL-6, IL-5, TNF, BLyS (B-lymphocyte stimulator) and APRIL (a proliferating inducing ligand)) as well as cell contact-mediated signals (for example, through the adhesion receptor VLA-4 (very late antigen 4) on plasma cells and VCAM-1 on stromal cells). Animal studies with TACI-Ig (fusion protein between one of the cellular receptors for BLyS and the Fc portion of human IgG) treatment suggest that BLyS and/or APRIL are necessary for terminal differentiation of marginal zone B lymphocytes in TI responses (Balazs et al., 2002). Also, in vitro studies have shown that BLyS selectively enhances the survival of plasmablasts generated from human memory cells (Avery et al., 2003). Survival niches providing such signals are apparently scattered throughout the bone marrow (Tokoyoda et al., 2004). Their number seems to be limited because the frequency of plasma cells in the bone marrow is constant at about 0.5% throughout adult life (McMillan et al., 1972).

Plasma cells in extra-lymphatic sites

Plasma cells have also been identified in several types of inflamed tissue in animal models and in humans, including in rheumatoid synovia (Munthe and Natvig, 1972, Kim et al., 1999, Cassese et al., 2001). Plasma cells found in inflammatory sites can arise locally from activated B lymphocytes but a proportion of them seem to be

generated in secondary lymphoid tissues and then migrate to the inflamed tissues (Kupp et al., 1991, Berek and Kim, 1997, Kim et al., 1999, Cassese et al., 2001). CXCR3 and its ligands CXCL9 and CXCL10 are involved in migration of antibody secreting cells to inflammatory sites (Kunkel and Butcher, 2003). These cells provide high local antibody concentrations in the inflamed tissue. These cells can produce autoantibodies but their specificity has been found not to be restricted to self-antigens and their presence in different inflammation sites has not been found to be dependent on the specificity of the antibodies they produce (Wahren et al., 1998, Munthe and Natvig, 1972, Cassese et al., 2001). Inflammatory cytokines produced locally such as IL-6 and TNF may contribute to plasma cell survival at these sites although it is not known for how long plasma cells can survive in inflammatory sites.

1.3.5 CD5 B lymphocytes

The CD5 antigen is expressed on the surface of thymocytes and T lymphocytes and at a lower density on a subpopulation of B lymphocytes (Small et al., 1990). In man, the CD5 marker was first identified on malignant B lymphocytes and only subsequently demonstrated as a marker of normal B lymphocytes (Caligaris-Cappio et al., 1982). In both man and mice, CD5 B lymphocytes are one of the major subpopulation in foetal life, in cord blood and in the peripheral blood of neonates (Small et al., 1990). Their frequency decreases with age. Small and colleagues described frequencies ranging from 20% to 69% (n = 9, median 34%) in cord blood, 10% to 30% (n = 9, median 22%) in children aged 2 to 12 years and 5% to 25% (n = 75, median 15%) in normal adults (Small et al., 1990).

Expression of CD5 on murine B lymphocytes

In mice, CD5 B lymphocytes have been studied extensively and they belong to the B-1 B-lymphocyte subpopulation (they constitute the B-1a subpopulation) (Janeway et al., 2001). B-1 lymphocytes are characterised by arising early in ontogeny and by distinctive receptor repertoire and functional properties. These cells express low affinity, polyreactive, surface immunoglobulins that frequently recognise carbohydrates present in common pathogens. This subpopulation is relatively sparse in lymph nodes and spleen but is the predominant subpopulation in serosal cavities (Berland and Wortis, 2002). It continues to be controversial whether these CD5 B lymphocytes are a distinct

lineage of cells, derived from committed precursors that arise only in the foetal and neonatal period and are thereafter capable of self-renewing, or whether they arise from common B-lymphocyte precursors in response to specific signals derived from the BCR and therefore dependent on the BCR specificity expressed by the B lymphocyte, particularly stimulation by self-antigens and TI-2 antigens (Janeway et al., 2001, Berland and Wortis, 2002).

It has been proposed that differences in the ability of different precursors to generate B-1 cells are due to differences in their repertoire (Berland and Wortis, 2002). There is evidence to suggest that there are differences between the repertoire generated during B-lymphocyte development in early versus later life. In the foetal liver, immunoglobulin heavy-chain rearrangements are dominated by those using variable heavy-chain (VH) gene segments closest to the D gene segments with few, if any, N-nucleotide insertions as TdT is not active in the prenatal period. In contrast, VH gene usage in adults reflects more the number of VH genes in each family. The BCR specificity-driven differentiation theory would help to explain differences found between B-1 cells in the neonate and in adult mice. B-1 cells that develop postnatally use a more diverse repertoire of VH genes and their rearranged immunoglobulin genes have abundant N-nucleotides.

The foetal and neonatal B-lymphocyte repertoires are skewed toward the expression of immunoglobulins that bind frequently encountered TI-2 antigens and also manifest weak autoreactivity (Hardy and Hayakawa, 2005). It is likely that before birth, the B-lymphocyte repertoire will be mainly selected by self-antigen. To what extent the characteristics of immunoglobulin gene rearrangements during foetal and neonatal life and possible selection by weak interactions with self-antigens influences development of the earlier B-lymphocyte repertoire is not known. It has been suggested that CD5-positive B lymphocytes express a repertoire enriched for weak autoreactivity selected by self-antigens (Berland and Wortis, 2002). The CD5 molecule may be functioning as a negative regulator of BCR signalling that may help prevent inappropriate activation of autoreactive B lymphocytes. Expression of low levels of CD5 by anergic B lymphocytes (cells in which BCR ligation does not induce calcium flux or proliferation) has been shown to help in maintaining anergy (Hippen et al., 2000). Induction of a B-1

phenotype could serve to tolerise B lymphocytes with certain specificities while still keeping them available for certain responses.

Marginal zone B lymphocytes are another phenotypically and functionally distinct subpopulation of cells in mice. Entry into the marginal zone subset also appears to be a consequence of BCR specificity (Berland and Wortis, 2002). How much does low-level activation through the BCR by self-antigen influences development of these two B-lymphocyte compartments is controversial. Also, it is not known whether the strength of the signal influences differential development into one or the other population. It has been suggested that a stronger signal is needed for B-1 differentiation when compared to marginal zone differentiation (Berland and Wortis, 2002). Both B-1a and marginal zone B-lymphocyte development are likely to need weaker signals than follicular B-lymphocyte development. Marginal zone B lymphocytes can be activated more rapidly by polyclonal stimuli than follicular B lymphocytes and they are thought to provide a rapid first line response to pathogens. B-1a lymphocytes are thought to be involved in responses to TI-2 antigens but what is their role in these responses in comparison with marginal zone B lymphocytes is not known (Berland and Wortis, 2002). B-1 and marginal zone B lymphocytes are thought to provide "natural memory" in mice (Baumgarth et al., 2005). In mice, one of the interesting differences between these two subsets is the fact that marginal zone B lymphocytes seem to be very dependent on BLyS while the opposite seems to occur with B-1 cells.

Expression of CD5 on human B lymphocytes

In humans, CD5 B lymphocytes are part of the normal B-lymphocyte repertoire and can be found circulating in peripheral blood as well as in secondary lymphoid tissues. CD5 B lymphocytes have not been extensively studied in humans and it is not clear to what extent they are comparable to B-1a cells in mice (Berland and Wortis, 2002). In humans, CD5 B lymphocytes are abundant early on ontogeny. CD5 B lymphocytes constitute frequently the majority of B lymphocytes in cord blood; their frequency in peripheral blood decreases with age until it reaches adult levels (5 to 25%) (Small et al., 1990, Youinou et al., 1999). In tonsils, 15-30% of B lymphocytes express CD5 while in the spleen this percentage is lower, around 10% (Youinou et al., 1999, Dono et al., 2004). The majority of CD5 B lymphocytes isolated both from the peripheral blood and from tonsils are naïve cells, expressing unmutated VH region genes (Fischer et al.,

1997, Brezinschek et al., 1997). In secondary lymphoid tissues, CD5 cells are found predominantly within the follicular mantle cell population (cells with CD23+ IgM+ IgD+ CD10- CD38+/- phenotype). These cells express both CD5 and CD23 while CD5-positive B lymphocytes in mice are characteristically CD23 negative (Berland and Wortis, 2002, Dono et al., 2004). Also, they express normal levels of IgD while in mice B-1 lymphocytes express low levels or no IgD ((Berland and Wortis, 2002, Dono et al., 2004)). In humans, the frequency of CD5 B lymphocytes does not seem to be affected by the lack of spleen in contrast to mice (Kruetzmann 2003). Somatic mutations can be found in some human CD5 B lymphocyte but not in peritoneal B-1 lymphocytes in mice (Ebeling et al., 1993).

Recently, it has been proposed that CD5 expression on B lymphocytes in humans can be either constitutive or induced by cell activation by various stimuli and that CD5 B lymphocytes are in fact two different subpopulations (Youinou et al., 1999). Only one of these subpopulations (presumably the one that expresses CD5 constitutively) appears to share other phenotypic properties with murine B-1 lymphocytes, such as the production of polyspecific autoreactive antibodies (Casali et al., 1987, Hardy et al., 1987).

CD5 B lymphoytes are thought to be involved in autoimmunity and are involved in early recovery after bone marrow transplantation and in malignant diseases (B-cell chronic lymphocytic leukaemia and mantle cell lymphoma). Some studies have found increased frequency of CD5 B lymphocytes in the peripheral blood of patients with autoimmune diseases, including RA, systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), Grave's disease and insulin dependent diabetes mellitus (Brennan et al., 1989, Plater-Zyberk et al., 1989, Munoz et al., 1995, Youinou and Lydyard, 2001). Some studies but not others have described a positive relationship between CD5 expression and disease activity in SLE and multiple sclerosis (Bongioanni et al., 1996, Huck et al., 1998, Seidi et al., 2002). CD5 B lymphocytes have been reported to produce low affinity autoantibodies, particularly IgM and thus suggested to be a source of high affinity pathogenic autoantibodies (Burastero et al., 1988, Pers et al., 1999, Youinou and Lydyard, 2001). However, most studies have shown that this is not exclusive of CD5 B lymphocytes (Munoz et al., 1995, Hou et al., 2003). In chronic immune thrombocytopaenic purpura both splenic CD5-positive and CD5-negative B

lymphocytes have been shown to produce IgG antibodies to platelet glycoproteins (Hou et al., 2003). Whether these cells could lose expression of CD5 upon stimulation and therefore be no longer traceable based on their surface or functional phenotype is not known.

Following bone marrow transplantation, an increased frequency of CD5 B lymphocytes is often found at B-lymphocyte repopulation (Small et al., 1990). This has been thought to reflect recapitulation of ontogeny and expression of an immature phenotype. In humans the expression of CD5 occurs late in the development of B lymphocytes most likely at the stage of immature B lymphocytes. The increased frequency of CD5 expressing B lymphocytes after bone marrow transplantation raises questions on the role of CD5 in B-lymphocyte development and function, and on the regulation of CD5 expression. Do all newly formed naïve B lymphocytes express CD5? If not, which ones do and is CD5 expression dependent on the specificity of their BCR? Are there differences in the repertoire generated when the bone marrow is regenerating after depletion when compared to normal B-lymphocyte development in adults? Is CD5 expression on B lymphocytes regulated differently when the bone marrow is regenerating when compared to when normal continuous B-lymphocyte development is occurring?

Polyreactive antigen-binding B lymphocytes were much more commonly found in the newborn B-lymphocyte repertoire than in the adult one (Chen et al., 1998). Patients with SS, RA and SLE had similar or slightly lower frequencies of polyreactive B lymphocytes than healthy adults. These polyreactive B lymphocytes expressed IgD, CD40 and HLA-DR but very few expressed CD80 or CD86. In newborns the majority expressed CD5 (93%) whereas, in adults, only 40% of these polyreactive B lymphocytes expressed this marker. This indicates that expression of the CD5 marker is not directly linked to polyreactivity.

CD5 B lymphocytes are thought to be the precursors of plasma cells or lymphoplasmacytoid cells that produce polyreactive antibodies which are primarily IgM, although IgA and IgG polyreactive antibodies have also been identified (Casali and Notkins, 1989a). Polyreactive antibodies are thought to be a major constituent of the so-called "natural antibody" found in serum. Polyreactive antibodies differ in

various ways from monoreactive antibody molecules. Polyreactive antibodies can bind to a variety of different self and non-self antigens, have low affinities (ranging from 10^{-4} to 10^{-7} mol/L) and utilise germ-line sequences with few, if any, mutations (Burastero et al., 1988, Casali and Notkins, 1989a, Avrameas, 1991, Harindranath et al., 1991). Some authors have suggested that germ-line immunoglobulin genes may encode proteins which display greater flexibility when encountering an antigen and be able to accommodate a number of different antigenic configurations in contrast with the more rigid structure from monoreactive antibodies (Wedemayer et al., 1997).

1.3.6 Marginal zone B lymphocytes

Marginal zone B lymphocytes were first characterized in mice. These cells are characterised by expression of high levels of IgM, low levels of IgD and high levels of CD21 (CR2). There are several differences between marginal zone B lymphocytes in mice and in humans (and other primates). The morphology of the marginal zone is different between the two species. Mouse splenic white pulp consists of a large T-lymphocyte zone surrounding the central arteriole (the periarteriolar lymphoid sheath zone) capped by smaller peripheral lymphoid follicles (Martin and Kearney, 2002, Pillai et al., 2005). The lymphoid follicles are surrounded by a marginal sinus containing marginal metallophillic macrophages, which separates the follicular B lymphocytes from the marginal zone B lymphocytes. In contrast, primate splenic white pulp lacks marginal zone sinuses and has large B-lymphocyte follicles with a prominent band of myofibroblasts that divides the outer marginal zone from the rest of the follicle (Steiniger et al., 2001). In mice, marginal zone B lymphocytes are naïve B lymphocytes, found only in the spleen and do not recirculate. In human, marginal zone B lymphocytes are found in other anatomical sites in addition to the spleen such as lymph nodes, consist primarily of memory cells in adults and recirculate (Spencer et al., 1998, Martin and Kearney, 2002, Pillai et al., 2005).

Marginal zone B lymphocytes are thought to include autoreactive B lymphocytes and may play a role in human autoimmune diseases pathogenesis (Batten et al., 2000, Segundo et al., 2001, Martin and Kearney, 2002, Mackay and Ambrose, 2003).

1.3.7 Further aspects of B-lymphocyte function

1.3.7.1 Thymus-independent and thymus–dependent antigens

Antigens can be classified as thymus- or T-lymphocyte-dependent (TD) or thymus- or T-lymphocyte independent (TI) antigens (Janeway et al., 2001).

Thymus-independent antigens

There are two classes of TI antigens that activate B lymphocytes by two different mechanisms (Janeway et al., 2001). TI type 1 (TI-1) antigens possess an intrinsic activity that can directly induce B-lymphocyte proliferation and they can activate both immature as well as mature B lymphocytes (for example, lipopolysaccharides). At high concentrations, these molecules cause the proliferation and differentiation of most B lymphocytes regardless of their antigen specificity (polyclonal activation). But at low concentrations (as in the earlier phases of an infection) only B lymphocytes whose antigen receptor binds the TI-1 antigen will be activated as only in the presence of specific binding will sufficient amounts of TI-1 antigen be concentrated on the B-lymphocyte surface. These B lymphocytes then mature into plasma cells which secrete antibodies to the TI-1 antigen. TI-1 antigens are inefficient inducers of isotype switching, affinity maturation or memory B cells, all of which require specific T-lymphocyte help.

The second class of TI antigens, consist of molecules such as capsular polysaccharides that have highly repetitive structures (for example, pneumococcal capsular polysaccharides). These TI type 2 (TI-2) antigens have no intrinsic B-lymphocyte stimulating activity and they can only activate mature B lymphocytes, presumably by multivalent cross-linking of the BCR. Immature B lymphocytes are characteristically inactivated by repetitive epitopes. After revaccination with TI-2 antigens antibody levels increase but an anamnestic response does not occur (Garner and Pier, 1989, Breukels et al., 2001, Musher et al., 1993). Response to TI-2 antigens does not require MHC class II-restricted T-lymphocyte help but does need T-lymphocyte factors as it is greatly diminished in animals with complete absence of T lymphocytes (Janeway et al., 2001). In immune reactions to TI-2 antigens both IgM and IgG antibodies are induced and somatically mutated antibodies can be found (Janeway et al., Musher et al., 1993). It is thought that T lymphocytes release cytokines that augment the production of

antibody against TI-2 antigens and induce isotype switching and somatic mutation. As TI-2 antigens bind the complement fragment C3d, it is possible that they can bind to follicular dendritic cells in germinal centres through CD21 (CR2) and thereby allow specific B lymphocytes to receive by-stander help from T lymphocytes. Antibodies against for example pneumococcal capsular polysaccharides (anti-PCP antibodies) usually persist for at least 5 years in healthy adults following vaccination or natural infection (Mufson et al., 1987). Whether this is due to the formation of long-lived plasma cells is not known.

Many common extracellular bacterial pathogens are surrounded by a polysaccharide capsule, which enables them to resist ingestion by phagocytes. This way the bacteria not only escape direct destruction by phagocytes but also avoid stimulating T-lymphocyte responses through the presentation of bacterial peptides by macrophages. B-lymphocyte responses to TI-2 antigens provide a prompt and specific response to an important class of pathogens. Responses to TI-2 antigens are deficient in infants. In mice, responses to several TI-2 antigens are prominent among marginal zone B lymphocytes. These cells are rare at birth and accumulate with age. As previously discussed, in humans, a subset with a similar function is thought to exist. These cells may be responsible for most physiological TI-2 responses, which also increase with age (Timens et al., 1989, Breukels et al., 2001).

Thymus-dependent antigens

TD antigens include proteins and hapten-protein conjugates for which MHC class II-restricted T-lymphocyte help is needed (Janeway et al., 2001). Immunological memory is the ability of the immune system to respond more rapidly and effectively to pathogens that have been encountered previously and reflects the pre-existence of a clonally expanded population of antigen-specific lymphocytes. In repeated exposures to the same antigen, the characteristics of the antibodies produced are distinct to those produced in the primary responses to the same antigen due to processes of somatic hypermutation, affinity maturation (selection for survival of B lymphocytes with high affinity for the antigen) and isotype switching. Immunological memory occurs typically following responses to TD antigens and is therefore T-lymphocyte dependent.

1.3.7.2 B lymphocyte tolerance to self

It has been established that when immature B lymphocytes encounter their antigen they either die by apoptosis, are rendered anergic or undergo receptor editing, a process which allows them to change the specificity of their BCR by new rearrangements in the available light chain immunoglobulin genes (Janeway et al., 2001). These are known to be important mechanisms by which tolerance to self-antigens is maintained in mice.

For mature B lymphocytes, a coherent theory to explain both cell responsiveness and tolerance to different antigens does not exist. The consensus model to explain tolerance to self-antigens is the two-signal hypothesis, which proposes that in the absence of T-lymphocyte help, BCR stimulation is a negative signal for B lymphocytes, leading to death or functional inactivation (Bretscher and Cohn, 1970). Therefore, as long as T-lymphocyte tolerance is maintained, tolerance of mature B lymphocytes will follow. However, mature B lymphocytes can respond to TI-2 antigens. So the question arises of how do B lymphocytes distinguish between TI-2 antigens and multimeric self determinants (Gavin et al., 2004). Several possible mechanisms have been suggested.

Lederberg proposed that the sensitivity to tolerance-induction mechanisms of B lymphocytes may be age-dependent with cells from very young individuals being tolerance sensitive, whereas those of adults would respond with activation (Lederberg, 2002). Several studies have indicated that neonatal cells may be especially susceptible to tolerance induction (Klinman, 1996). These cells do not appear to undergo receptor editing like adult newly formed bone marrow cells and are tolerised mainly by an apoptotic process (Hertz and Nemazee, 1997). Interesting, it is well known that the ability of B lymphocytes to respond to TI-2 antigens is acquired relatively slowly after birth, usually within the first 2 years of age.

Nemazee and collaborators have postulated that mature B lymphocytes use NK-like missing self recognition to provide the self/non-self discrimination needed to distinguish foreign TI-2 antigens from multivalent self antigens (Nemazee and Gavin, 2003). They have proposed that self antigens, even those that are displayed in a multimeric array, are associated with one or more widely expressed "self markers" that are capable of stimulating a B-lymphocyte's inhibitory receptor. Putative "self markers" include ligands for CD22. Tissue heterogeneity in the expression of self markers and

possible allelic variation, could lead to a situation in which in certain individuals particular tissues are more vulnerable than others to a breakdown in tolerance. This could explain how even general defects in lymphocytes can lead to specific autoantibody production (Okazaki et al., 2002). The suggestion is that B-lymphocyte recognition of self is mediated by inhibitory receptors, a cell intrinsic mechanism, which facilitates the self/non-self discrimination. The outcome of signalling through the BCR, including B-lymphocyte anergy or activation, survival or death, will reflect thresholds of positive and negative signals, along with extrinsic signals that shift these thresholds.

Other factors are also known to be important, at least in mouse models. In transgenic mouse models, the fate of autoreactive B lymphocytes is dependent on the presence of competing, non-autoreactive B lymphocytes, indicating that B lymphocytes compete for limiting survival factors or niches (Schmidt and Cyster, 1999, Lang and Nemazee, 2000).

Three main mechanisms influence the peripheral repertoire of B lymphocytes. First, the potential diversity in lymphocyte repertoire is created largely by a well-defined, developmentally regulated process in the bone marrow through the recombination of the genes coding for the variable region of the heavy (VH) and the light (VL) chains with insertation of templated and non-templated nucleotides at the junctions (Yancopoulos and Alt, 1986, Alt et al., 1987). Second, several selection processes take place based on the antigen-binding domain of the antibody expressed on the cell surface (Grandien et al., 1994, Tarlinton, 1994). This way autoreactive clones are deleted in the bone marrow, excluded from access to survival signals in the peripheral organs, or induced into an anergic state (Nemazee et al., 1991, Cyster and Goodnow, 1995). Third, antigen-driven responses during life induce the proliferation of specific clones, introduce somatic diversification by mutation and generate a pool of memory B lymphocytes (McHeyzer-Williams, 1997, Healy and Goodnow, 1998). Together, these processes result in alterations of the antibody repertoire during B-lymphocyte development as well as during ontogeny and senescence (van Dijk-Hard et al., 1997).

After birth, serum IgM and IgG reactivity patterns diversify until 1-2 years of age, after which they are relatively stable. Reactivity patterns change from expressing a high

degree of homogeneity among neonates to being more diverse in children, young adults and elderly individuals. Self-reactive repertoires are more conserved between individuals and in time than anti-bacterial repertoires (Mouthon et al., 1995b, Mouthon et al., 1995a, Mouthon et al., 1996, Lacroix-Desmazes et al., 1999).

At a molecular level, several studies suggest that expression of the VH gene family may not be random, nor a simple reflection of genomic complexity (Huang et al., 1996, Davidkova et al., 1997). The genes coding for the VH region are divided into seven families, based on nucleotide sequence homology and are located on chromosome 14q32.33. Restricted VH gene family usage has been shown early in fetal development, in malignant B lymphocytes, in CD5-positive B1 B lymphocytes and in autoantibody repertoires (Sanz et al., 1989, Casali and Notkins, 1989b, Kipps et al., 1989, Logtenberg et al., 1989b, Cuisinier et al., 1990, Schroeder and Wang, 1990, Pascual et al., 1990). Adult peripheral repertoires show a VH gene family utilization pattern that correlates approximately with their genomic complexity, with the VH3 gene family most frequently used (Tonnelle et al., 1995, Huang et al., 1996, Davidkova et al., 1997). However, some of the smaller gene families are over-represented, while only a few members of the VH3 family are preferentially used (Raaphorst et al., 1992, Schroeder et al., 1998).

Van Dijk-Hard and Lundkvist found a high degree of stability in the VH gene family B-lymphocyte repertoire over time in the same individual as well as between individuals with a Caucasian background (Van Dijk-Hard and Lundkvist, 2002). Primary usage of VH3 and VH5 family was found. In one individual at one-time point, a deviating pattern was found with decreased expression of VH3 and increased expression of VH4 and VH5. This pattern resembled the pattern observed in naturally polyclonal activated B lymphocytes. The fluctuation correlated with the presence in serum of RhF. This could reflect the possible influence of polyclonal, transient stimulation of B lymphocytes on VH gene repertoires in circulating B lymphocytes.

Other authors suggest that the expression pattern of the different VH families in the adult peripheral blood B lymphocytes merely reflects their respective sizes (Logtenberg et al., 1989a, Guigou et al., 1990). VH3, which is the largest family accounts for about 60% of the heavy chains, whereas the VH6 single-member family represents less than

5%. Very early in ontogeny, VH6 and VH5 have been shown to occur first and to be transiently overexpressed (Berman et al., 1991). This seems, at least in part, to be due to a topological effect as VH6 and one of the VH5 are localised at the 3' end of the VH locus but association with selection of a particular repertoire by antigen, particularly self-antigen, cannot be excluded

1.3.7.3 BLyS, APRIL and B-lymphocyte homeostasis

B-lymphocyte stimulator (BLyS), a member of the TNF family of cytokines, is an essential survival factor for B lymphocytes and has also been implicated in B-lymphocyte development (Moore et al., 1999, Cancro, 2004). BLyS is also known as BAFF (B-cell activator factor belonging to the TNF family), TALL-1 (TNF- and ApoL-related leukocyte-expressed ligand 1), THANK (TNF homologue that activates apoptosis, NF-kB and JNK) and zTNF4. BLyS exists in a membrane bound and in a soluble form. It is predominantly expressed by cells of myeloid lineage (monocytes, macrophages and dendritic cells), and not by B or T lymphocytes or NK cells (Moore et al., 1999, Nardelli et al., 2001). Activated neutrophils and stromal cells from lymphoid tissues may also produce BLyS (Scapini et al., 2005, Gorelik et al., 2003). Interferon-γ and less IL-10 increase BLyS expression or secretion (Nardelli et al., 2001).

BLyS binds to three receptors, BR3 (BLyS receptor 3, also known as BAFF-R), TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor) and BCMA (B-cell maturation antigen). BR3 and BCMA expression is restricted to B lymphocytes with BR3 being the predominant receptor expressed by B lymphocytes (Gras et al., 1995, Moore et al., 1999, Thompson et al., 2000, Thompson et al., 2001, Avery et al., 2003). TACI has been found on resting B lymphocytes and a subset of activated T lymphocytes (von Bulow and Bram, 1997). BR3 seems to be the receptor mediating the major pro-survival and development signals as mice with a mutation in this receptor have a similar phenotype to BLyS knockout mice (Yan et al., 2001). BR3 is upregulated upon activation of B lymphocytes through the BCR (Smith and Cancro, 2003) and is downregulated as cells differentiate into plasma cells (Avery et al., 2003). TACI seems to be involved in negative signalling as TACI knockout mice have increased numbers of B lymphocytes and develop a lymphoproliferative disorder as they age (Yan et al., 2001, Seshasayee et al., 2003). Nevertheless, recent descriptions of

mutations in the TACI gene in patients with primary immunodeficiencies suggest that TACI role, at least in humans, may be more complex (Salzer and Grimbacher, 2005). BCMA is upregulated during plasma cell development and has been implicated in long-term plasma cell survival in the bone marrow (Avery et al., 2003, Tarte et al., 2003, O'Connor et al., 2004). BLys signalling results in upregulation of anti-apoptotic bcl-2 family members.

Both TACI and BCMA bind another ligand from the TNF family known as APRIL. The function of APRIL is not fully understood and it is not known to what extent it may contribute or even, be responsible for some of the activities attributed to BLyS (Baker, 2004, Dillon et al., 2006, Ramanujam et al., 2006). It is known to be involved in tumour growth (Ware, 2000).

BLyS seems to have a role in protecting both resting and activated B lymphocytes from apoptosis and presumably has a role both in conservation of naive B-lymphocyte homeostasis and in normal B-lymphocyte immune responses (Do et al., 2000, Avery et al., 2003). Some results suggest that BLyS may have a preferential effect on activated cells and on cells with a marginal zone phenotype, which express higher levels of BR3 (Mackay et al., 1999, Avery et al., 2003, Vugmeyster et al., 2006). BLyS signalling is important in antibody responses to TI-2 antigens and may also influence the survival of both short-lived and long-lived plasma cells (Mackay et al., 1999, Do et al., 2000, Avery et al., 2003, O'Connor et al., 2004).

BLyS has no effect on B-lymphocyte precursors before the immature B-lymphocyte stage (Mackay et al., 1999). BLyS knockout mice have normal numbers of B-lymphocyte precursors and T1 transitional immature B lymphocytes but very few mature B-2 lymphocytes, with reduced immunoglobulin serum levels and normal B-1 lymphocyte populations (Gross et al., 2001). These findings suggest that the point of arrested differentiation of B-2 lymphocytes (T1 transitional state) is the earliest step at which BLyS is essential for normal B-lymphocyte development in mice. Studies in mice deficient in BLyS or BR3 suggest that BLyS is not essential for the initiation of a germinal centre reaction but is required for the maintenance of the response (Rahman et al., 2003, Vora et al., 2003).

It has been suggested that BLyS plays a role in autoimmunity. Two main lines of evidence link BLyS to autoimmunity, namely studies in animal models and the finding of elevated BLyS levels in patients with autoimmune diseases. Results of treatment with anti-BLyS strategies provide further putative evidence, even though its effects may be independent of whether BLyS disturbances play a primary role in disease pathogenesis. Abnormalities in the BLyS pathway could presumably lead to abnormal survival of autoreactive B lymphocytes allowing them to escape normal regulatory mechanisms and to less controlled humoral immune reactions and therefore contribute to autoimmunity. Autoantigen-binding B lymphocytes may also have an increased dependence on survival signals delivered by BLyS (Lesley et al., 2004). The B lymphocyte development arrest in BLyS knockout mice is at a point in which negative selection of autoreactive immature B lymphocytes is thought to occur (Batten et al., 2000). Also, BLyS seems to have a particularly marked effect on marginal zone B lymphocytes, which are considered to be a potential reservoir for autoreactive B lymphocytes (Mackay et al., 1999). In vitro, BLyS increases the proliferation observed with CD40 ligand stimulation or surface immunoglobulin (M) cross-linking (Moore et al., 1999, Do et al., 2000). In vitro, BLyS enhances production of IgM and IgA (Moore et al., 1999, Avery et al., 2003). BLys enhances humoral immune responses to both TI-2 and TD antigens (Do et al., 2000).

Mice administered recombinant BLys show increased numbers of B lymphocytes and plasma cells in the spleen and produce increased amounts of IgM and IgA antibodies (Moore et al., 1999). Mice transgenic for BLyS show increased numbers of B lymphocytes and increased serum levels of immunoglobulins. Prolonged BLyS overexpression in these mice has been associated with production of autoantibodies, mainly antinuclear antibodies, including anti-dsDNA, and RhF, immunoglobulin deposition in the kidneys and glomerulonephritis. These mice have shortened life spans and, as they age, develop a Sjogren's syndrome-like phenotype but without the presence of anti-Ro or anti-La antibodies (Mackay et al., 1999, Khare et al., 2000, Groom et al., 2002). BLyS is elevated in autoimmune-prone mice such as NZB/WF1 and MRL*lpr* strains (Gross et al., 2000).

BLys has been found to be elevated in patients with SLE, rheumatoid arthritis and particularly in SS (Cheema et al., 2001, Zhang et al., 2001). In RA, BLyS levels in the

synovium were found to be higher that in the serum (Tan et al., 2003). In SS patients, BLyS expression was found to be increased in inflamed salivary glands (Groom et al., 2002).

Several approaches are being taken to antagonise BLyS. These include a fully human antibody, belimumab, and several fusion proteins between the different BLyS receptors and the Fc domain of immunoglobulin (TACI-Ig, BR3-Fc and BCMA-Ig) (Baker et al., 2003, Xia et al., 2000, Thompson et al., 2000, Kayagaki et al., 2002). Treatment of mice with TACI-Ig inhibited TI and TD humoral immune responses (Xia et al., 2000). In mice with no autoimmune background, short-term treatment with BCMA-Ig led to a two-fold reduction in B lymphocyte numbers in the peripheral blood (Thompson et al., 2000). In animal models of autoimmune disease, BLyS antagonists reduce disease severity and delay progression. TACI-Ig and BR3-Fc administration in NZB/WF1 mice reduced the severity of the glomerulonephritis and improved survival (Gross et al., 2000, Kayagaki et al., 2002). TACI-Ig was effective at reducing inflammation and preventing joint damage in collagen-induced arthritis and in preventing and reducing hypergammaglobulinemia and in reducing glomerulonephritis and improving survival in MRL.*lpr/lpr* mice (Wang et al., 2001, Liu et al., 2004). In a chimaeric mouse model of implanted human RA synovium, treatment with TACI-Ig led to collapse of the ectopic synovial lymphoid structures (Seyler et al., 2005). Some of these approaches are currently being tested in clinical trials in patients with SLE and RA.

There are differences between the different reagents and these may turn out to be clinically relevant. TACI-Ig and BCMA-Ig will block both BLyS and APRIL, while BR3-Fc will only bind BLyS. Expression of the different receptors differs in the various B-lymphocyte subpopulations and on plasma cells (Avery et al., 2003, Zhang et al., 2005). Also, differences between autoimmune diseases on the dependence of autoreactive and normal B lymphocytes on signals delivered by BLyS or APRIL probably exist and will influence the response to therapeutic blockade of these cytokines.

1.3.7.4 *Soluble CD23 antigen*

The CD23 antigen is expressed mainly on B lymphocytes and monocytes, but can also be expressed by a variety of other haematopoietic cells (Delespesse et al., 1991). It is a low affinity receptor for the Fc region of IgE (FcεRII). CD23 is frequently considered a marker of B-lymphocyte activation but although its expression is up-regulated when naïve B lymphocytes are activated, it is a B-lymphocyte differentiation marker and is expressed by normal resting cord blood B lymphocytes and by follicular mantle B cells that have otherwise a naïve resting phenotype (Bohnhorst et al., 2001, Yawetz et al., 1995, Small et al., 1990, Dono et al., 2004). The soluble form, sCD23, is a multifunctional cytokine that is thought to participate in inflammatory processes and in the mechanisms of apoptosis (Ribbens et al., 2000). Soluble CD23 in serum is thought to be derived from cleavage of sCD23 from different cells, but predominantly from B lymphocytes (Bansal et al., 1994). Membrane CD23 expression is lost on B lymphocytes after isotype switching (Delespesse et al., 1991, Yawetz et al., 1995). Some studies have found increased levels of sCD23 in the serum and synovial fluids of patients with RA (Bansal et al., 1994, Ribbens et al., 2000).

1.4 B-lymphocyte depletion therapy

B lymphocytes are targeted non-specifically by many of the immunosuppressive therapies used in the treatment of RA. Effective, specific B-lymphocyte depletion has been made possible by the availability of rituximab, a monoclonal antibody developed for the treatment of B-cell non-Hodgkin's lymphoma (NHL).

1.4.1 Rituximab (anti-CD20)

Rituximab (IDEC-C2B8) is a genetically engineered, chimaeric human-mouse monoclonal antibody containing human IgG1 heavy-chain and kappa light-chain constant region sequences and murine variable region sequences directed against the CD20 antigen (Reff et al., 1994). Rituximab was developed by IDEC Pharmaceuticals for the treatment of B-cell lymphoma (San Diego, CA) and is currently commercialised by Genentech (Rituxan; San Francisco, CA, USA) and by Roche (Mabthera; F. Hoffmann-La Roche, Basel, Switzerland). It is produced by transfection of the relevant gene constructs into Chinese hamster ovary cells.

Rituximab binds specifically to the CD20 antigen with high affinity ($5x10^{-9}$ mol/L) (Reff et al., 1994). The CD20 antigen is expressed in the vast majority of B-cell NHL. The CD20 antigen is also expressed on the membrane of most normal cells of B-lymphocyte lineage with the exception of the early precursors in the bone marrow (pro-B lymphocytes) and the terminally differentiated plasma cells (Anderson et al., 1984). Treatment with rituximab causes rapid depletion of malignant and normal CD20-positive B lymphocytes in the peripheral blood. Data on the degree of depletion achieved in solid tissues, including bone marrow and secondary lymphoid tissues (lymph nodes and spleen) is limited. Normal B lymphocytes repopulate the peripheral blood, starting six to nine months after therapy in most patients (Maloney et al., 1997b). During the period of peripheral blood B-lymphocyte depletion, antibody production is thought to be maintained by long-lived plasma cells.

Rituximab was the first monoclonal antibody approved by the United States Food and Drug Administration for treatment in malignancy in 1997. It was initially approved for treatment of relapsed or refractory low-grade or follicular, CD20-positive B-cell NHL but is now also licensed for the first-line treatment of diffuse large-cell CD20-positive B-cell NHL in combination with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) or other anthracyclin-based chemotherapy regimen. The overall response in the earlier trials was around 50% when rituximab was used as a single agent (McLaughlin et al., 1998). Response rates were significantly higher (more than 90%) when it was used in combination with chemotherapy (Czuczman et al., 1999). In clinical practice, rituximab is now used in the treatment of most lymphoproliferative diseases of B cell origin usually in combination with chemotherapy.

1.4.1.1 The CD20 antigen

Following the identification of surface immunoglobulin, CD20 (previously named B1) was the first cell-surface differentiation antigen of human B lymphocytes to be identified by a monoclonal antibody (Stashenko et al., 1980). Previously specific and associated B-lymphocyte antigens had been identified by heteroantisera. The antigen identified by anti-B1 monoclonal antibodies was specific for B lymphocytes in the peripheral blood and was not found on T lymphocytes, natural killer (NK) cells or monocytes (Stashenko et al., 1980). It was also found to be expressed on malignant B

lymphocytes but not on tumour cells of T-lymphocyte or myeloid origin (Nadler et al., 1981).

CD20 is a membrane nonglycosylated protein with a molecular weight between 33 and 37 kDa, containing multiple hydrophobic domains that probably span the plasma membrane four times (Einfeld et al., 1988, Stamenkovic and Seed, 1988). The long N- and C-terminal ends of the molecule are located within the cytoplasm, with only a minor portion of the molecule exposed on the cell surface. The cytoplasmic domains contain multiple consensus sequences for phosphorylation rich in serine and threonine but with no tyrosine (Oettgen et al., 1983). Differential phosphorilation results in the three immunoprecipitable forms of CD20 (33, 35 and 37 kDa) (Tedder and Engel, 1994). The human CD20 gene is encoded by eight exons located on chromosome 11 at position q12-q13 (Tedder et al., 1989).

CD20 expression on B lymphocytes is regulated during development (Reff et al., 1994). Uncommitted hematopoietic-precursor stem cells and the majority of pro-B lymphocytes do not express CD20 (Reff et al., 1994). CD20 expression is first detected usually at the pre-B lymphocyte stage, just before or at the time of production of cytoplasmic μ-heavy chain. CD20 is expressed consistently by all B-lymphocyte subpopulations until it is lost on differentiation into plasma cells. B-lymphocyte activation is associated with increased CD20 expression. CD20 surface expression is high on resting mature B lymphocytes (approximately 1.5 X 10^5 molecules/cell) in peripheral blood and lymphoid organs and even higher (4-fold) on activated germinal centre B lymphocytes (Ledbetter and Clark, 1986, Einfeld et al., 1988). Cells described as lymphoplasmacytoid, the earlier plasma cell precursors, can still express CD20 although at lower levels but normal mature plasma cells do not (Banchereau and Rousset, 1992).

The CD20 antigen is generally described as being specific for B lymphocytes but low expression of CD20 has been described in a small proportion of bone marrow and peripheral blood T lymphocytes as well as in rare T-lymphocyte malignancies (Hultin et al., 1993, Quintanilla-Martinez et al., 1994, Algino et al., 1996). These cells have been described to express CD4 more frequently than CD8 on their surface (Algino et al., 1996).

The precise function of CD20 in humans is not known and in vitro studies using different antibodies and different experimental conditions have revealed varied possibilities. CD20 is thought to be involved in B-lymphocyte activation, regulation of B-lymphocyte growth and transmembrane calcium flux (Tedder and Engel, 1994). Most studies suggest that CD20 participates in regulation of a step in the activation process important for cell-cycle initiation and differentiation, probably as part of a signal transduction complex but its relative importance in B-lymphocyte development and function is not known (Tedder et al., 1985, Tedder et al., 1986, Golay et al., 1985, Clark et al., 1985). No natural ligand for CD20 has been identified. Mice deficient in CD20 do not show any detectable abnormalities in B cell development or function (O'Keefe et al., 1998).

The CD20 molecule may exist in the cell surface in a heteromolecular complex that may include major histocompatibility complex class II, CD40 and possibly other cell-surface proteins (Leveille et al., 1999, Riley and Sliwkowski, 2000). The composition of the complex appeared to be partly dependent on cell type (Leveille et al., 1999). Further, adjacent CD20 molecules may exist in a homo-oligomeric form (Bubien et al., 1993, Polyak and Deans, 2002). At least some effects of CD20 ligation may be propagated via ligation of these oligomeric complexes (Polyak and Deans, 2002).

The structure of CD20 as a transmembrane protein suggests a function as a membrane transporter or ion channel. It appears to act as a calcium channel and it is possible that homo-oligomeric complexes of the CD20 molecule form Ca^{2+} conductive channels in the plasma membrane of B lymphocytes (Einfeld et al., 1988, Bubien et al., 1993, Tedder and Engel, 1994). CD20 regulates membrane Ca2+ current however, in general, binding of CD20 by antibodies does not result in a significant increase in intracellular calcium, suggesting that ligand binding to CD20 does not lead to a significant release of calcium from intracellular stores (Bubien et al., 1993).

Several studies suggest that CD20 is functionally regulated by phosphorylation (Einfeld et al., 1988, Tedder and Engel, 1994). CD20 is not phosphorylated in resting B lymphocytes but becomes heavily phosphorylated following mitogen stimulation of B lymphocytes, and is a dominant phosphoprotein in activated B lymphocytes. It has been

suggested that differential phosphorilation of CD20 may contribute to regulate transmembrane Ca^{2+} conductance.

Binding of monoclonal antibodies to CD20 generates membrane signals that can result in enhanced phosphorylation of the molecule, induction of *c-myc* and homotypic adhesion of cells; induction of serine/threonine and tyrosine phosphorylation of cellular proteins; or translocation of CD20 to lipid rafts (Kansas and Tedder, 1991, Deans et al., 1993, Deans et al., 1998).

It has been suggested that CD20 may regulate cell-cycle progression, since binding of several monoclonal antibodies to CD20 has been shown to inhibit B-lymphocyte progression from the G1 phase of the cell cycle into the S phase following mitogen stimulation (Tedder et al., 1985, Tedder et al., 1986). Monoclonal antibody binding also inhibits B cell differentiation and EBV- or pokeweed mitogen-induced immunoglobulin secretion (Tedder et al., 1985, Golay et al., 1985, Tedder et al., 1986, Golay and Crawford, 1987). The mechanism whereby monoclonal antibody binding to CD20 alters normal cell-cycle progression may involve the altering of cell-cycle-dependent calcium homeostasis and it might also explain differential effects from different antibodies. Calcium influx is required for B-lymphocyte activation but cell-cycle progression from the G1 to the S phase is dependent on decreased calcium levels. Binding of 1F5 to CD20 immediately increases Ca^{2+} conductance, delivering an activation signal to B lymphocytes. 1F5 appears to be unique in its ability to activate resting B lymphocytes driving them into the G1 stage of the cell cycle (Golay et al., 1985, Clark et al., 1985). However, on its own, this signal is not sufficient to drive the cell into the S stage and induce clonal differentiation. Binding of anti-B1 to CD20 increases calcium conductance but its effect is delayed in time. The consequent increase in cytosolic calcium may explain why this antibody inhibits cell-cycle progression (Bubien et al., 1993).

The CD20 antigen is appealing for targeted therapy because it is not shed and there are no detectable soluble CD20 molecules which might block binding to therapeutic antibodies. CD20 does not internalize and its expression is not modulated when it is bound by antibody (Liu et al., 1987, Press et al., 1994, Grillo-Lopez, 2000).

There are approximately 20 monoclonal antibodies directed to the human CD20 antigen. Anti-CD20 antibodies include mouse antibodies (like tositumomab = anti-B1, and 1F5), human/mouse chimaeric antibodies (like rituximab = IDEC-C2B8) and, more recently, humanised and fully human antibodies (like HuMax-CD20 = Genmab-2F2). All chimaeric and fully humanised anti-CD20 antibodies are very efficient at inducing antibody-dependent cell-mediated cytotoxicity (ADCC). In vitro studies showed that the antibody fine specificity controls in vitro effector mechanisms of anti-CD20 reagents, which certainly underlines the higher efficacy of rituximab when compared at least with the older anti-CD20 monoclonal antibodies (Cragg and Glennie, 2004). Antibodies like rituximab, HuMax-CD20 and 1F5, lead to the redistribution of the CD20-antigen molecules into lipid rafts, bind C1q and are very efficient at inducing complement activation while antibodies like tositumomab (anti-B1) do not cause redistribution of the CD20 molecules into lipid rafts and do not induce complement activation but cause homotypic adhesion of B lymphocytes which then tend to undergo apoptosis (Polyak and Deans, 2002, Cragg and Glennie, 2004, Teeling et al., 2004). It is not fully understood why some of these differences exist but may have to do with the Fc regions of different antibodies and the recognition of slightly different epitopes. The mouse antibody 1F5 is reported to bind a unique CD20 epitope (Clark et al., 1985). The new humanized anti-CD20 antibody HuMax-CD20 recognizes a different epitope from rituximab (Glennie M, personal communication). All anti-CD20 antibodies have been shown or are expected to cross-react with each other, most likely due to a limited exposure of the molecule on the cell surface (Polyak and Deans, 2002).

The use of chimaeric and humanised monoclonal antibodies leads to higher serum concentrations and longer half-lifes and more efficient recruitment of effector mechanisms in humans when compared with mouse monoclonal antibodies with the same specificity. 2B8, rituximab's parent murine monoclonal anti-CD20 antibody does not lead to complement-dependent cytotxicity (CDC) and ADCC in vitro against human B-lymphocyte malignancies whereas rituximab clearly does (Reff et al., 1994, Golay et al., 2000, Harjunpaa et al., 2000).

1.4.1.2 Mechanisms of action of rituximab

Rituximab has been shown to mediate cytotoxicity of malignant B lymphocytes in vitro via several mechanisms. These include ADCC, CDC, induction of apoptosis, inhibition of cell proliferation and sensitization to chemotherapy (Reff et al., 1994, Taji et al., 1998, Clynes et al., 2000, Flieger et al., 2000, Golay et al., 2000, Harjunpaa et al., 2000, Hofmeister et al., 2000, Shan et al., 2000, Rose et al., 2002).

In vivo studies in both animals and humans suggest that ADCC and CDC are the most important mechanisms that mediate the anti-tumour activity of rituximab (Grillo-Lopez et al., 1999, Gopal and Press, 1999). The IgG4 version of the C2B8 antibody is ineffective in cynomologus monkeys (Anderson et al., 1997). Which of these mechanisms is dominant in vivo is not entirely clear.

Several studies suggest that ADCC may be the more important mechanism that mediates rituximab activity. In vitro studies of normal B lymphocytes and fresh patient-derived chronic lymphocytic leukaemia (CLL) tumour cells showed minimal cytotoxic effect with the use of autologous complement, whereas the addition of CD56+CD14+ mononuclear cells significantly increased cell lysis (Voso et al., 2002). In a report by Clynes and colleagues, an Fc common γ-chain knockout (lacking stimulatory FcγRI and FcγRIII) mouse showed reduced protection conferred by rituximab against Raji xenografts compared with the wild type (Clynes et al., 2000). Differences in activation of ADCC by rituximab have been correlated with a genetic dimorphism in the gene that encodes the FcγRIIIa. This genetic dimorphism results in either a phenylalanine or a valine residue at amino acid position 158, located in the region of the receptor that binds IgG1. The homozygous valine FcγRIIIa (FcγRIIIa-158V) has a higher affinity for human IgG1 and mediates increased ADCC in vitro relative to homozygous phenylalanine FcγRIIIa (FcγRIIIa-158F) or heterozygous (FcγRIIIa-158F-carrier) receptors (Wu et al., 1997, Koene et al., 1997). The FcγRIIIa genotype has been found to correlate with response to treatment in patients with follicular NHL, Waldenstrom's macroglobulinemia and with the degree of B-lymphocyte depletion in the peripheral blood in patients with SLE (Cartron et al., 2002, Anolik et al., 2003, Treon et al., 2005).

There is evidence that complement activation occurs following administration of rituximab but its precise role in causing B-lymphocyte cytotoxicity in vivo is not

known. In vitro sensitivity to complement of fresh tumour cells from patients with B-lymphocyte malignancies varies following rituximab exposure (Bellosillo et al., 2001, Treon et al., 2001). Cell sensitivity to rituximab-induced complement activation may correlate with CD20-antigen density, particularly in tumour cells with low CD20 expression, for example, CLL (Golay et al., 2001). In addition, increased expression of the complement regulatory proteins, CD55, CD59 and CD46, confers resistance to rituximab-induced CDC to tumour cell lines and primary lymphoma cells (Golay et al., 2000, Treon et al., 2001). Treatment with antibodies that block complement regulatory proteins' activity abrogate complement resistance in vitro (Golay et al., 2000, Harjunpaa et al., 2000). In a mouse model, depletion of CD20-positive murine lymphoma cells was dependent on the presence of C1q suggesting that complement activation is fundamental for rituximab therapeutic activity in vivo (Di Gaetano et al., 2003). However the significance of complement and complement regulatory proteins expression was recently challenged in B-cell NHL by the observation that expression of these proteins and CDC in vitro in primary B-cell NHL tumour cells did not correlate with clinical response to rituximab (Weng and Levy, 2001). CLL cells are often resistant to CDC and this has been attributed in part to overexpression of CD55 and CD59 (Golay et al., 2000). Nevertheless, no correlation with clinical response was found with the expression levels of CD55 and CD59 on CLL cells (Bannerji et al., 2003).

It is likely that ADCC is the main mechanism of B-lymphocyte killing by rituximab in vivo in the sense that CDC as an isolated phenomenon does not seem to be very important. However, the capacity to activate complement seems to contribute to the higher efficacy of rituximab when compared to some of the other anti-CD20 antibodies. There are some mechanisms by which complement activation could contribute to facilitating ADCC, including release of anaphylotoxins like C3a, C4a and C5a that induce inflammation and recruit and activate macrophages and NK cells and opsonisation of cells by C3 fragments.

Recent studies suggest that the relative importance of the two mechanisms may vary depending on tumour cell types, B-lymphocyte subpopulations and particularly on specific microenvironments (Gong et al., 2005). Differences will probably also reflect a combination of pharmacokinetic factors, the level of CD20 expression, susceptibility to

CDC, availability and efficiency of ADCC but also susceptibility to apoptosis (where different mechanisms may be important) and state of activation.

It has been shown that rituximab can cause apoptosis or growth arrest in several B-cell NHL cell lines (Taji et al., 1998, Rose et al., 2002). The in vivo significance of rituximab's ability to signal growth arrest and cell death is not known. The induction of growth arrest and apoptosis may be more important in tumour cells that express only low levels of the CD20 antigen like CLL cells. In CLL, cellular and genetic features that disrupt apoptosis are associated with diminished response (Bannerji et al., 2003, Byrd et al., 2003). It has been suggested that caspase-dependent apoptosis may be the predominant mechanism by which tumor elimination occurs (Byrd et al., 2002) (Byrd JC 2002). Induction of apoptosis seems to play a role in in vitro sensitisation of lymphoma B-lymphocyte lines to chemotherapy agents and glucorticorticoids (Demidem et al., 1997).

Rituximab with other agents

In vitro, rituximab sensitizes lymphoma cell lines to the cytotoxic and apoptotic effects of different chemotherapeutic drugs (including cisplatin, fludarabine, vinblastine and doxorubicin) (Demidem et al., 1997, Alas et al., 2001). A potential mechanism of sensitization is downregulation of IL-10 (maybe mediated by inhibition of p38 mitogen-activated protein kinase activity), resulting in decreased activity of the transcriptional activator, Stat3 protein, which decreases the expression of *bcl-2*, an inhibitor of apoptosis (Alas et al., 2001).

Glucocorticoids are often administered in conjunction with rituximab in chemotherapeutic regimens or as premedication to reduce infusion-related side effects. Studies in vitro showed additive effects on antiproliferative and apoptotic effects when rituximab and glucocorticoides were used together or in sequence (Rose et al., 2002). No effect on ADCC and CDC was observed when rituximab and dexamethasone were used concurrently but preincubation of effector and lymphoma cells with dexamethasone led to a decrease in specific cell lysis in ADCC assays and to increased cell sensitivity to CDC in some lymphoma cell lines. This suggests that administration of glucocorticoids should occur at approximately the same time as the rituximab infusion to optimize patient benefit.

In a study in cynomolgus monkeys, CD20 ligation with rituximab decreased CD40 expression and increased CD21 expression (Vugmeyster et al., 2003b). It was suggested that these changes might affect the composition of the heteromolecular complexes that include CD20 or the oligomerisation status of CD20 and consequently the susceptibility of the cell to anti-CD20 killing. Interestingly, longer incubation with rituximab of cells with lower expression of CD20 did not augment depletion once CD21 and CD40 levels of expression had been modified. This may be one of the mechanisms that influences acquired resistance or sensitization to anti-CD20 in monkeys and humans and it may also contribute to the differences between different anti-CD20 antibodies.

Similarly, in a study of a panel of lymphoma cell lines, CD20 expression had no correlation with the susceptibility to rituximab-induced CDC and ADCC in vitro (Flieger et al., 2000). However, in freshly isolated cells from CLL, prolymphocytic leukaemia and mantle cell lymphoma, a statistically significant correlation has been found between levels of CD20 expression and anti-CD20 mediated CDC (Golay et al., 2001). These differences between studies may be due in part to differences in the experimental systems and malignancy status of cells.

The pharmacokinetic of rituximab is also important in determining its effect on individual B-lymphocyte subpopulations in different tissues. Low doses are adequate for depletion of circulating B lymphocytes but higher doses and higher serum levels are required to coat tumour cells in extravascular sites, including bone marrow and secondary lymphoid tissues (Press et al., 1987). Rituximab has a long half-life and can be found circulating and bound to cells for a long period after its administration (more than 6 months) (Berinstein et al., 1998). Rituximab dissociates rather quickly from cells with a *t* ½ of 2 hours (Ghetie et al., 2001). In some patients rituximab is more rapidly consumed but the reason is unknown (Berinstein et al., 1998; Grillo-Lopez et al., 2000). Patients with this pharmacokinetic profile usually do not respond to treatment with rituximab.

A complete understanding of rituximab's mechanisms of action is of primary importance when considering strategies for increasing rituximab efficacy and designing combination therapies in malignancies. It may also be important in understanding or

predicting different susceptibilities to rituximab killing of different B-lymphocyte subpopulations, which can influence its effect in different autoimmune diseases. Different strategies to develop more effective therapeutic strategies and optimize patient response include combination with neutralizing antibodies to complement regulatory proteins CD55 and CD59 (Cerny et al., 2002) and structural changes on rituximab that can increase its affinity for the low affinity FcγRIIIa among others (Cartron et al., 2004).

1.4.1.3 Animal studies

Animal studies can provide information on quantitative and qualitative aspects of the depletion in vivo of the normal B-lymphocyte subpopulations, on the functional consequences of B-lymphocyte depletion and on in vivo factors that regulate B-lymphocyte therapy based on monoclonal antibodies. However, among other factors, the pattern of expression of the CD20 antigen on B lymphocytes, specific characteristics of B-lymphocyte subpopulations and efficacy of rituximab killing differ between species, even between primates and need to be taken into account.

In the early study in cynomolgus monkeys, rituximab was shown to cause a marked B-lymphocyte depletion in the peripheral blood, bone marrow and lymph nodes (Reff et al., 1994). In this study, doses that induced a greater than 98% depletion of B lymphocytes in the peripheral blood caused a varied degree of depletion in the lymph nodes (ranging from 34% to 78%) even in individuals treated with the same dose. Following treatment with 4 daily infusions, most depletion in lymph nodes occurred before day 15 with repeated biopsies on day 29 showing quite similar numbers of B lymphocytes.

Recovery of B-lymphocyte numbers in peripheral blood occurred more rapidly in some monkeys than others and did not seem to be dose dependent (Reff et al., 1994). Treatment of a small number of monkeys with very high doses in a toxicity study (described as equivalent to 4 weekly infusions of 1000mg of rituximab in a 60Kg individual) consistently induced a higher degree of depletion in lymph nodes (ranging from 69% to 87% 3 to 5 weeks after the last drug infusion) but was still incomplete. Depletion in the bone marrow was also significant, ranging from 74% to 95% (here

comparison with normal individuals as taken in the study may be more inaccurate due to higher variability). No side effects were detected in these animals. In this study, all antibodies produced against rituximab were found to be exclusively anti-idiotypic and anti-joining region specific.

In baboons, 4 weekly infusions of rituximab 20 mg/kg yielded only approximately 70% depletion of B lymphocytes in the lymph nodes and up to 92% depletion in the bone marrow (Alwayn et al., 2001).

A more recent study in cynomolgus monkeys showed that these monkeys had two different subpopulations of B lymphocytes in the peripheral blood with different levels of expression of CD20 and different sensitivities to rituximab-induced depletion in a whole blood matrix (Vugmeyster et al., 2003). Interestingly, it also suggested that differences in the sensitivity to rituximab killing, may not be explained exclusively on the basis of differences in the levels of expression of CD20.

In an immunisation study of baboons with a hapten (dinitrobenzene = DNP), treatment with rituximab at doses that induced over 99% depletion of B lymphocytes in the peripheral blood, blocked primary and secondary humoral responses to the immunogen (Gonzalez-Stawinski et al., 2001). Again, data suggested that B-lymphocyte depletion occurred rather rapidly as immunisation took place 48 hours after rituximab administration. The question was raised whether the limited repertoire of B lymphocytes that respond to DNP may be particularly susceptible to rituximab killing and whether rituximab will interfere in the same way with a B-lymphocyte response to an antigen that recruits a larger repertoire of B lymphocytes. The authors report that total levels of serum immunoglobulins did not change.

In a study in Macaca fascicularis individuals used in cardiac transplant studies, treatment with rituximab induced major depletion of B lymphocytes in the peripheral blood, lymph nodes, spleen, bone marrow and tonsils four weeks after starting treatment with 4 or 2 weekly infusions of 20 mg/kg of rituximab (Schroder et al., 2003). Three animals had previously rejected a cardiac transplant and one animal was undergoing subacute vascular rejection of the transplant. Again, treatment with the same dose induced different degrees of depletion in different individuals but the pattern of B-

lymphocyte depletion as detected by immunohistochemistry was consistent within an individual. The data suggested that lymph nodes might be more difficult to clear of B lymphocytes than the spleen. In secondary lymphoid organs, remaining B lymphocytes were usually localised in the central areas of germinal centres. Plasma cell numbers were not reduced as assessed by semi-quantitative analysis. In the animal with less depletion, at 28 days, the number of cells of B-lymphocyte lineage (CD19-positive cells by flow cytometry) in the bone marrow was at the high end of the normal range. At the same time point no B lymphocytes were detected in the peripheral blood. No data was provided on the time of B-lymphocyte repopulation of the peripheral blood. Before treatment, all animals had detectable anti-donor IgM and IgG antibodies. Anti-donor IgM levels remained stable during the first 2 weeks and then slowly declined. IgG levels remained relatively constant during anti-CD20 treatment. The animal being treated for subacute vascular rejection of the transplant received only 2 doses of rituximab and showed residual B lymphocytes in the lymph nodes and tonsils. In this animal, graft explant was carried out at day 7. Treatment with rituximab did not prevent an abrupt rise in both IgM and IgG anti-donor antibodies following graft explant (IgG rise was detected at day 14 and of IgM at day 21).

Studies of the effects of specific B-lymphocyte depletion in animal models of autoimmune diseases have been hampered by the lack of an effective agent to be used in adult mice. Rituximab and 2H7 do not bind mouse CD20 (Hamaguchi et al., 2005). Recently, transgenic mice expressing human anti-CD20 able to be targeted by efficient anti-human CD20 antibodies and also effective anti-mouse CD20 antibodies have been developed and they will most certainly change this (Gong et al., 2005; Hamaguchi et al., 2005).

A recent study by Gong and colleagues on human CD20 transgenic mice treated with rituximab and with 2H7 (a different mouse anti-human CD20 monoclonal antibody) has demonstrated the great complexity involving the circulatory dynamics of B lymphocytes and survival factors provided by the microenvironment as critical regulators that seem to determine the mechanisms of depletion through complement or ADCC and the reticulendothelial system and also the relative sensitivities to depletion of the different B lymphocyte subpopulations (Gong et al., 2005). A hierarchy of B-lymphocyte sensitivities to anti-CD20 was found with different B-lymphocyte

subpopulations in the spleen and in Peyer's patches showing different degrees of depletion. In the spleen, follicular, immature and transitional B lymphocytes were very sensitive to anti-CD20 therapy (> 90% depletion). Marginal zone B lymphocytes were more resistant (50% depletion only). Germinal centre B lymphocytes both in Peyer's patches or spleen (in this case following immunisation) demonstrated the greatest resistance to treatment (no detectable depletion described). In both the case of marginal zone and germinal centre B lymphocytes, relative resistance to depletion with anti-CD20 could not be attributed to differences in the expression of the human CD20 molecule (both marginal zone and germinal centre B lymphocyte subsets express even higher levels of CD20 than follicular B lymphocytes) or lack of accessibility of the monoclonal antibody and was not overcome by treatment with higher doses or repeated administration of the anti-CD20.

In the same study, the kinetics of depletion paralleled the kinetics of lymphocyte circulation with peripheral blood B lymphocytes being depleted quicker than B lymphocytes in the lymph nodes and both of these subpopulations quicker than B lymphocytes in the peritoneum even when the anti-CD20 was administered intraperitoneally. Mobilisation of marginal zone B lymphocytes into the vasculature (with anti-integrin antibodies) but not into the follicles (with lipopolysaccharide) rendered them sensitive to depletion mediated by anti-CD20. Conversely, if lymphocyte egress from lymph nodes was inhibited (with SEW2871) depletion of lymph nodes B lymphocytes was significantly decreased.

Contributions of the microenvironment to B-lymphocyte depletion by anti-CD20 were studied with competition experiments (Gong et al., 2005). In chimaeric mice in which bone marrow had been reconstituted from a mixture 50:50 of human CD20-positive and human CD20-negative cells, > 90% of human CD20-positive marginal zone B lymphocytes were depleted by anti-CD20, suggesting that cellular competition possibly for survival factors with marginal zone B lymphocytes not expressing human CD20 has rendered the human CD20-positive marginal zone B lymphocytes more susceptible to anti-CD20 killing. The contribution of BLyS for the relative resistance of marginal zone B lymphocytes to depletion by anti-CD20 was shown by the fact that all spleen B-lymphocyte subsets, including the marginal zone, showed major depletion in animals

treated with a combination of the fusion protein BR3-Fc and anti-human CD20 monoclonal antibody.

The study also showed that different effector mechanisms appear important for depletion of distinct splenic B-lymphocyte compartments with complement-dependent mechanisms playing a dominant role in the marginal zone compartment and Fc receptor-mediated mechanisms in the elimination of circulating, lymph node and splenic follicular B lymphocytes. As far as Fc receptor-mediated killing was concerned, monocytes/macrophages (the reticuloendothelial system) were found to be the most important effector cell population, with the liver and, to a lesser degree, the spleen, representing the major site of depletion of circulating B lymphocytes.

Altogether, the data from this study support the requirement for B lymphocytes in mice to access the circulation for efficient depletion but also the important role played by local survival factors in determining susceptibility to anti-CD20 mediated depletion. These survival factors may be especially important in populations that do not recirculate or recirculate less or with different kinetics. The relation of these patterns of recirculation with rituximab pharmacokinetics and therefore length of availability of sufficient amounts in peripheral blood to deplete cells that recirculate will certainly be important.

A study published on treatment of mice with anti-mouse CD20 antibodies showed some interesting differences when compared to the study on transgenic mice (Hamaguchi et al., 2005). In this study, the B-lymphocyte depletion observed was much more significant, occurred more quickly and was much more predictable than what the studies in non-human primates or in humans show. There was little intermouse variability in solid tissue B-lymphocyte depletion. With the higher doses most B lymphocytes in the peripheral blood, spleen, peripheral lymph nodes, mesenteryc lymph nodes, Peyer's patches and in gut intraepithelial and lamina propria areas were depleted. Again, B-lymphocyte depletion occurred very quickly, with depletion of circulating B lymphocytes occurring within 1 hour and maximal spleen and lymph node depletion requiring approximately 2 days. With lower doses, maximal depletion of lymph node and spleen B lymphocytes occurred later but still early, within 7 days. Depletion in

lymph nodes and spleen was similar despite coating of spleen and peritoneal cavity B lymphocytes occurring before coating of lymph node B lymphocytes.

Depletion of all B-lymphocyte subsets by anti-mouse CD20 was FcγRc-dependent but not complement-dependent (Uchida et al., 2004, Hamaguchi et al., 2005). When B-lymphocyte subsets were analysed in the spleen, the depletion of marginal zone B lymphocytes was similar to follicular B lymphocytes, but B1a-B lymphocytes (CD5-positive) were relatively resistant to depletion (only reduced 60%). B lymphocytes in the peritoneal cavity were the most resistant to anti-CD20 mediated depletion with data suggesting that this was mainly due to the absence of effector cells in the peritoneum.

Extrapolation of these murine studies to humans is limited. Human marginal zone B lymphocytes do not seem to be limited to the spleen and recent studies suggest that at least some human marginal zone B lymphocytes recirculate and therefore can be expected to be sensitive to anti-CD20 (Spencer et al., 1998, Pillai et al., 2005, Martin and Kearney, 2002, Weller et al., 2004). Also, the extent of depletion within the marginal zone compartment has been said to vary among different mouse genetic backgrounds and may also reflect differences between anti-human and anti-mouse CD20 antibodies (Gong et al., 2005; Hamaguchi et al., 2005). Differential sensitivity of germinal centre and marginal zone B lymphocytes to anti-human CD20 monoclonal antibodies has also been described in cynomologous monkeys, though these differences appear more prominent in the lymph node than the spleen (Vugmeyester et al., 2003).

1.4.1.4 Lymphoma studies

The CD20 antigen proved to be a useful target for therapeutic monoclonal antibodies in oncology due to its expression by the majority (more than 90%) of B-cell lymphomas (Anderson et al., 1984). Also, as mentioned previously, it does not shed from the cell surface and therefore is not present free in the serum, it does not modulate in response to monoclonal antibody binding and it is described as not binding to any normal tissues other than B lymphocytes. The fact that CD20 is expressed by all normal B lymphocytes but not bone marrow multipotent stem cells, early B-lymphocyte precursors (pro-B lymphocytes) and terminally differentiated plasma cells has proved very important to the safety of this therapeutic approach. Treatment with anti-CD20

leads to depletion of normal B lymphocytes as well as malignant B lymphocytes but new B lymphocytes repopulate the peripheral blood starting several months after treatment. During the period of B-lymphocyte depletion, immunoglobulin levels are reasonably maintained due to the survival of plasma cells.

1F5 was the first anti-CD20 antibody used to treat 4 patients with refractory B-cell lymphoma (Press et al., 1987). 1F5, a mouse anti-human CD20 monoclonal antibody, was administered by continuous intravenous infusion during five to ten days. The results were promising but very large amounts of antibody had to be given to achieve an in vivo effect. Smaller doses of 1F5 sufficed to deplete circulating tumour cells but higher doses were needed to achieve penetration into extravascular sites such as bone marrow and, particularly, the lymph nodes. Even at very high doses, the intranodal distribution of 1F5 was heterogeneous. Clinical response appeared to correlate with dose of monoclonal antibody administered, peak-serum monoclonal antibody concentration achieved and degree of extravascular tissue penetration obtained. Serum elimination half-lifes varied between 24 to 52 hours. The patients with higher numbers of circulating tumour cells showed decreased serum 1F5 levels. Depletion of normal B lymphocytes was observed. One of the four patients (the patient treated with the lower dose) developed human anti-mouse antibodies (HAMA) five months after therapy. Toxicity was limited to minor fever and mild to moderate cytopenias. No patient demonstrated a decrease in any immunoglobulin class total serum levels. The patients with higher numbers of circulating tumour cells showed evidence of complement consumption.

However, in vitro, 1F5 activates a G0 to G1 cell cycle transition in resting B lymphocytes (Clark et al., 1985). It was felt that this effect of ligation to CD20 may not be desirable and so other antibodies against anti-CD20 that did not show this effect were developed further. It was in this context that rituximab was developed. The production of human/mouse chimaeric antibodies such as rituximab, allowed for the development of more effective therapies with unconjugated antibodies. Chimaeric antibodies have a longer half-life, effectively mediate normal host effector functions and are less immunogenic than murine antibodies (Liu et al., 1987).

A single dose, dose escalating, phase I open-label trial of rituximab in patients with relapsed B-cell NHL demonstrated both efficacy in terms of transient B-lymphocyte depletion and a good safety profile (Maloney et al., 1994). Fifteen patients were treated with a single infusion of rituximab in doses ranging from 10 to 500 mg/m^2. Modest tumour responses (2 partial remissions lasting 8.1 and 8.6 months and 5 minor responses lasting 0.9 to 6 months) were observed in 7 of the 9 patients treated with single doses of at least 100 mg/m2. Infusion reactions were mild to moderate and there was no dose-limiting toxicity. There was a dose-dependent, rapid (24 to 72 hours) and specific depletion of B lymphocytes in the peripheral blood especially in those patients treated with doses greater than 100 mg/m2. Depletion persisted for 1 to greater than 3 months. IgG, IgM and IgA total serum levels did not change for the 3 months of the study. There was no effect on the numbers of T lymphocytes. The mean half-life of the antibody was 4.4 days in the patients treated with doses equal to or greater than 100 mg/m2, ranging between 1.6 to 10.5 days. No patient developed specific antibodies to rituximab. Post-treatment tumour biopsies specimens taken 2 weeks after treatment showed tumour cells coated with non-saturating amounts of rituximab in several of the cases. In some of the biopsies an increased infiltrate of macrophages was observed.

A phase I, multiple dose, dose ranging, open label study was undertaken in 20 patients with relapsed lymphoma (Maloney et al., 1997a). Patients were treated with 4 weekly infusions of doses of 125, 250 or 375 mg/m2 each. The treatment was well tolerated and 6 of 18 evaluable patients had a partial response with no dose-limiting toxicity identified. The 375 mg/m2 for 4 weeks dose was selected for open label phase II evaluation in patients with relapsed or refractory follicular or low-grade B-cell lymphoma.

In a first phase II open label study on 37 patients, 25 of the 34 evaluable patients (74%) had evidence of anti-tumour effect with 50% showing partial or complete responses (9% complete responses, 41% partial responses) (Maloney et al., 1997b). The median time to onset of clinical response was 50 days (range, 7 to 112 days). Measured tumour masses continued to regress with maximum response occurring 3 to 4 months after antibody therapy. Tumour responses were noted in extra-nodal masses as well as in primary and secondary lymphoid tissues and peripheral blood. The median duration of response was 8.2 months. The median time to progression for the clinical responders

was 10.2 months, with 5 patients exceeding 20 months and 2 patients exceeding 30 months. Treatment related side effects were seen most frequently with the first infusion (67%) and dramatically decreased during the second, third and fourth infusions. All infections reported were mild to moderate and resolved without complications after appropriate therapy. The risk of infection appeared less than with other lymphoma single-agent therapy, which also affect T lymphocytes or other cells in the immune system. No clinically significant change in serum complement levels was observed. Only one patient had detectable, low titre human anti-chimaeric antibodies (HACA) titer 7 months post-treatment. The average dose of antibody administered during each infusion was 711 mg (range, 562 to 825 mg), with a cumulative dose over 4 infusions of 2,730 mg (maximum cumulative dose of 3,300 mg). Most patients exhibited increasing pre-infusion antibody concentration with each subsequent infusion. However, some patients had low pre-infusion levels (less than 10 μg/ml) suggesting that they did not reach steady state (possibly due to higher tumour burden) or cleared the antibody at a faster rate. Clinical response correlated with the median values of rituximab serum levels before the second infusion. Mean serum antibody half-life was 226 hours (range, 13 to 371 hours).

The pivotal (phase II/III) study of rituximab monotherapy in 166 patients resulted in a 48% overall response rate of which 6% were complete responses and a median duration of response of 11.2 months (McLaughlin et al., 1998). The majority of the patients classified as non-responders showed a net decrease in measurable disease (56 of 75). These results were considered good results in the context of therapy with single agents in lymphoma. Toxicity was mostly mild with no severe infections reported. Only one patient developed HACA (detected at day 50). In the majority of patients B-lymphocyte counts in the peripheral blood decreased with treatment to undetectable levels, usually after the first infusion. Fifteen of the 16 patients who did not deplete circulating B lymphocytes to undetectable levels did not respond to treatment. Recovery of B lymphocytes started between 6 and 9 months, with recovery to normal counts between 9 and 12 months. Mean serum total IgG and IgA levels remained within the normal range throughout the study. The mean serum total IgM level decreased to slightly below the lower limit of the normal range (to 41.5 mg/dL; normal range, 45 to 145 mg/dL) at 6 months posttreatment and recovered (to 65 mg/dL) at the 8-month follow up point. Twenty-three patients (out of 166) had reductions in immunoglobulin levels by ≥ 50%

to subnormal levels. Serum rituximab levels correlated with clinical response with medium serum antibody levels being higher for responders than nonresponders. Attainable serum antibody concentrations correlated negatively with the number of circulating B lymphocytes at baseline and sizes of tumour masses. Median absolute T-lymphocyte (using anti-CD3, anti-CD4 and anti–CD8 antibodies) and natural-killer cell counts remained stable throughout the study. In 18 patients a decrease ≥20% from baseline in serum complement (C3) was detected.

The etiology of infusion related symptoms following rituximab administration is not completely understood but may represent the rapid depletion of B lymphocytes with lysis in the reticuloendothelial systems of the lung, liver and spleen. The marked decrease in the incidence of infusion related symptoms with subsequent infusions likely reflects the elimination of most circulating B lymphocytes and the saturation of antigenic sites with the first infusion.

As expected, patients with B-lymphocyte malignancies undergoing treatment with monoclonal antibodies seldom develop HAMA, in contrast to patients with T-lymphocyte malignancies (Schroff et al., 1985, Shawler et al., 1985). Rituximab has been described to be associated with development of specific HACA in 1% or less of the patients treated for B-cell lymphoma, with no increased risk of side effects and no correlation with clinical response having been observed (Grillo-Lopez et al., 1999).

In lymphoma studies, the presence of extra-nodal disease significantly reduces the period of progression-free survival (Igarashi et al., 2002) and overall response rates (McLaughlin et al., 1998). These results suggest reduced penetration of the antibody into these extra-nodal sites or less efficient depleting mechanisms within these sites. Rituximab can deplete lymphoma cells present in pleura and peritoneum (Perez and Rudoy, 2001, Takao et al., 2004).

Re-treatment with rituximab monotherapy was effective in 40% of 58 patients with relapsed low-grade or follicular lymphoma who had previously responded to rituximab (11% complete responses) (Davis et al., 2000). Following retreatment a longer duration of response was observed when compared to response after the first treatment (O'Brien et al., 2001, Byrd et al., 2001). No increased toxicity was observed (Davis et al., 2000).

Five patients received their third course of rituximab and 3 responded. No patient developed HACA after rituximab re-treatment. Development of resistance is characteristic of B-cell lymphomas after treatment with all other available therapies. Possible mechanisms of resistance to rituximab include reduction of CD20 expression below some critical threshold necessary for cell death, uncoupling of the apoptotic signal that is initiated by rituximab's binding to the CD20 molecule or progressive inhibition, by the disease or by antilymphoma therapy, of the immune response that is recruited by bound rituximab, including CDC and ADCC. Relapse with CD20-negative B-cell NHL has been reported only very rarely (Davis et al., 2000).

When rituximab monotherapy is used in previously untreated patients with lymphoma, response rates are higher than in relapsed or refractory cases (70-75% compared to around 50%) and median progression-free survival is approximately 18 months (compared with around 12 months) (Ghielmini, 2005).

Based on in vitro studies that showed synergistic effects between rituximab and certain chemotherapeutic agents, an independent activity and a favourable safety profile, various clinical trials have combined rituximab with chemotherapy. Combination trials with the standard chemotherapy regimen (CHOP) have reported better results with higher rates of response and longer duration of response, including in more refractory types of B-lymphocyte malignancies when compared to rituximab monotherapy trials (Czuczman et al., 1999, (Vose et al., 2001, Coiffier et al., 2002). The combination of CHOP with rituximab in patients with low-grade or follicular lymphoma increased response rates from about 50% to frequently more than 90% (Czuczman et al., 1999). Studies are in progress to determine the utility of rituximab given prior to chemotherapy or as a maintenance therapy following chemotherapeutic regimens.

The good results obtained with rituximab monotherapy and its good safety profile led to trials using higher doses and increased dose frequency (extended and maintenance dosing schedules). Various alternative dosing schedules have been used, including same single dose weekly infusions for 8 weeks, repeat of the standard dose regimen every 6 months (up to a maximum of 4) in patients who have responded, further single doses every 2 months for a total of four additional infusions and increase in infusion frequency with or without increase in single dose particularly in B-lymphocyte

malignancies that are associated with lower surface densities of the CD20 antigen (small lymphocytic lymphoma and CLL). In general these studies have reported better results when compared with the standard 4-week course with no increased toxicity. Studies that have used maintenance regimens (scheduled, intermittent re-treatment) have reported significantly longer duration of response to treatment with median progression-free survivals varying between 22 and 37 months (Hainsworth et al., 2002, Hainsworth et al., 2005). When maintenance therapy was compared with re-treatment with the standard 4-week dose at the time of lymphoma progression the actuarial duration of rituximab benefit was similar but the final overall and complete responses were higher in the maintenance group (Hainsworth et al., 2005). Studies using higher dose and frequency schedules have improved rates of response in malignancies with lower expression of CD20, in particular CLL (O'Brien et al., 2001).

Rituximab has also been used in combination with myeloablative therapy and autologous bone marrow transplantation (BMT) with good results (Magni et al., 2000, Brugger, 2002, Horwitz et al., 2004). Rituximab has been used either before stem cell collection for in vivo purging or after BMT with the objective of targeting minimal residual disease that remains in many patients and is responsible for relapse (observed in 40 to 60% of patients with diffuse large cell lymphoma). The incidence of infection does not appear to be increased by the addition of rituximab to these regimens despite some reports of delayed neutropaenia and hypogammaglobulinaemia (Rastetter et al., 2004, Uchida et al., 2004, Lim et al., 2004, Sesin and Bingham, 2005, Lim et al., 2005).

Some immunisation studies have been reported in patients with B-lymphocyte malignancies treated with rituximab. Van der Kolk and colleagues, found significantly decreased responses to recall antigen tetanus toxoid and polio virus, 4 weeks after rituximab monotherapy (van der Kolk et al., 2002). Responses to primary antigens (keyhole limpet hemocyanin and hepatitis A) were absent even before treatment and so no conclusions can be drawn.

In a combination study of rituximab following BMT in which patients received 4 weekly infusions starting at day 42 after BMT and a repeated course at 6 months, patients were immunised at 12 and at 15 months against haemopilus influenzae (conjugated vaccine), pneumococcus (unconjugated vaccine) and tetanus toxoid

(Horwitz et al., 2004). Fewer patients had protective levels of anti-PCP antibodies 3 months after the first and second immunisations than protective levels to tetanus and to haemophilus influenzae. The authors suggest that this may reflect immaturity of the B-lymphocyte subsets on repopulation. Storek et al had previously described poor T-lymphocyte independent B-lymphocyte responses up to 2 years after BMT and so it was unclear whether rituximab adversely influences these humoral responses (Storek et al., 1993).

Rituximab is now nearly universally used as part of the first-line treatment of B-cell lymphoma. Overall, B-cell lymphoma or leukaemia types that show lower responses to other chemotherapy regimens also show lower responses to rituximab. One of rituximab's advantage is its better toxicity profile. Patients with lymphoma will have different tumour burdens and distribution, different numbers of circulating malignant cells acting to absorb the monoclonal antibodies and different surface densities of the CD20 antigen. Consequently, the dose levels and kinetic data found in a group of patients may not be applicable in another group. At the present, the cost associated with the higher doses is prohibitive and combination therapy is seen as the way forward, exploring the potential synergy between rituximab and chemotherapeutic agents.

1.4.1.5 Treatment of other autoimmune diseases with rituximab preceding its use in rheumatoid arthritis

The first publications of the use of rituximab to treat autoimmune diseases involved patients with cold agglutinin disease and IgM-associated polyneuropathy (Lee and Kueck, 1998, Levine and Pestronk, 1999). In both these diseases, clinical manifestations are a consequence of the pathogenicity of an IgM paraprotein and are therefore associated with clonal proliferation of B lymphocytes.

In cold agglutinin disease, the pathogenic autoantibodies are typically monoclonal IgMk immunoglobulins against carbohydrate antigen 1 on red blood cells (Berentsen et al., 2001). Frequently, a lymphoplasmacytic clone is identified in the bone marrow. These cells typically express CD20 and have low rates of proliferation. Conventional immunosuppressive or cytotoxic treatment is often unable to control the disease.

In IgM-associated polyneuropathies, monoclonal antibodies to GM1 ganglioside or to Myelin-associated glycoprotein (MAG) have been identified (Levine and Pestronk, 1999). These patients frequently respond to intravenous immunoglobulin (IVIG) and to a combination of plasmapheresis and cyclophosphamide. Reduction in serum autoantibody titers is usually associated with an amelioration of the neuropathy.

The rationale for B-lymphocyte depletion in these diseases was the removal of the cellular source of the antibodies with the consequent decrease in autoantibody titers expected to lead to clinical improvement. Despite the fact that in lymphoma rituximab had been reported to have only a minimal impact on serum total levels of IgA, IgG and IgM, there were reasonable expectations that rituximab might lead to a decrease in pathogenic autoantibody production. In cold agglutinin disease, it was likely that the paraprotein was derived from cells that expressed CD20. In IgM-associated polyneuropathies, the kinetics of their response to other treatments suggested that there was continuous proliferation and differentiation of clonal B lymphocytes into autoantibody producing plasma cells and that killing the B lymphocytes would stop the formation of new autoantibody-producing plasma cells. If these plasma cells had a short half-life, it should result in a decrease in the pathogenic autoantibody titer.

1.4.1.6 Rituximab in the treatment of rheumatoid arthritis

1.4.1.6.1 Rationale for B-lymphocyte depletion in rheumatoid arthritis

In 1998, Edwards and Cambridge hypothesized that if particular autoreactive B lymphocyte clones and certain species of autoantibodies were responsible for the initation and perpetuation of the inflammation in patients with RA then specific B-lymphocyte depletion might be an effective therapy in RA (Edwards and Cambridge, 1998). Involvement of pathogenic B-lymphocyte clones both in the afferent and efferent arms of abnormal immune reactions involved in disease pathogenesis were suggested. If the pathogenic B-lymphocyte clones were depleted and if the depletion of B lymphocytes lasted long enough to allow plasma cells to die and serum levels of pathogenic autoantibodies to decrease significantly or even disappear, then long-term improvement of rheumatoid arthritis should be achieved.

1.4.1.6.2 First open label trial

An initial open-label study at UCL/University College London Hospitals (UCLH) in 5 patients with active RA who were refractory to standard therapy suggested that treatment with a B-lymphocyte depletion protocol based on rituximab could lead to significant improvement in disease manifestations with a good safety profile (Edwards and Cambridge, 2001). The 5 patients were treated with the standard rituximab dose and schedule (as in lymphoma) combined with cyclophosphamide and oral prednisolone. All 5 patients achieved major improvement in symptoms and signs of active disease following treatment. In 2 of the five patients clinical relapse coincided with the return of B lymphocytes to the peripheral blood while in the other 3 clinical response continued beyond B-lymphocyte repopulation.

1.4.2 B-lymphocyte reconstitution after therapeutic procedures

Depletion of B lymphocytes occurs following therapeutic procedures such as total body irradiation and myeloablative chemotherapy. With the more aggressive regimens bone marrow stem cells are irreversibly damaged and reconstitution of B lymphocytes as well as the other bone marrow lineages occurs following autologous or allogeneic bone marrow or peripheral stem cell transplantation. In all of these situations T lymphocytes are also depleted. The same happens with the use of therapeutic monoclonal antibodies that target antigens expressed by all lymphocytes and other myeloid cells like CD52 (CAMPTH-1). Mature plasma cells are considered to be mostly resistant to all these treatments.

1.4.2.1 B-lymphocyte reconstitution after bone marrow transplantation

In normal ontogeny, the peripheral blood B-lymphocyte count is believed to be very low during the first trimester because B-lymphopoiesis begins only during the third month of gestation (Abe, 1989). B-lymphocyte counts reach the normal adult range during the second trimester and then overshoot above the normal adult range, reaching peak levels at birth or during the first year of life (Rainaut et al., 1987, Lucivero et al., 1991). They subsequently drop over several years to the normal adult range (Kotylo et al., 1990, Hannet et al., 1992). Around birth, the majority of circulating B lymphocytes are immature, naïve cells. Compared with normal adults, these cells are larger, usually

do not express L-selectin and more than 90% of them express high amounts of CD38, mIgM and mIgD; CD5 B lymphocytes are abundant (Durandy et al., 1990, Hannet et al., 1992).

Studies of B-lymphocyte reconstitution after BMT in patients without chronic graft versus host disease (GVHD) suggest a recapitulation of ontogeny based on the pattern of quantitative recovery of circulating B lymphocytes (low → high → normal B-lymphocyte counts), the increased B-lymphocyte size observed and the constellation of surface antigens on B lymphocytes which resemble neonatal cells (Small et al., 1990, Storek et al., 1993). At repopulation, the majority of circulating B lymphocytes express a surface phenotype compatible with immature, naïve B lymphocytes. They express CD38, mIgM and mIgD and express CD38 at higher levels than the majority of circulating B lymphocytes in normal adults. Circulating earlier B-lymphocyte precursors have not been detected at B-lymphocyte repopulation (Small et al., 1990; Storek et al., 1993). The complete pattern reminiscent of ontogeny is not observed in all patients without chronic GVHD and some variation occurs. An increased frequency of CD5 expressing B lymphocytes was observed in the majority of studies but not in all (Drexler et al., 1987, Bengtsson et al., 1989, Kagan et al., 1989, Small et al., 1990). Expression of CD23 and L-selectin also varies between the studies. Differences in the expression of CD5 and other markers at repopulation may be due to differences in the extent of myeloablation achieved or in detection methods and reagents used.

The observed phenotype at B-lymphocyte reconstitution in patients who received different types of transplant show that B-lymphocyte reconstitution occurs always from stem cells rather than from grafted mature B lymphocytes (Small et al., 1990, Koehne et al., 1997).

The naïve nature of the majority of the B lymphocytes at reconstitution has been confirmed by studies looking at the frequency of somatic mutations. Suzuki and colleagues reported a general low incidence of somatic mutations up to 1 year post-BMT, equal to the level of mutations in the preimmune repertoire in healthy controls consistent with the majority of the cells being naïve (Suzuki et al., 1996).

In one of the studies, an increased frequency (> 15%) of CD19-positive cells that had low or absent CD20 antigen expression is described in several of the samples at repopulation (Storek et al., 1993). This phenotype is characteristic of circulating plasma cell precursors.

Following myeloablative therapy and BMT, B-lymphocyte repopulation occurs earlier in peripheral stem cell transplantation when compared to bone marrow stem cell transplantation and earlier following an autologous transplant when compared with an allotransplant (Zintl et al., 1989, Storek et al., 1993, Roberts et al., 1993, Ashihara et al., 1994, Koehne et al., 1997). Some variation occurs between the studies and also within each group of patients but, in general, the period of profound B-lymphocyte depletion in the peripheral blood is short (1 to 3 months) in the majority of autotransplant patients, longer (3 to 4 months) in allograft recipients without chronic GVHD and the longest (4 to >12 months) in patients with chronic GVHD, in whom B-lymphocyte repopulation can be severely delayed (Witherspoon et al., 1981, Zintl et al., 1989, Storek et al., 1993). The speed at which B-lymphocyte counts reach the normal range also varies between patients. More frequently, B-lymphocyte counts normalise between 4 and 12 months after BMT but in patients with autologous transplants they can normalise earlier (between 2 and 4 months) (Storek and Saxon, 1992, Storek et al., 1997a, Steingrimsdottir et al., 2000).

Functional studies have suggested that the majority of B lymphocytes at repopulation are immature cells, owing to several similarities with functional characteristics of B lymphocytes in cord blood and young children. In vitro, these cells show suboptimal responses to B-lymphocyte mitogens and other polyclonal activators (including TI antigens) and growth factors, as well as failure to terminally differentiate into antibody-producing cells (Lum et al., 1981, Witherspoon et al., 1981, Pahwa et al., 1982, Witherspoon et al., 1982, Bengtsson et al., 1989, Kagan et al., 1989, Small et al., 1990).

Usually, the pattern of surface antigen expression characteristic of immature, naïve B lymphocytes tends to normalise during the second year post-transplant. The circulating B-lymphocyte memory population seems to reconstitute only later (Suzuki et al., 1996, Storek et al., 1997b). Similar to the change in surface phenotype, mature functional characteristics such as normal responses to mitogens and growth factors tend to be

appear within 1 year but terminal differentiation particularly into IgG- and IgA-producing plasma cells usually occurs only later (Witherspoon et al., 1981, Pahwa et al., 1982, Kagan et al., 1989, Small et al., 1990). Glas and colleagues described that even 1 year after grafting, allogeneic BMT patients B lymphocytes lacked the capacity to accumulate somatic mutations in vivo in a T-lymphocyte dependent manner compared with healthy subjects (Glas et al., 2000).

Normal levels of circulating IgM and IgG antibodies are achieved during the first year after BMT (Storek et al., 1992), but specific humoral immunity often remains impaired more than 1 year post-BMT (Lum, 1990, Storek and Saxon, 1992). IgM levels are usually within the normal range at 2-6 months after transplantation (Storek et al., 1992). Several reports have demonstrated that the return of serum immunoglobulin isotypes and IgG subclasses post-transplant follows a pattern similar to that seen in normal children over the first 1 to 2 years of life (Aucouturier et al., 1987, Lum, 1987, Velardi et al., 1988). IgM, IgG1 and IgG3 recover earlier while recovery of IgG2, IgG4 and IgA is delayed. This is again compatible with recapitulation of ontogeny. Although the ability to secrete IgM in response to a variety of polyclonal B-lymphocyte activators is present at birth, the ability to secrete IgG is gradually attained between birth and two years of age (Andersson et al., 1981, Miyawaki et al., 1981). Velardi et al. found that serum IgG2 and IgG4 were deficient for more than 18 months after BMT with elevated IgG1 levels accounting for the normal or increased levels of total IgG (Velardi et al., 1988). Abnormally low or borderline subclass (mostly IgG2 and IgG4) levels were found after transplant as long as 25 months, in association with low or borderline IgA levels in 78% of 31 cases. The dominant antibody response to capsular polysaccharide antigens such as PCP occurs predominantly in the IgG2 subclass (Siber et al., 1980). Decreased proliferative responses to TI antigens and deficient production of IgG2 are thought to account for the fact that survivors of BMT, as well as normal infants, have an increased susceptibility to infections with encapsulated bacteria (Atkinson et al., 1982).

Changes in B-lymphocyte and serum immunoglobulin repertoires following BMT have been studied. Transient preferential expression of smaller VH families (VH6 and VH5) in grafted patients similar to ontogeny has been described (Berman et al., 1991, Fumoux et al., 1993). Fumoux F and colleagues studied the pattern of VH usage in BM lymphoid cells in the first 3 months after BMT and compared to BM from healthy

controls (Fumoux et al., 1993). In normal controls the following gradient of expression was detected VH3>VH1>VH4>VH5 with VH2 and VH6 below the level of detection, similar to that reported in peripheral blood on adult B-lymphocyte clones. During the first 3 months after BMT, VH3 usage was decreased with a corresponding increase in the usage of the other families. In foetal tissues this phenomenon is thought to be due either to a topological effect in relation to their localisation at the 3' end of the IgVH locus or to selection by antigen, particularly self-antigen. The minor VH groups may be more involved in self-recognition (Sanz et al., 1989, Pascual and Capra, 1991).

Several authors have described that reconstitution of the B-lymphocyte repertoire after BMT follows an oligoclonal pattern (Nasman and Lundkvist, 1996, Nasman-Bjork and Lundkvist, 1998, Gokmen et al., 1998). This restriction of the B-lymphocyte repertoire seems to apply mainly to memory B lymphocytes with evidence suggesting that the processes involved in generating and selecting the primary antibody repertoire are largely functional within 1 year after BMT. Appropriate V-region gene usage has been described and complementarity determining region (CDR) 3 complexity following BMT is similar in BMT recipients and healthy subjects 1 year after transplantation. Omazic and colleagues have studied the pattern of CDR3 size distribution and have found a significant restriction of the memory B-lymphocyte repertoire at 3 and 6 months after transplantation but not of the naïve B-lymphocyte repertoire (Omazic et al., 2003). Twelve months after transplantation, both memory and naïve B-lymphocyte repertoires were as diverse as in healthy controls. In some patients, oligoclonality of the B-lymphocyte repertoire can remain for years (Gerritsen et al., 1993).

It is suggested that this restriction in the memory B-lymphocyte repertoire after BMT may be associated with a slow recovery of memory B lymphocytes as documented by Storek and colleagues (Storek et al., 1997b). A possible explanation is the lack of helper T lymphocytes to support activation of naïve B lymphocytes or the simple fact that it takes time to reconstitute the memory B-lymphocyte repopulation. It has been suggested that oligoclonal expansion of B lymphocytes similar to that of T lymphocytes after BMT may occur (Nasman et al., 1996, Nasman-Bjork et al., 1998). However, there is an important difference between B- and T-lymphocyte reconstitution. After BMT it appears that B lymphocytes do not expand from transferred mature cells as T lymphocytes do but from cells developed from the donated stem cells.

Even though serum IgM concentration is usually within the normal range 6 months after BMT, a study of circulating antibodies repertoires after allogeneic BMT showed that 60% of BMT patients had severely reduced diversity in the IgM repertoire during and after the first year after BMT when compared with healthy controls (Bjork et al., 2000). In contrast, the majority of patients had a polyclonal IgG repertoire similar to that of healthy controls. It is likely that antibodies produced by host long-lived plasma cells, that have survived, contribute to this.

Recipient pretransplantation antibody levels against polio, tetanus, *haemophilus influenzae* and *Streptococcus pneumoniae* correlated with the post-transplantation levels particularly during the first 6 months after BMT (Storek et al., 2003) (Storek et al., 2003). Patient age, conditioning regimen and the presence of GVHD or its treatment had no effect. These results suggest that antibody levels in the first 6 months following BMT are primarily produced by residual host plasma cells that are not depleted by the conditioning regimen.

1.4.2.2 B-lymphocyte reconstitution after rituximab

Treatment with rituximab in patients with lymphoma is associated with a period of peripheral blood B-lymphocyte depletion that lasts usually 6 to 9 months (McLaughlin et al., 1998). To our knowledge, at the time of start of the work presented in this thesis, there was no published data in the literature regarding the phenotype or functional characteristics of the repopulating B lymphocytes following treatment with rituximab. In patients with lymphoma, treatment with rituximab had not been associated with a significant decrease in mean serum total immunoglobulin levels.

1.5 Hypothesis and aims of this thesis

1.5.1 Hypothesis

The apparent benefit of BLDT in RA renews the interest in studying the complex role of B lymphocytes in the pathogenesis of this autoimmune disease.

It is proposed that recurrence of signs and symptoms of active disease following an earlier response to BLDT is due either to the persistence of pathogenic B-lymphocyte clones or to the persistence of long-lived plasma cells producing pathogenic autoantibodies or to both and that the main role of B lymphocytes in the perpetuation of inflammation in RA is the production of pathogenic species of autoantibodies capable of inducing inflammation and of leading to aberrant interactions between B and T lymphocytes and abnormal B-lymphocyte survival.

1.5.2 Aims

A first open label trial of combination therapy with rituximab, cyclophosphamide and prednisolone in five patients with RA suggested that B-lymphocyte depletion might be an effective therapy in these patients. Interesting kinetics of clinical response were observed with no patient relapsing before B-lymphocyte return to the peripheral blood was detected and relapse occurring either at the time of B-lymphocyte repopulation or at a variable time thereafter. The possibility that, at least in some patients with RA, disease perpetuation may be B-lymphocyte dependent has raised many questions regarding the pathogenic role of B lymphocytes in the initiation and particularly in the perpetuation of inflammation in this disease.

From birth, B lymphocytes are known to have two main roles in the adaptive immune system of humans. They are responsible for the production of antibodies and they are efficient APC for T lymphocytes, particularly of soluble antigens. Treatment of patients with RA with rituximab, a B-lymphocyte specific agent, provides a unique opportunity to study B-lymphocyte biology in this disease, in particular, the role of B lymphocytes and of autoantibodies in their pathogenesis.

The primary aims of this project are to investigate:

1) why do patients with RA relapse after responding to BLDT;
2) whether it is possible to prevent relapse and improve the long-term results of this therapeutic approach.

Relapse in this context means true relapse following disease remission as defined by absence of clinical and laboratorial manifestations of active disease or disease flare following an earlier response to treatment. Several possibilities exist to explain relapse in patients who respond to B-lymphocyte depletion. Relapse may occur because B-lymphocyte depletion is not complete and important pathogenic B-lymphocyte clones are left behind, because certain species of pathogenic autoantibodies are produced by long-lived plasma cells and these cells are not depleted by rituximab or because the memory for the disease resides in other cells, in particular autoreactive T lymphocytes. It is possible that different pathogenic B-lymphocyte clones or different species of pathogenic autoantibodies are either associated with disease persistence or with disease re-initiation, or that the differences that underlie clinical response and relapse are mainly quantitative. Ultimately, relapse following B-lymphocyte depletion may be inevitable, for example, if the memory for the disease resides in autoreactive T lymphocytes or if abnormalities in B-lymphocyte central or peripheral tolerance are responsible for re-initiation of the pathological process once formation of mature B lymphocytes is re-established.

Elucidation of the dynamics of different B-lymphocyte subpopulations and of disease-related autoantibodies following treatment with rituximab will allow a better understanding of what causes relapse in these patients and, eventually, of what is the role of B lymphocytes and of autoantibodies in the disease process. Potentially, this knowledge may lead to the development of more effective B-lymphocyte targeting therapeutic strategies.

1.5.3 Experimental approaches

To achieve the primary aims the following experimental approaches were chosen:

1) a study of clinical response of patients with RA to BLDT of varying intensity in a larger group of patients;

2) the measurement of serial humoral changes after BLDT in patients with RA;
3) the investigation of changes in peripheral blood B lymphocytes following BLDT;
4) the determination of the presence and characteristics of cells of B-lymphocyte lineage (B-lymphocyte precursors, mature B lymphocytes and plasma cells) in bone marrow aspirates 3 to 4 months after BLDT.

CHAPTER 2 RESULTS: CLINICAL STUDIES

2.1 Study design

An initial open-label study at UCL/UCLH in 5 patients with active RA refractory to standard therapy suggested that treatment with a B-lymphocyte depletion protocol based on rituximab could lead to significant improvement in disease manifestations with a good safety profile (Edwards and Cambridge, 2001). All 5 patients achieved major improvement in symptoms and signs of active disease following treatment. In 2 of the 5 patients clinical relapse coincided with the return of B lymphocytes to the peripheral blood while in the other 3 clinical response continued beyond B-lymphocyte repopulation.

The 5 patients were treated with the standard rituximab dose and schedule (4 weekly infusions of 375 mg/m^2 as in lymphoma) combined with cyclophosphamide and oral corticosteroids. In B-cell NHL, treatment with the standard dose of rituximab leads not only to killing of malignant cells but also to almost complete depletion of normal B lymphocytes in the peripheral blood lasting usually 6 to 9 months (McLaughlin et al., 1998). It was not known whether in RA the standard dose of rituximab used in lymphoma might be excessive as in RA there is no extra population of malignant B lymphocytes.

The open-label trial of BLDT based on rituximab in 5 patients was extended to include a total of 22 patients with active refractory RA. In this extended trial, the effect of diffęrent doses of rituximab with or without cyclophosphamide or oral prednisolone were investigated. The main objectives were to investigate: 1) whether a positive clinical response could be reproduced in a larger group of patients; 2) whether there was a dose-response to rituximab and whether cyclophosphamide or oral prednisolone had additive effects; 3) the duration of clinical response to BLDT in a larger group of patients and the time relationship between clinical relapse and B-lymphocyte repopulation of the peripheral blood; 4) the tolerability and side effect profile of the different protocols in this group of patients. A different schedule of administration of rituximab and the effects of re-treatment were also studied. The study design was based on the oncology concept of rolling mini-phase I studies of 5 patients each. Some cohorts (cohorts II and V) were smaller as before recruitment of the 5 patients it was felt that its

purpose had already been served. One of the cohorts (cohort IV) was larger as it was also designed to gain more data on which to base the design of the phase II double blind, placebo-controlled trial run by Roche (F. Hoffman-La Roche Ltd) that took place afterwards (Edwards et al., 2004).

Subsequently, a further 18 patients with active RA were treated with BLDT and several patients were retreated leading to a total of 77 treatments altogether. Details on response to treatment and in particular, extended data on efficacy and safety of re-treatment and of time of clinical relapse following repeated courses of treatment were collected.

2.2 Patients and Methods

2.2.1 Patient selection

The initial open-label trial of BLDT in 5 patients with RA was extended to 22 patients. All patients satisfied the American College of Rheumatology (ACR) diagnostic criteria for RA and were treated for active, erosive disease not adequately controlled by standard DMARDs (Arnett et al., 1988). The UCLH Ethics Committee approved the study and all patients gave informed consent before entering the study. Two patients were male and 20 female. At study entry, mean age was 58 years (range 33 to 81) and mean disease duration was 18 years (range 5 to 40). The number of DMARDs previously failed (due to inefficacy or toxicity) ranged from 2 to 6 (mean 4). Table 2.1 summarises patient characteristics at study entry.

The following patients had salient features that might have influenced outcome. Patient 11 had secondary amyloidosis with renal involvement and had been receiving monthly intravenous cyclophosphamide after oral chlorambucil had failed. Patient 18 was functional grade IV with multiple osteoporotic fractures, but still had inflammatory symptoms. Patient 8 was seronegative and antinuclear antibody (ANA) positive, but with erosive disease and no features of lupus. Patient 20 was seronegative and had psoriasis but a symmetrical rheumatoid pattern of disease and no nail dystrophy and had previously been diagnosed as seronegative RA.

Table 2.1 Extended open-label trial of BLDT in RA: details of the 22 patients at study entry

Patient	Age (years)	Sex	Duration of RA (years)	Erosive	Global functional status	RAPA (ever +)	ANA (ever +)	Extra-articular features	DMARD failure	DMARD/steroid at entry
1	57	F	25	+	III	Yes	No	Anaemia	ADGMS	P 7.5 mg/day
2	42	F	21	+	III	Yes	Yes	Anaemia	ADGMS	A 100 mg/day
3	69	F	40	+	III	Yes	Yes	-	ADGMS	G 50 mg/month
4	50	F	11	+	III	Yes	Yes	Anaemia	AGMS	P 10 mg/day
5	59	F	17	+	III	Yes	Yes	Nodules	ADGHMS	M 12.5 mg/week
6	69	F	27	+	III	Yes	Yes	Anaemia	ADGHMS	A 100 mg/day P 5 mg/day
7	55	F	5	+	II	Yes	No	Anaemia	HMS	P 5 mg/day
8	57	F	11	+	II	No	Yes	Anaemia	ADGHMS	-
9	63	F	13	+	II	Yes	No	Anaemia Nodules	AGMS	A 100 mg/day G 50 mg/month
10	70	F	18	+	III	Yes	Yes	Anaemia	AGS	G 50 mg/month
11	60	F	28	+	III	Yes	Yes	Anaemia Amyloidosis	ChCyDMS	Cy 750 mg/month P 5 mg/day
12	69	F	39	+	II	Yes	Yes	-	ADGHMS	M 10 mg/week
13	51	F	15	+	II	Yes	No	-	AMS	S 3 g/day A 100 mg/day
14	33	F	17	+	III	Yes	Yes	Anaemia	AGS	-
15	60	M	7	+	III	Yes	No	Anaemia Nodules	CyaMS	M 30 mg/week Cya 150 mg/day P 10 mg/day
16	63	M	20	+	II	Yes	Yes	-	GHM	M 20 mg/week H 400 mg/day

Patient	**Age** (years)	**Sex**	**Duration of RA** (years)	**Erosive**	**Global functional status**	**RAPA** (ever +)	**ANA** (ever +)	**Extra-articular features**	**DMARD failure**	**DMARD/steroid at entry**
17	51	F	14	+	III	Yes	Yes	Anaemia SS	HM	M 20 mg/week H 400 mg/day
18	50	F	20	+	IV	Yes	Yes	-	ACyaDGMS	P 10 mg/day
19	54	F	9	+	III	Yes	Yes	-	AGMS	-
20	64	F	14	+	II	No	No	-	AGLMS	P 7.5 mg/day
21	49	F	5	+	III	Yes	Yes	Anaemia	AGLMST	P 5 mg/day
22	81	F	23	+	III	Yes	Yes	Skin vasculitis Nodules	ADG	A 100 mg/day

Abbreviations: F, female; M, male; RAPA, rheumatoid arthritis particle agglutination test; ANA, antinuclear antibodies; A, azathioprine; Ch, chlorambucil; Cy, cyclophosphamide; Cya, cyclosporine; D, *D*-penicillamine; DMARD, disease modifying anti-rheumatic drugs; G, gold; H, hydroxychloroquine; L, leflunomide; M, methotrexate; P, prednisolone; S, sulfasalazine; SS, Sjögren's syndrome; T, anti-TNF agents.

Subsequently a further 18 patients were treated with BLDT and several patients were retreated, on the basis of clinical need. Eight new patients were male, eight female. At study entry, mean age was 61 years (range 32 to 80) and mean disease duration was 18 years (range 5 to 45). The number of DMARDs previously failed (due to inefficacy, toxicity or contraindication) ranged from 1 to 7 (mean 4). Table 2.2 summarises the 18 patients characteristics at study entry.

Several patients were retreated with BLDT and the 40 patients received a total of 77 courses of treatment.

2.2.2 Treatment protocols

Within the extended open-label trial the 22 patients received a total of 29 treatments (seven re-treatments). The first 5 patients had been treated in the initial open label trial with a combination protocol that included 4 weekly intravenous infusions of rituximab, 2 intravenous infusions of cyclophosphamide and oral prednisolone (Edwards and Cambridge, 2001). Further patients were treated with protocols involving reduction in one or more of the components of the original protocol. As previously mentioned, study design was based on the oncology concept of rolling mini-phase I studies. Patients were treated primarily according to individual clinical need with a protocol judged reasonably likely to provide an optimum cost-benefit profile at that time. Minor modifications of protocol were dictated by logistic or toxicity considerations, but treatments were matched as far as possible within five sequential cohorts (table 2.3). All patients were pre-medicated with paracetamol (1g oral) and chlorpheniramine (10 mg intravenous) before each rituximab infusion to decrease the risk of infusion reactions. DMARDs were discontinued from day 0. All patients used NSAIDs or analgesics as needed. Patients on oral prednisolone continued this medication with further decreases or eventual stopping dictated by clinical response. Patients were retreated either following relapse after improvement or if a reduced dose protocol had failed to produce significant improvement.

Cohort I comprised the first 5 patients that had been treated with 4 intravenous infusions of rituximab on days 2, 8, 15 and 22, of 300, 600, 600 and 600 mg (approximately 200, 375, 375 and 375 mg/m^2), respectively, (patient 5 omitted dose 4 because of an episode

Table 2.2 Details of patients 23 to 40 at study entry

Patient	**Age** (years)	**Sex**	**Duration of RA** (years)	**Erosive**	**Global functional status**	**RAPA** (ever +)	**ANA** (ever +)	**Extra-articular features**	**DMARD failure**	**DMARD/steroid at entry**
23	59	F	16	+	II	Yes	Yes	Anaemia Nodules	ACyaHLMST	P 10 mg/day
24	63	M	10	+	II	Yes	Yes	-	CyaMST	Cy 50 mg/day P 7.5 mg/day
25	77	F	45	+	IV	Yes	NK	Pulmonary fibrosis Pericarditis	AG	A 100 mg/day P 7.5 mg/day
26	57	M	9	+	II	Yes	No	Anaemia Sec amyloidosis	MS	-
27	60	M	15	+	III	Yes	NK	-	GMS	M 10mg/week
28	69	F	15	+	IV	No	No	-	M	M 10 mg/week
29	65	F	14	+	III	No	No	Anaemia	M	M 15 mg/week
30	58	M	26	+	III	Yes	No	-	ADGMST	-
31	71	F	24	+	III	Yes	Yes	Anaemia Nodules Sec amyloidosis	AGMS	-
32	32	M	5	+	II	Yes	NK	-	GM	M 10 mg/week
33	38	F	12	+	III	Yes	NK	Anaemia	DGHMT	M 10 mg/day
34	65	M	15	+	II	Yes	NK	-	GMS	M 15 mg/day
35	79	F	30	+	III	Yes	No	-	DGM	M 7.5 mg/week
36	64	M	12	+	III	Yes	NK	-	ACyaGMS	M15 mg/week Cya 75 mg/day
37	80	M	20	+	III	Yes	NK	-	DGMS	M 12.5 mg/week

Patient	**Age** (years)	**Sex**	**Duration of RA** (years)	**Erosive**	**Global functional status**	**RAPA** (ever +)	**ANA** (ever +)	**Extra-articular features**	**DMARD failure**	**DMARD/steroid at entry**
38	47	F	20	+	III	Yes	Yes	Anaemia	ACyaHMS	P 15 mg/day
39	55	F	16	+	III	Yes	Yes	-	AHLMS	M 7.5 mg/day P 10 mg/day
40	58	M	17	+	III	Yes	Yes	Anaemia	AGLMS	P 10 mg/day

Abbreviations: F, female; M, male; RAPA, rheumatoid arthritis particle agglutination test; ANA, antinuclear antibodies; NK, not known; A, azathioprine; Ch, chlorambucil; Cy, cyclophosphamide; Cya, cyclosporine; D, *D*-penicillamine; DMARD, disease modifying anti-rheumatic drugs; G, gold; H, hydroxychloroquine; L, leflunomide; M, methotrexate; NK, not known; P, prednisolone; S, sulfasalazine; SS, Sjögren's syndrome; T, anti-TNF agents.

of fever and pleuritic pain), 2 intravenous infusions of 750 mg of cyclophosphamide (days 3 and 16) and oral prednisolone 60 mg on days 1 to 22, reducing in the 3 older patients (patients 1, 3 and 5, perceived to be at higher risk of toxicity) to 30 mg on days 11 to 22 and then withdrawn over 3 weeks in subjects not previously taking prednisolone and, in the other cases, reduced to 5 mg daily over six weeks.

Cohort II comprised 4 patients (patient 2, retreated from cohort I and patients 6 to 8) who received either one or two 300-350 mg/m^2 doses of rituximab 14 days apart without cyclophosphamide (table 2.3). Patients 7 and 8 received oral prednisolone cover (60 mg orally for five consecutive days starting the day before the rituximab infusion).

Cohort III comprised 10 patients (patients 9 to 12 and patients 2, 4, 6 and 7, retreated from previous cohorts) who received two 300-350 mg/m^2 doses of rituximab and, in most cases, 2 doses of 750 mg of cyclophosphamide, under oral prednisolone cover (table 2.3). Patients received 60 mg of prednisolone for 5 days twice unless individual tolerance to corticosteroids dictated otherwise. Patient 2 received 3 doses of 750 mg of cyclophosphamide. Patients 7 and 10 received 2 doses of 500 mg of cyclophosphamide in view of their small body surface area.

Cohort IV comprised 6 patients (patients 13, 14 and 16 to 19) treated with a protocol as in cohort I but without oral prednisolone cover (except patient 16 who had 30 mg for 4 days twice for anti-emesis) and with compression of rituximab treatment into two infusions (table 2.3).

Cohort V comprised 4 patients (patients 15 and 20 to 22) who received one 500 mg/m^2 dose of rituximab with two 750 mg doses of cyclophosphamide under variable prednisolone cover (table 2.3).

After the open-label trial exploring different B-lymphocyte depletion protocols based on rituximab, treatment of new patients (patients 18 to 40) and re-treatments (a total of 30 re-treatments) consisted of a total dose of 1500 mg (earlier treatments) or 2000 mg of rituximab divided in 2 infusions (table 2.4). The first patients still received 1 or 2 infusions of 750 mg of cyclophosphamide but this was later abandoned due to safety concerns in view of the need for repeated courses of treatment. Oral prednisolone cover

Table 2.3 Extended open-label trial of BLDT in RA: details on drug dosage used and on clinical response to treatment

Cohort	Patient	Rituximab (mg/m^2)	Cyclophosphamide (mg)	Prednisolone (mg)	ACR grade of improvement at 6 months	Time to B-lymphocyte repopulation (months)	Time to relapse (months)
I	1	1400	1500	850	70	21	34
	2	1400	1500	1300	70	7	7
	3	1400	1500	1250	70	9	26
	4	1400	1500	1425	50	7	8
	5	900	1500	1100	50	11	21
II	2a	300	-	-	0	0	NA
	6	700	-	-	0	5	NA
	7	700	-	640	20	7	7
	8	600	-	605	0	4	NA
III	2b	600	2250	400	70	7	7
	4a	600	1500	600	70	10	10
	6a	600	1500	500	20	NK (> 18)	6
	7a	700	1000	300	20	8	11
	9	600	1500	420	70	7	7
	10	700	1000	400	70	9	9
	10a	700	1000	400	70	6	20
	11	600	1500	500	70	10	37
	12	600	1500	600	50	7	12
	12a	600	1500	600	50	7	14
IV	13	1200	1500	-	70	12	44
	14	1200	1000	-	50	10	12
	16	1200	1500	240	70	6	7
	17	1200	1500	-	20	17*	NA*
	18	1200	1000	-	20	10	17
	19	1200	1500	-	50	6	13

Cohort	Patient	Rituximab (mg/m^2)	Cyclophosphamide (mg)	Prednisolone (mg)	ACR grade of improvement at 6 months	Time to B-lymphocyte repopulation (months)	Time to relapse (months)
V	15	500	1500	210	0	9	NA
	20	500	1500	300	0	7	NA
	21	500	1500	340	0	9	NA
	22	500	1500	150	0	5	NA

Abbreviations: NA, not applicable; NK, not known; * chemotherapy for breast cancer starting at 4 months after BLDT; a, first retreatment; b, second re-treatment.

Table 2.4 BLDT in patients 18 to 40 and a further 30 re-treatments: details on drug dosage used and on clinical response to treatment

Patient	Rituximab (mg)*	Cyclophosphamide (mg)	Prednisolone (mg)**	Methotrexate (mg)	Response to treatment	Time to B-lymphocyte repopulation (months)	Time to relapse (months)
1a	1500	750	685	-	Yes	12	24
1b	2000	-	625	-	Yes	8	18
3a	2000	750	685	-	Yes	11	19
3b	***			-	NA	NA	NA
4b	1500	750	625	-	Yes	10	11
4c	2000	-	625	-	Yes	9	11
5a	2000	-	625	7.5	Yes	9	> 12
8a	2000	-	250	-	No	2	NA
10b	2000	-	370	-	Yes	5	7
12c	2000	-	625	-	Yes	9	15
12d	2000	-	625	-	Yes	9	>10
14a	2000	-	625	-	Yes	NK	NK
15a	2000	-	625	17.5	Yes	12	17
16a	2000	-	625	17.5	Yes	6	6
17a	1500	-	625	-	Yes	5	8
17b	2000	-	625	-	Yes	5	>7
18a	1500	750	625	-	Yes	NK	NK
19a	2000	-	625	-	Yes	10	20
19b	2000	-	625	-	Yes	11	>15
21a	1500	750	625	-	No	11	NA
22a	2000	-	625	-	Yes	6	6
23	1500	1500	1030	-	Yes	7	7
23a	3000	-	710	-	Yes	13	13
23b	2000	-	370	-	Yes	12	12
24	2000	-	370	-	Yes	9	15

Patient	Rituximab (mg)*	Cyclophosphamide (mg)	Prednisolone (mg)**	Methotrexate (mg)	Response to treatment	Time to B-lymphocyte repopulation (months)	Time to relapse (months)
24a	2000	-	250	15	Yes	NK	16
25****	1500	750	40	-	No	NK	NA
26	2000	-	745	-	Yes	9	9
26a	2000	1500	370	-	Yes	11	11
26b	2000	-	370	-	Yes	11	11
27	2000	-	650	10	Yes	NK	NK
28	2000	-	250	10	No	12	NA
29	2000	-	250	15	No	NK	NA
30	2000	-	250	-	Yes	6	16
30a	2000	-	250	-	Yes	6	18
31	2000	-	250	-	Yes	6	6
31a	2000	-	250	-	Yes	5	6
32	2000	-	500	10	Yes	5	7
33	2000	-	250	10	Yes	8	13
34	2000	-	250	12.5	Yes	8	>17
35	2000	-	250	7.5	Yes	7	13
36	2000	-	250	15	Yes	6	8
36a	2000	-	250	20	Yes	5	6
37	2000	-	250	12.5	Yes	7	12
38	2000	1500	625	-	Yes	3	5
39	2000	-	250	10	Yes	7	>13
40	2000	-	250	-	Yes	>12	>12

Abbreviations: NA, not applicable; NK, not known; a, first re-treatment; b, second re-treatment; c, third re-treatment; d, fourth re-treatment.

* Rituximab used at a fixed dose (two infusions of 1000 mg, approximately 1200-1400 mg/m^2BSA).

** Or equivalent when methylprednisolone used.

*** Third treatment of patient 2 interrupted because of allergic reaction.

**** Lost to follow up.

for rituximab infusions was substituted for one 100 (more frequently) or 250 mg intravenous infusion of methylprednisolone before each rituximab infusion. In patients 32 to 40 and in some re-treatments, methotrexate was continued if patients had been taking it and tolerating it well before study entry as at that time data from the phase IIa randomised controlled trial was already available and showed that this approach was safe and more effective than to stop methotrexate and treat with only rituximab monotherapy (Edwards et al., 2004).

2.2.3 Assessment of patients

In the open-label trial, the 22 patients were assessed at recruitment, before treatment, monthly for the first 6 months after treatment, every 2 months until 1 year and every 2 to 3 months afterwards.

Further patients and further re-treatments were assessed before treatment, 1 month after treatment, every 1 to 3 months until B-lymphocyte repopulation of the peripheral blood and every 2 to 3 months afterwards.

Maria Leandro or Professor Jonathan Edwards assessed the patients.

2.2.3.1 Clinical assessment

In the open-label trial clinical assessment included duration of early morning stiffness in minutes, patient's assessment of pain on a 100 mm visual analogue scale, number of swollen joints (out of 28 joints), number of tender joints (out of 28 joints), and physician's and patient's global assessment of disease activity on a 100 mm visual analogue scale as well as assessment of side effects. Global functional status was defined based on the classification criteria revised by the ACR in 1991 (Hochberg et al., 1992). Class I patients are completely able to perform usual activities of daily living (self-care, vocational and avocational). Class II patients are able to perform usual self-care and vocational activities, but limited in avocational activities (recreational or leisure). Class III patients are able to perform usual self-care activities, but limited in vocational (work, school, homemaking) and avocational activities. Class IV patients are limited in ability to perform usual self-care, vocational and avocational activities.

For patients 23 to 40 and further re-treatments clinical assessment was as in normal clinical practice with careful assessment of side effects. Clinical assessment also included all the measures of clinical disease activity described above whenever logistic aspects related to the clinic allowed it.

2.2.3.2 Laboratory assessment

In the open-label trial laboratory measurements included erythrocyte sedimentation rate (ESR), serum C reactive protein (CRP) concentration, full blood count, renal and liver function tests, serum immunoglobulin levels, RhF by latex fixation (positive or negative), titre of rheumatoid arthritis particle agglutination (RAPA) test, and ANA titre by immunofluorescence. Circulating B lymphocytes were assessed by CD19 positive cell counts by flow cytometry. All tests were performed at the Central Pathology Laboratory at UCLH. The normal range for peripheral blood CD19 positive cell counts in this laboratory is 0.03 to 0.40 x 10^9/l (CD19 positive cell count is calculated based on the frequency of positive cells detected in 2,000 cells in the lymphocyte gate). Depletion of B lymphocytes was deemed to have occurred when the number of CD19 positive cells was less than 0.005 x 10^9/l and was reported as zero. B-lymphocyte repopulation was deemed to have started when B lymphocytes were again detectable in the peripheral blood.

Further patients and further re-treatments baseline assessment included all the preceding measurements with the exception of ANA titre by immunofluorescence.

The FcγRIIIa-158V/F polymorphism was genotyped by direct sequencing in 18 patients (patients 1 to 5, 7, 9 to 13, 15, 16 and 18 to 22) (Morgan et al., 2000). Staff at the Molecular Medicine Unit at St. James's University Hospital in Leeds performed this assay.

2.2.4 Definition of response to treatment, remission and relapse

In the open-label trial (first 22 patients), response at 6 months was defined as a score of ACR20, 50 or 70, representing a 20, 50 or 70% minimum percentage improvement in five of eight measures, including swollen and tender joint counts, as defined by the American College of Rheumatology (ACR) (Felson DT 1995). In long-standing disease,

ACR70 can be considered an optimal response as presence of deformities and joint damage frequently makes achievement of remission by accepted criteria extremely difficult. Clinical response at 6 months was also assessed by the modified disease activity score (DAS28, calculated as 0.56 x 28T + 0.28 x 28S + 0.70 x lnESR + 0.014 x GH; T, tender joint count out of 28 joints; S, swollen joint count out of 28 joints; GH, patient's global health assessment on a 100 mm visual analogue scale) (Prevoo et al., 1995). Relapse was defined as deterioration to less than ACR20 having achieved at least this level of improvement.

In patients 23 to 40 and further re-treatments, clinical response and relapse were defined applying the same ACR criteria as above whenever the appropriate sequential measurements had been made. Otherwise, a patient was considered to have responded to treatment if there was a definite improvement in signs and symptoms of disease with a documented improvement in swollen joint count and in CRP levels. A patient was considered to be relapsing if a non-transient recurrence or worsening in manifestations of active disease was noted including an increase in CRP levels.

2.2.5 Statistical analysis

For descriptive statistics, mean and standard deviation (or mean and range) were used for data that followed a normal distribution and median and range for data that did not. Comparisons between different groups of data were performed using the paired or unpaired t test for matched or unmatched groups, respectively, if the data followed a normal distribution. When the data were not normally distributed or the equal variance test was not satisfied, non-parametric testing was performed by the Wilcoxon signed rank test for paired data and by the Mann-Whitney rank sum test for non-paired data. Correlations were determined by Pearson product moment correlation for normally distributed data or by Spearman rank order correlation for data that did not follow a normal distribution. P values equal to or less than 0.05 were considered statistically significant. All the analyses were performed using GraphPad PRISM software (GraphPad Software, Inc., San Diego, CA, USA).

2.3 Results

2.3.1 Open label trial – 22 patients

2.3.1.1 Improvement following B lymphocyte depletion – open label trial

Baseline disease characteristics for the 22 patients included in the open-label trial are summarised in table 2.5.

Table 2.5 Baseline disease characteristics in the 22 patients included in the extended open-label trial

Variable	Mean	Range
Swollen joint count (0-28)	18	7-26
Tender joint count (0-28)	7	0-23
VAS pain (0-100)	65	27-100
VAS disease activity (patient) (0-100)	66	15-100
VAS disease activity (physician) (0-100)	69	29-91
EMS (min)	88	0-240
ESR (mm/1st h)	53	11-92
CRP (mg/l)	43	4.4-103.0
DAS28	4.4	2.5-6.4

Abbreviations: VAS, visual analogue scale; EMS, early morning stiffness; ESR, erythrocyte sedimentation rate; CRP, C reactive protein; DAS28, modified disease activity score.

Cohort I:

Cohort I comprised the first 5 patients with RA treated with B lymphocyte depletion. At six months, three of the five patients achieved ACR70 and the other 2 achieved ACR50 (table 2.3). Patient 5 had rheumatoid nodules and these regressed with treatment.

Cohort II:

Patients in cohort II were treated with a smaller dose of rituximab without cyclophosphamide and results were disappointing; only 1 patient achieved ACR20 at 6 months. Cyclophosphamide was retained in further protocols in the open-label trial.

Cohort III:

Cohort III showed broadly comparable responses at 6 months to those in cohort I (table 2.3), although subject 6 remained relatively refractory. Patient 7 achieved only ACR20 at 6 months but improved further to ACR50 at 10 months. Five patients achieved

ACR70 at 6 months with patient 10 repeating this score on re-treatment. Patient 12 improved to ACR50 with first treatment and after re-treatment. Patient 9 had a subcutaneous rheumatoid nodule in one of the forearms that decreased in size.

Cohort IV:

In cohort IV 2 patients achieved ACR70 and 2 others ACR50 at 6 months. Patient 17 had highly refractory disease, with severe Sjögren's syndrome. At 3 months she achieved ACR20 but owing to persistently active disease methotrexate was reintroduced at 4 months. At 6 months she continued to achieve only ACR20. Patient 18 only achieved ACR20 but was functional class IV. When this was taken into account, cohort IV showed responses close to those of cohorts I and III.

Cohort V:

In cohort V clinical benefit was short lived, all cases having worsened by 6 months.

Figure 2.1 shows DAS28 results at baseline and at 3 and 6 months following treatment for each of the patients in the different cohorts.

The median duration of response to a single course of treatment in cohorts I, III and IV was 11.5 months (range 6 to 44).

The two patients who were seronegative at study entry (patients 8 and 20) did not respond to treatment.

2.3.1.2 Laboratory results

Peripheral blood B-lymphocyte counts fell to undetectable levels in all cases except on patient 2 in cohort II when re-treated with only one dose of 300 mg/m^2 of rituximab. The median period of B-lymphocyte depletion in peripheral blood following treatment was 7 months (range 4 to 21). In cohorts I to V, median periods of B-lymphocyte depletion were 9 months (range 7 to 21), 5 months (range 4 to 7), 7 months (range 6 to 10), 10 months (range 6 to 17) and 8 months (range 5 to 9), respectively. The time to reach normal B-lymphocyte counts varied considerably between patients and took from some weeks to several months. Total lymphocyte counts showed no consistent trend.

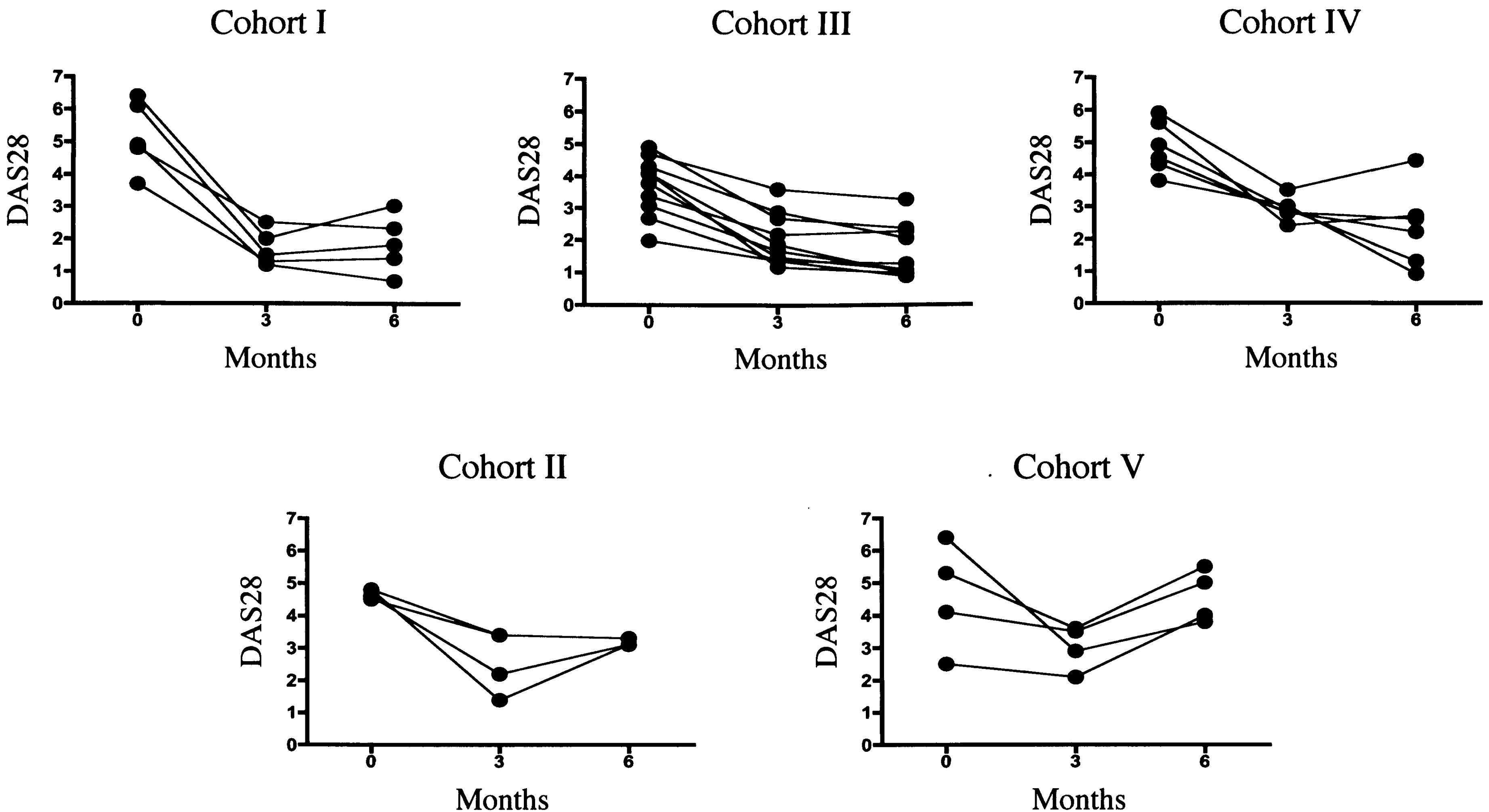


Figure 2.1 DAS28 scores at baseline (0 months) and at 3 and 6 months following BLDT in individual patients in cohorts I to V.

Serum total immunoglobulin levels decreased significantly but median levels remained within the normal range ($P < 0.0001$ for the 3 classes when baseline and nadir levels were compared, Wilcoxon signed rank test) (table 2.6 and figure 2.2). At baseline, 3 patients had low IgA levels (patients 3, 11 and 12) and 1 had low IgG level (patient 11). Following BLDT IgG levels dropped below normal in 3 patients (patients 2, 3 and 5) and IgM levels fell below normal in 7 patients (patients 5, 7, 10-13 and 17).

Table 2.6 Maximum decreases in serum total immunoglobulin levels, IgA, IgG and IgM, in cohorts I to V following BLDT

Cohort	IgA		IgG		IgM	
	g/l median (range)	% mean (range)	g/l median (range)	% mean (range)	g/l median (range)	% mean (range)
I	0.7 (0.2-3.0)	44 (15-75)	3.1 (1.8-8.0)	35 (26-51)	0.7 (0.4-1.4)	50 (22-70)
II	0.1 (0.0-9.0)	15 (0-50)	2.6 (0.0-8.2)	22 (0-53)	0.2 (0.0-4.0)	26 (0-67)
III	0.4 (0.2-2.3)	34 (15-58)	2.8 (0.9-5.6)	27 (9-41)	0.3 (0.1-0.8)	46 (19-70)
IV	1.0 (0.7-2.0)	28 (18-40)	2.6 (1.1-5.3)	20 (8-37)	0.7 (0.3-1.5)	55 (30-71)
V	0.6 (0.1-0.9)	17 (3-31)	3.1 (1.6-3.6)	22 (12-27)	0.5 (0.3-0.9)	33 (14-47)
All cohorts	0.6 (0.0-3.0)	30 (0-75)	2.8 (0.0-8.2)	26 (0-53)	0.4 (0.0-1.5)	43 (0-71)

RAPA titres decreased consistently following treatment but only rarely became negative. ANA titres fluctuated, with no consistent pattern identified. RAPA titres were frequently seen to increase before or at the time of clinical relapse.

Several patients had normochromic normocytic anaemia. Haemoglobulin levels followed the pattern of clinical response, normalising when disease was significantly controlled.

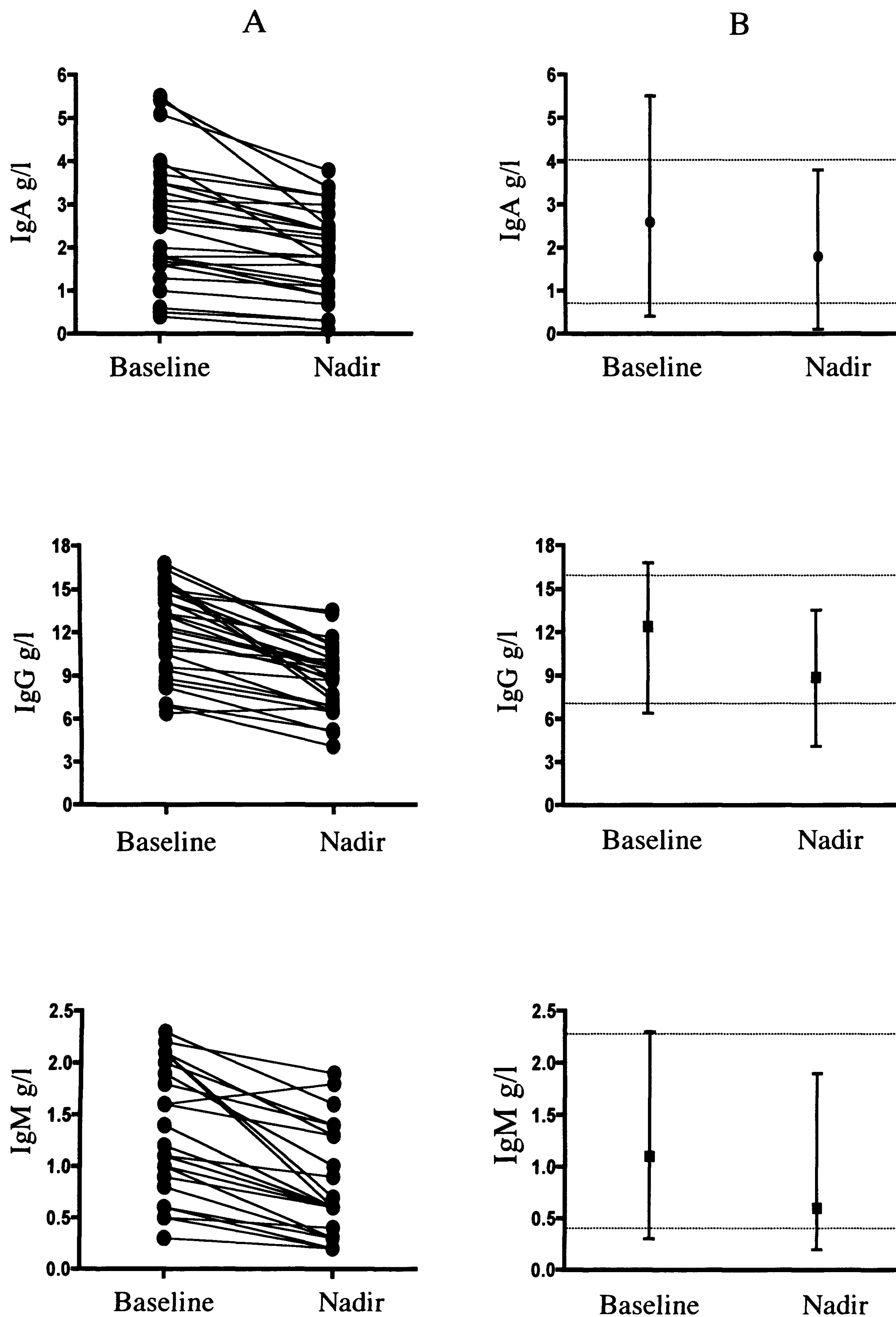


Figure 2.2 Serum total immunoglobulin levels, IgA, IgG and IgM, at baseline and at nadir following BLDT in the extended clinical trial (n= 22). A) each individual patient shown. B) dot and line represent median and range; dotted lines represent upper and lower limits of normal range (IgA 0.7-4.0 g/l; IgG7.0-16.0 g/l; IgM 0.4-2.3 g/l).

Patient 11 had secondary amyloidosis with renal involvement and chronic renal failure diagnosed 3 years earlier. Serum amyloid A (SAA) protein and CRP levels were measured regularly at the National Amyloidosis Centre at the Royal Free Hospital in London. SAA serum levels normalised 4 months after treatment and remained within the normal range for most of the time until 33 months after treatment (figure 2.3A). CRP levels generally followed SAA. Serum amyloid P scan before treatment showed moderate uptake in the kidneys and spleen. Repeated serum amyloid P scan 2 years after treatment with BLDT showed almost complete regression of amyloid deposits with only equivocal signs of uptake in the kidneys and spleen.

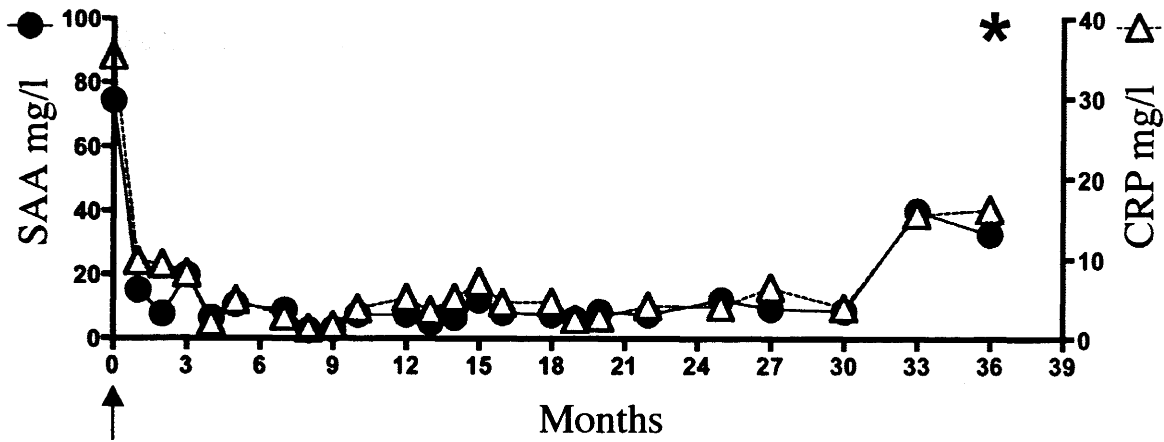
2.3.1.3 Differences between treatment protocols

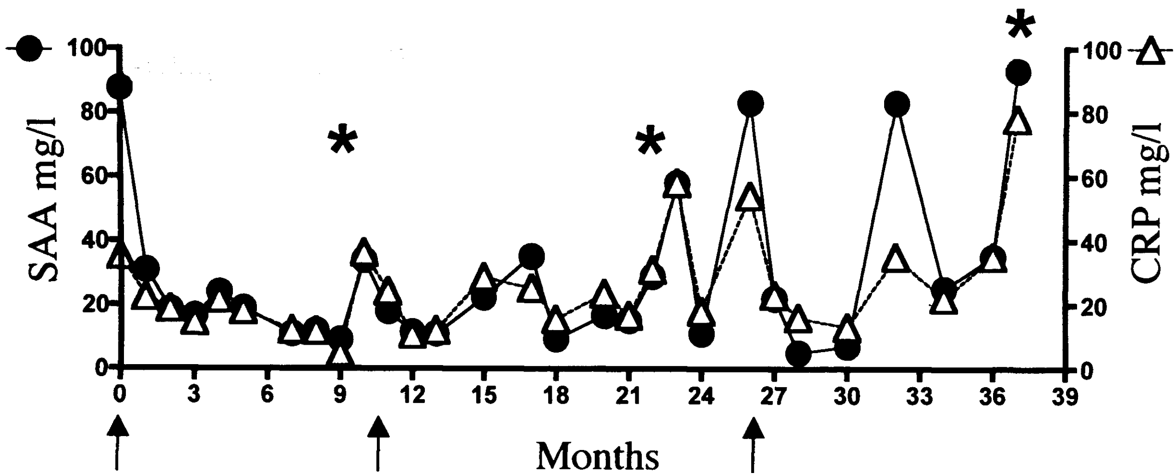
In this open-label trial, patients treated with protocols that included full dose rituximab with cyclophosphamide with or without prednisolone, or half dose rituximab with cyclophosphamide and oral prednisolone, showed a significant improvement in disease manifestations as measured by ACR criteria. On the other hand, patients treated with half dose rituximab with oral prednisolone but without cyclophosphamide, or with approximately one third of full dose rituximab with cyclophosphamide and oral prednisolone, did not respond significantly to treatment.

When a single dose of 300 mg/m^2 of rituximab was used without cyclophosphamide (patient 2a) the CD19 positive count did not decrease below the limit of detection. Median time to repopulation was shorter in cohort II than in the other cohorts but the differences were not statistically significant ($P > 0.05$, Mann-Whitney rank sum test).

The patients treated with cyclophosphamide and prolonged course of steroids in the initial open label trial tended to start responding to treatment fairly early (within 1 month) but patients treated with subsequent protocols took generally longer to respond to therapy, usually 2 to 3 months. Some patients continued to improved beyond this time point.

Changes in total serum immunoglobulin levels in the different cohorts are detailed in table 2.6. IgM decreased significantly less in patients in cohort II when compared to





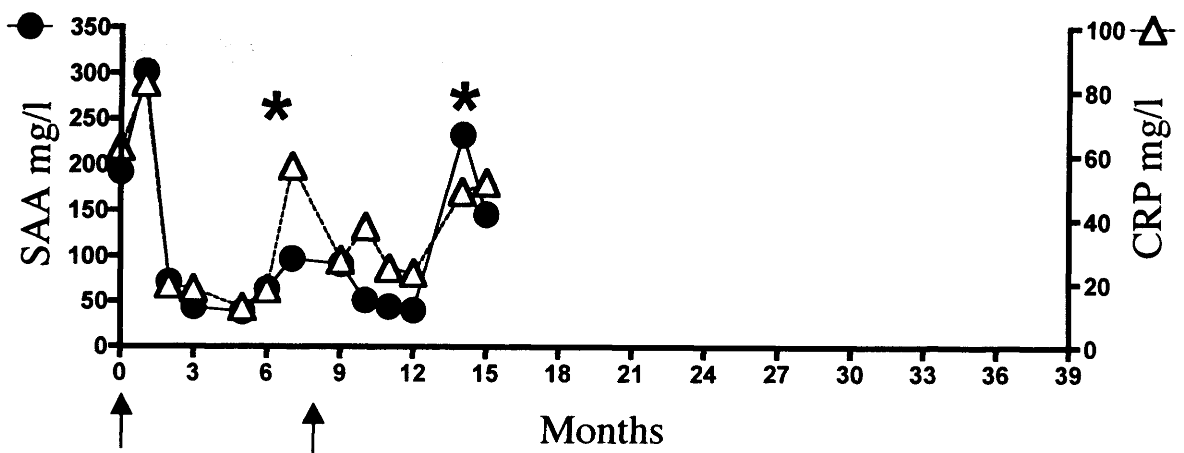


Figure 2.3 Serial measurements of serum CRP (normal range < 10 mg/l) and SAA protein (normal range < 10 mg/l) in A) patient 11, B) patient 26 and C) patient 31. Solid bar indicates period of B-lymphocyte depletion, arrow indicates course of treatment and asterisk indicates clinical relapse (details on tables 2.3 and 2.4).

patients in cohorts I and IV ($P = 0.02$ and $P = 0.03$, respectively, Mann-Whitney rank sum test). There were no other significant differences.

Eighteen of the patients were genotyped for the FcγRIIIa-158V/F polymorphism. No apparent relationship was observed in this group of patients between the FcγRIIIa genotype and the degree of improvement or the duration of clinical response following BLDT (table 2.7). Also, no apparent relationship was apparent between the FcγRIIIa genotype and the length of the period of B-lymphocyte depletion in the peripheral blood (table 2.8).

2.3.2 Patients 23 to 40 clinical response to BLDT

Following the open-label trial a further 18 new patients were treated (tables 2.2 and 2.4). Fourteen patients responded to treatment with major improvement in signs and symptoms of active disease. This improvement was reflected in the decrease in CRP serum levels (figure 2.4). Four new patients did not respond to treatment. Two of these 4 patients were seronegative (patients 28 and 29) and one had end-stage disease (patient 25).

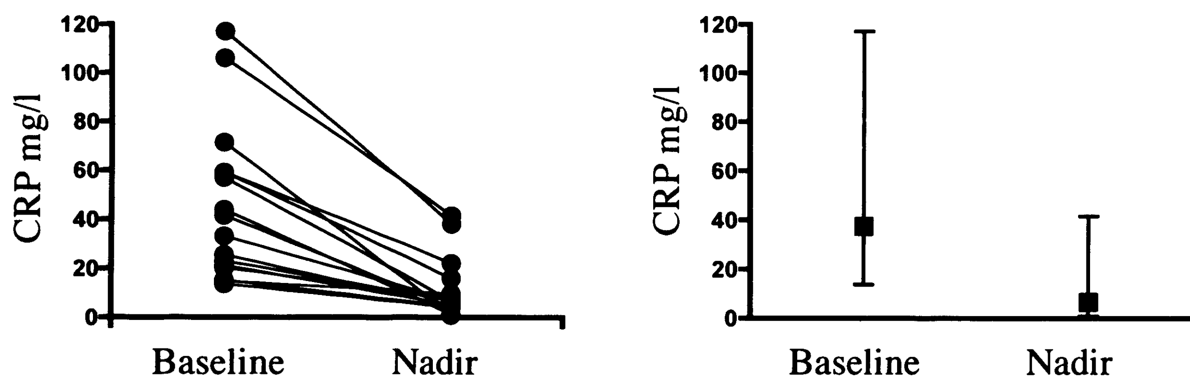


Figure 2.4 CRP serum levels at baseline and nadir following BLDT in patients 18 to 40 who responded to treatment (n = 14; details on table 2.4). A) individual Patients. B) median (square) and range (lines).

Table 2.7 FcγRIIIa-158V/F polymorphism and length of period of B-lymphocyte depletion in the peripheral blood (in months) (n=18, total of 20 treatments).

Cohort	Homozygous 158V	Heterozygous	Homozygous 158F
I	9	7	21
			7
			11
III	10	10	7
		8	9
		7	6
		7	
		7	
IV	6	10	12
		6	
V			9
			7
			9
			5

Table 2.8 FcγRIIIa-158V/F polymorphism and maximum clinical response (ACR grade of improvement: 0 (<20), 20, 50 or 70) following BLDT (n=18, total of 20 treatments).

Cohort	Homozygous 158V	Heterozygous	Homozygous 158F
I	70	50	70
			70
			70
III	70	70	70
		70	70
		70	70
		50	
		50	
IV	70	50	70
		50	
V			70
			20
			0
			0

Two more patients in this group had secondary amyloidosis with renal involvement and chronic renal failure (patients 26 and 31). Patient 26 also had ankylosing spondylitis diagnosed 40 years earlier, which could confound both CRP and SAA response to BLDT. Both patients responded clinically to treatment but relapsed at the start of B-lymphocyte repopulation. Patient 26 received a total of 3 courses of treatment (the second course included cyclophosphamide) and patient 31 a total of 2 (table 2.4). Serial measurements of SAA and CRP for both these patients are presented in figure 2.3 B and C.

2.3.3 Relapse following BLDT in patients 1 to 40

All patients who responded to BLDT eventually experienced clinical relapse. Patients relapsed at the time or after B-lymphocyte repopulation. No clinical relapse was observed during the period of B-lymphocyte depletion. Only on patient 6, following her second course of treatment, worsening of symptoms was noted at 6 months with B lymphocytes still undetectable in the peripheral blood. This patient was relatively refractory to treatment and only achieved transient improvement fulfilling ACR20 criteria at the 6 months time point.

The time of B-lymphocyte repopulation and of relapse were available in 31 of the 34 patients who responded to treatment with effective protocols of BLDT. After their first course of effective treatment, half of the patients (15) relapsed at the time or shortly after B-lymphocyte repopulation, and the other half (16) relapsed at varying times up to 32 months after B-lymphocyte return (figure 2.5A).

2.3.4 Re-treatment

Twenty-four patients were re-treated with BLDT following clinical relapse. A total of 37 re-treatments took place and patients received up to 5 cycles of treatment. All patients who responded to rituximab on their first treatment responded to re-treatment with no secondary treatment failures observed. All of these patients achieved levels of depletion and clinical improvement following repeat cycles comparable with the first cycle. Percentage drops in CRP following repeated courses of treatments were similar in

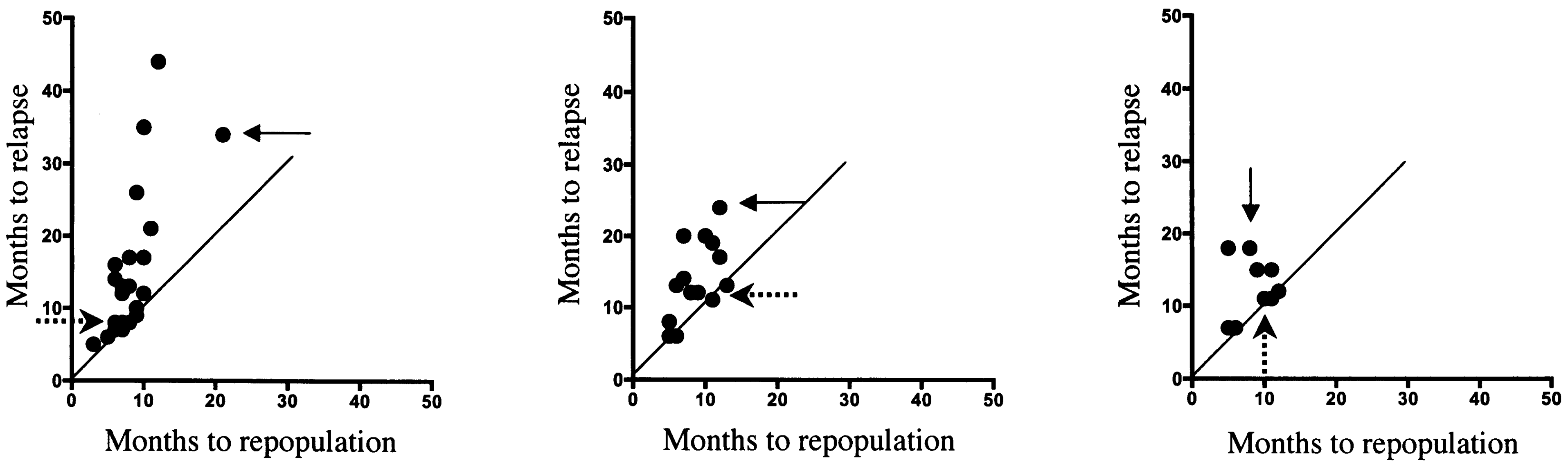


Figure 2.5 Relation between B-lymphocyte repopulation and clinical relapse following BLDT. A) first course of treatment. B) second course of treatment. C, third course of treatment. The two types of arrows indicate patients 1 (full line) and 4 (dashed line) on repeated courses of treatment.

the group of patients who responded to treatment (figure 2.6). The median period of benefit was 14 months (range 6 to 44). Following re-treatment, patients tended to show the same pattern of relapse (either at B-lymphocyte repopulation or at a variable time afterwards) (tables 2.3 and 2.4 and figure 2.5).

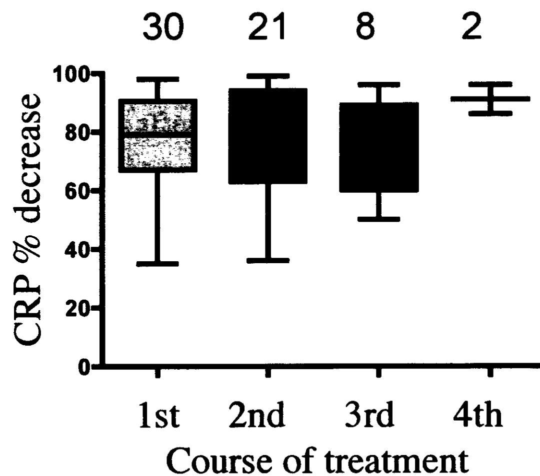


Figure 2.6 Maximal percentage decrease in CRP levels following repeated courses of BLDT. Line, box and whiskers indicate mean, standard deviation and range, respectively. Numbers indicate patients included in each treatment sample.

Only one patient (patient 3) has been withdrawn from repeat BLDT at the start of her third course of treatment because of secondary intolerance, with an urticarial infusion reaction. Only one patient (patient 8) failed to deplete as expected following her first re-treatment. This patient was first treated with only 600 mg/m^2 of rituximab without cyclophosphamide (table 2.3, cohort II). She repopulated at 4 months and did not respond to treatment. Following her second treatment with 1200 mg/m^2 of rituximab monotherapy (table 2.4) she did not deplete well and started repopulating at 2 months and again did not respond to treatment. Interestingly, she was seronegative before her first treatment but was anti-CCP positive. Before her second treatment she was found to be seropositive.

2.3.5 Side effects

2.3.5.1 Open label trial – 22 patients

No major adverse events attributable to the treatment occurred. Only two mild cases of infusion related toxicity were seen: patient 21 had fever (38.5°C) after the first rituximab infusion that responded to paracetamol; patient 19 had mild tachycardia (pulse rate 108/min) during the first rituximab infusion that resolved when the infusion rate was decreased. Patient 4 had phlebitis secondary to cyclophosphamide extravasation. Seven patients complained of mild to moderate nausea and occasional vomiting lasting one to two days after cyclophosphamide infusion.

During the open-label trial there were four cases of lower respiratory episodes with cough, fever and sometimes sputum production that were treated as infections (patients 1, 18 and 19) and one case of acute bacterial maxillary sinusitis (patient 13) that responded to antibiotic treatment. One patient in the earlier trial had developed fever, cough and chest pain suggestive of serositis following the 3rd rituximab infusion (patient 5). Patient 3 had three episodes of mild transient thrombocytopaenia (platelet counts over 100×10^9/l), with associated minor bruising only on the first episode. Patient 9 had a mild asymptomatic thrombocytopaenia (platelet count 123×10^9/l). Patient 2 had self-limited acne rosacea at two months. Patient 7 had a self-limited erythematosus rash over her nose at 6 months.

2.3.5.2 Patients 23 to 40 and further re-treatments

Treatment with B-lymphocyte depletion protocols based on rituximab was generally well tolerated. Only a small number of mild infusion reactions were observed. One patient developed an urticarial skin reaction to the infusion on her third course of therapy and had to discontinue treatment (patient 3).

Eight patients developed lower respiratory episodes with cough, fever and sometimes sputum production that were treated as infections. In 4 cases these episodes followed the second rituximab infusion. All symptoms resolved with antibiotics but one patient (patient 25) had to be admitted to hospital and spent a period in intensive care (concomitant diagnosis of pulmonary fibrosis). One patient had an episode of herpes

zoster. No other serious infections and no other evidence of opportunistic infections were observed.

Three new cases of carcinoma of the breast were diagnosed following early usage of rituximab with cyclophosphamide (patients 1, 13 and 17; in patient 17 the disease almost certainly predated BLDT). A further patient who also received combined protocol with cyclophosphamide developed disseminated carcinoma of the ovary (patient 29) and a patient treated with rituximab alone was diagnosed with renal cell carcinoma (patient 34). None of the patients with a previous history of malignancy experienced recurrence of malignant disease (patient 22, tubulo-villous adenoma of rectum; patient 36, MALT gastric lymphoma).

Table 2.9 Maximum decrease in total immunoglobulin levels, IgA, IgG and IgM, following repeated courses of BLDT.

		Treatment 1 (n=37)	**Treatment 2** (n=20)	**Treatment 3** (n=9)	**Treatment 4** (n=2)*
IgA decrease	g/l				
	median	0.7	0.5	0.5	-
	(range)	(0.0-3.0)	(0.0-2.3)	(0.0-1.9)	0.0;1.4
	%				
	mean	25	21	19	-
	(range)	(0-75)	(0-58)	(3-42)	0; 38
IgG decrease	g/l				
	median	3.2	1.6	2.2	-
	(range)	(0.7-8.2)	(0.3-4.5)	(0.9-5.5)	0.0; 4.9
	%				
	mean	25	15	22	-
	(range)	(6-53)	(3-37)	(8-41)	0; 37
IgM decrease	g/l				
	median	0.6	0.3	0.4	-
	(range)	(0.1-1.5)	(0.1-0.8)	(0.2-0.8)	0.2; 0.5
	%				
	mean	43	35	34	-
	(range)	(14-71)	(9-67)	(15-50)	56; 100

Abbreviations: n, number of patients.
* Values represent individual patients (n=2).

A skin lesion diagnosed as cutaneous lupus on the biopsy was observed in patient 23 coinciding with B-lymphocyte repopulation following her first treatment. This lesion was transient and did not require specific treatment.

Although falls in total immunoglobulin levels following a single cycle of BLDT were modest, repeated cycles led occasionally to hypogammaglobulinemia. One patient developed low IgA after her third treatment. IgG levels fell to as low as 3.5gm/L in two cases and IgM levels fell to undetectable levels in 3 cases. Percentage drops in Ig serum levels were similar following repeated courses of treatment. Hypogammaglobulinaemia was observed (table 2.9 and figure 2.7) either in patients who relapsed at the time of B-lymphocyte repopulation and were treated shortly after or in patients who relapsed only some time after B-lymphocyte repopulation but whose immunoglobulin serum levels did not show a definite increase following B-lymphocyte return. It was more frequent in patients who had lower baseline Ig levels.

2.4 Summary

Extension of the early open-label trial in 5 patients to a total of 22 patients provided further evidence that BLDT based on rituximab could lead to major improvement in disease manifestations in patients with active refractory RA. Treatment was generally well tolerated. The 2 patients seronegative at study entry did not respond to treatment. Results suggested a dose-response to rituximab and additive or synergistic effect of cyclophosphamide with patients treated with half-dose rituximab without cyclophosphamide or a third of the rituximab dose with cyclophosphamide not responding to treatment while patients treated with half-dose or full dose rituximab with cyclophosphamide responded to treatment. There was no apparent major additive or synergistic effect from oral corticosteroids as patients treated with full dose rituximab and cyclophosphamide without corticosteroids responded to treatment.

Treatment of a further 18 patients extended the experience with B lymphocyte depletion. On the whole only 8 patients did not respond to treatment. These 8 patients included the 4 seronegative patients and another patient with end-stage erosive disease.

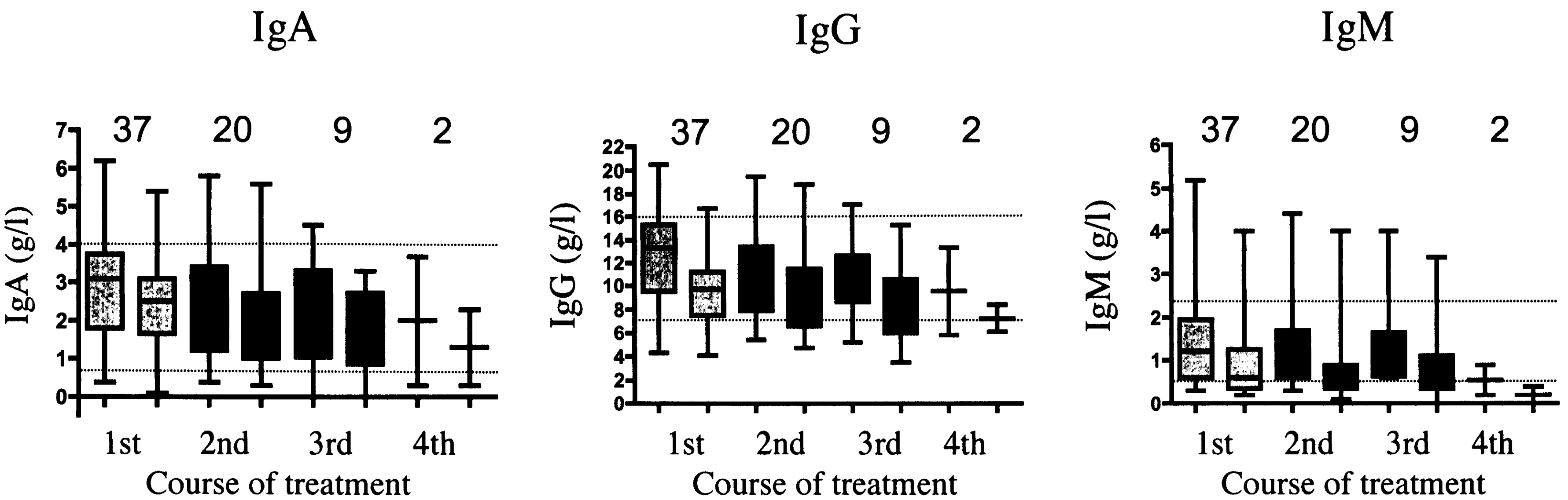


Figure 2.7 Serum total immunoglobulin levels, IgA, IgG and IgM, at baseline and nadir following repeated courses of BLDT. Line, box and whiskers indicate mean, standard deviation and range, respectively. Numbers indicate patients included in each treatment sample. Dashed lines represent upper and lower limit of normal (IgA 0.7-4.0 g/l; IgG 7.0-16.0 g/l; IgM 0.4-2.3 g/l).

All patients eventually relapsed after having responded to BLDT. Patients relapsed either at the time of B-lymphocyte repopulation of the peripheral blood or at a varying time after repopulation. Re-treatment was effective. After re-treatment, patients tended to show the same pattern of relapse.

B-lymphocyte depletion was usually well tolerated.

CHAPTER 3 RESULTS: SEROLOGICAL CHANGES FOLLOWING B-LYMPHOCYTE DEPLETION THERAPY

3.1 Study design

The results of the extended open-label clinical trial presented before suggested that BLDT could be an effective therapy in RA. The observations that patients who responded to treatment did not relapse before B-lymphocyte repopulation but could either relapse at that time point or at a variable time thereafter contributed to focusing of the research on which mechanisms underlined clinical relapse in these patients. The data suggested that the presence of B lymphocytes was necessary for relapse to occur but not sufficient, and that relapse could therefore depend on the return or the re-expansion of particular pathogenic B-lymphocyte populations.

A series of serological studies were designed to study the effects of BLDT on autoantibody profiles and on the levels of soluble factors associated with B-lymphocyte development and maturation, to investigate possible associations with clinical relapse and with patterns of relapse. Patients were selected for the different serological studies depending on the time of initiation of the study and availability of individual sequential serum samples at the important time points.

1) In the 22 patients with RA treated with BLDT as part of the extended clinical open-label trial, mean serum immunoglobulin levels decreased significantly following treatment but remained within the normal range. RAPA titres were noticed to decrease consistently following treatment but only rarely became negative. An increase in RAPA titres frequently preceded or accompanied clinical relapse.

Serial serum samples of the 22 patients were tested: i) to investigate the changes in serum levels of disease-associated autoantibodies, IgA-, IgG-, and IgM-RhF and anti-CCP antibodies, following BLDT; ii) to study the relationship between the time course of these changes and B-lymphocyte depletion and B-lymphocyte repopulation of the peripheral blood; iii) to find whether any changes observed correlated with clinical response particularly time of clinical relapse; iv) to investigate whether there were differences between the different disease-associated autoantibodies; v) to compare the changes in disease-associated autoantibodies to changes in total serum immunoglobulin levels and anti-microbial antibodies.

To study anti-microbial responses, specific IgG serum antibodies to tetanus toxoid (anti-TT) and to pneumococcal polysaccharide capsular antigens (anti-PCP) were chosen as they are part of the evaluation of B-lymphocyte immune function in primary and acquired immunodeficiency disorders (Ballow, 2002). They allow study of specific antibody responses to T-dependent and T-independent antigens, respectively.

This study was later extended to include a total of up to 35 patients and particularly to include changes following re-treatment.

2) In the clinical studies, the available peripheral blood B-lymphocyte counts did not distinguish between patients who responded to treatment and those who did not as almost all patients depleted to below the limit of detection of the Central Pathology Laboratory. There was also no apparent correlation between total numbers of circulating B-lymphocytes at repopulation and the different times of relapse.

Membrane CD23 antigen is expressed by naïve B lymphocytes and cleaved from the cell surface as B lymphocytes participate in a germinal centre reaction (Delespesse et al., 1991). Soluble CD23 levels were measured in serial samples, as it was possible that changes in sCD23 could reflect the degree of depletion or repopulation of the naïve B-lymphocyte subpopulation and also the degree of naïve B-lymphocyte activation (Bansal et al., 1994, Yawetz et al., 1995).

BLyS is one of the key factors regulating peripheral B-lymphocyte development, survival and expansion (Moore et al., 1999). Changes in BLyS serum levels following BLDT could possibly reflect the degree of B-lymphocyte depletion or repopulation and allow differentiation of patients. Changes in BLyS levels could also possibly correlate with expansion of autoreactive B-lymphocyte clones and relapse, as it has been suggested that autoreactive B-lymphocyte clones may be more dependant than non-autoreactive ones on survival signals provided by BLyS (Lesley et al., 2004).

3.2 Patients and methods

3.2.1 Patients

a) Serial serum samples from the 22 patients included in the extended clinical trial were studied except for patient 17 who was diagnosed with breast cancer and treated with chemotherapy before B-lymphocyte repopulation occurred. Patient 23 who had been already treated when the study was performed was also included. Changes in serum levels of IgA-, IgG- and IgM-RhF and of anti-CCP, anti-TT and anti-PCP antibodies following the first course of BLDT were investigated.

Circulating B-lymphocyte counts had been measured by flow cytometry (CD19 positive cells), serum concentration of CRP by nephelometry and serum total immunoglobulin levels by immunoturbidometry at UCLH Central Pathology Laboratory as part of the laboratorial assessment of patients for the clinical studies (normal ranges for CD19 count 0.03-0.40 x 10^9/l, CRP < 5 mg/dl, IgA 0.7-4.0 g/l, IgG 7.0–16.0 g/l and IgM 0.4-2.3 g/l).

b) Serum total immunoglobulin levels were also measured in sequential samples of a further 12 patients and IgA-, IgG- and IgM-RhF and anti-TT and anti-PCP antibodies in a further 7 patients. Results from patients 1 to 40 were then pooled to provide more extensive data and, in particular, comparison between different doses of rituximab and between rituximab monotherapy and combination protocols. Sequential changes in serological parameters were also studied following repeated courses of treatment in several patients.

c) Serum levels of soluble CD23 antigen were measured in sequential samples from 17 patients following their first course of treatment (patients 1-3, 5-8, 11, 13-16, 18, 19, 21 and 22).

d) BLyS serum levels were measured in sequential samples in 15 patients (patients 2, 4-6, 10, 11, 13-16, 19, 30, 32, 33 and 36). Seven of these patients were retreated at least once and re-treatment samples were also studied.

Tables 2.1 and 2.2 include patients' clinical characteristics at the time of entry to the study. Tables 2.3 and 2.4 include treatment protocols, time to repopulation and to clinical relapse for the different patients.

The UCLH Ethics Committee approved the study. All patients gave informed consent. Serum samples were kept undiluted at -80°C until tested.

3.2.2 Measurement of IgA, IgG and IgM rheumatoid factors by ELISA

IgA-, IgG- and IgM-RFs were measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available RhF-isotype detection kit (catalog: EL-RF/3; TheraTest Laboratories, Chicago, IL, USA). This test is based on the binding of RhF to rabbit IgG. The TheraTest ELISA utilizes horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG F(ab')2 to detect IgG-RhF, and prior to testing for IgG-RhF, samples are digested with pepsin to avoid interference from IgM-RhF and IgA-RhF. Completeness of pepsin digestion is checked by confirming the absence of binding of anti-IgM-HRP conjugate to pepsin-digested samples and controls. Results are expressed in IU/ml. The cut-off values defined by the manufacturer for normal control sera are ≤ 35 IU/ml, ≤ 20 IU/ml and ≤ 25 IU/ml for IgA-, IgG- and IgM-RhF, respectively. The intra- and inter-assay coefficients of variation for the IgA-RhF, IgG-RhF and IgM-RhF ELISA reported by the manufacturer were less than 10%. The kit was used according to the manufacturer's instructions. All the sequential samples from each patient were run simultaneously. Maria Leandro and Dr. Geraldine Cambridge performed the assays.

3.2.3 Measurement of IgG anti-CCP antibodies by ELISA

IgG anti-CCP antibodies were measured by ELISA (catalog: Immunoscan RA; Euro-Diagnostica, Arnhem, The Netherlands). This is a first generation anti-CCP test. Results are expressed as unit equivalents to the standard serum, as determined by reading on a calibration curve. Positive values are ≥ 50 U/ml. The intra- and inter-assay coefficients of variation for the anti-CCP antibodies ELISA reported by the manufacturer were less than 14 and 12%, respectively. The kit was used according to the manufacturer's

instructions. All the sequential samples from each patient were run simultaneously. Maria Leandro and Dr. Geraldine Cambridge performed the assays.

3.2.4 Measurement of IgG anti-tetanus toxoid and IgG anti-PCP antibodies

Specific IgG antibodies to TT and to PCP (combination of 23 common serotypes), were measured by ELISA (Binding Site Limited, Birmingham, UK). Results are expressed as unit (anti-TT) or mg (anti-PCP) equivalents to the standard serum, as determined by reading on a calibration curve. For anti-TT antibodies, levels ≥ 0.1 IU/ml were considered to be optimally protective (Galazka, 1993). Anti-PCP levels ≥ 50 mg/dl were considered to be protective (Dr. David Webster, Department of Clinical Immunology, Royal Free Hospital, London, UK, oral information). The intra- and inter-assay coefficients of variation for the anti-TT ELISA reported by the manufacturer were less than 5 and 9%, respectively. The intra- and inter-assay coefficients of variation for the anti-PCP ELISA reported by the manufacturer were less than 6 and 8%, respectively. The kits were used according to the manufacturer's instructions. All the sequential samples from each patient were run simultaneously. In the first 23 patients, anti-TT and anti-PCP antibodies were measured in serum samples from before treatment, 3 to 4 months after the first rituximab infusion and at or shortly after B-lymphocyte repopulation to the peripheral blood. Samples tested were from the patients' first course of BLDT. These measurements were performed in the Clinical Immunology Laboratory at the Royal Free Hospital in London, UK. Subsequently, anti-TT and anti-PCP antibodies were measured in sequential samples from 18 patients. Maria Leandro and Dr. Geraldine Cambridge performed these last assays.

3.2.5 Measurement of soluble CD23

Soluble CD23 serum levels were measured by ELISA (catalog: BMS227/2CE, Bender MedSystems, Vienna, Austria). Results are expressed as unit equivalents to the standard serum, as determined by reading on a calibration curve. The limit of detection of the assay is 3.3 U/ml. The intra- and inter-assay coefficients of variation for the sCD23 ELISA reported by the manufacturer were 5.9% and 12.5%, respectively. The kit was used according to the manufacturer's instructions. All the sequential samples from each

patient were run simultaneously. Maria Leandro and Dr. Geraldine Cambridge performed these assays.

3.2.6 Measurement of BlyS serum levels

Serum levels of BLyS were measured by ELISA (Cheema GS A&R 2001) (Propriety of Human Genome Sciences, Rockville, MD, USA). The assay uses Fab fragments of the capture antibody rather than the whole antibody to reduce assay interference from RhF. Samples with BLyS concentrations below the lower limit of detection were assigned a value of 0 ng/ml. Staff at Human Genome Sciences performed these assays.

3.2.7 Statistical analysis

For descriptive statistics, mean and standard deviation (or mean and range) were used for data that followed a normal distribution and median and range (or median and interquartile range) for data that did not. Comparisons between different groups of data were performed using the paired or unpaired t test for matched or unmatched groups, respectively, if the data followed a normal distribution. When the data were not normally distributed or the equal variance test was not satisfied, non-parametric testing was performed by the Wilcoxon signed rank test for paired data and by the Mann-Whitney rank sum test for non-paired data. Correlations were determined by Pearson product moment correlation for normally distributed data or by Spearman rank order correlation for data that did not follow a normal distribution. P values equal to or less than 0.05 were considered statistically significant. All analysis were performed using GraphPad PRISM software (GraphPad Software, Inc., San Diego, CA, USA).

3.3 Results

3.3.1 Effects of BLDT on disease-associated autoantibodies, total immunoglobulins and anti-microbial antibodies and relationship with clinical response

3.3.1.1 Pre-treatment parameters and analysis of results

Twenty of the 22 patients included in this study were seropositive (positive latex test and RAPA); all of these seropositive patients were found by ELISA to have IgM-RhF, 18 (82%) had IgA-RhF and 13 (59%) had IgG-RhF (table 3.1). The two patients who were seronegative by latex test and RAPA were also negative by ELISA (patients 8 and 20). Thirteen patients (59%) had anti-CCP antibodies, including one of the seronegative patients (patient 8).

Since all patients treated with the different protocols achieved B-lymphocyte depletion in the peripheral blood, changes in CRP, total serum immunoglobulin, autoantibodies and anti-microbial antibody levels were combined and analysed together. Only changes following the first course of treatment were included in this initial study. For comparison of serologic responses to treatment, patients were classified as responders (those achieving and maintaining an ACR level of improvement of $\geq 20\%$ at 6 months) (15 of 22 patients) or non-responders (ACR level of improvement $< 20\%$ at 6 months) (7 of 22 patients).

3.3.1.2 Comparison of the effect of BLDT on the levels of total serum Igs and autoantibodies

Following treatment, B lymphocytes were undetectable ($< 0.005 \times 10^9$/l) in the peripheral blood of patients for a mean of 8.4 months (range 3.5 to 20.8) (table 3.1). Total serum immunoglobulin levels decreased moderately but significantly ($P < 0.0001$ for the 3 classes when baseline levels were compared with levels at nadir, Wilcoxon signed rank test).

In order to compare the effect of BLDT on the levels of individual autoantibodies and their respective classes of serum immunoglobulins, the data were normalised by expressing the lowest value attained for each parameter as a percentage of the pre-treatment value. Differences between the percentage drop in the 3 classes of RhF measured became apparent (figure 3.1). The percentage drop in IgG-RhF levels was greatest and was also significantly greater than that in IgA-RhF levels ($P = 0.007$, unpaired t test). No other significant differences were found between percentage drop in the different autoantibodies, including any of the RhF classes and anti-CCP antibodies.

Table 3.1 Relationship between B-lymphocyte return, levels of circulating autoantibodies and clinical relapse in 22 patients following BLDT.

Patient	**Autoantibodies present at baseline**	**ACR response at 6 months**	**Time to B-lymphocyte return** (months)	**Time to clinical relapse** (months)	**Time to autoantibody rise** (months) (responding antibody)
1	M, A, CCP	70	21	34	30 (M, A, CCP)
2	M, A, CCP	70	7	7	6 (M, A, CCP)
3	M, G	70	9	26	26 (M)
4	M, G, A, CCP	50	7	8	6 (A, CCP, M)
5	M, G, A, CCP	50	11	21	21 (M, G, A)
7	M, A	20	8	7	7 (M)
9	M, G, A	70	7	7	6 (M, A)
10	M, G, A, CCP	70	9	9	8 (A, M, G)
11	M, G, A	70	10	37	30 (M, A)
12	M, CCP	50	7	12	10 (M, CCP)
13	M, A, CCP	70	12	44	28 (M, A)
14	M, G, A, CCP	50	10	12	12 (M)
16	M, G, A, CCP	70	6	7	6 (M, G, A,CCP)
18	M, G, A	20	10	17	-
19	M, G, A	50	6	14	13 (M, A)
6	M, A, CCP	<20	5	NA	-
8	CCP	<20	4	NA	4 (CCP)
15	M, G, A, CCP	<20	9	NA	15
20	-	<20	7	NA	-
21	M, G, A	<20	9	NA	-
22	M, G, A	<20	5	NA	5 (A, M)
23	M, A, CCP	<20	7	NA	-

Abbreviations: M, IgM-RhF; G, IgG-RhF; A, IgA-RhF; CCP, anti-CCP antibodies; NA, not applicable.

When decreases in the different autoantibodies and in their respective immunoglobulin classes were compared, the mean percentage decrease in IgA-RhF and IgG-RhF, but not in IgM-RhF, was significantly greater than that found in the corresponding total immunoglobulin levels ($P < 0.001$, $P < 0.001$ and $P = 0.68$, respectively, unpaired t test) (figure 3.1). Anti-CCP antibodies (IgG) also decreased significantly more than total IgG ($P = 0.001$, unpaired t test).

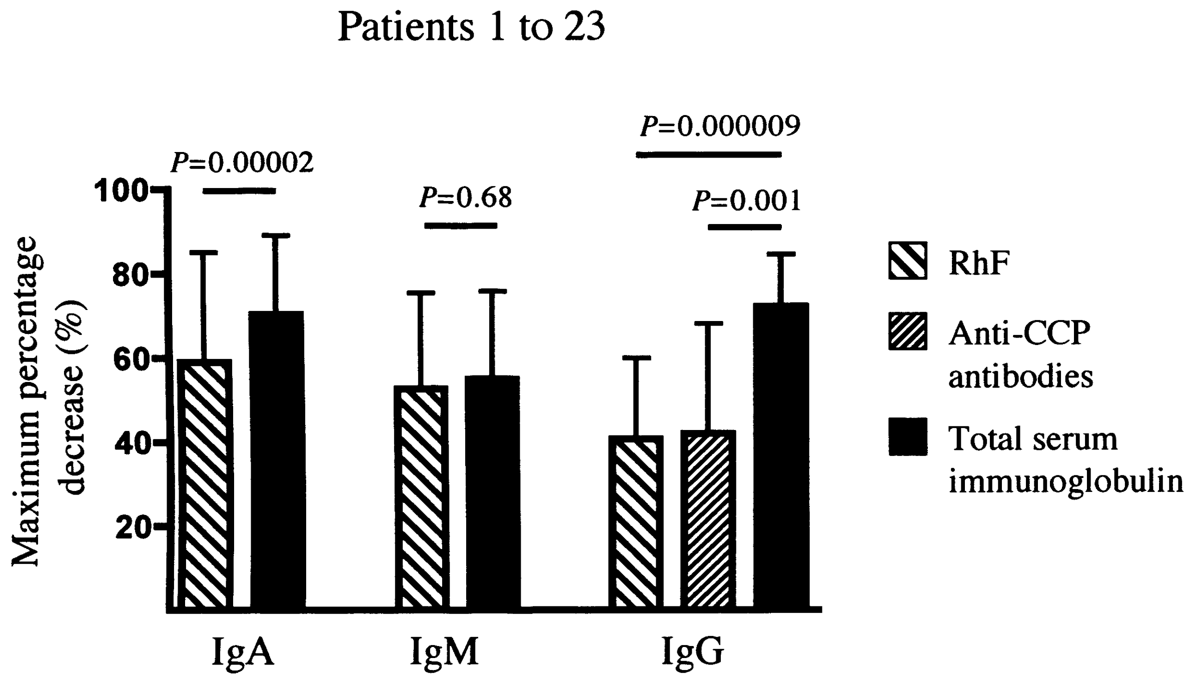


Figure 3.1 Comparison between maximum percentage decrease from pre-treatment levels in IgA-, IgM- and IgG-RhF, anti-CCP antibodies and total serum immunoglobulin of the respective classes (IgA, IgM and IgG) (unpaired *t* test).

The kinetics of changes observed in autoantibody and CRP serum levels following BLDT were studied. Figure 3.2 compares the T50 (median time taken for serum levels to fall by 50% of their maximum fall) and T80 (the equivalent for 80%) for each autoantibody and for CRP. The T50 for IgG-RhF, anti-CCP and CRP was within 5 weeks of treatment. The T80 for the fall in all autoantibody levels was longer than that for the CRP levels, although the T80 for IgG-RhF and anti-CCP was achieved sooner than that for IgA-RhF and IgM-RhF.

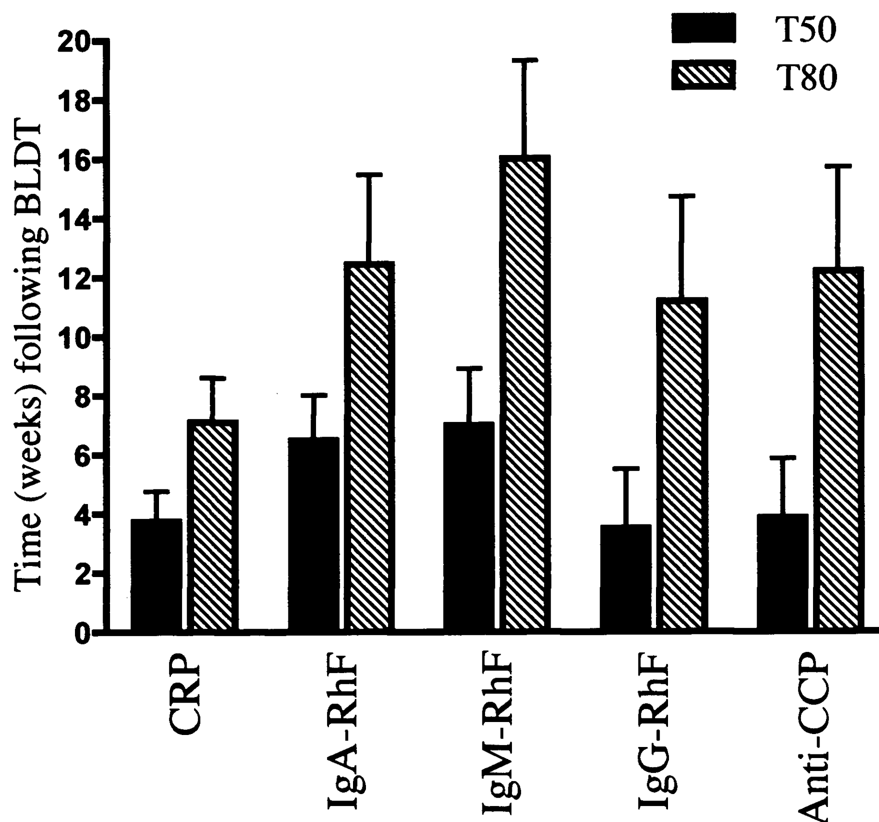


Figure 3.2 Comparison of kinetics of change in CRP, IgA-, IgG- and IgM-RhF and anti-CCP serum levels following BLDT. T50, median time taken for serum levels to fall by 50% of their maximum fall. T80, equivalent to T50 for 80% fall. Bars indicate median levels, lines indicate interquartile range.

3.3.1.3 Comparison between responder and non-responder patient groups

When the pre-treatment values of the serologic parameters in responders and non-responders were compared, there were no significant differences between the groups (table 3.2). Similarly, there were no significant differences between the nadir levels in responders and non-responders, except, as expected, when CRP levels were compared ($P = 0.01$, Mann-Whitney rank sum test). There was a highly significant difference between CRP levels at baseline and at nadir in the responding patients, but less so in the non-responding patients ($P < 0.001$ and $P = 0.02$, respectively, Mann-Whitney rank sum test). In the responder group, the levels of all classes of RhF and of anti-CCP antibodies

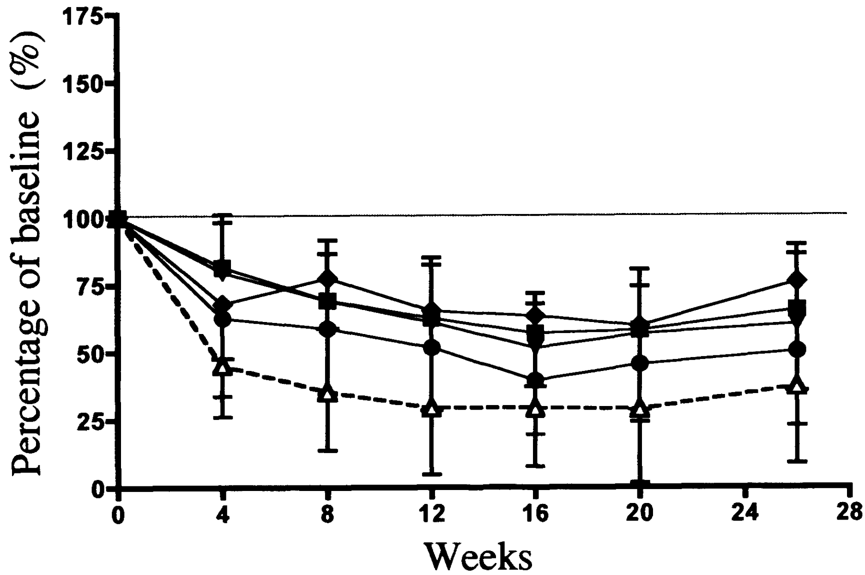
decreased significantly relative to their pre-treatment values (table 3.2). Identical analysis in the non-responder group revealed non-significant changes (table 3.2).

Table 3.2 Serologic parameters in 22 patients before treatment and at nadir following BLDT.

Parameter **Response to therapy** (number of patients per group)	**Pre-treatment Value** (median, range)	**Lowest level attained after treatment** (median, range)	***P***
IgM-RhF			
Responders (15)	126 (62-2200)	50 (11-560)	0.00006
Non-responders (5)	122 (82-1900)	90 (39-900)	0.06
IgG-RhF			
Responders (10)	77 (25-297)	16 (9-117)	0.002
Non-responders (3)	50 (20-147)	25 (16-83)	0.13
IgA-RhF			
Responders (13)	147 (34-2573)	78 (28-1120)	0.0002
Non-responders (5)	158 (74-573)	118 (52-242)	0.06
Anti-CCP			
Responders (8)	950 (122-5200)	236 (50-4240)	0.008
Non-responders (4)	2282 (105-2665)	1350 (50-1800)	0.25
CRP			
Responders (15)	38 (4-97)	2 (1-18)	0.00006
Non-responders (7)	40 (9-142)	14 (4-38)	0.02

For comparison of the kinetics of the fall in serologic parameters following BLDT, post-treatment values at each time point were expressed as a percentage of pre-treatment values. The results are shown in responding (figure 3.3A) and non-responding (figure 3.3B) patients. In the responder group, a gradual decline in CRP and autoantibody levels was observed, with an apparent plateau reached at approximately 16 weeks post-treatment (figure 3.3A). In the non-responder group (figure 3.3B), the course of CRP and autoantibody decline was more erratic, although the levels of the different RhF did reach an apparent plateau, also at approximately 16 weeks post-

A. Responders



B. Non-responders

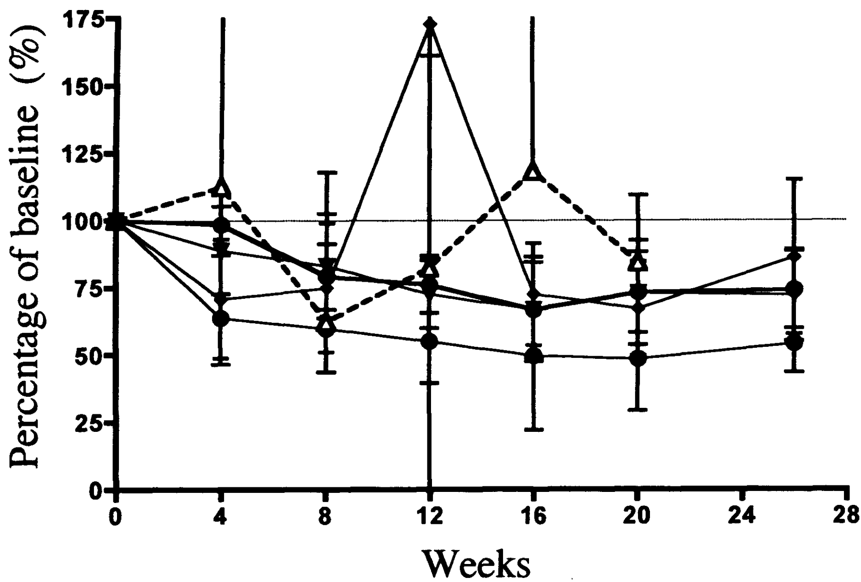


Figure 3.3 Mean (± SD) percentage change from pre-treatment levels in serum CRP, IgA-, IgG and IgM-Rh and anti-CCP antibodies following BLDT in A) patients who achieved at least a sustained ACR20 at 6 months (n = 15) and in B) those who did not (n= 7).

treatment. Comparison of these percentages of pre-treatment values for any of the autoantibodies and CRP at the specific post-treatment time points revealed no significant differences between the 2 groups of patients, except for a higher percentage drop in IgA-RhF at 4 weeks and in CRP at 26 weeks post-treatment in the responder group when compared to the non-responder group ($P = 0.03$ and $P = 0.04$, respectively, unpaired t test).

3.3.1.4 Effect of BLDT on anti-microbial antibody levels

Anti-TT and anti-PCP antibodies were measured at 3 time points: baseline, 3 to 4 months after BLDT and at, or shortly after, the time of B-lymphocyte repopulation. Before treatment, 7 patients had sub-protective levels of anti-TT antibodies (< 0.1 IU/ml) and 11 patients had sub-protective levels of anti-PCP antibodies (< 50 mg/L).

At 3 months, anti-TT antibody levels had decreased significantly, with no further decrease evident at the time of B-lymphocyte repopulation ($P = 0.02$ and $P = 0.31$, respectively, Wilcoxon signed rank test). However, when anti-TT antibody levels at baseline were compared with levels at B-lymphocyte repopulation the difference was not statistically significant ($P = 0.05$, Wilcoxon signed rank test) suggesting some recovery of anti-TT antibodies. Comparison between percentage decreases in anti-TT antibodies with that in total IgG at the three time points showed no significant differences (figure 3.4). In those subjects with baseline antibody levels within the protective range, levels remained protective at the second and third time points.

The levels of anti-PCP antibodies did not change significantly with treatment ($P = 0.07$ and $P = 0.91$ when baseline values were compared with levels at 3 to 4 months and at B-lymphocyte repopulation, respectively, Wilcoxon signed rank test) (figure 3.4). However, in 3 patients with baseline levels within the lower protective range, anti-PCP antibodies levels had decreased to sub-protective levels at or shortly after B-lymphocyte repopulation. In 1 patient, anti-PCP antibodies levels increased 4-fold at 3 months (from 363 to 1471 mg/dl), at the time of a respiratory infection.

Anti-microbial antibodies

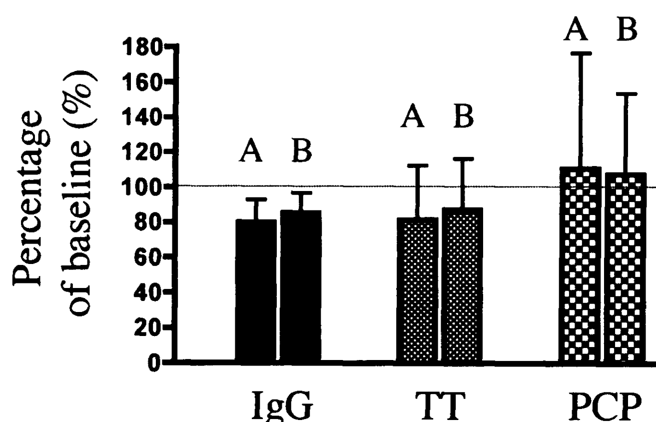


Figure 3.4 Comparison of mean (±SD) percentage change from baseline levels in total serum IgG with anti-TT and anti-PCP antibody levels A) 3 to 4 months following BLDT and B) at B-lymphocyte repopulation, in 22 patients.

3.3.1.5 Factors associated with relapse

At the time of this study (May 2002), all except 2 patients (patient 11 and 13) had clinically relapsed. All patients who had relapsed had done so at or up to 17 months after B lymphocytes were again detectable in the circulation (CD19 counts > 0.005 x 10^9/L). In all patients except 1 (patient 18), clinical relapse was preceded by a detectable rise in autoantibody levels. Rises in autoantibody levels, usually IgM-RhF, in the absence of relapse were rarely seen (on 4 occasions only) and were transient in nature. Figure 3.5 shows the absolute values of IgA-, IgG- and IgM-RhF and anti-CCP antibodies in samples from individual responding patients at the pre-treatment time point, at the time when patients had reached their maximum ACR levels of improvement and at relapse. Levels of all autoantibodies were found to decrease following treatment and, in most cases, to show a relative increase in titer at or close to the time of relapse. There was no clear preference for rises in any particular class of RhF or anti-CCP antibodies in association with relapse (table 3.1). In addition, no new autoantibody classes or specificities were found to emerge during the study. However, in later studies, when patient 8, who was seronegative (no RhF detectable on latex test, RAPA or ELISA) and anti-CCP antibody positive before her first treatment, was

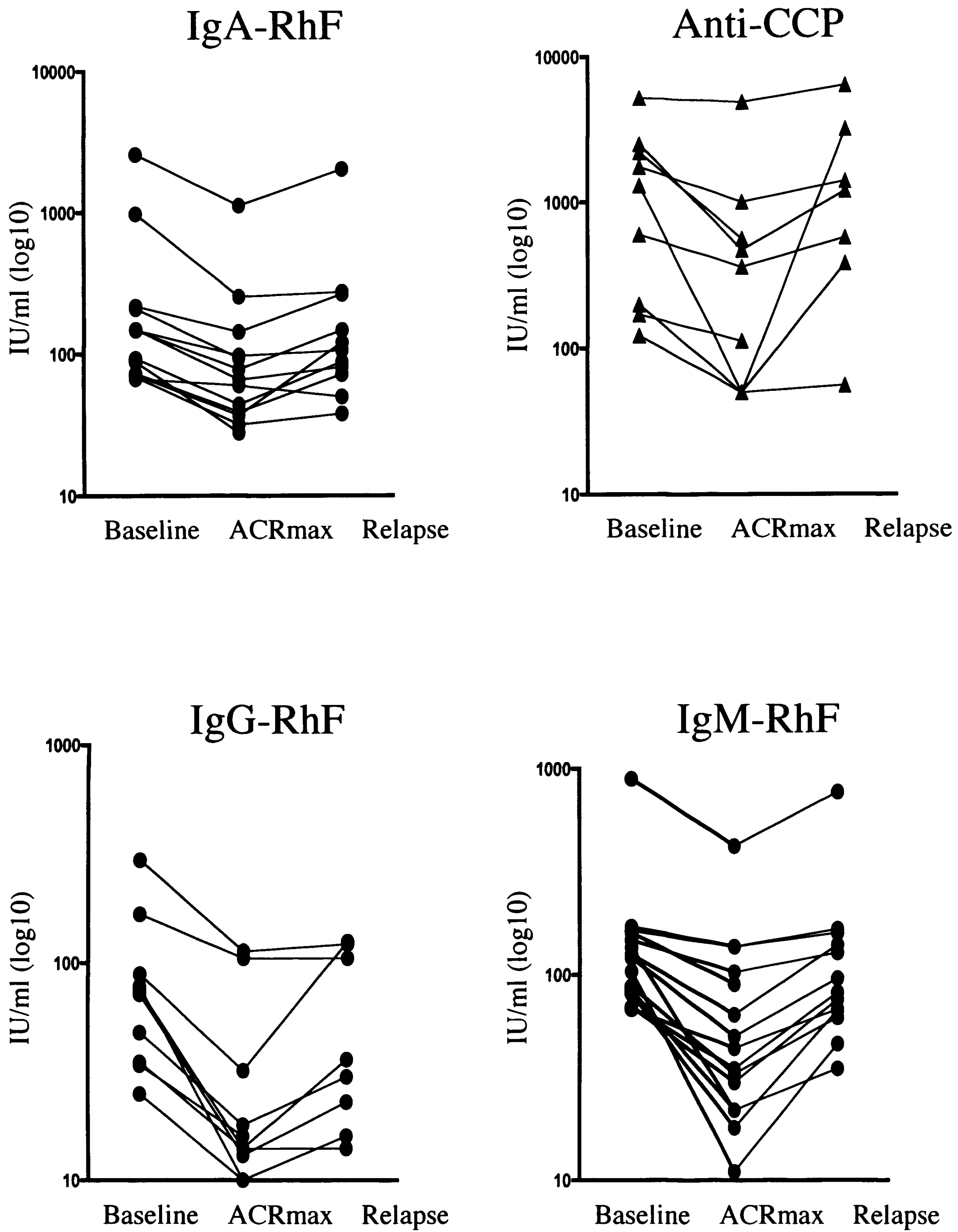


Figure 3.5 Absolute values of IgA-, IgG- and IgM-RhF and of anti-CCP antibodies pre-treatment, at the time of maximum ACR response and at clinical relapse.

assessed for re-treatment with full dose rituximab, 40 months after her first treatment, she was found to be seropositive (RAPA 1/1280 and IgA-, IgG- and IgM-RhF positive on ELISA).

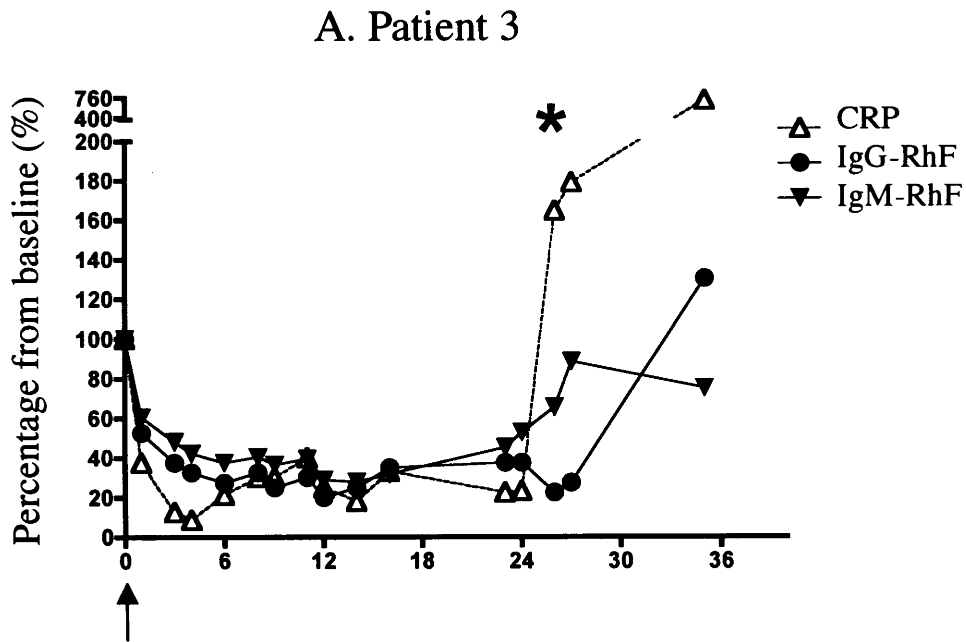
3.3.1.6 Serial studies of 2 responding patients

Figure 3.6 shows the changes in serologic parameters over time following BLDT in 2 patients. Patient 3 (figure 3.6A), relapsed at 26 months, 15 months after B-lymphocyte repopulation (table 2.3). Following initial falls in autoantibodies levels, IgM-RhF can be seen to rise before clinical relapse. In patient 4 (figure 3.6B), both B-lymphocyte repopulation and a rise in autoantibody levels occurred shortly before clinical relapse (table 2.3). As exemplified by the time courses of serologic responses in these 2 representative patients and the responder group data shown in figure 3.3A, reduction in CRP levels had the general appearance of an exponential decay curve. The pattern of changes in autoantibody levels generally paralleled the CRP profiles in each patient. Although the slopes of the curves differed from patient to patient, there tended to be an early drop in autoantibody and CRP levels to a plateau, which was sustained until relapse.

3.3.2 Changes on total immunoglobulin levels, on IgA-, IgG- and IgM-RhF and on anti-microbial antibodies in patients 1 to 40

3.3.2.1 Effects on total immunoglobulin levels (IgA, IgG and IgM)

Results from patients 1 to 40 were pooled to provide a more comprehensive series and to compare the effects of different doses of rituximab and also between rituximab monotherapy and combination treatment protocols. In 34 patients (all patients except patients 17, 24, 25, 27, 28 and 34), serial measurements of serum total immunoglobulin levels were available from before and following their first course of BLDT. Following treatment, total serum IgA, IgG and IgM levels decreased significantly ($P < 0.0001$ for the 3 classes when baseline levels were compared with levels at nadir, Wilcoxon signed rank test) but median levels remained within the normal range (table 3.3).



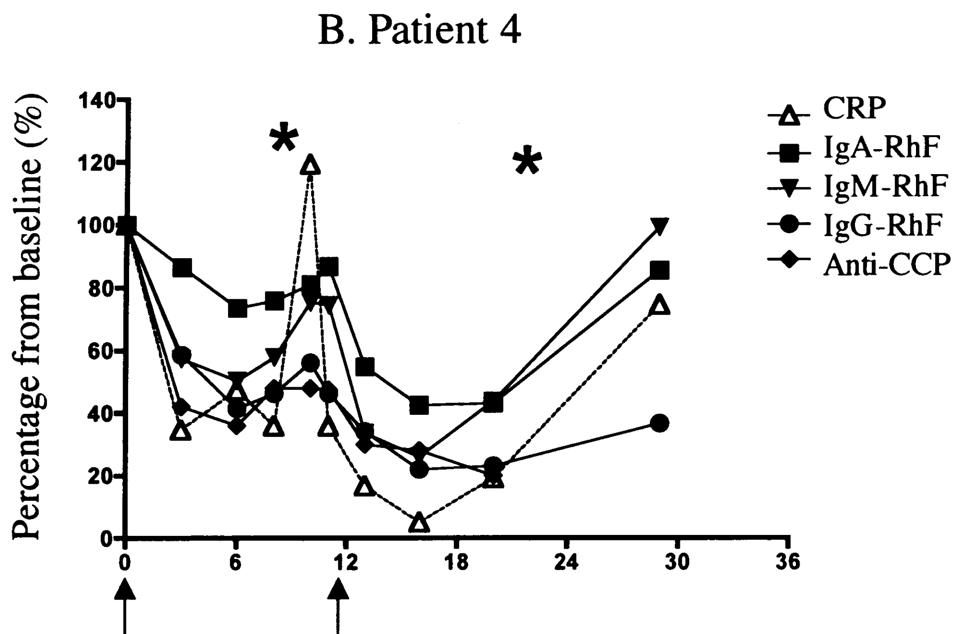


Figure 3.6 Percentage changes from baseline in serologic parameters in 2 individual patients. A) patient 3. B) patient 4. Bars indicate period of B-lymphocyte depletion, arrow indicates course of treatment and asterisk indicates clinical relapse.

Table 3.3 Changes in total immunoglobulin, IgA, IgG and IgM, IgA-, IgG- and IgM-RhF and anti-CCP antibody levels following BLDT.

Antibody	**Number of patients included* / total tested**	**Baseline** (median and range)	**Nadir** (median and range)	**Statistics** (Wilcoxon signed rank test)	**Percentage decrease** (mean ± SD)
Total IgA (g/L)	35	3.10 (0.40-6.20)	2.45 (0.10-5.40)	P = 0.0001	25.10±16.21
Total IgG (g/L)	35	13.30 (6.40-20.5)	9.65 (4.10-16.70)	P = 0.0001	24.99±11.31
Total IgM (g/L)	35	1.20 (0.50-5.20)	0.60 (0.20-4.00)	P = 0.0001	43.46±17.22
IgA RhF (IU/ml)	25/28	149.20 (33.40-2573)	78.20 (22.00-1148.00)	P = 0.0001	44.83±17.44
IgG RhF (IU/ml)	19/28	62.00 (24.70 to 297.00)	23.00 (8.8-116.60)	P = 0.0001	52.99±5.14
IgM RhF (IU/ml)	26/28	130.20 (68.40-919.00)	89.20 (11.00-422.40)	P = 0.0001	46.43±23.96
Anti-CCP (U/ml)	13/22	1200 (122-5720)	360 (50-4240)	P = 0.0002	56.28±21.10

* For autoantibodies, only positive patients included.

At baseline, 3 patients had low and 7 patients had high IgA, 2 patients had low and 5 patients had high IgG and 2 patients had high IgM. Following the first course of BLDT, no new patient developed low IgA, 4 patients developed low IgG and 9 patients developed low IgM. IgA and IgM remained high in one patient each. In all other cases, raised IgA, IgG and IgM levels decreased with treatment to within the normal range.

When patients treated with protocols including cyclophosphamide (20 patients) were analysed separately and compared with patients treated without cyclophosphamide (14 patients) no significant differences were found on total IgA, IgG or IgM percentage decrease. It was noted that in patients treated with rituximab and cyclophosphamide, nadir levels for total IgA and IgG but not for total IgM were attained significantly earlier than in patients treated without cyclophosphamide (P = 0.03, P = 0.02 and P = 0.38, respectively, Mann-Whitney rank sum test). In patients who received cyclophosphamide, median times to nadir were 3.5 months for IgA (range 1 to 12), 2.7 months for IgG (range 1 to 14) and 5.1 months for IgM (range 1 to 14). In patients who did not receive cyclophosphamide, median times to nadir were 7.2 months for IgA (range 1 to 17), 7.2 months for IgG (range 1 to 14) and 6.0 months for IgM (range 1 to 14).

No significant differences were found when patients who received full dose rituximab (23 patients) were compared with patients who received half-dose rituximab or less (11 patients).

3.3.2.2 Rheumatoid factor

Serial measurements of IgA, IgG and IgM-RhF were performed in serum samples from a total of 28 patients following their first course of BLDT (patients 1 to 16, 18 to 23, 30, 32 to 34, 36 and 37). Twenty-six patients were seropositive. All seropositive patients had IgM-RhF and at least one other isotype. Twenty-five patients (89%) had IgA-RhF and 19 (68%) had IgG-RhF.

Serum levels of RhF of all three isotypes decreased significantly following treatment (table 3.3). IgA-RhF decreased from a median of 149 IU/ml (range 33 to 2573) at baseline to a median of 78 (range 22 to 1148) ($P < 0.001$, Wilcoxon signed rank test).

IgG-RhF decreased from a median of 62 IU/ml (range 25 to 297) at baseline to a median of 23 (range 9 to 117) ($P < 0.001$, Wilcoxon signed rank test). IgM-RhF decreased from a median of 130 IU/ml (range 68 to 919) at baseline to a median of 89 (range 11 to 422) ($P < 0.001$, Wilcoxon signed rank test). Mean percentage decreases following BLDT were 44.8±17.4% (mean ± SD) for IgA-RhF, 53.0±5.1% for IgG-RhF and 46.4±24.0% for IgM-RhF. As observed in the first 22 patients, IgA-RhF and IgG-RhF decreased significantly proportionately more than total IgA and total IgG, respectively ($P < 0.001$ for both comparisons, unpaired t test). There were no significant differences between the percentage decrease in IgM-RhF and total IgM ($P = 0.52$, unpaired t test) (figure 3.7).

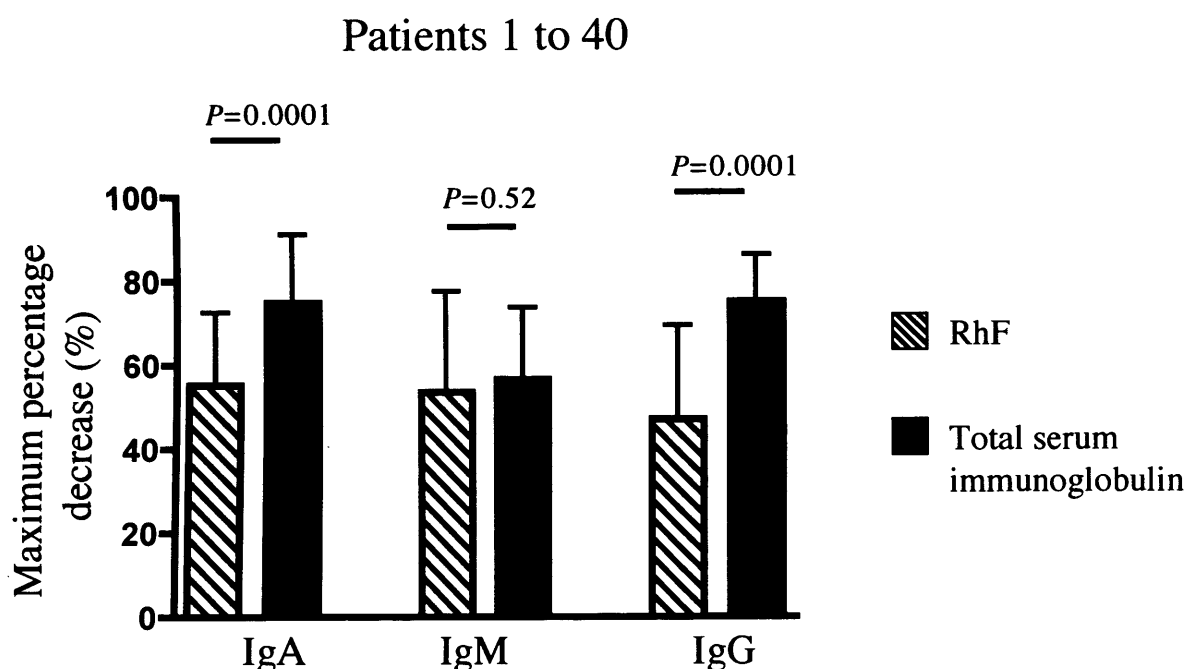


Figure 3.7 Comparison between maximum percentage decrease from pre-treatment serum levels in IgA-, IgM- and IgG-RhF and respective total immunoglobulins classes (n = 25 for IgA; n = 19 for IgG; n = 26 for IgM)(unpaired *t* test).

In some cases RhF decreased to below the upper limit of the normal range. This happened in 3 patients for IgA-RhF, 8 patients for IgG-RhF and 5 patients for IgM-RhF. In patients 3 and 11 the three isotypes decreased to within the normal range. In patient 7, both IgA-RhF and IgM-RhF normalised and in patient 5 both IgG-RhF and IgM-RhF normalised.

When patients treated with rituximab and cyclophosphamide were compared with patients treated with only rituximab, the patients who received cyclophosphamide were seen to have higher levels of IgG-RhF at baseline (P = 0.05 test Mann-Whitney rank sum test) and showed a bigger drop in IgG-RhF following treatment (P = 0.04, Mann-Whitney rank test). No significant differences were found when IgA-RhF and IgM-RhF levels at baseline and at nadir and total decrease were compared. In both groups of patients, IgA-, IgG- and IgM-RhF decreased significantly.

When the group of patients who responded clinically to treatment (21 patients) were compared with patients who did not respond to treatment (7 patients), once again IgA-, IgG- and IgM-RhF were seen to decrease significantly in the responders group but not in the non-responders (responders $P < 0.001$ for the 3 comparisons; non-responders P = 0.06, P = 0.25 and P = 0.06, respectively, Wilcoxon signed rank test).

3.3.2.3 Anti-CCP antibodies

As described earlier 13 out of 22 patients tested had anti-CCP antibodies. Anti-CCP antibodies decreased significantly following BLDT from a median of 1200 U/ml (range 122 to 5720) to a median of 360 U/ml (range 50 to 4240) (P = 0.002, Wilcoxon signed rank test) (table 3.3). As described before, anti-CCP antibodies decreased proportionally more than total IgG (figure 3.1).

In 2 cases anti-CCP antibodies decreased to within the normal range (patients 1 and 12).

3.3.2.4 Effects on anti-microbial antibodies

In 29 patients, anti-TT and anti-PCP antibodies were measured in serum samples from before treatment, 3 to 4 months after treatment and at the time or shortly after B-lymphocyte repopulation. Samples tested were from the patients' first course of BLDT.

Anti-TT antibodies

Anti-TT antibodies decreased significantly from a baseline median of 0.34 IU/ml (range 0 to 7.1) to a median of 0.18 IU/ml (range 0 to 7.2) 3-4 months after treatment (P = 0.026, Wilcoxon signed rank test) (figure 3.8). Differences between anti-TT antibodies

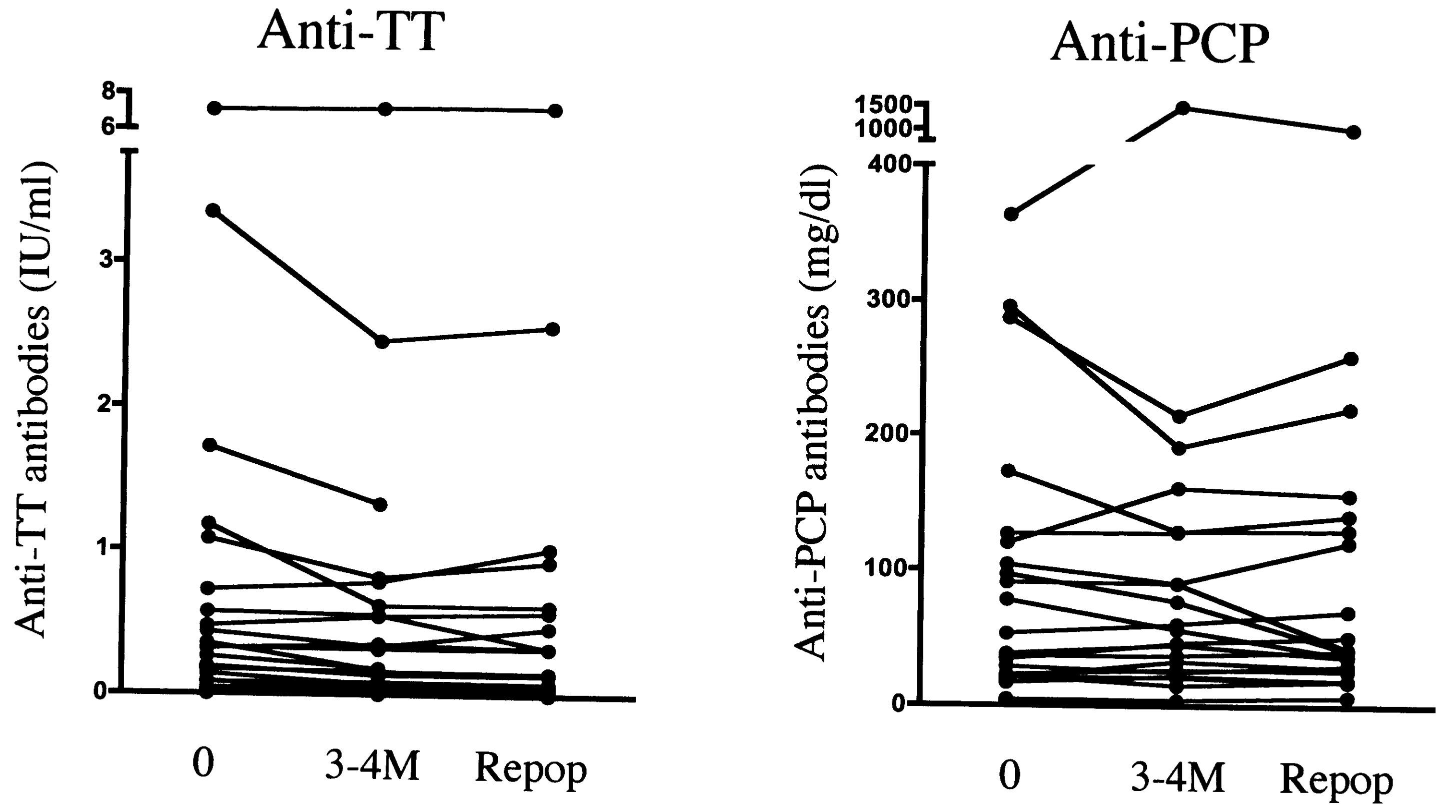


Figure 3.8 Anti-TT and anti-PCP antibody levels pre-treatment (0), 3 to 4 months (3-4M) and at the time of B-lymphocyte repopulation (Repop) following BLDT (n = 29).

at 3-4 months and at or shortly after repopulation (median 0.24, range 0 to 7.3) and again between baseline and at or shortly after repopulation were not significant (P = 0.28 and P = 0.30, respectively, Wilcoxon signed rank test) (figure 3.8).

Anti-TT antibodies levels at 3-4 months after treatment were a mean of 81.6% (range 150.0 to 25.0) of baseline values. At repopulation, anti-TT antibodies levels were a mean of 91.3% (range 150.0 to 33.3%) of baseline values. At both time points, no significant difference was found between the percentage decrease in anti-TT antibodies and in total IgG (P = 0.87 and P =0.93, respectively, Mann-Whitney rank sum test).

At baseline, 16 patients (72.7%) had protective levels of anti-TT antibodies (≥ 0.1 IU/ml). At 3-4 months after BLDT and at or shortly after repopulation all these patients maintained levels within the protective range.

Anti-PCP antibodies

Anti-PCP antibodies did not decrease significantly following BLDT (figure 3.8). Median anti-PCP antibodies levels were 91 mg/dl at baseline (range 3 to 407) and 77 mg/dl (range 3 to 1471) 3-4 months after treatment (P = 0.07, Wilcoxon signed rank test).

Anti-PCP antibodies levels 3-4 months after treatment were a mean of 90.4% (range 64.8 to 405.2) of baseline values. At repopulation, anti-PCP antibodies levels were a mean of 86.0% (range 42.3 to 279.3) of baseline values.

At baseline, 18 patients (62.1%) had protective levels of anti-PCP antibodies (≥ 50 mg/dl). At 3-4 months after rituximab and after repopulation 15 patients maintained levels within the protective range. In 3 patients with baseline levels within the lower protective range, anti-PCP antibodies levels had decreased to sub-protective levels at or shortly after B-lymphocyte repopulation as described before.

Response to immunization

Two patients (patient 3 and 23) were immunised with tetanus toxoid and pneumococcal capsular polyssacharides vaccines (Adsorbed tetanus vaccine and Pneumovax, respectively) while still depleted and no increase in anti-TT and anti-PCP antibodies

were detected 4 weeks after immunisation. Two patients (patient 10 and 36) were immunised before re-treatment with the same preparations. Patient 10 was immunised 15 months after B-lymphocyte return was first detected: anti-PCP antibodies increased from 78 to 132 mg/dl and anti-TT from 1.32 to 1.56 IU/ml 4 weeks after immunisation. Patient 36 was immunised 3 months after B-lymphocyte return: anti-PCP antibodies did not increase (239 mg/dl before immunisation; 187 mg/dl, 1 month after immunisation; 212 mg/dl 2 months after re-treatment) and anti-TT antibodies increased from 0.16 to 0.41 IU/ml 4 weeks after immunisation.

3.3.3 Effects of repeated cycles of BLDT on total Ig levels, IgA-, IgG- and IgM-RhF and anti-microbial antibodies

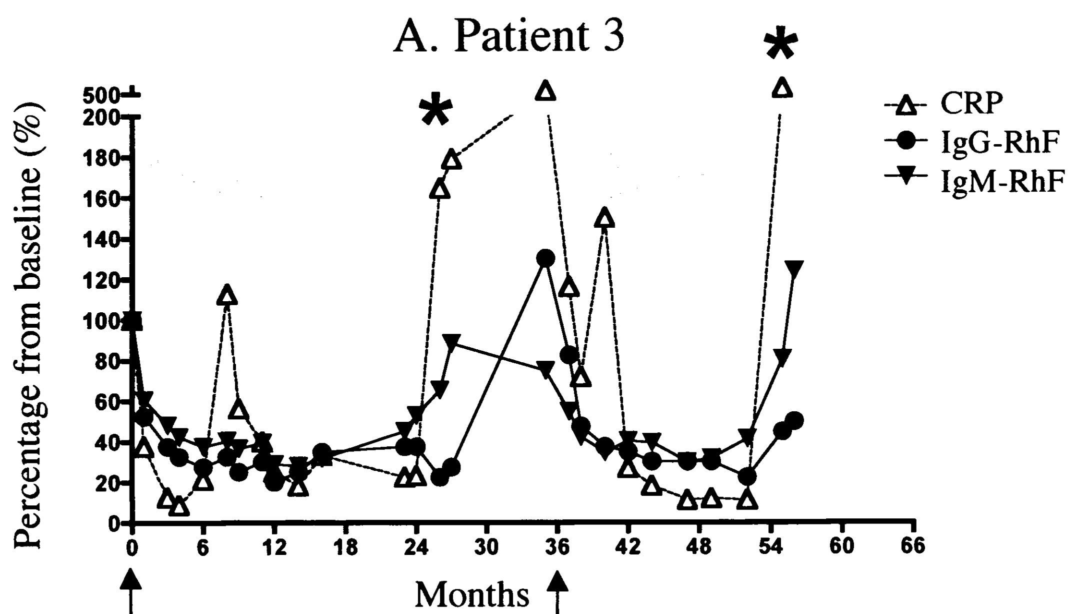
Effects on serum total immunoglobulin levels

As described in the previous chapter, although falls in total immunoglobulin levels following a single cycle of BLDT were modest, repeated cycles led occasionally to hypogammaglobulinemia, particularly low, occasionally, undetectable IgM. This was observed (table 2.9 and figure 2.7) either in patients who relapsed at the time of B-lymphocyte repopulation and were treated shortly after or in patients who relapsed only some time after B-lymphocyte repopulation but whose immunoglobulins serum levels did not show a definite increase following B-lymphocyte return. It was more frequent in patients who had lower baseline immunoglobulin levels.

B-lymphocyte repopulation of the peripheral blood was associated with an increase in total immunoglobulin levels in many patients but not in all. In some patients immunoglobulin levels tended to stay stable for a variable period of time. Changes in immunoglobulin levels did not always correlate with changes in serum autoantibody levels or with clinical relapse.

Effects on IgA-, IgG- and IgM-RhF

The pattern of change for the three isotypes of RhF was similar following repeated courses of BLDT (figure 3.9).



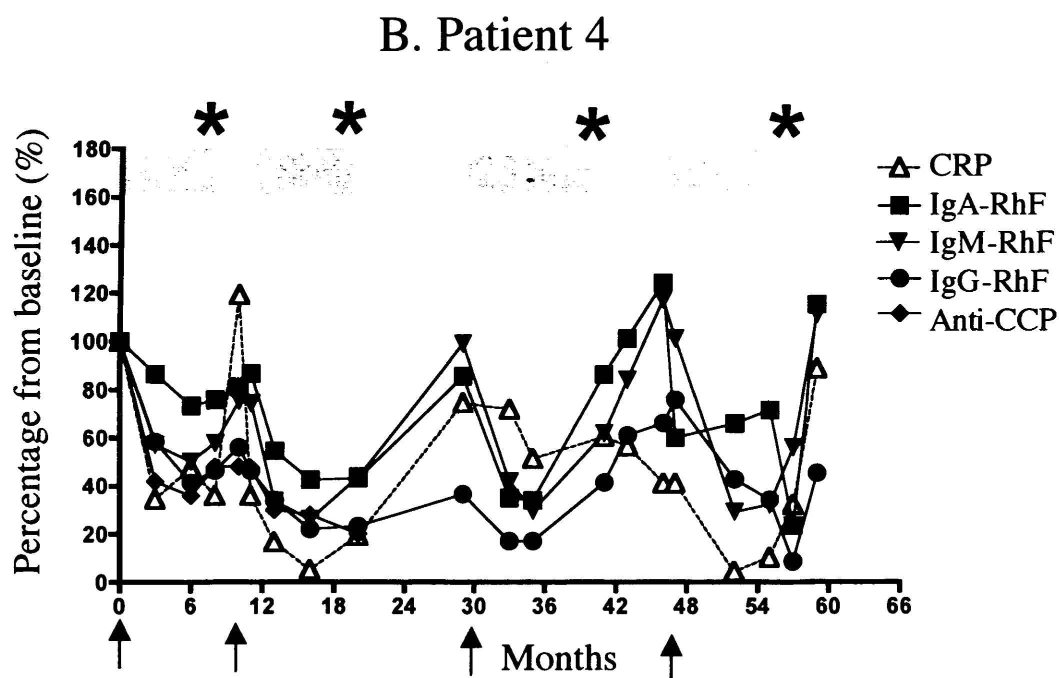


Figure 3.9 Percentage changes from baseline in CRP and autoantibodies levels following repeated courses of BLDT when compared to baseline levels before the first treatment. A) patient 3. B) patient 4. Bar indicates period of B-lymphocyte depletion, arrow indicates course of treatment and asterisk indicates clinical relapse.

3.3.4 Soluble CD23

Serum levels of sCD23 were measured in sequential samples from 17 patients following their first course of BLDT (patients 1-3, 5-8, 11, 13-16, 18, 19, 21 and 22).

At baseline, sCD23 levels correlated positively with peripheral blood CD19 counts (r = 0.75, P = 0.01, Spearman rank order correlation coefficient) (figure 3.10).). In 2 patients (patients 6 and 21) sCD23 antigen was undetectable at baseline and remained so following treatment and at repopulation. In the other 15 patients sCD23 antigen levels decreased rapidly following BLDT coincident with the disappearance of B lymphocytes from the circulation. Levels decreased significantly from a median of 75 U/ml at baseline (range 12 to 350) to a median of 9 U/ml at nadir (range 0 to 93 units) ($P < 0.001$, Mann-Whitney rank sum test) (figure 3.11). Mean percentage decrease from baseline was 76% (range 14 to 100%). Mean percentage decrease from baseline was 80% in patients who responded to treatment (range 16 to 100) and 59% in patients who did not respond to treatment (range 14 to 89) with the difference not being statistically significant (P = 0.27, unpaired t test).

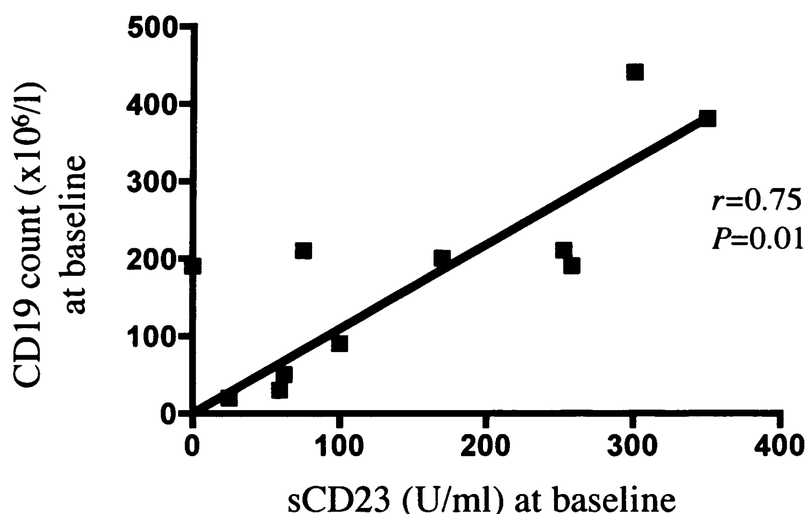


Figure 3.10 Correlation between serum sCD23 levels (U/ml) and peripheral blood CD19 count (nx10^6/l) at baseline (n = 15 patients) (Spearman rank order correlation coefficient).

Serum levels of sCD23 increased at the time of B-lymphocyte repopulation in 6 of the 15 patients who had detectable levels at baseline (figure 3.11). When clinical relapse did not occur at the same time as repopulation no changes in the serum levels of sCD23 were noted when compared with the previous sample.

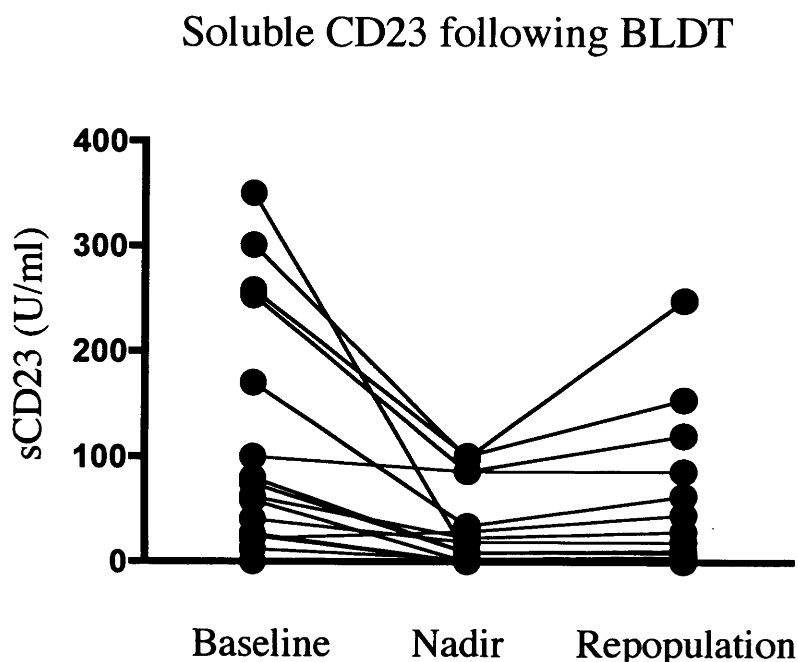


Figure 3.11 Serum sCD23 levels at baseline and at nadir and at B-lymphocyte repopulation, following BLDT (n = 17 patients).

3.3.5 Effects on serum Blys levels

Serum BLyS levels were measured in sequential samples from 15 patients (23 treatments) treated with BLDT (patients: 2, 2a; 3, 3a; 4, 4a, 4b; 5; 6; 10, 10a; 11; 13; 15; 16, 16a; 19, 19a; 30; 32; 33; 36, 36a) (tables 2.1 to 2.4). Seven of these patients were retreated at least once. Thirteen of the 15 patients responded to BLDT (tables 2.3 and 2.4). In 7 patients (Group A: patients 3, 5, 11, 13, 19, 32 and 33), there was at least a 5-month interval between B-lymphocyte repopulation and clinical relapse (median 11 months). In the other 6 patients (Group B: patients 2, 4, 10, 16, 30 and 36), clinical relapse ensued no later (and often sooner) than 2 months after B-lymphocyte repopulation (tables 2.3 and 2.4). As previously described, pattern of relapse following re-treatment was similar to that following the initial course of BLDT.

Individual patients varied in the way B-lymphocyte recovery occurred after B lymphocytes first re-appeared in the peripheral blood. Some patients underwent rapid B-lymphocyte repopulation with peripheral B-lymphocyte counts reaching the normal range within 4 to 8 weeks, whereas numbers of B lymphocytes rose more slowly, over months, in other patients. There was a similar distribution of patients who repopulated their periphery rapidly and those whose B-lymphocyte counts took longer to reach the normal range after B-lymphocyte repopulation in Groups A and B.

Serum BLyS and antibody levels following BLDT

BLyS was not detectable in the sera of 9 of the 15 RA patients prior to their first course of BLDT. In the 6 patients with detectable BLyS at baseline median serum level was 1.06 ng/ml (range 0.92 to 5.12). At baseline, BLyS serum levels showed a negative correlation with total IgA levels ($r = -0.55$, $P = 0.035$, Spearman rank order correlation coefficient). There were no correlations between baseline BLys serum levels and baseline serum IgA-, IgG- and IgM-RhF, anti-CCP, total IgG and IgM, anti-PCP or anti-TT or B-lymphocyte counts (Spearman rank order correlation coefficient) (figures 3.12, 3.13 and 3.14).

In every case, BLyS serum levels rose markedly following BLDT (figure 3.15). By 1-2 months following treatment, BLys serum levels had increased significantly to a median of 5.10 ng/ml (range 1.42 to 8.04) ($P < 0.001$, Wilcoxon signed rank test). At 3-4 months post-treatment, serum BLys levels, in aggregate, remained elevated compared to those at baseline (median 5.86 ng/ml, range 0 to 9.61; $P = 0.001$, Wilcoxon signed rank test) and were not significantly different from levels at 1-2 months ($P = 0.693$, Wilcoxon signed rank test).

Relationship between serum BLyS levels, B-lymphocyte depletion and B-lymphocyte repopulation

As described above, a strong inverse relationship between BLyS levels and depletion of B lymphocytes in the peripheral blood was found but the relationship between BLyS levels and B-lymphocyte repopulation was more complex. To illustrate this relationship, serial studies of BLyS levels and CD19 counts in 2 patients, representative of the two response patterns, are shown in figure 3.16A (Group A) and figure 3.16B (Group B). B-

BLyS and CD19 count and total immunoglobulins at baseline

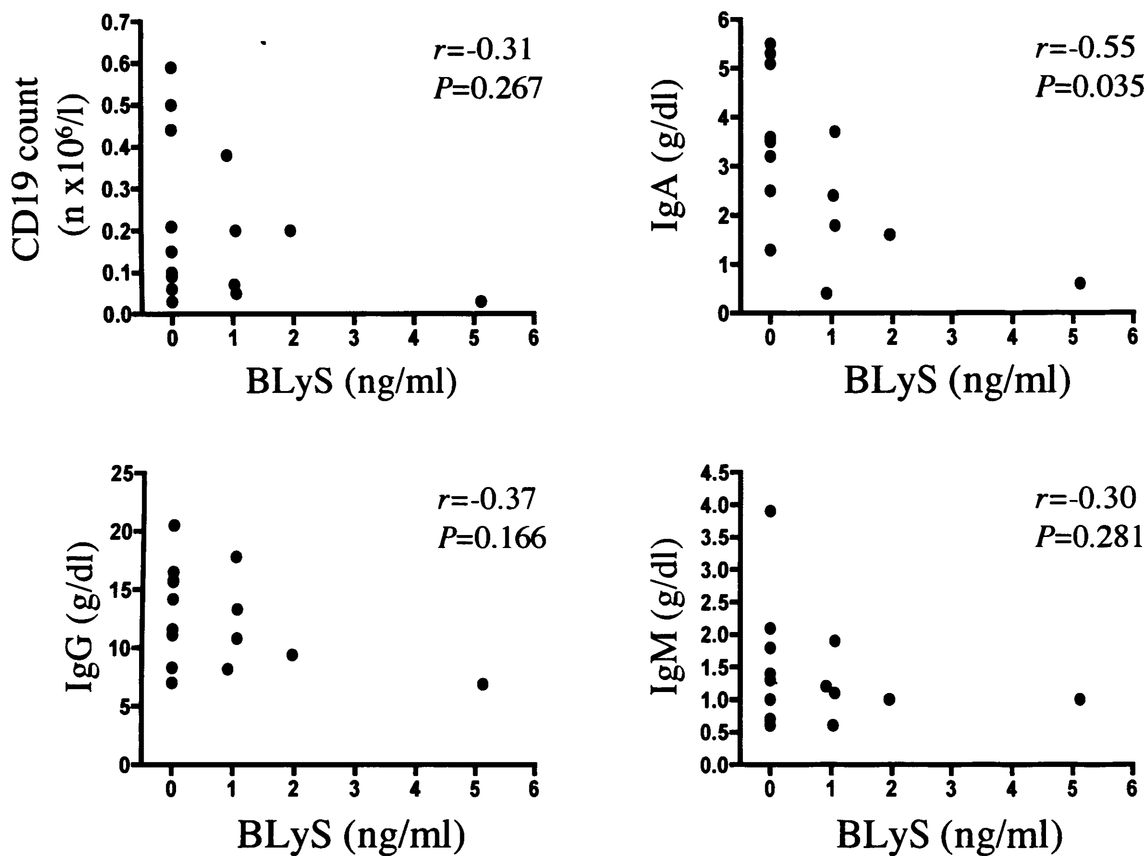


Figure 3.12 Relationship between serum BLyS levels and CD19 counts and total serum immunoglobulins (IgA, IgG and IgM) at baseline (Spearman rank order correlation coefficient).

BLyS and autoantibodies at baseline

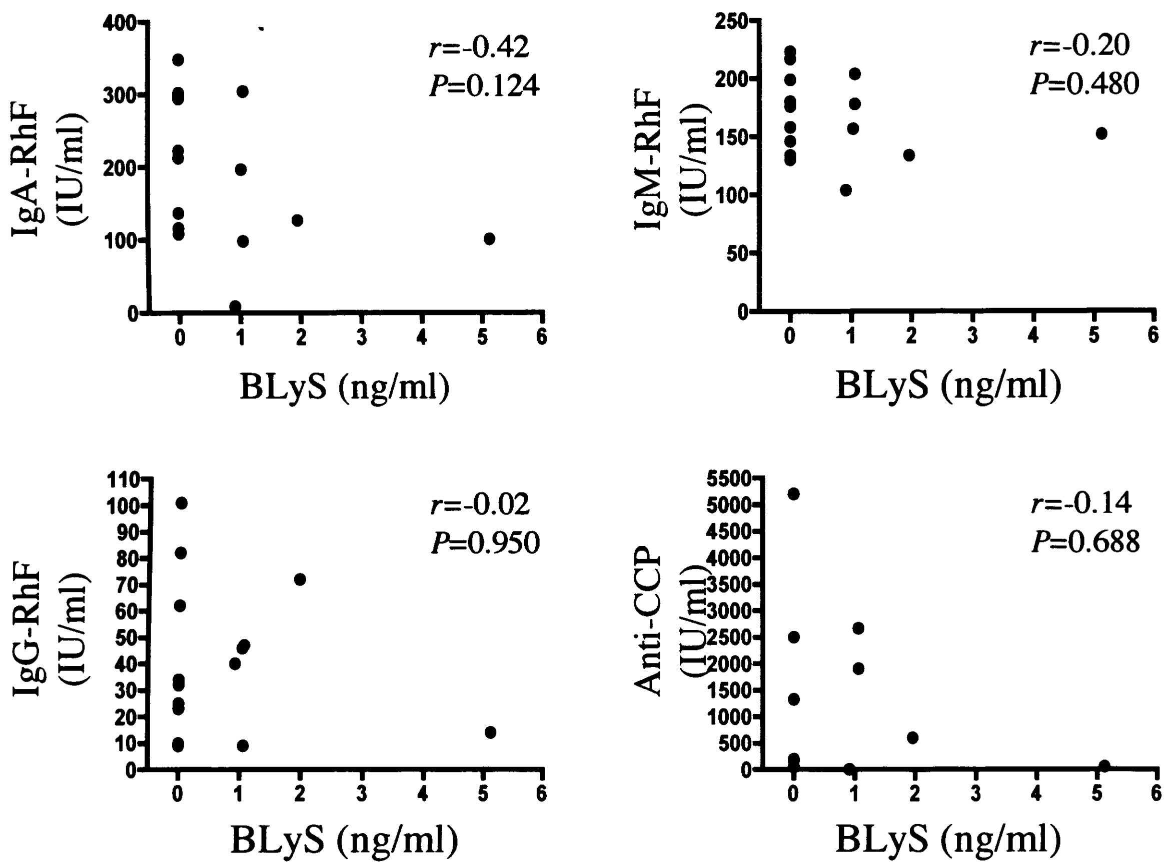


Figure 3.13 Relationship between serum BLyS levels and IgA-, IgG- and IgM-RhF and anti-CCP antibodies levels at baseline (Spearman rank order correlation coefficient).

BLyS and anti-microbial antibodies at baseline

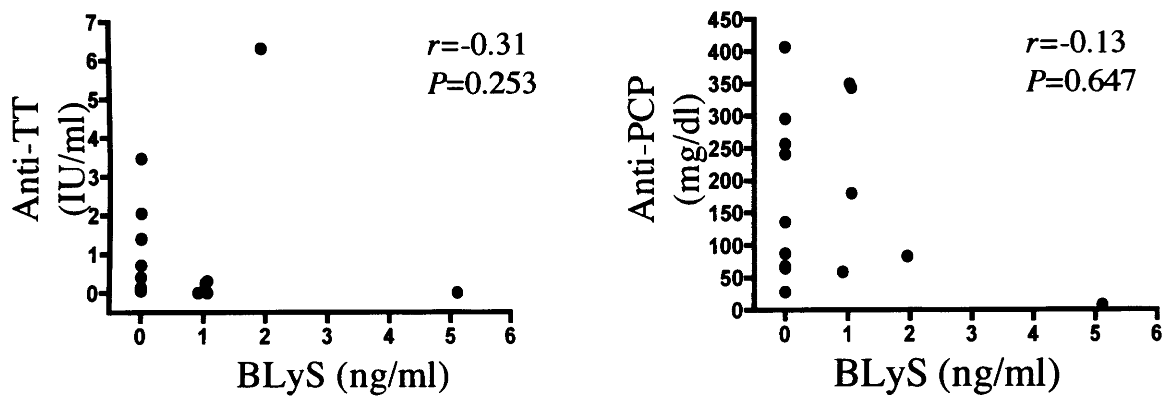


Figure 3.14 Relationship between serum BLyS levels and anti-TT and anti-PCP antibodies levels at baseline (Spearman rank order correlation coefficient).

BLyS and antibodies up to 4 months after BLDT

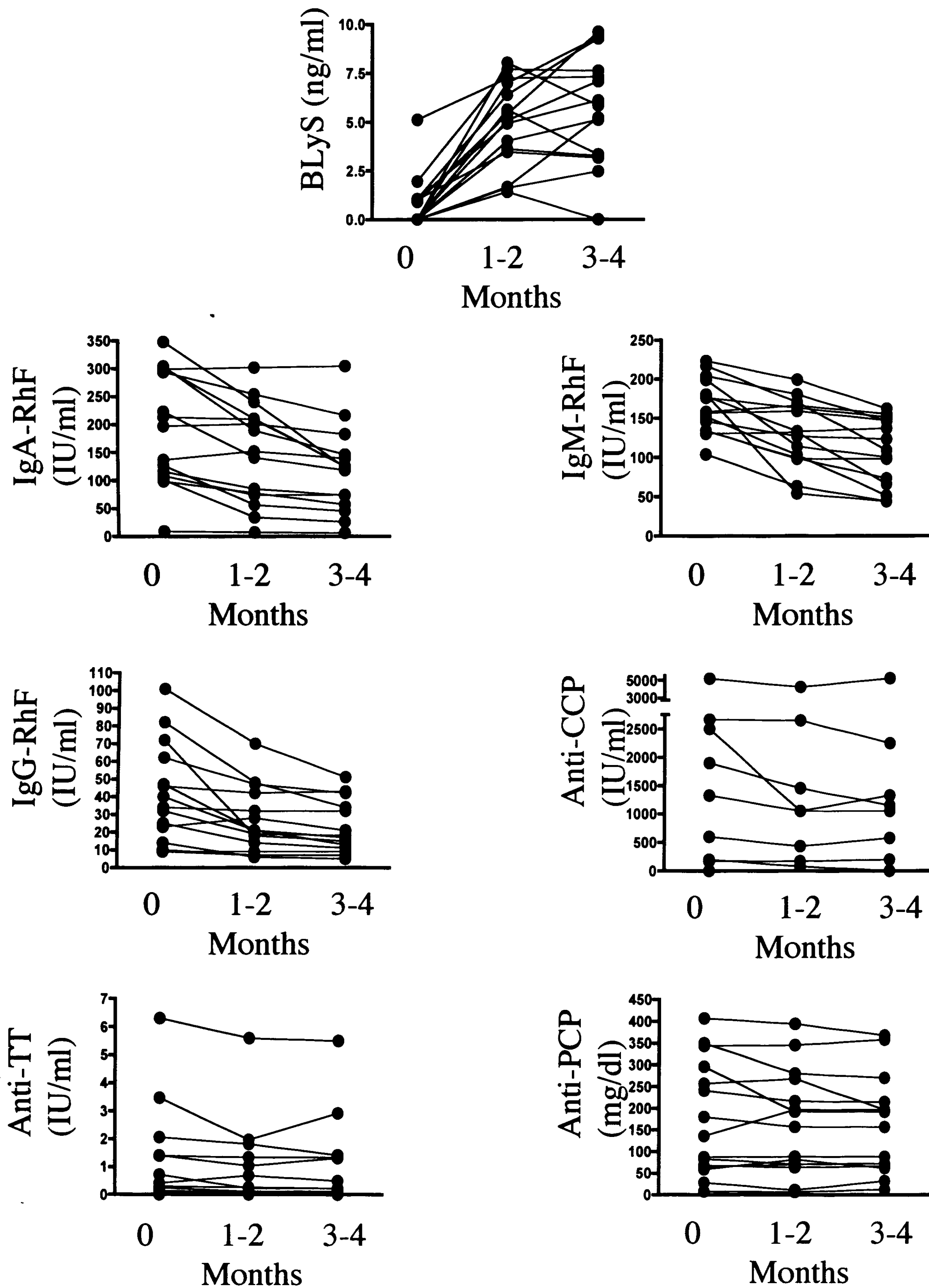


Figure 3.15 Serum BLyS, IgA-, IgM- and IgG-RhF and anti-CCP antibodies, anti-TT and anti-PCP antibody levels at baseline and at 1-2 and 3-4 months following BLDT.

lymphocyte numbers in both patients shown reached the normal range within weeks of the first recorded positive CD19 count post-treatment but the patients relapsed at different times. Similar patterns and timing of B-lymphocyte repopulation and fluctuations in BLyS levels were observed in these and the other retreated patients.

In patient 4 (Group B patient), the drop in BLyS level to its pre-treatment value was very closely associated with increasing peripheral B-lymphocyte numbers (Figure 3.16B). In patient 3 (Group A patient) BLys levels also decreased towards pre-treatment levels following B-lymphocyte repopulation but the decrease was more gradual and slower than in the Group B patient (figure 3.16A). Correlation of B-lymphocyte numbers and BLyS levels, followed until clinical relapse 17 months after B-lymphocyte repopulation, revealed a significant inverse correlation (r =-0.80; P = 0.003, Spearman rank order correlation coefficient) in this patient. However similar analysis of B-lymphocyte numbers versus BLyS levels in the other Group A patients from whom sufficient samples were available for analysis revealed a significantly inverse relationship in only 2 of the other 5 individuals. In addition, the presence of raised pre-treatment serum BLyS levels did not correlate with the time taken for B lymphocytes to return to the peripheral blood (P = 0.079, Spearman rank order correlation coefficient).

Changes in autoantibodies, CRP and BLyS levels and correlation with clinical relapse

The relationships between BLyS levels, CRP levels, circulating antibodies and clinical relapse are shown for representative patients from each group in figures 3.17 (group A) and 3.18 (group B). In all patients, serum BLyS levels rose and IgM-RhF levels fell, the latter frequently reaching nadirs prior to peripheral blood B-lymphocyte repopulation. IgM-RhF levels usually rose before or at the time of relapse and of rise in CRP in both groups of patients (A and B) as previously mentioned. Serum IgM-RhF levels began to rise in patient 16 (figure 3.18 A-B) even while B-lymphocyte return to the peripheral blood was not yet detectable but serum BLyS levels were already undetectable.

Similar patterns of serological responses were observed following subsequent treatment courses in patients 3 (figure 3.17 A-B) and patient 4 (figures 3.18 C-D).

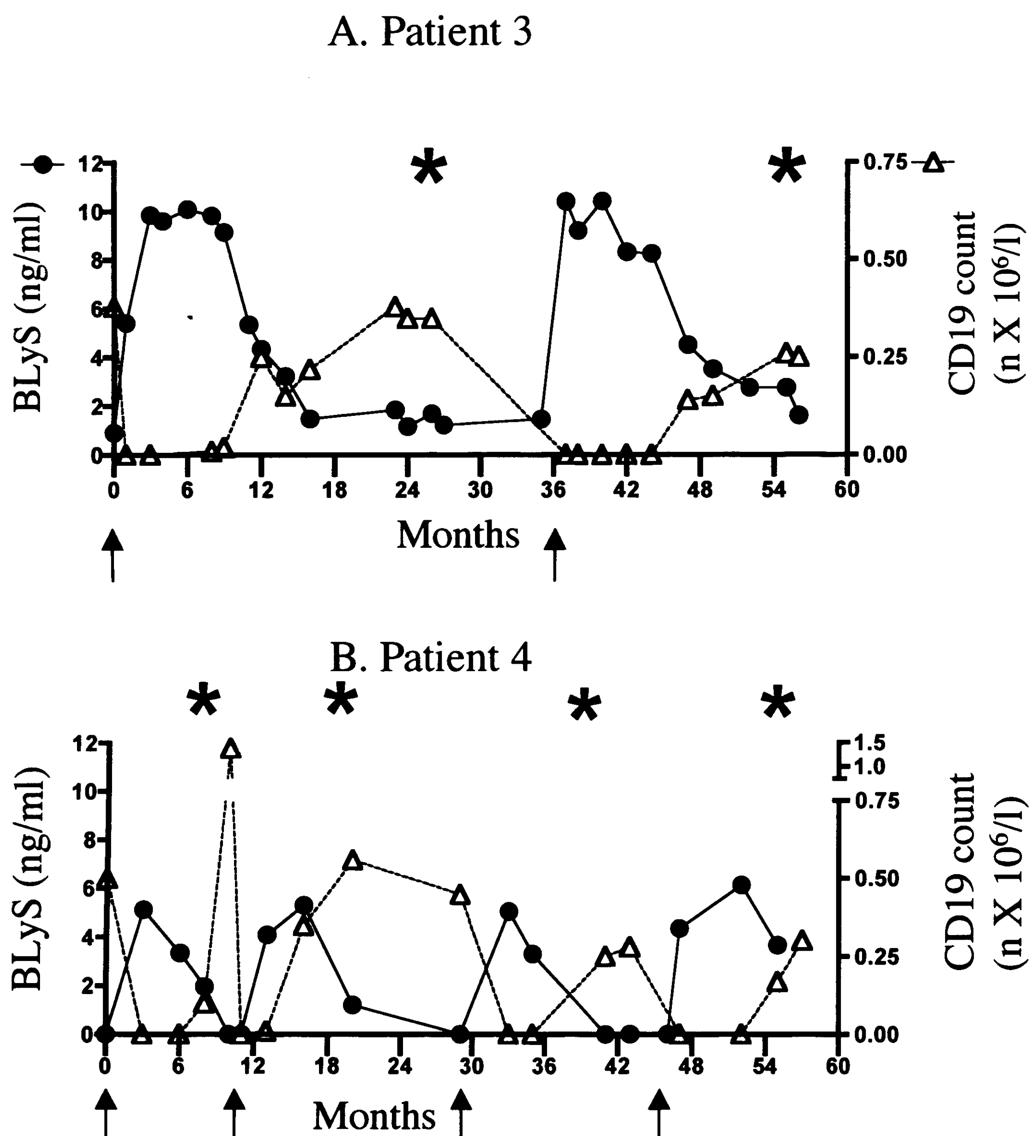
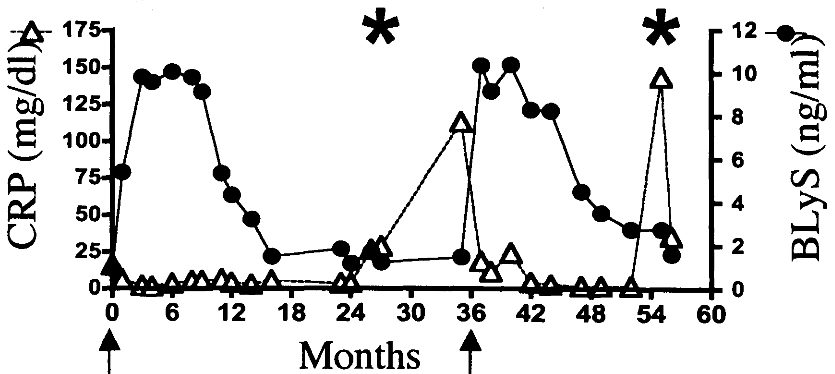
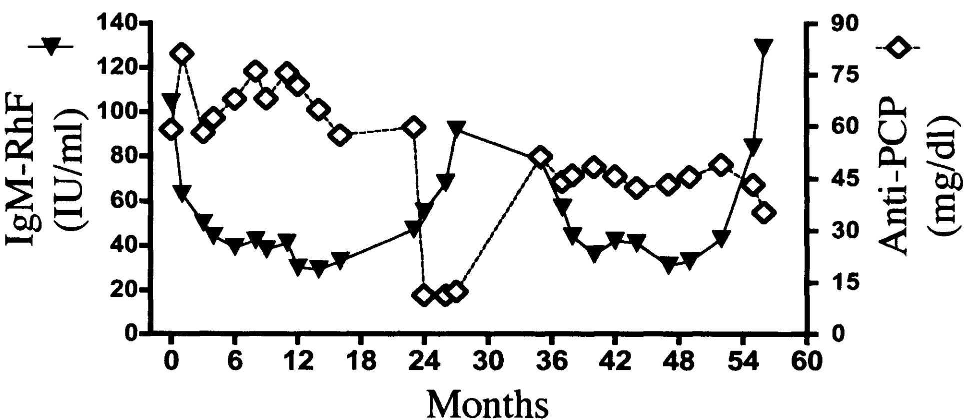
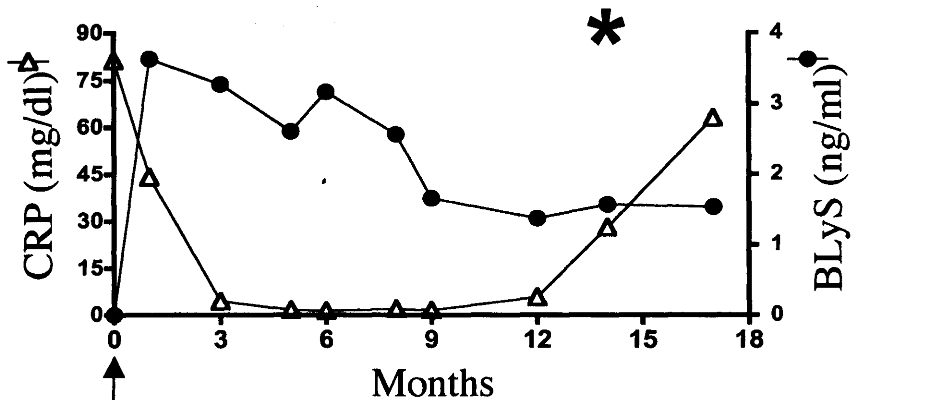


Figure 3.16 Changes in BLyS serum levels and CD19 counts following BLDT. A) patient 3, representative of the group of patients who relapsed more than 5 months after B-lymphocyte repopulation (group A). B) patient 4, representative of the group of patients who relapsed at B-lymphocyte repopulation (group B).







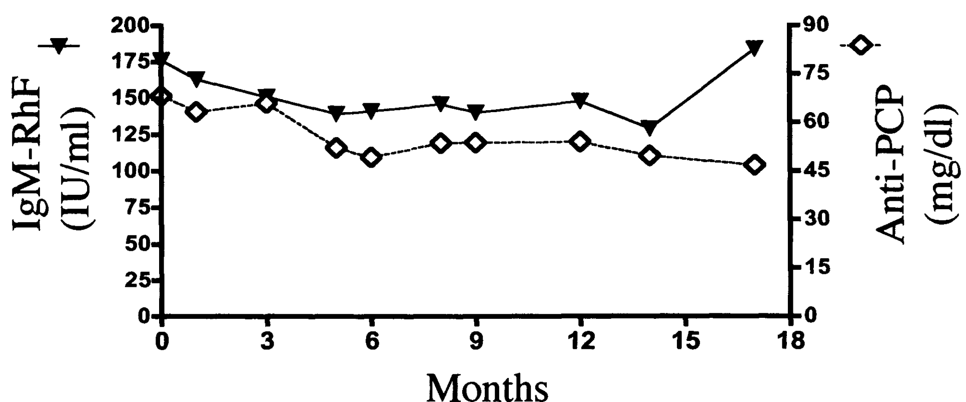
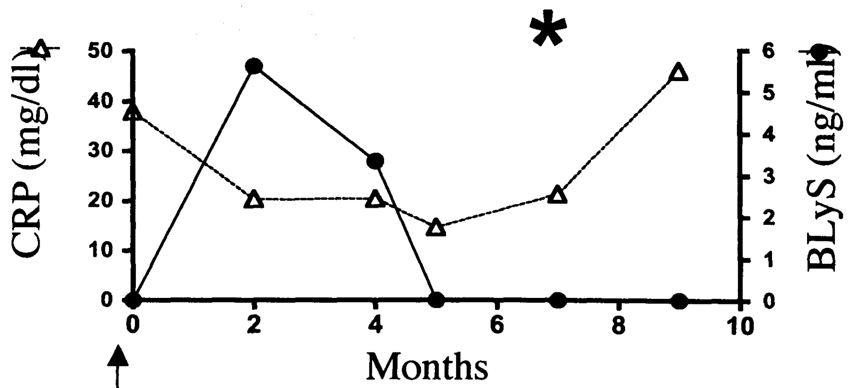
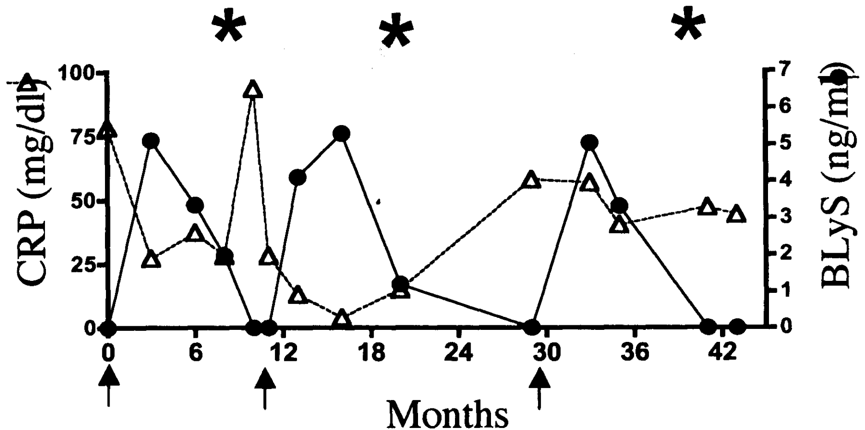
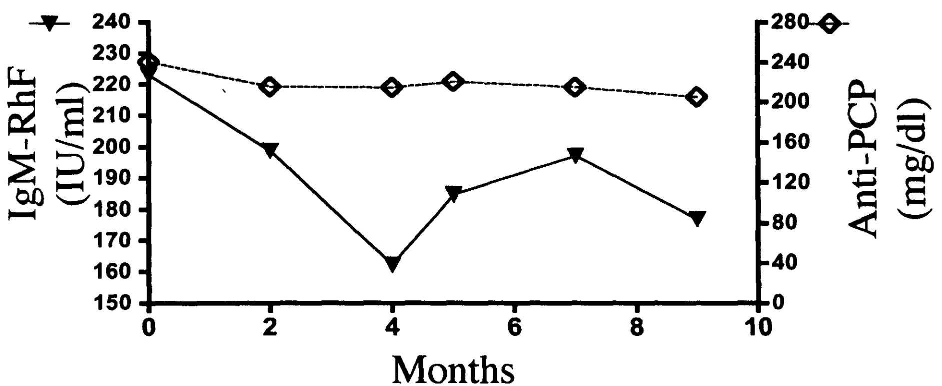


Figure 3.17 Relationship between peripheral blood B-lymphocyte depletion, serum levels of CRP, BLyS, RhF and anti-PCP antibodies in two representative patients from group A. A) and B) patient 3. C) and D) patient 33. Bar indicates period of B-lymphocyte depletion, arrow indicates course of treatment and asterisk indicates clinical relapse.







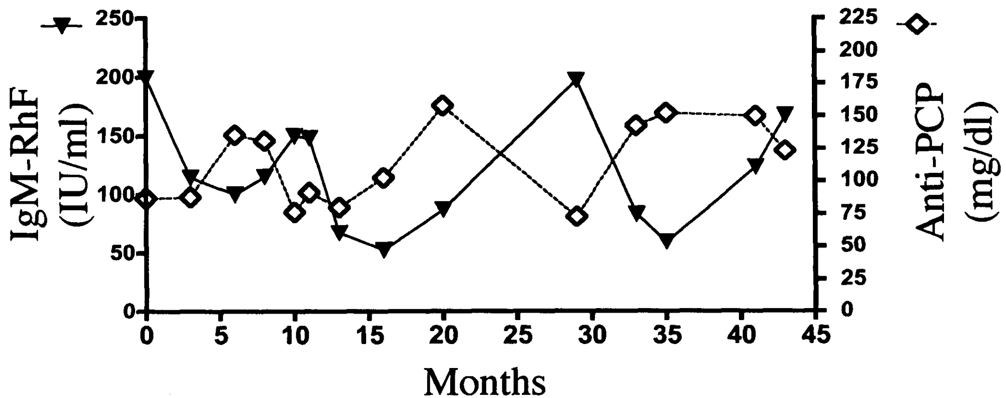


Figure 3.18 Relationship between peripheral blood B-lymphocyte depletion, serum levels of CRP, BLyS, RhF and anti-PCP antibodies in two representative patients from group B. A) and B) patient 16. C) and D) patient 4. Bar indicates period of B-lymphocyte depletion, arrow indicates course of treatment and asterisk indicates clinical relapse.

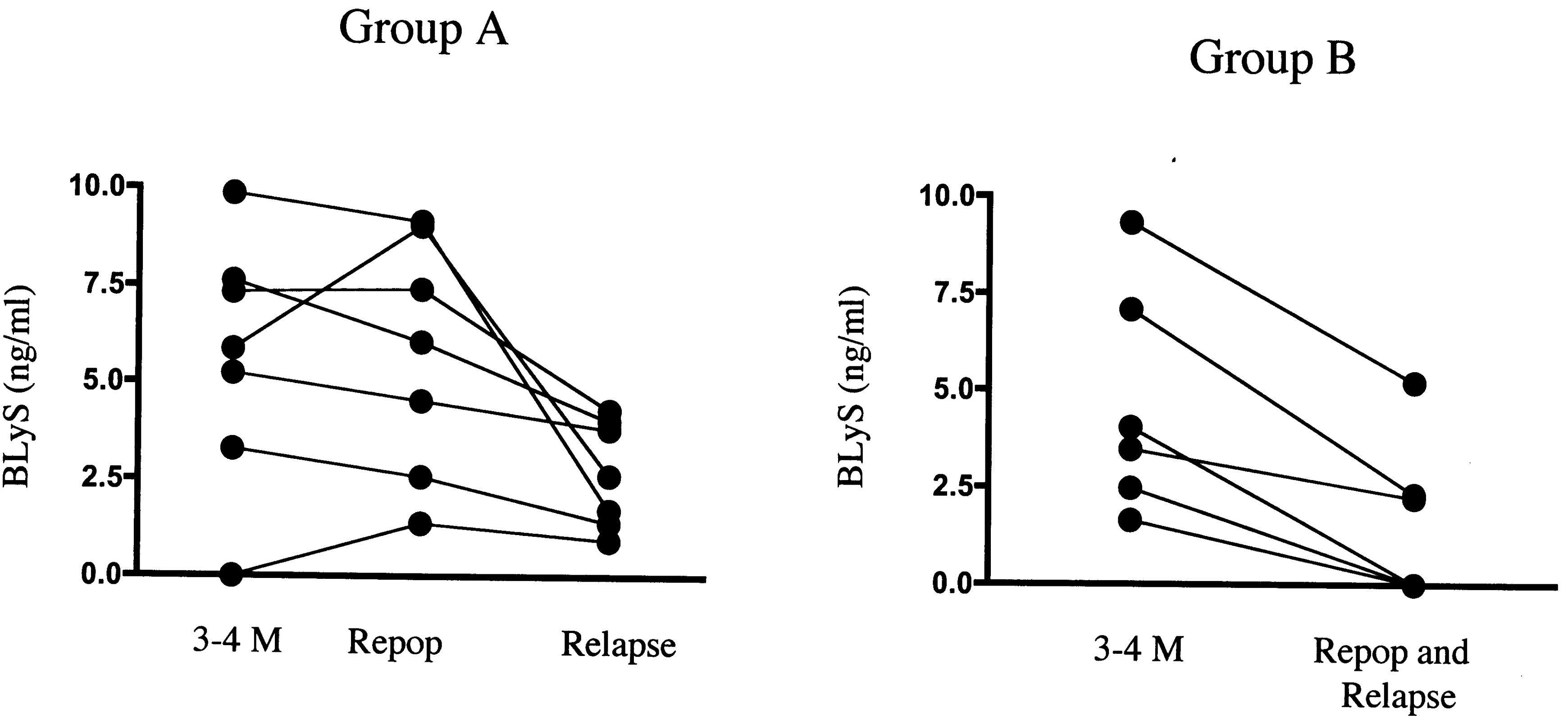


Figure 3.19 Serum BLyS levels in group A patients who relapsed only 5 months or more after B-lymphocyte repopulation (n = 7) and group B patients who relapsed at B-lymphocyte repopulation (n = 6) at 3 to 4 months following BLDT (3-4 M),at the time of B-lymphocyte repopulation (repop) and at clinical relapse.

Relationship between serum BLyS levels and timing of clinical relapse

Among the 7 group A patients in whom clinical relapse occurred months (range 5-32 months) after B-lymphocyte repopulation, pre-treatment, 1-2 months and 3-4 months post-BLDT serum BLyS levels were no different from those in the Group B patients (n=6) in whom clinical relapse occurred at the time of B-lymphocyte repopulation (P = 0.445, P = 0.453 and P = 0.588, respectively, Mann-Whitney rank sum test). However, there was a striking difference in BLyS levels between Group A and B patients at the time of B-lymphocyte repopulation (figure 3.19) with levels being significantly higher in Group A (P = 0.018; median BLyS values 6.04 and 1.1 ng/ml in Groups A and B, respectively, Mann-Whitney rank sum test). At the point in time when Group A patients did experience clinical relapse, there was no significant difference between the two groups in serum BLyS levels (P = 0.299).

3.4 Summary

The main findings in this study were that:

(i) All seropositive RA patients had detectable IgM-RhF as expected. The presence of IgM-RhF was always associated with the presence of IgA-RhF and/or IgG-RhF;

(ii) Autoantibodies serum levels decreased in all patients following BLDT. This decrease was statistically significantly in patients who responded to treatment. Although IgM-RhF decreased to the same extent as serum total IgM, IgA-RhF, IgG-RhF, and IgG anti-CCP antibodies decreased significantly more than the corresponding immunoglobulin class;

(iii) Anti-TT antibodies decreased to the same extent as total IgG and anti-PCP antibodies did not decrease following BLDT;

(iv) In all except one patient, clinical relapse was preceded or occurred simultaneously, with an increase in serum levels of one or more disease-related autoantibodies;

(v) Serum levels of sCD23 decreased following BLDT and increased with B-lymphocyte repopulation;

(vi) Patients who responded to treatment showed a tendency to have higher percentage decreases from baseline in sCD23 serum levels when compared to patients who did not respond to treatment;

(vii) Serum BLyS levels rose markedly following BLDT and declined after re-emergence of B lymphocytes in the peripheral blood;

(viii) Serum BLyS levels at the time of B-lymphocyte repopulation were significantly lower in the group of patients who relapsed at the time of B-lymphocyte repopulation when compared to the group of patients who relapsed only later.

CHAPTER 4 RESULTS: DEPLETION AND RECONSTITUTION OF PERIPHERAL BLOOD B LYMPHOCYTES FOLLOWING RITUXIMAB

4.1 Study design

The results presented so far suggested that there was a quantitative threshold that needs to be reached in patients with RA for patients to respond to BLDT. In summary, the clinical studies showed that there was a dose response to rituximab and the serological studies showed that patients who did not respond to BLDT experienced smaller, non-significant decreases in autoantibodies levels. Patients were found to relapse clinically either at the time of B-lymphocyte return to the peripheral blood or at a varying time afterwards and, on re-treatment, patients tended to show the same pattern of relapse. The results were interpreted to mean that there might exist, qualitative and quantitative differences in the depletion itself that underline the differences in serological and clinical responses. There were no data available in the literature on the phenotype of B lymphocytes during depletion and on repopulation after B-lymphocyte depletion with rituximab in patients with lymphoma or autoimmune diseases.

It was therefore decided to study peripheral blood B-lymphocyte immunophenotypic changes associated with treatment with BLDT using three- and four-colour flow cytometry with combinations of different monoclonal antibodies. The objectives were to study:

(1) The degree of B-lymphocyte depletion in the peripheral blood in patients with RA treated with BLDT and whether differences between patients could be detected;

(2) Whether different circulating B-lymphocyte subpopulations had different sensitivities to depletion with rituximab;

(3) How patients repopulated following rituximab, whether from naïve B lymphocytes, memory B lymphocytes or a mixture of both;

(4) And whether any of these findings correlated with clinical response to treatment and particularly, time of relapse.

B-lymphocyte subpopulations were studied using a panel of monoclonal antibodies that allowed the distinction between naïve and memory B-lymphocyte phenotypes, the detection of cells with a plasma cell precursor phenotype, and also CD5 positive B lymphocytes. Some patients were studied at repopulation with a panel of monoclonal

antibodies allowing the distinction between immature and mature subsets of naïve B lymphocytes. Each patient served as her/his own control as data after B-lymphocyte repopulation was compared with data from before treatment. Data on peripheral blood T lymphocytes and natural killer (NK) cells was also collected.

4.2 Patients and methods

4.2.1 Patients

Twenty-four patients from the UCLH cohort entered the study with a total of 32 treatments (patients and treatments: 1a and b; 2b; 4d; 5a; 8a; 10a and b; 12c and d; 14a; 15a; 16a; 19a and b; 23a and b; 26a, b and c; 30 and 30a; 32 to 35; 36 and 36a; 37 to 40). UCLH Ethics Committee approved the study and all patients gave informed consent before entering the study. Fifteen patients were female, 9 were male. At study entry, mean age was 59 years (range 32 to 80) and mean disease duration was 20 years (range 5 to 42). More detailed patient characteristics are included in tables 2.1 and 2.2.

Whenever possible, patients were assessed before treatment, at one month after the first rituximab infusion, every two months until B-lymphocyte repopulation of the peripheral blood was first detected and every two to three months thereafter. All patients treated with BLDT at UCLH between September 2002 and July 2004 who gave consent entered the study. Patients treated before September 2002 who were still B-lymphocyte depleted, at the start of the study, were also included. At the end of the study in July 2004, a small number of patients had not yet repopulated. In summary, in 20 patients (22 treatments) data was available at baseline and at 1 and 3 months after treatment. Twenty-one patients (27 treatments) were studied during depletion and repopulation. Baseline data was available in 14 of these 21 patients (15 treatments). Courses of treatment for which data both at baseline and at repopulation were not available were not included in the analysis comparing B-lymphocyte subpopulations pre-treatment and at repopulation.

Most patients were treated with two infusions of 1000mg of rituximab given two weeks apart under steroid cover (100mg intravenous methylprednisolone with each infusion) (table 2.4). Eight patients continued oral methotrexate (patients 5, 16, 32, 33, 34, 36, 37,

39). Mean dose of methotrexate at baseline was 14mg (range 7.5 to 20). Fifteen patients had been treated with rituximab previously. Previous course of rituximab had been administered a median of 20 months before (range 8 to 46).

4.2.2 Flow cytometry

4.2.2.1 Principles of flow cytometry

Flow cytometry is a system for measuring and then analysing the signals that result as particles flow in a liquid stream through a beam of light. For each particle multiple variables can be measured including forward and side scatter of light and fluorescence signals emitted by reagents that have been allowed to bind to the particles before collection of data.

4.2.2.2 Sample preparation

Six millilitres of whole blood was collected by venepuncture in tubes containing EDTA (ethylenediaminetetraacetic acid; potassium salt). Sample preparation was carried out (and finished) within 7 hours after sample collection. A lysed whole-blood technique was used. Red cells were lysed by adding 30 ml of red cell lysis reagent (appendix I) to 6 ml of whole blood. The mixture was gently mixed and incubated for 6 minutes at room temperature. After centrifugation, the cell pellet was washed twice in phosphate-buffered saline (PBS) (appendix I) by centrifugation at 300xg for 5 minutes, and then re-suspended in cold PBS with 2% heat-inactivated foetal calf serum (appendix I). The cells were incubated with each monoclonal antibody combination at 4°C for 20 minutes (50μl of cell suspension containing approximately 10^6 cells with 20μl of each antibody or equivalent amount for 10^6 cells, as recommended by the manufacturers for the relevant batches; manual cell counting was done using a Neubauer counting chamber). The samples were subsequently washed twice in cold PBS by centrifugation at 300xg for 5 minutes. Subsequently, the cells were fixed by incubation with 50 μl of PBS with 2% paraformaldehyde (appendix I) for 5 minutes at room temperature. The samples were again washed twice by centrifugation at 300xg for 5 minutes, and re-suspended in 200 μl of cold PBS. Samples were kept protected from light and at 4° until data collection. Data collection was carried out either on the same day or the day after sample collection and preparation.

Cell viability was checked (in all samples) before incubation with the monoclonal antibodies, by the trypan blue assay. Twenty microliters of the cell suspension were mixed with 20 μl of trypan blue solution (0.4% in PBS) (appendix I) and left at room temperature for 3 minutes. Twenty microliters of the mixture were then applied in the Neubauer counting chamber. The cells were observed under a light microscope and viable cells (unstained) and dead cells (stained) were counted. Cell viability was more than 90%.

4.2.2.3 Monoclonal antibodies used

Three-colour immunophenotyping of B and T lymphocytes and NK cells in peripheral blood was performed using matched combinations of anti-human murine monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or phycoerythrin-cyanin 5 (PE-Cy5). For analysis of B lymphocytes, combinations of anti-IgD (FITC), anti-CD5 (PE-Cy5), anti-CD19 (PE), anti-CD20 (FITC), anti-CD27 (FITC) and anti-CD38 (PE-Cy5) were used (table 4.1). For analysis of T lymphocytes and NK cells, anti-CD3 (PE), anti-CD4 (FITC), anti-CD8 (FITC), anti-CD20 (FITC or PE-Cy5), anti-CD25 (PE-Cy5), anti-CD45RA (PE or PE-Cy5), anti-CD45RO (PE) and anti-CD56 (PE-Cy5) were used (table 4.1).

Four-colour immunophenotyping of B lymphocytes of some samples on repopulation was performed using matched combinations of anti-human murine monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll *a* complex protein (PerCP) or allophycocyanin (APC). Antibodies used included anti-IgD (PerCP), anti-CD10 (APC), anti-CD19 (PE or APC), anti-CD24 (PE), and anti-CD38 (FITC) (table 4.1).

All antibodies were purchased from Pharmingen (BD biosciences, San Diego, California). Table A in appendix II contains detailed data on the monoclonal antibodies used. Table B in appendix II contains details on fluorochromes used. Table C in appendix II characterizes the CD antigens identified by the monoclonal antibodies used.

Table 4.1 Combinations of monoclonal antibodies used to analyse lymphocyte subpopulations in the peripheral blood.

Lymphocyte population	Monoclonal antibodies			
B lymphocytes	**FITC**	**PE**	**PE-Cy5**	
	Anti-IgD	Anti-CD19	Anti-CD38	
	Anti-CD27	Anti-CD19	Anti-CD5	
	Anti-CD20	Anti-CD19	Anti-CD38	
	FITC	**PE**	**PerCP**	**APC**
	CD38	CD24	IgD	CD19
	CD38	CD19	IgD	CD10
T lymphocytes	**FITC**	**PE**	**PE-Cy5**	
	Anti-CD20	Anti-CD3	Anti-CD56	
	Anti-CD4	Anti-CD3	Anti-CD25	
	Anti-CD4	Anti-CD3	Anti-CD45RA	
	Anti-CD4	Anti-CD3	Anti-CD45RO	
	Anti-CD8	Anti-CD3	Anti-CD25	
	Anti-CD8	Anti-CD3	Anti-CD45RA	
	Anti-CD8	Anti-CD3	Anti-CD45RO	
	Anti-CD4	Anti-CD3	Anti-CD20	
	Anti-CD8	Anti-CD3	Anti-CD20	
NK cells	Anti-CD20	Anti-CD3	Anti-CD56	

Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; PE-Cy5, phycoerythrin-indodicarbocyanine, also known as Cy-chrome; PerCP, peridinin-chlorophyll *a* complex protein; APC, allophycocyanin.

4.2.2.4 Data acquisition

Data was acquired on a FACSCalibur flow cytometer using Cellquest software (BD Biosciences Immunocytometry Systems). Linear amplification was used for forward and side scatter of light signals. Logarithmic amplification was used for fluorochrome signals as it allows distinction between a wider range of intensities. Signal intensity and amplifier gain were adjusted in a two-parameter dot plot of forward versus side scatter of light to allow separation of the different cell populations present in peripheral blood so that a region of interest could be drawn on the lymphocyte population (figure 4.1). Lymphocytes are cells with a low to moderate forward scatter and low side scatter of light. The red cell lysing solution enabled a good separation of lymphocytes and red blood cell debris. The lymphocyte gate was drawn so that it would almost reach the monocyte region to allow inclusion of activated lymphocytes and plasma cell precursors, which are larger cells (figure 4.1).

Compensation for auto-fluorescence of cells was achieved using a sample with unstained cells and adjusting amplifier gains so that the signal coming from unstained cells would fall in the first decade of channels for all fluorochromes. Compensation for emission spectra overlap between the different fluorochromes used in combination was achieved using samples with cells labelled with only one of the directly conjugated monoclonal antibodies used. Two-parameter dot plots including the corresponding channel of the fluorochrome in question and each of the other channels used in the experiments, were used to change amplifier gain so that a sample with one fluorochrome would only give a positive signal in the appropriate channel. Compensation was based on signals obtained from the cells in the lymphocyte gate. During the period of B-lymphocyte depletion each patient sample was tested in parallel with another sample where B lymphocytes were present (patient or control).

For B-lymphocyte subpopulation analysis a minimum of 20,000 events were collected in the lymphocyte gate. For T-lymphocyte subpopulation and NK cell analysis a minimum of 10,000 events were collected in the same gate. Data on all cell populations was stored.

Lymphocyte gate in peripheral blood

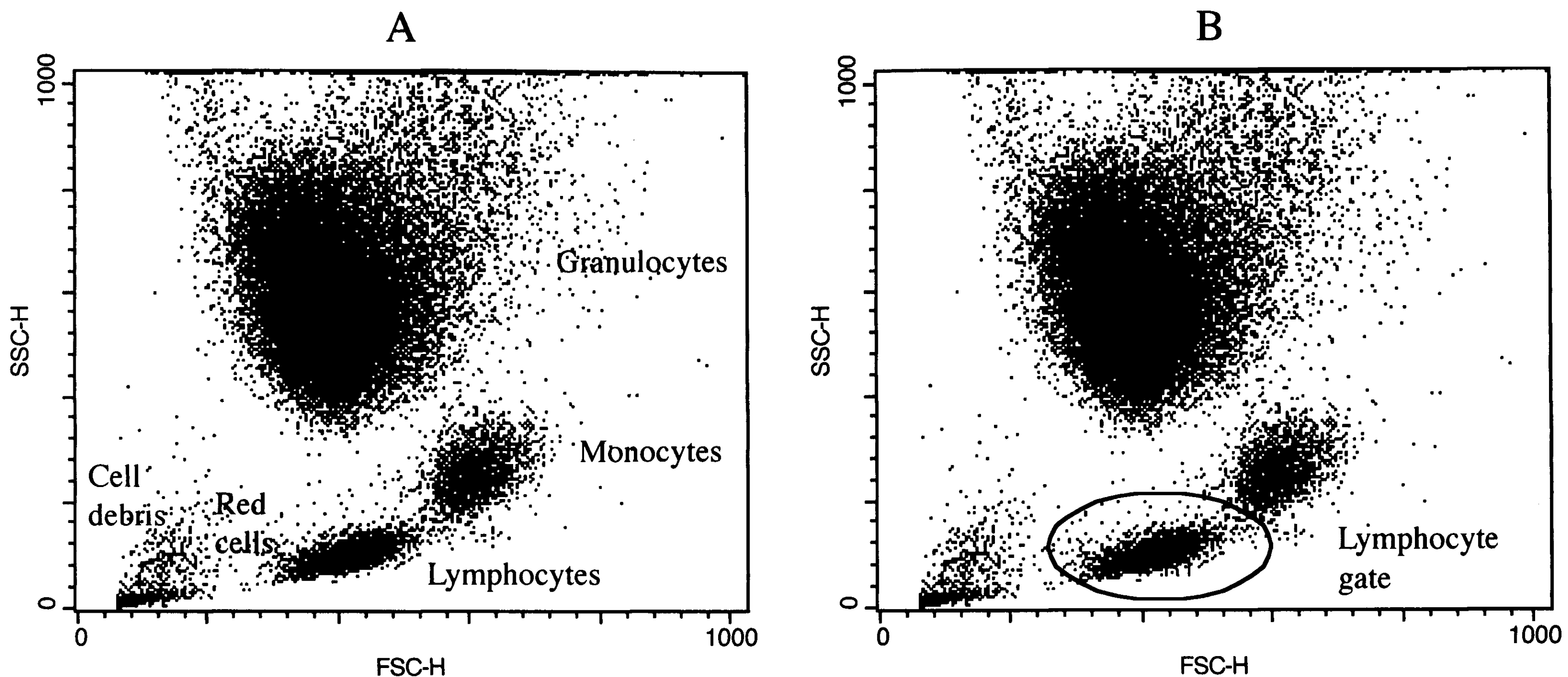


Figure 4.1 Dot plot of linear forward and linear side light scatter of peripheral blood after red blood cell lysis. A) The position of granulocytes, monocytes, lymphocytes, residual red cells and cell debris are indicated in the figure. B) Lymphocyte gate used in acquisition and analysis of data.

4.2.2.5 Data analysis

Data analysis was performed using Cellquest software (BD Biosciences Immunocytometry Systems) starting with manual gating around the lymphocyte population. Again, the lymphocyte gate was drawn so that it would almost reach the monocyte region to allow inclusion of larger cells (activated lymphocytes and plasma cell precursors) (figure 4.1). Results were expressed as percentages of B, T or NK lymphocytes positive for each marker. Absolute number values were derived using an absolute lymphocyte count performed at the UCLH Haematology Laboratory. As the lymphocyte gate was drawn larger to include activated lymphocytes and plasma cells precursors, the probability of including contaminants within the lymphocyte gate increased. As this can influence the calculated proportion of cells positive for one marker within the lymphocyte gate, the "lymphogram" (summation of the percentage of cells within the lymphocyte gate that were CD19+, CD3+ or CD56+) was calculated in each sample (Lowdell, 2001). The "Lymphogram" was 94% or above in almost all samples.

Isotype-control antibodies were not used routinely during the experiments. For markers that showed a clear separation between unstained and stained cells, a region of interest was drawn based on this distinction (cluster analysis) (Lowdell, 2001). These included CD3 and CD19 that characterise T and B lymphocytes, respectively (figure 4.2). For markers that showed a continuous distribution on the lymphocyte population studied, such as CD5 and CD27 on B lymphocytes and CD25, CD45RA and CD45RO on T lymphocytes, positive cells were classified based on the distinction between stained and unstained cells found in other lymphocyte populations not being specifically studied but included in the sample (figure 4.3). It was felt that this was better than comparing changes in fluorescence intensities for these markers within the population of interest as the samples were collected and analysed over a relatively long period of time. To correctly compare fluorescence intensities from samples from the same patient, obtained at different points in time, careful and frequent calibration of the flow cytometer would need to have been carried out (Lowdell, 2001). This was not practically possible. Isotype-control monoclonal antibodies were used in only a few samples to control for the way the regions of interest were drawn for some of the markers that showed a

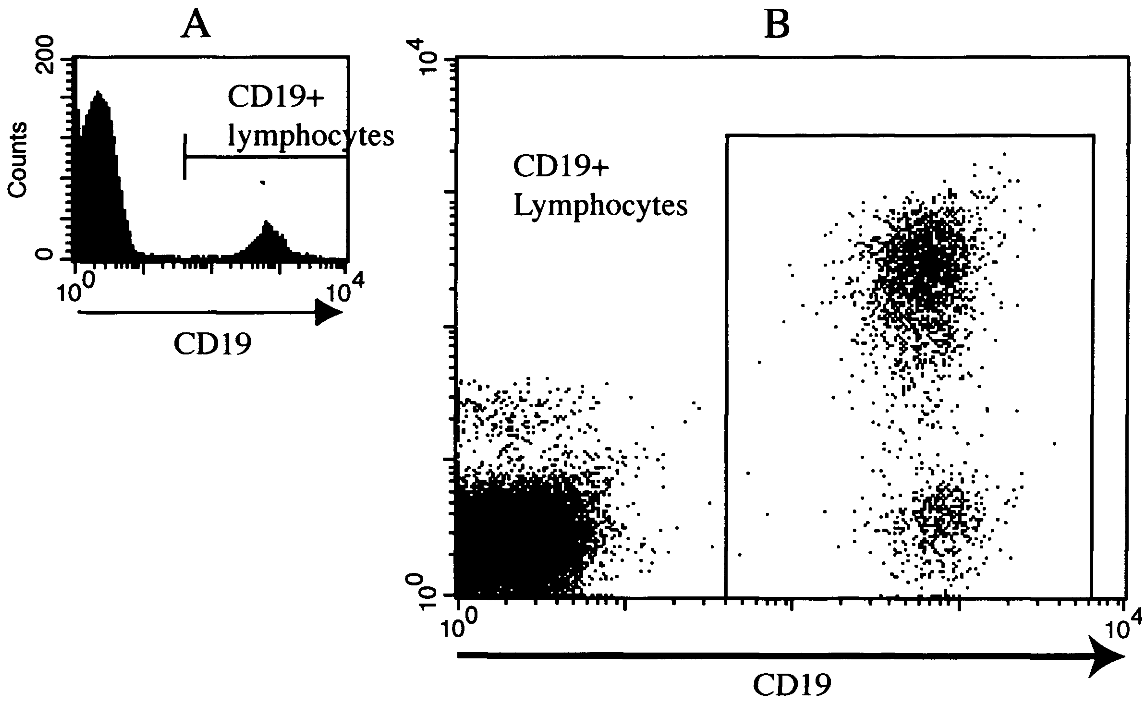


Figure 4.2 Use of "cluster" analysis to define CD19 positive cells in the lymphocyte gate. The negative lymphocyte population is used to discriminate between "positive" and "negative" events. A) One-parameter histogram showing CD19 negative and CD19 positive cells versus cell counts. B) Two-parameter dot plot of CD19 versus CD20 expression on cells in the lymphocyte gate showing CD19 negative and CD19 positive cells (region of interest).

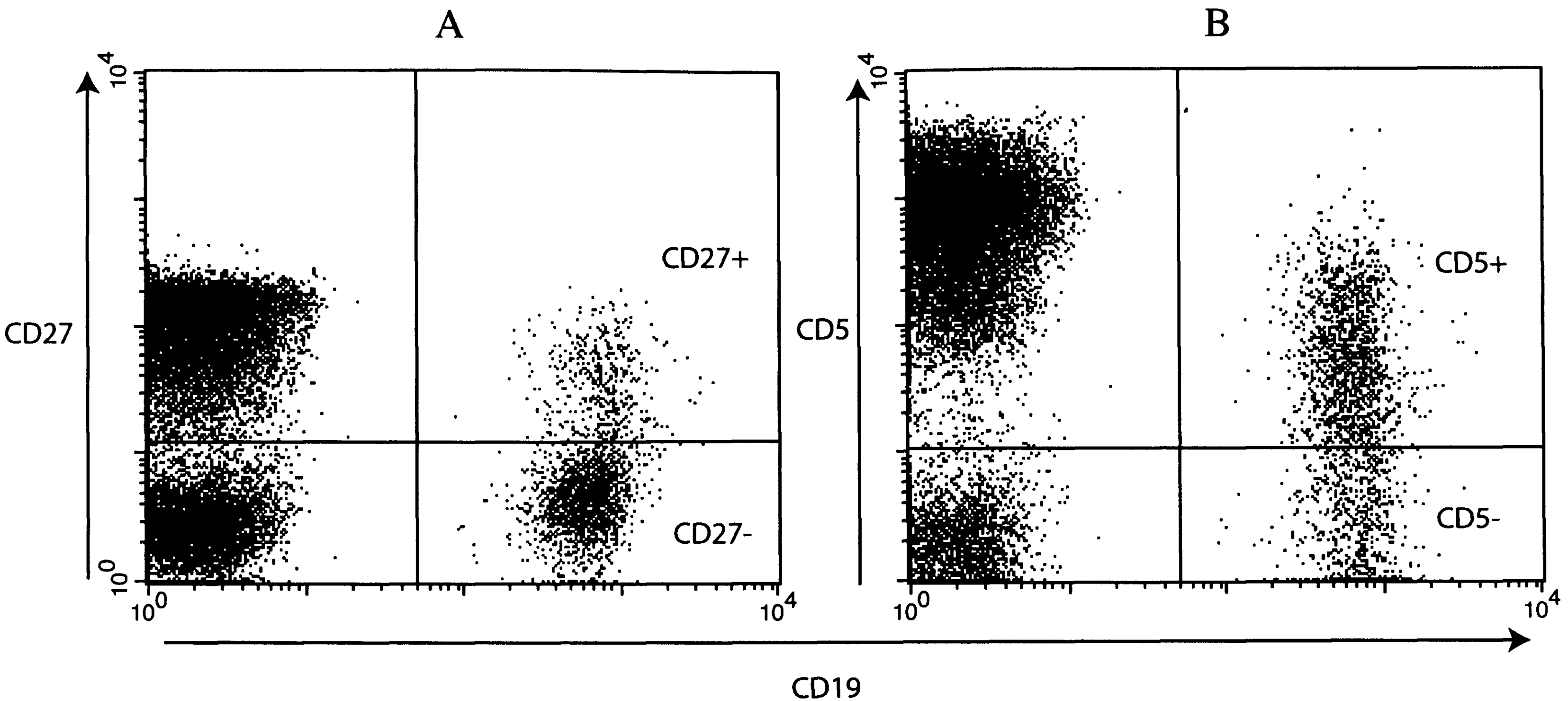


Figure 4.3 Example of drawing of region of interest to discriminate between CD19+ cells positive or negative for a second antibody when expression of this antibody on CD19+ cells shows a continuous distribution. The existence of "negative" and "positive" cell populations for the same antibody in the CD19- lymphocyte population was used for the analysis.

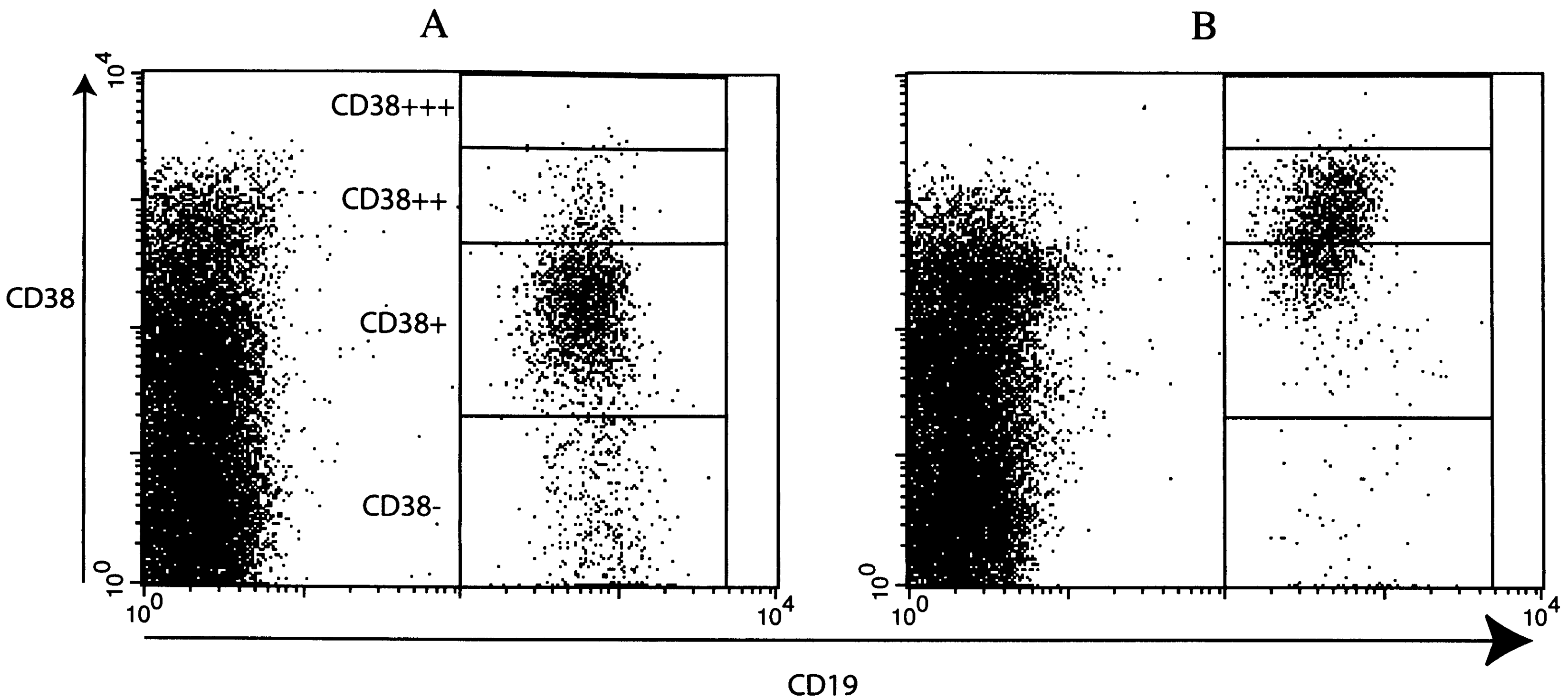


Figure 4.4 Drawing of region of interest to discriminate between CD19+ cells negative or positive for CD38 and between CD19+ cells expressing diferent degrees of CD38. A) Pre-treatment sample. B) Sample at the time of B-lymphocyte repopulation of the peripheral blood.

continuous distribution within the lymphocyte population studied (CD5 on B lymphocytes, CD25, CD45RA and CD45RO on T lymphocytes). Only in the case of CD38 expression on B lymphocytes, and despite the limitations discussed above, signal intensity was used to distinguish different subpopulations of CD19+ cells (B lymphocytes) that expressed different levels of the CD38 antigen and also cells that did not express the antigen (figure 4.4). Regions of interest were drawn based on this distinction and the same regions were used to describe the increase on CD38 expression seen at repopulation (figure 4.4). The CD19 negative lymphocyte population did not provide a clear separation between the different subpopulations with different degrees of CD38 expression and was therefore not helpful in this situation.

4.2.2.6 Quality control in flow cytometry

Calibration of the flow cytometer used in these experiments was carried out, by the personnel responsible, every 3 weeks during the time the data was being collected. As described before, a sample with unstained cells was used as a negative control to compensate for autofluorescence of cells and samples incubated with only one directly conjugated monoclonal antibody were used to compensate for overlap between the different fluorochromes spectra.

During the period of B-lymphocyte depletion each patient sample was tested in parallel with another sample where B lymphocytes were present to allow for compensation between the different antibodies used.

Inter-observer variation was nonexistent as only Maria Leandro collected and prepared the samples, collected and analysed the data. Subjective items like compensation and region of interest for analysis drawing were kept to the minimum possible, particularly by constant comparisons of data from the same patient and from patients studied on the same day.

4.2.3 Definition of lymphocyte immunophenotypes used

B lymphocytes

CD19 was used as a marker for all circulating B lymphocytes and plasma cell precursors within the lymphocyte gate (Macey, 2001). Expression of IgD versus CD38 on CD19+ cells was used to separate different B lymphocyte subpopulations (Pascual et al., 1994, Bohnhorst et al., 2001). IgD+CD38- cells include a mixture of naïve and memory cells (Klein et al., 1988). IgD+CD38+ cells were naïve cells. Other authors had previously classified IgD+CD38++ cells as circulating germinal centre founder cells (Pascual et al., 1994, Arce et al., 2001, Bohnhorst et al., 2001, Odendahl et al., 2003). IgD-CD38++/+++ cells were plasma cell precursors (Pascual et al., 1994, Bohnhorst et al., 2001, Medina et al., 2002). IgD-CD38+ cells were post-germinal memory cells and IgD-CD38- were resting memory cells (Pascual et al., 1994, Bohnhorst et al., 2001).

Expression of CD24 and CD10 was analysed on the different B-lymphocyte subpopulations defined by the expression of IgD versus CD38 to further characterise CD19+IgD+CD38++ cells (Galibert et al., 1996, Brady et al., 1999, Ingvarsson et al., 1999, Suzuki et al., 2001).

Expression of CD27 was used as a marker of memory B lymphocytes but high expression of CD27 was used as a marker of plasma cell precursors (Klein et al., 1998, Odendahl et al., 2003). CD5 expressing B lymphocytes were analysed. Circulating plasma cell precursors were defined as cells expressing CD19 that were IgD-CD38++/+++, CD27++ or CD20-CD38++/+++ (Horst et al., 2002, Medina et al., 2002, Odendahl et al., 2003).

T and NK lymphocytes

CD3 was used as a marker for all T lymphocytes within the lymphocyte gate (Macey, 2001). CD4 was used as a marker of helper cells and CD8 as a marker of cytotoxic cells (Janeway et al., 2001). CD25 was used as a marker of activated (CD25+) and regulatory (CD25++) cells (Wing et al., 2002). CD45RA was used as a marker of predominantly naïve cells and CD45RO as a marker of predominantly memory cells (Janeway et al., 2001). Cells expressing both CD3 and CD56 were described as NK-T lymphocytes (Janeway et al., 2001).

Cells within the lymphocyte gate expressing CD56 but not expressing CD3 were described as NK lymphocytes (Janeway et al., 2001).

Table 4.2 shows the different cell populations distinguished with the different monoclonal antibodies combinations.

Table 4.2 Identification of lymphocyte subpopulations based on their immunophenotype.

Cells	Subpopulation	Immunophenotype
B lymphocytes		CD19+
	Naive	CD19+CD27- CD19+IgD+CD38+/++
	Memory	CD19+CD27- CD19+IgD-CD38-/+
	Plasmablasts	CD19+CD20-CD38++/+++ CD19+IgD-CD38++/+++ CD19+CD27++
	Immature (transitional)	CD19+IgD+CD38++CD10+ CD19+IgD+CD38++CD24++
T lymphocytes		CD3+
	Helper	CD3+CD4+
	Cytotoxic	CD3+CD8+
	Activated	CD3+CD4+/8+
	"Regulatory"	CD3+CD4+CD25++
	Naïve	CD3+CD4+/8+CD45RA+
	Memory	CD3+CD4+/8+CD45RO+
NK cells		CD56+CD3-

4.2.4 Statistical analysis

Wilcoxon signed rank test was used to compare frequencies and total numbers of the different lymphocyte subpopulations before depletion and at repopulation in the same patients (paired data). Mann-Whitney rank sum test was used to compare different groups of patients (non-paired data). Correlations were determined by calculating

Spearman rank order correlation coefficient (data did not follow a normal distribution). P values equal to or less than 0.05 were considered statistically significant. All analysis were performed using GraphPad PRISM software (GraphPad Software, Inc., San Diego, CA, USA).

4.3 Results

4.3.1 Rituximab efficiently depleted all subpopulations of B lymphocytes in the peripheral blood

All patients except one (patient 8a) showed almost complete B-lymphocyte depletion in the peripheral blood for more than three months with CD19 counts decreasing a median of 97% to less than 1.4 x 10^6/L (figures 4.5 and 4.6). All patients who depleted well improved clinically following treatment achieving at least an ACR20 grade of improvement. Serum levels of CRP decreased from a median of 31 mg/l at baseline (range 8 to 101) to a median of 5 mg/l at nadir (range 1 to 18) (figure 4.7).

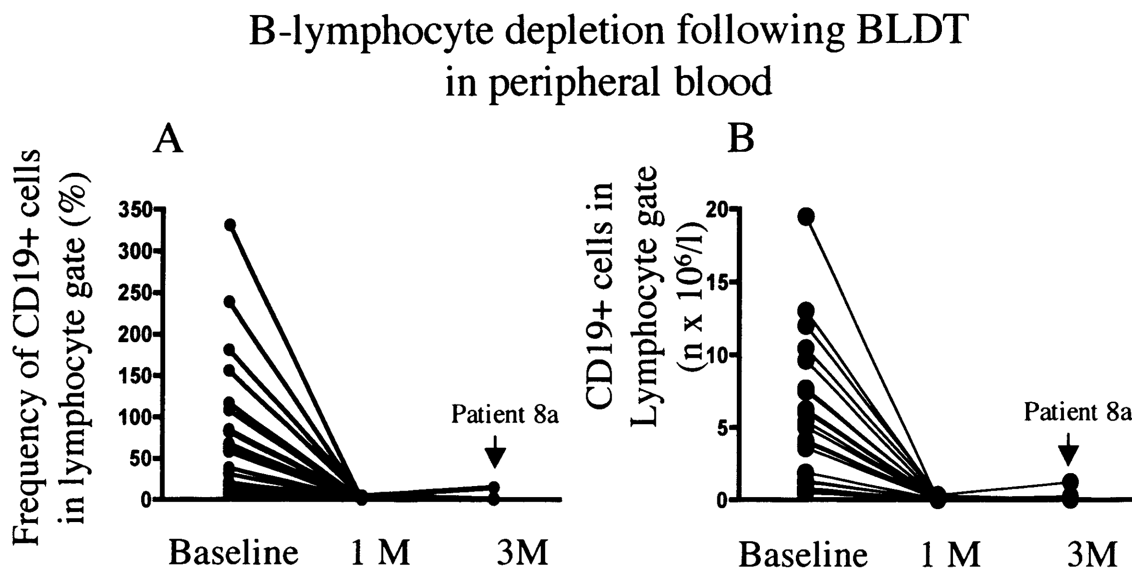


Figure 4.5 Depletion of B lymphocytes in the peripheral blood following BLDT. A) Total numbers of CD19 positive cells in the lymphocyte gate pre-treatment (baseline) and at 1 (1M) and 3 months (3M) after treatment (n =22; 20 patients).

B-lymphocyte depletion in the peripheral blood following BLDT

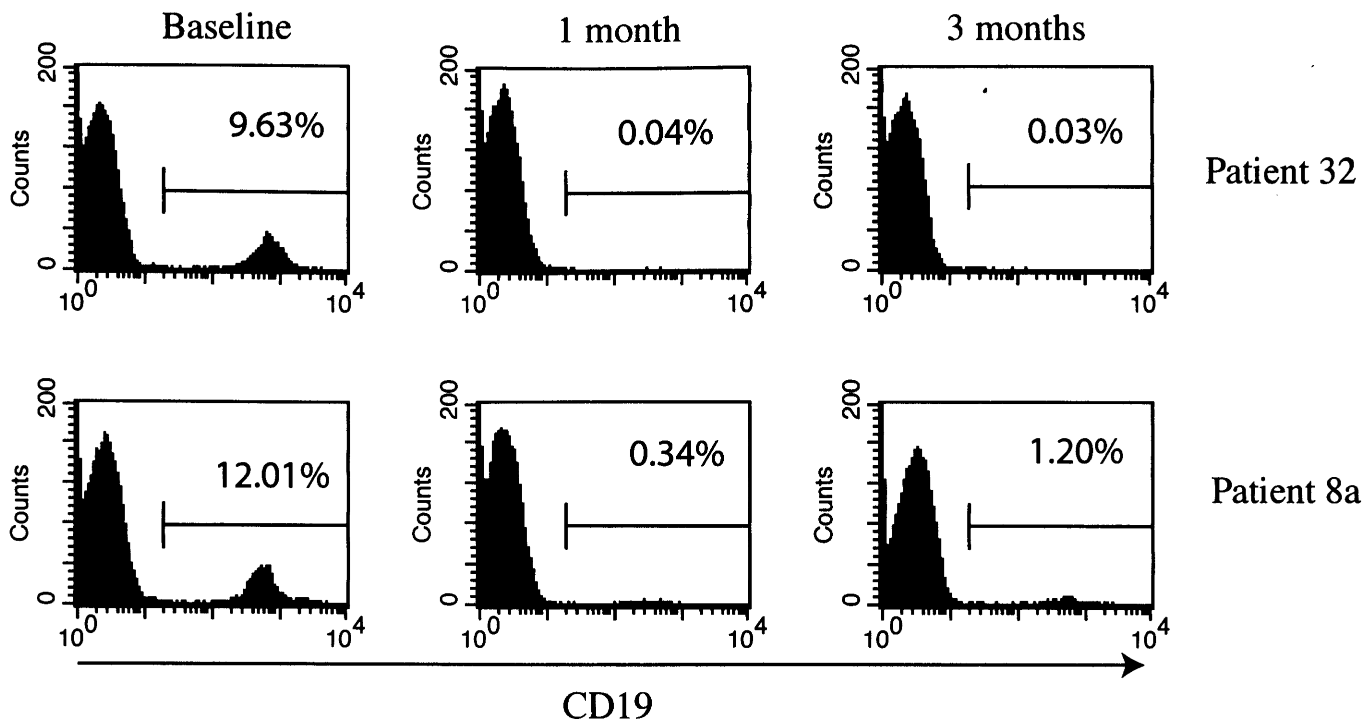


Figure 4.6 CD19 histograms pre-treatment (baseline) and at 1 and 3 months following BLDT in a representative patient who depleted well (patient 32) and in the patient who did not deplete well (patient 8a).

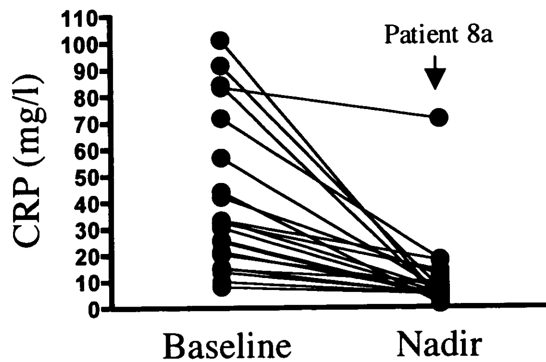


Figure 4.7 CRP serum levels at baseline and nadir following BLDT (n = 22; 20 patients).

Total CD19+ cells in the peripheral blood decreased from a median of 65.0 x 10^6/l (range 6.8-331.0) to a median of 0.7 x 10^6/l at 1 month (range 0.2-5.1) ($P < 0.001$, Wilcoxon signed rank test) after treatment. The frequency of CD19+ cells in the peripheral blood decreased from a median of 4.6% (range 0.5 to 19.5) at baseline to a median of 0.04% (range 0.01 to 0.34) at 1 month ($P < 0.001$, Wilcoxon signed rank test) after treatment. The total number and frequency of CD19+ cells did not change significantly between 1 and 3 months ($P = 0.77$ and $P = 0.98$, respectively, Wilcoxon signed rank test).

The total number of CD19+ cells in the peripheral blood pre-treatment was a median of 68.0 x 10^6/l (range 17.4-239.0) in the group of patients being treated with BLDT for the first time (n = 9) and a median of 39.0 x 10^6/l (121.4, range 6.8-331.0) in the group being retreated with BLDT (n = 11 patients; 13 treatments). The frequency of CD19+ cells was a median of 5.4% (range 0.8-9.6) in the group of patients being treated with BLDT for the first time and a median of 1.9% (range 0.5-19.5) in the group being retreated. The differences between the two groups at baseline were not statistically significant ($P = 0.3$ and $P = 0.6$ for frequency and total number of CD19+ cells, respectively, Mann-Whitney rank sum test). Depletion was similar in both groups of patients.

All subpopulations of B lymphocytes were depleted following treatment with rituximab (figure 4.8 A and B). During the period of depletion a very small number of CD19+ cells were detected (usually 5-20 cells per 20,000 cells in the lymphocyte gate). Approximately 80%, of this small number of cells, were IgD- and CD27+/++ (memory B lymphocyte or plasma cell precursor phenotype) (figure 4.8 III B).

During the period of depletion CD20 expression was absent or very low in the small number of circulating CD19+ cells detected, even in the ones expressing low levels or no CD38 and, therefore, not plasma cell precursors. CD20 expression was apparently normal in the patient that did not deplete well (patient 8a) (figure 4.9).

4.3.2 Repopulation occurred mainly from naïve B lymphocytes with an increased expression of CD38 and CD5

Repopulation occurred a mean of 8 months after treatment (range 5 to 13). Repopulation occurred mainly with naïve B lymphocytes (IgD+, CD27-) with increased expression of CD38 and CD5 (figure 4.8 A and C). When compared to baseline, the frequency of CD19+IgD+CD38++ cells increased from a median of 7.2% (range 0.2 to 29.3) to a median of 51.1% (range 15.3 to 79.3) ($P < 0.001$, Wilcoxon signed rank test) and the frequency of CD19+CD5+ cells increased from a median of 33.7% (range 20.4 to 67.1) to 78.7% (range 46.2 to 93.7) ($P = 0.001$, Wilcoxon signed rank test) (n = 14; patient 36a excluded as re-treated 1 month after B-lymphocyte repopulation) (figure 4.10). In the patient that did not deplete as expected (patient 8a) and who started repopulating at 2 months, the same pattern of increased expression of CD38 and CD5 was observed.

As previously discussed in the clinical studies, no patient relapsed before repopulation of the peripheral blood with CD19 positive cells started. In half of the cases (12 out of 24) patients relapsed at the time or shortly (within 2 months) after B-lymphocyte repopulation was first observed in the peripheral blood. In the other half, clinical relapse occurred at a variable time after B-lymphocyte repopulation (mean 8 months, range 5 to 12; 3 out of 12 patients in this group had not yet relapsed at the end of the study). Patients who relapsed at the time of B-lymphocyte repopulation tended to repopulate with a higher frequency of B lymphocytes with a memory phenotype (figure 4.11A).

B-lymphocyte immunophenotype pre-treatment and at repopulation

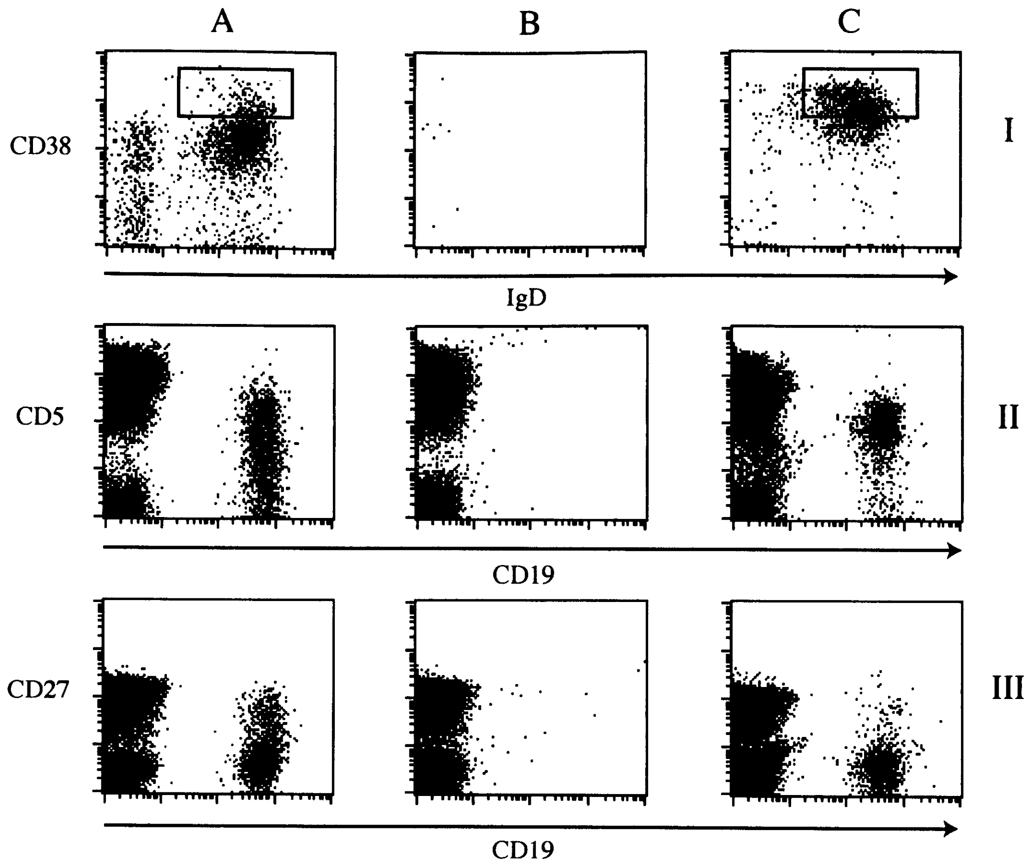


Figure 4.8 Dot plots of I) IgD versus CD38 expression on CD19 positive cells and of II) CD19 versus CD5 and III) CD19 versus CD27 expression on cells in the lymphocyte gate in a representative patient. A) Pre-treatment samples. B) Depletion samples at 3 months. C) Samples at repopulation.

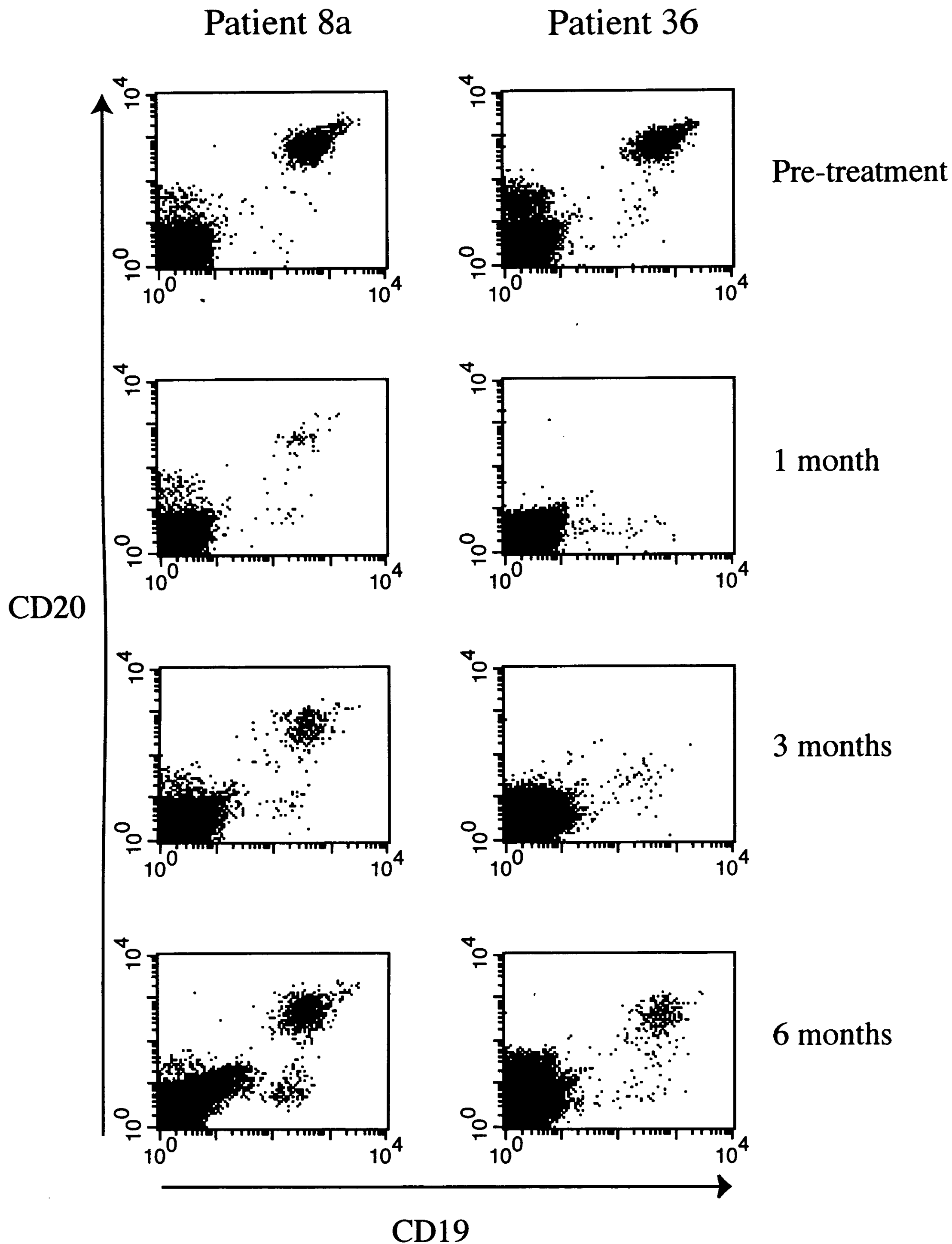


Figure 4.9 Dot plots of CD19 versus CD20 expression on cells in the lymphocyte gate pre-treatment and 1, 3 and 6 months following treatment with rituximab in a representative patient, patient 36a, and in patient 8a who did not deplete well. In patient 8a CD19 positive cells expressing CD20 can be detected at 1 and at 3 months. This was not observed in a representative patient, patient 36a.

Frequency of IgD+CD38++ and CD5+ B lymphocytes at repopulation

A

Frequency of IgD+CD38++ B lymphocytes (%)

100
80
60
40
20
0

P=0.0001

Baseline Repopulation

B

Frequency of CD5+ B lymphocytes (%)

100
80
60
40
20
0

P=0.001

Baseline Repopulation

Figure 4.10 Comparison between the frequency of specific subsets of CD19 positive cells (B lymphocytes) in the lymphocyte gate at baseline and at repopulation (n = 14). A) frequency of IgD+CD38++ B lymphocytes (%). B) frequency of CD5+ B lymphocytes (%). Lines represent median values.

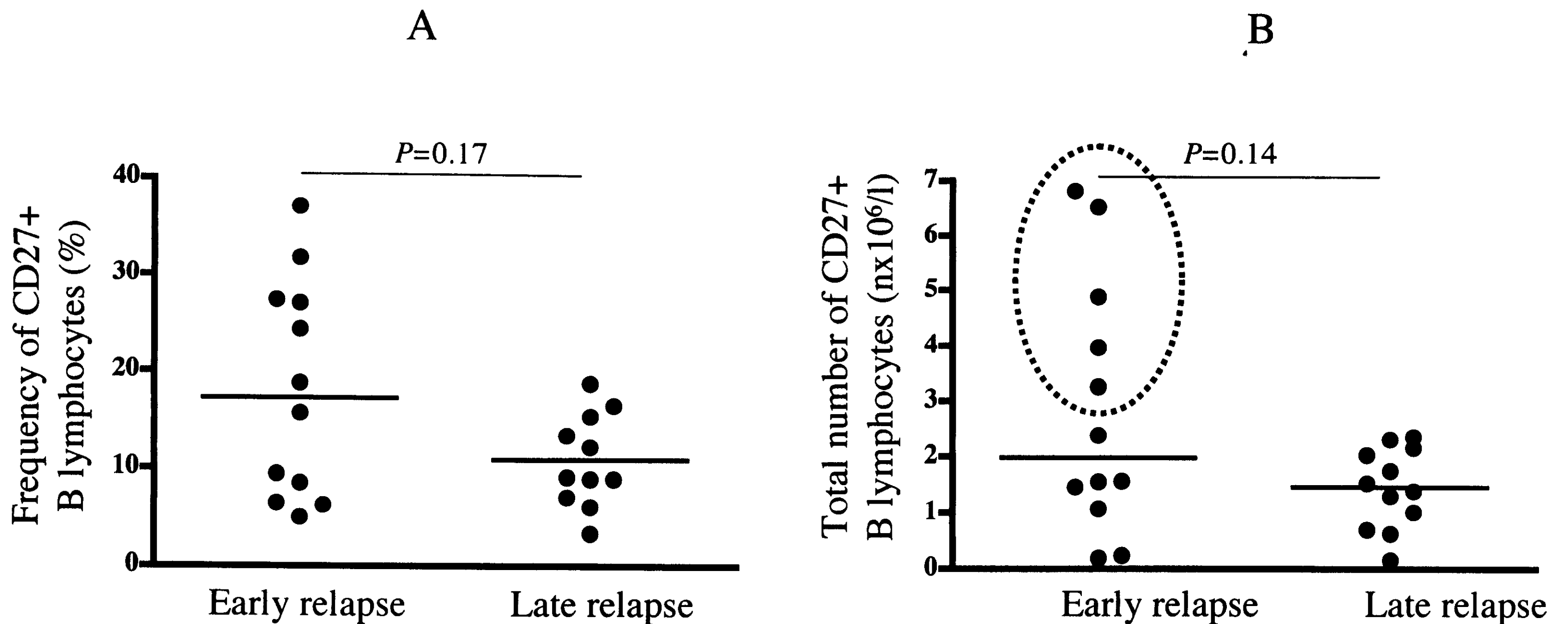


Figure 4.11 Comparison between the frequency and total numbers of CD27 positive B lymphocytes (memory B lymphocytes) in the patients who relapsed at B-lymphocyte return (early relapse) (n = 12) and the patients who relapsed only after B-lymphocyte repopulation (late relapse) (n = 12). A) Frequency of CD27+ B cells (%). B) Total numbers of CD27+ B cells (x10^6/L). Lines represent median values.

Patients who relapsed earlier showed a median of 17.3% CD27+ B lymphocytes (range 5.0 to 31.8), while patients who relapsed only later showed a median of 9.1% CD27+ B lymphocytes (range 3.3 to 18.8) (P = 0.17, Mann-Whitney rank sum test) at repopulation. Patients who relapsed earlier showed a median of 1.98 x 10^6/l CD27+ B lymphocytes (range 0.19 to 6.81), while patients who relapsed only later showed a median of 1.46 x 10^6/l CD27+ B lymphocytes (range 0.16 to 2.36) (P = 0.14, Mann-Whitney rank sum test) at repopulation (figure 4.11B). Eventhough, no statistically significant differences in median levels were found between the 2 groups patients who at repopulation showed memory B lymphocyte numbers higher than 3 x 10^6/L all relapsed at B-lymphocyte return to the peripheral blood (figure 4.11B).

When patients being first treated with rituximab were compared with patients being retreated, and when patients on concomitant methotrexate were compared with patients not on methotrexate, no differences in the pattern of B cell repopulation were observed, with similar frequencies of CD19+IgD+CD38++ and CD19+CD5+ cells in the different subgroups of patients.

Identification of circulating immature B lymphocytes at repopulation

CD19+IgD+CD38++ cells were identified as being immature naïve B lymphocytes based on their expression of low levels of CD10 and high levels of CD24 (figure 4.12). Although median levels of expression for IgD in CD19+IgD+CD38++ cells were usually slightly lower than for CD19+IgD+CD38+ in the same sample, in the majority of the immature cells (CD19+IgD+CD38++) the level of IgD expression was similar to that found on mature naïve B cells (CD19+IgD+CD38+ cells) (figure 4.13).

Repopulation occurred at different rates in different individuals

Total numbers and frequency of CD19+ cells in the lymphocyte gate returned to the normal range very quickly (within one month of repopulation) in a few patients but, more frequently, took several months (figure 4.14).

Twenty-one of the patients were followed up for a period longer than one year after treatment. In eight of these 21 patients high CD19 counts were detected one to four years after treatment. CD19+ cell frequencies from the central haematology laboratory

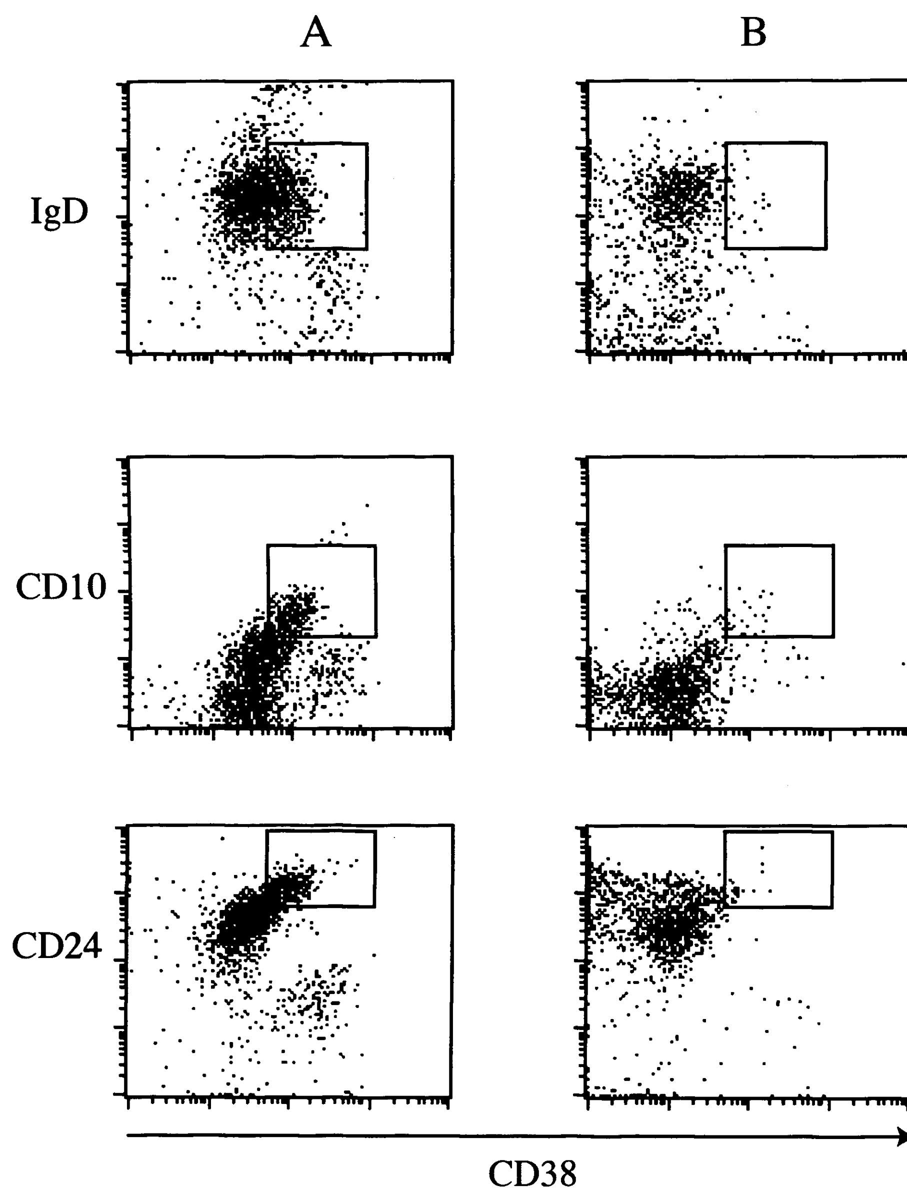


Figure 4.12 Phenotype of immature B lymphocytes at repopulation. Expression of IgD, CD10 and CD24 versus CD38 on CD19 positive cells (B lymphocytes) in A) a patient with rheumatoid arthritis at repopulation and in B) a normal control. Immature B cells are IgD+CD38++CD10+CD24high (square region).

IgD level of expression on immature B lymphocytes at repopulation

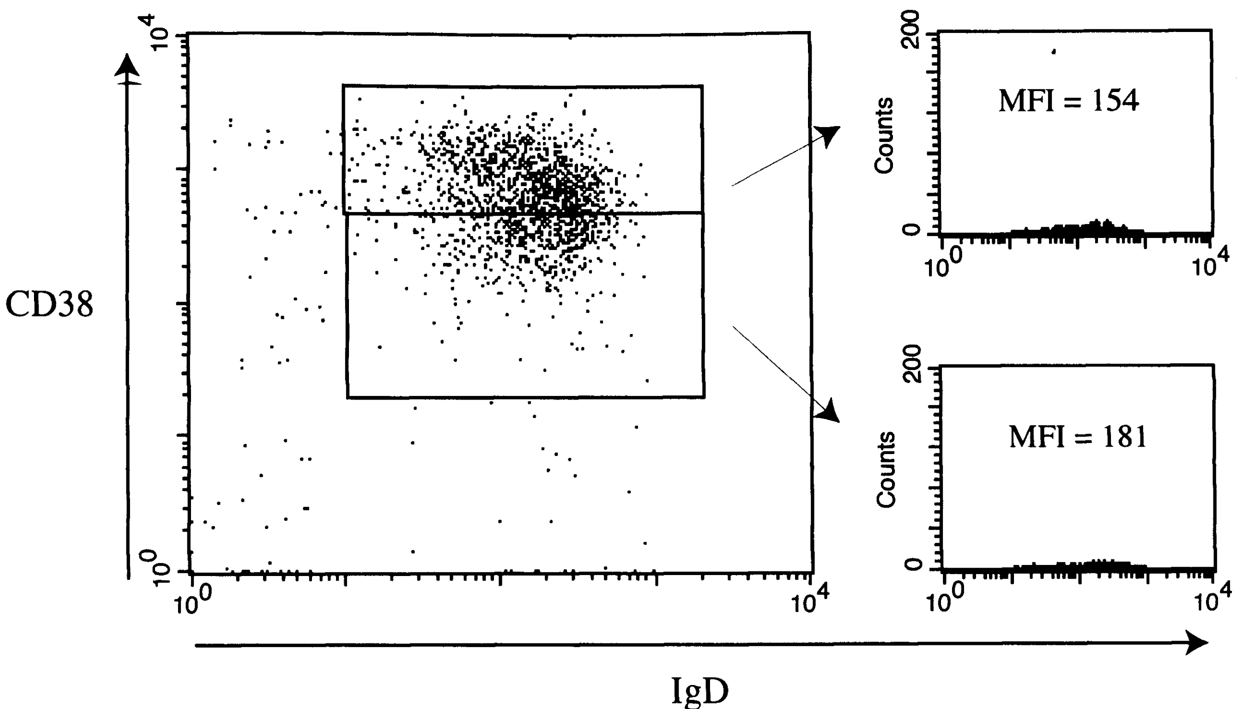


Figure 4.13 Comparison between level of IgD expression between IgD+CD38++ (immature/transitional) and IgD+CD38+ B lymphocytes at repopulation following BLDT.

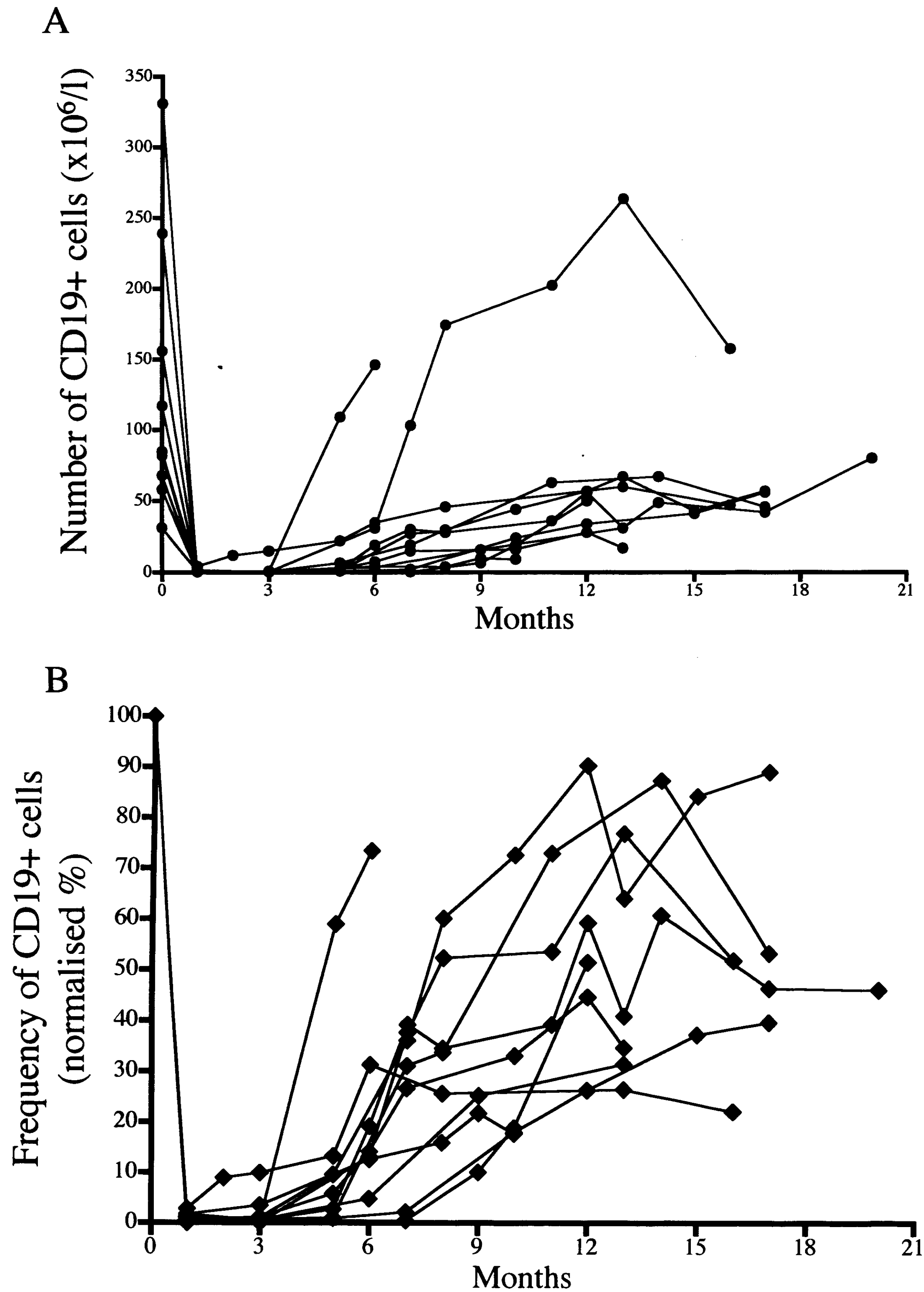


Figure 4.14 Recovery of CD19 positive cells (B lymphocytes) following BLDT (n = 11). A) Total number of CD19+ cells in the lymphocyte gate (n x 10^6/l). B) Normalised frequency of CD19+ cells in the lymphocyte gate following BLDT (results following treatment expressed as a percentage of pre-treatment values).

from these patients at the same time points showed frequencies above the normal range (greater than 15%).

In the majority of patients during repopulation, frequency of CD19+CD5+ cells followed the same course as frequency of CD19+IgD+CD38++ cells but more CD19+ cells expressed CD5+ than IgD and moderate levels of CD38 (figure 4.15). In some patients, the increased expression of CD38 by naïve B lymphocytes (CD19+IgD+ cells) seemed to return to pre-treatment levels earlier than the increased expression of CD5 by circulating B lymphocytes (CD19+CD5+ cells) (figure 4.16).

The frequency and particularly the total numbers of memory B lymphocytes (CD27+CD19+ cells) remained below baseline values for longer periods in several patients (figure 4.17). These changes over time after repopulation did not appear to be dependent on the total number of circulating B lymphocytes or on the total number of cells of other subsets.

In several patients an increased frequency of plasma cell precursors could be found at repopulation (figure 4.18). This could be present only the first time that repopulation was detected or persist for a variable time afterwards. No association between increased circulating plasmablasts and time of relapse was found.

In two patients that were immunised with tetanus toxoid (secondary immunisation) after repopulation and before retreatment, the expected transient increase in the frequency of circulating plasmablasts was detected one week after immunisation (figure 4.19).

4.3.3 A small proportion of T lymphocytes and of NK cells expressed low levels of CD20 and were depleted by rituximab

A small proportion of T lymphocytes (CD3+ cells) and of NK cells (CD56+CD3- cells) in the peripheral blood expressed low levels of CD20 (figure 4.20A). Treatment with rituximab depleted both these subpopulations (figure 4.20). CD3+CD20low cells decreased from a median of 3.2% (range 0.4 to 20.8) at baseline to a a median of 0.02% (range 0.0% to 0.8%) ($p < 0.001$, Wilcoxon signed rank test) 1 month after treatment.

CD5+ and IgD+CD38++ B lymphocytes following repopulation I

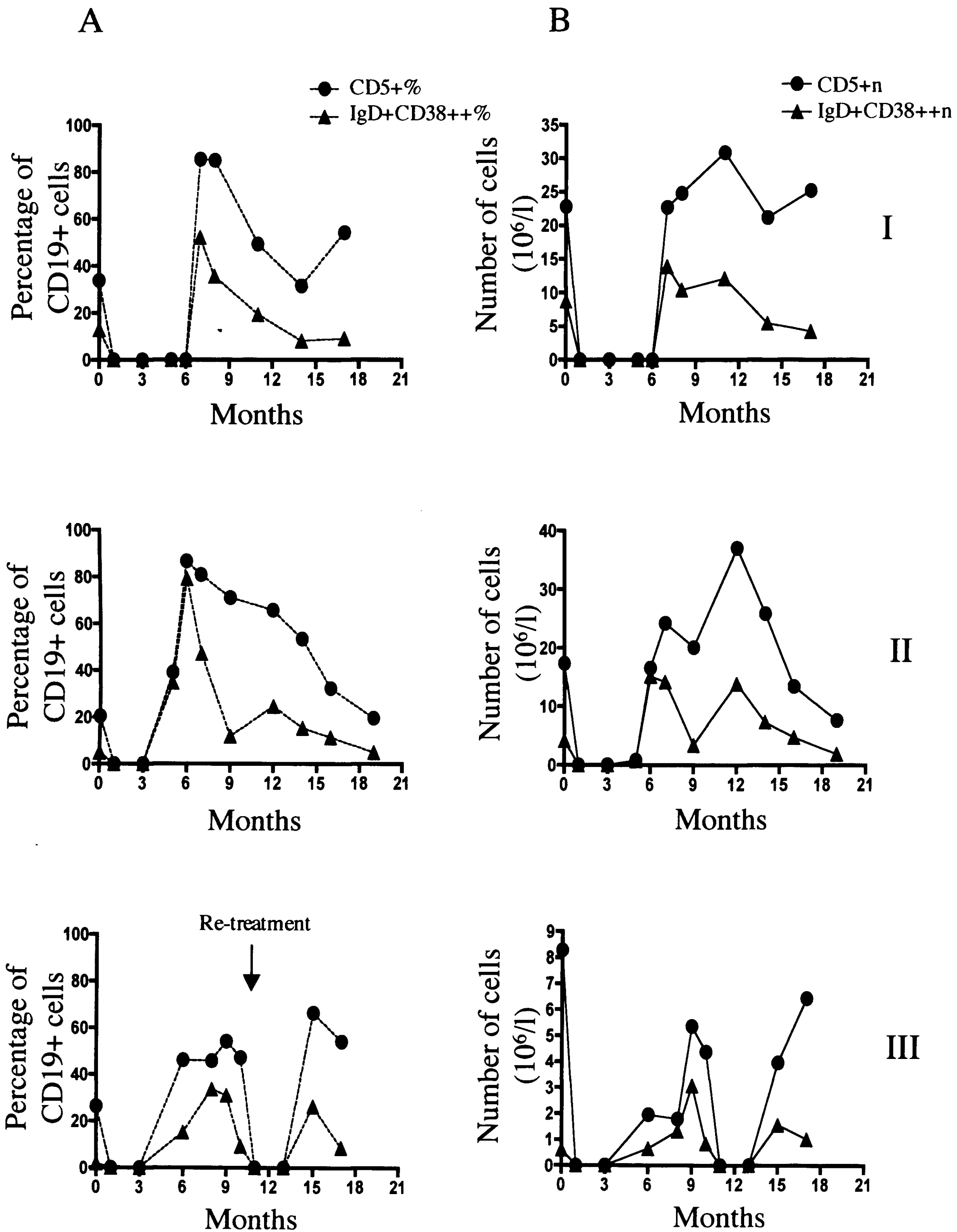


Figure 4.15 Changes over time in A) frequency and B) total numbers of CD5+ and IgD+CD38++ B lymphocytes following BLDT. I) Patient 33. II) Patient 30. III) Patient 36.

CD5+ and IgD+CD38++ B lymphocytes following repopulation II

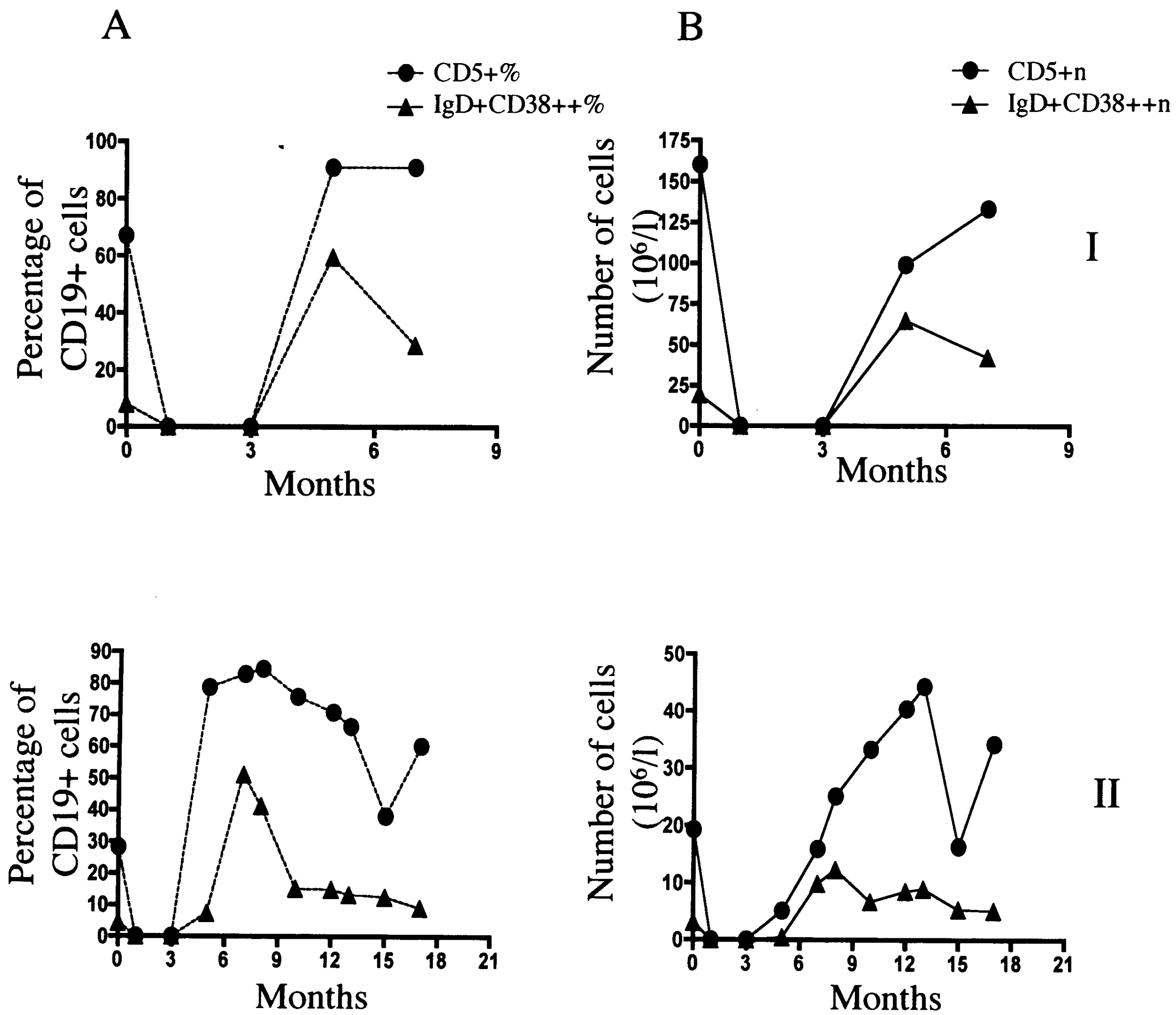


Figure 4.16 Changes over time in A) frequency and B) total numbers of CD5+ and IgD+CD38++ B lymphocytes following BLDT. I) Patient 32. II) Patient 35.

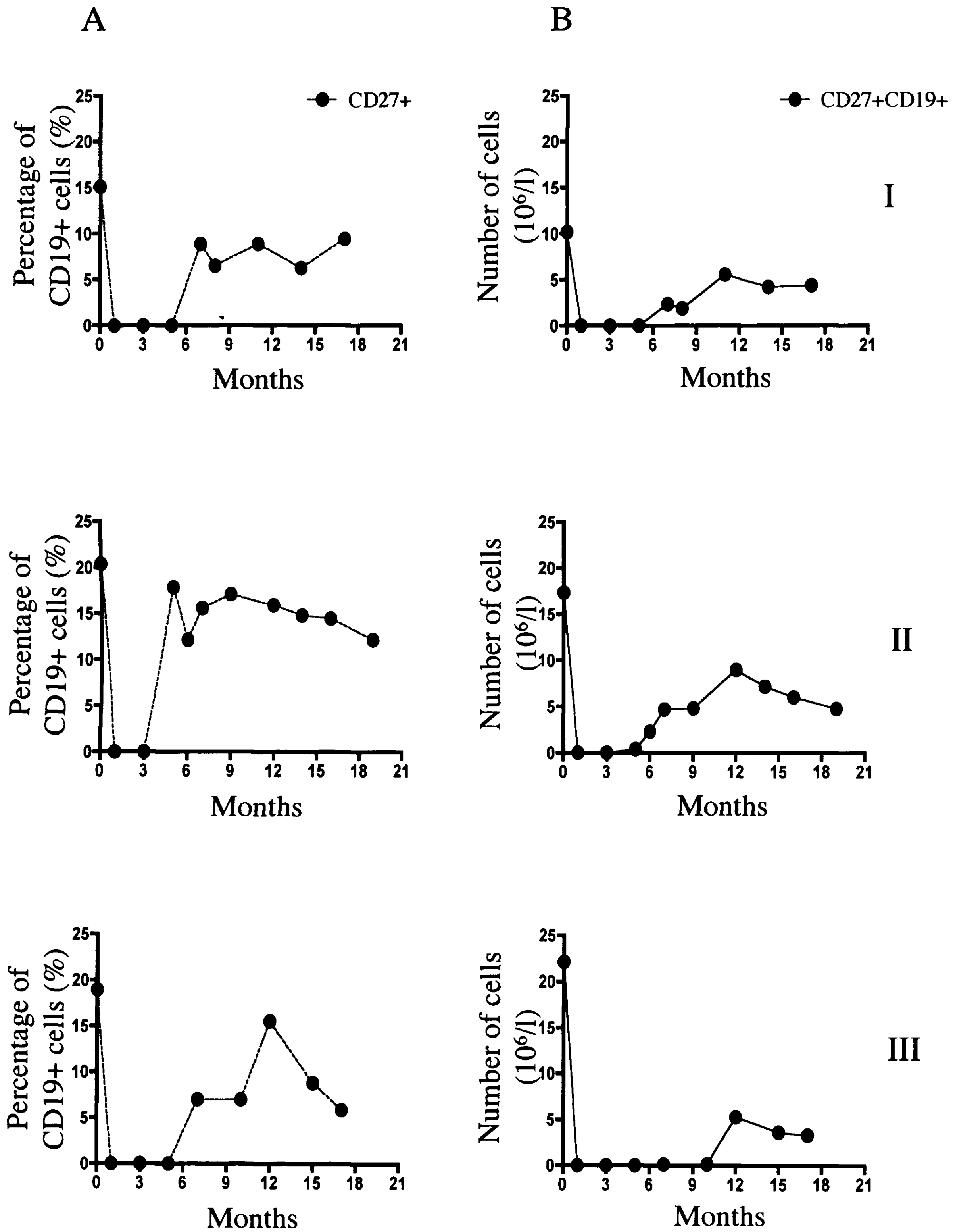


Figure 4.17 Changes over time in A) frequency and B) total numbers of CD27+ B lymphocytes following BLDT. I) Patient 33. II) Patient 30. III) Patient 34.

Plasma cell precursors at repopulation

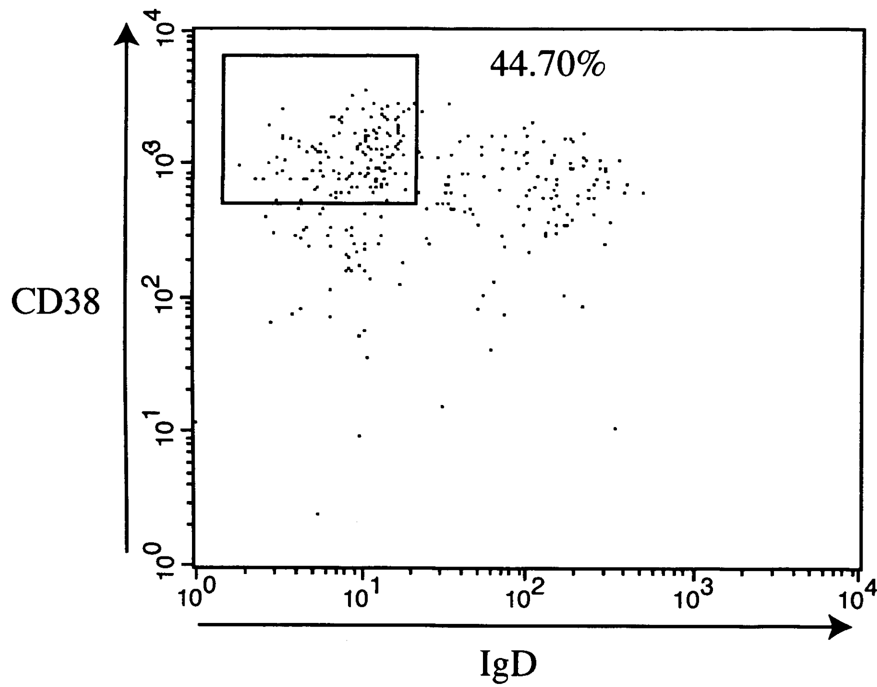


Figure 4.18 Increased frequency of circulating plasma cell precursors at repopulation (patient 30).

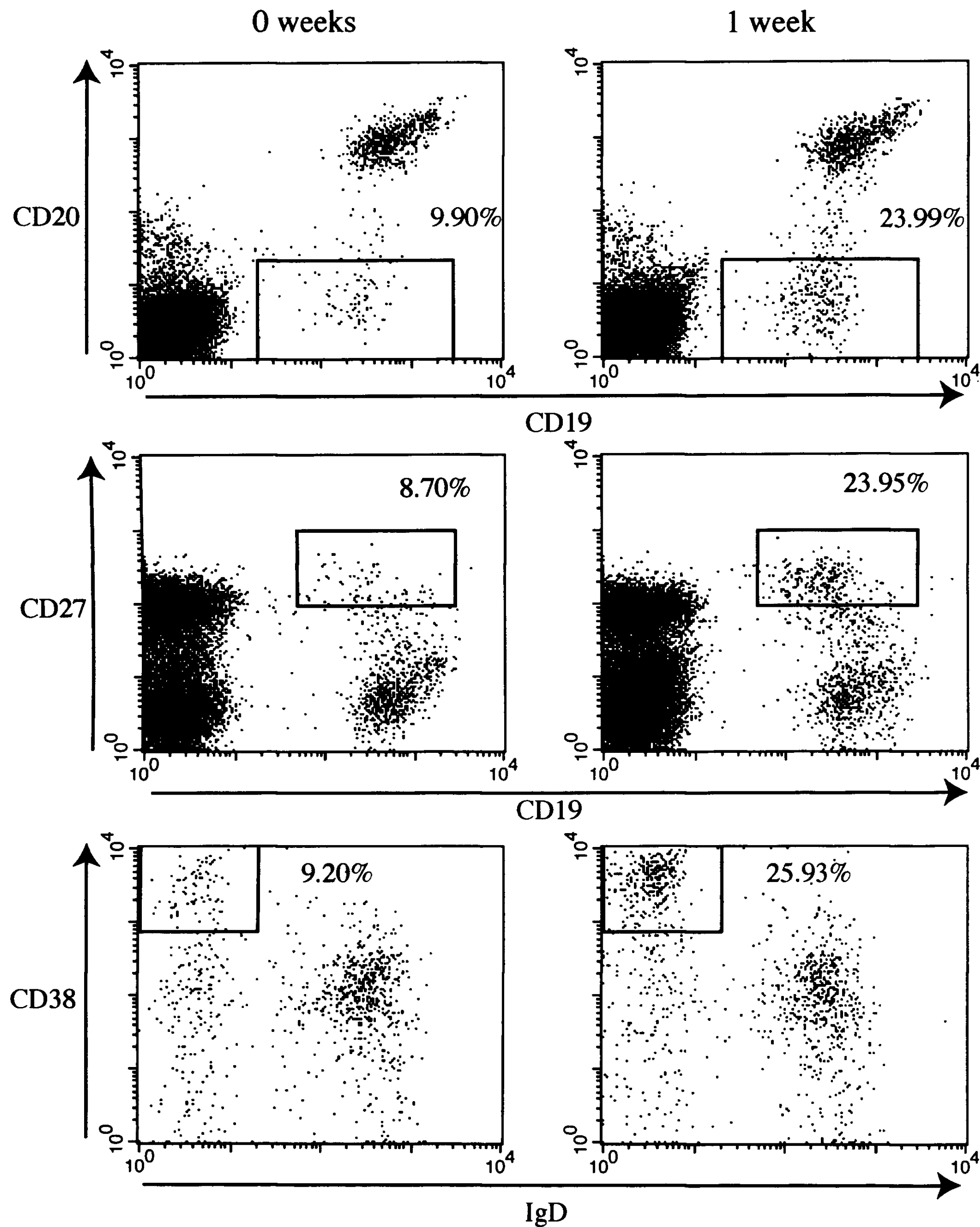


Figure 4.19 Increased frequency of circulating plasma cell precursors one week after immunisation with tetanus toxoid vaccine (patient 10).

Depletion of CD20+ T lymphocytes and NK cells

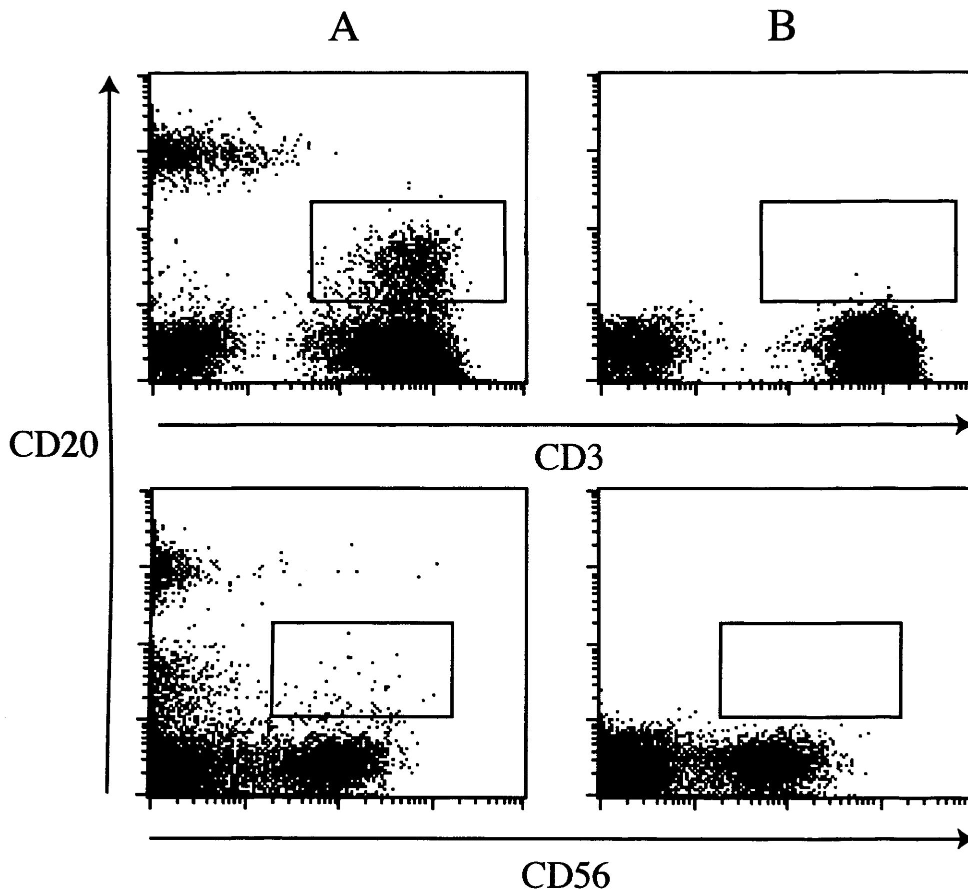


Figure 4.20 Depletion of CD3 positive and CD56 positive cells expressing low levels of CD20 in patients with rheumatoid arthritis following treatment with rituximab. A) Baseline. B) Repopulation. CD56 positive cells do not include CD3+CD56+ cells.

CD56+CD3-CD20low cells decreased from a median of 1.7% (range 0.2 to 14.2) at baseline to a median of 0.1% (range 0.0 to 1.5) ($P < 0.001$, Wilcoxon signed rank test), 1 month after treatment. Repopulation of both these subpopulations occurred before B-lymphocyte return, at a mean of 5 months. Studies in a small number of patients after repopulation showed that CD3+CD20low cells could express CD4 (more frequently), CD8 or the NK marker CD56 (less frequently).

Other T lymphocyte subpopulations and NK cells did not change significantly following treatment with rituximab

Frequency and absolute numbers of T lymphocytes (CD3+) and of NK cells (CD56+CD3-) did not change significantly 1 month and 3 months after rituximab.

No significant differences were detected in different subpopulations of T lymphocytes (CD4+ cells, CD8+ cells, CD4+ cells expressing CD25 (CD25+), CD4+ or CD8+ cells expressing CD45RA, CD45RO or both markers) except for the subpopulation that expressed low levels of CD20 described above. Frequency of CD25++ CD4 positive T lymphocytes decreased 1 month after treatment ($P < 0.05$, Wilcoxon signed rank test) but total numbers did not, and no significant differences were found at 3 months.

4.4 Summary

The study of immunophenotypic changes on circulating B lymphocytes following treatment with rituximab in patients with RA showed that the majority of patients achieved major depletion of B lymphocytes in the peripheral blood. The only patient who did not respond to treatment in this cohort did not deplete as well as the others.

All circulating B lymphocyte subpopulations were depleted by rituximab but during depletion a small number of cells could be detected, usually expressing a memory or plasma cell precursor immunophenotype.

B-lymphocyte repopulation of the peripheral blood occurred mainly with naïve B lymphocytes showing increased expression of CD5 and an immature phenotype with increased expression of CD38 and CD24 and expression of CD10.

Patients did not relapse before return of B lymphocytes to the peripheral blood. Patients who relapsed at the time of B-lymphocyte repopulation tended to show increased frequency and total numbers of memory B lymphocytes at repopulation when compared with patients who relapsed only later.

The data suggested that there was a quantitative threshold that needed to be achieved for BLDT to be effective in the treatment of RA. It also demonstrated that repopulation of the peripheral blood was dependent on formation of naïve B lymphocytes. The tendency to show increased frequency and total numbers of memory B lymphocytes at repopulation from patients who relapsed at or near the time of B-lymphocyte return when compared with patients who relapsed only later suggested that there might be quantitative and or qualitative differences between the degree of depletion achieved in solid lymphoid tissues in different patients that influence the time of relapse.

CHAPTER 5 RESULTS: BONE MARROW B-LINEAGE CELLS FOLLOWING RITUXIMAB

5.1 Study design

In the peripheral blood study described in the previous chapter, it was observed that patients who relapsed at the time of B lymphocyte repopulation tended to repopulate with higher frequencies of memory B lymphocytes. These cells might have been residual B lymphocytes not depleted by rituximab suggesting that these patients may have depleted less well than the patients who relapsed only several months to years after B-lymphocyte return to the peripheral blood. As no major differences were found between the degree of B-lymphocyte depletion in the peripheral blood following treatment and at the time of relapse, the peripheral blood data could be explained by differences in the degree of depletion in solid tissues. Very little published human data existed on degree of depletion of normal B lymphocytes in solid lymphoid tissues and animal studies had shown that B-lymphocyte depletion in these tissues was not complete and that it varied between individuals even if treated with the same dose of rituximab (Reff et al., 1994).

It was felt justified to look into the degree of B-lymphocyte depletion in solid lymphoid tissues in patients who had benefited substantially from rituximab, had relapsed and were being retreated. Bone marrow was chosen because of its accessibility and because it might provide additional information on mechanisms of B-lymphocyte repopulation following treatment with rituximab. Treatment with rituximab is followed by depletion of B lymphocytes in the peripheral blood lasting usually six to nine months (McLaughlin et al., 1998). The reason for such prolonged period of depletion is not known. Stem cells and the earlier B-lymphocyte precursors, pro-B lymphocytes, do not express CD20 and therefore should not be depleted (Reff et al., 1994). Repopulation of the peripheral blood after chemotherapy (bone marrow ablating protocols) occurs within the first three to four months after treatment (Witherspoon et al., 1981, Zintl et al., 1989, Storek et al., 1993). The prolonged period of depletion after rituximab might be due to availability of rituximab not allowing full development of B-lymphocyte precursors or to some other effect on precursor B-lymphocyte subpopulations. It was hoped that the study of bone marrow samples after treatment with rituximab could provide some useful information on why repopulation takes so long.

The objective was to collect qualitative data on the depletion of cells of B-lymphocyte lineage in the bone marrow following treatment with rituximab, looking for the presence of CD20- and CD20+ B-lymphocyte precursors, mature recirculating B lymphocytes and also plasma cells using four-colour flow cytometry. The three months after treatment time point was chosen to collect the samples as it was thought that at this time point rituximab would have been mostly cleared from the circulation and from the tissues and repopulation would not have started yet. A drug half-life of approximately 9 days had been described in patients with lymphoma treated with rituximab (Berinstein et al., 1998).

5.2 Patients and methods

5.2.1 Patients

Six patients from the UCL cohort (patients 1b, 14a, 19b, 26b, 30a, 36a) entered this study. UCLH Ethics Committee approved the study and all patients gave informed consent before entering the study. Three patients were male, three female. Mean age was 57 years (range 37 to 62 years) and mean disease duration was 19 years (range 10 to 27 years) at study entry. Details of individual patients at entry to the study and other clinical characteristics are given in table 5.1. Other details incuding treatment protocol are included in tables 2.1, 2.2 and 2.4.

All patients were being re-treated with rituximab, a mean of 23 months after their previous treatment (range 10-46). Patients were assessed before treatment, at one and at three months after treatment. Before treatment and at one month, whole peripheral blood was collected for B-lymphocyte immunophenotyping. Three months after treatment, a bone marrow aspirate was collected. A peripheral blood sample was collected at the same time of the bone marrow aspirate to evaluate the degree of depletion of B-lymphocyte subpopulations in the peripheral blood and help in excluding significant contamination of the bone marrow sample with peripheral blood. A bone marrow sample available from a lymphoma patient treated two years before with rituximab, with no evidence of disease at the time of sampling and full peripheral blood B cell repopulation, was used for qualitative comparison with the rheumatoid arthritis patients as an index of the B-lymphocyte lineage profile expected at full reconstitution.

Table 5.1. Patients' clinical characteristics.

Patient	1 (36a)	2 (1b)	3 (26b)	4 (14a)	5 (19b)	6 (30a)
Sex	Male	Female	Male	Female	Female	Male
Age at study entry (years)	64	62	57	37	58	58
Disease duration at study entry (years)	12	30	9	21	13	26
Date of previous treatment with rituximab (months)	11	26	13	45	23	21
Concomitant immunosupressive treatment	M	-	-	-	-	-
Time to B cell repopulation (months)	5	8	11	10	11	6
Time to clinical relapse (months)	5	18	11	11	>15	>13

Abbreviations: M, methotrexate; a, first retreatment; b, second retreatment.

5.2.2 Flow cytometry

5.2.2.1 Sample preparation

Twelve milliliters of bone marrow aspirate were collected in tubes containing EDTA. Sample preparation was carried out within 2 hours after sample collection. The bone marrow aspirate was diluted 3:1 with PBS with 20% heat-inactivated foetal calf serum (appendix I). Red cells were lysed, by adding 30 ml of red cell lysis buffer (appendix I) to 6 ml of the diluted bone marrow aspirate sample. The mixture was gently mixed and incubated for 6 minutes at room temperature. After centrifugation, the cell pellet was sieved, washed twice in PBS by centrifugation at 300 x g for 5 minutes and then re-suspended on cold PBS with 2% heat-inactivated fetal calf serum (appendix I). The

cells were incubated with each monoclonal antibody combination at 4°C for 20 minutes (50μl of cell suspension containing approximately 10^6 cells with 20μl of each antibody or equivalent amount for 10^6 cells, as recommended by the manufacturers for the relevant batches). The samples were subsequently washed twice in cold PBS by centrifugation at 300 x g for 5 minutes. The cells were then fixed by incubation with 50 μl of PBS with 2% paraformaldehyde (appendix I) for 5 minutes at room temperature. The cells were washed twice by centrifugation at 300 x g for 5 minutes, and re-suspended in 200 μl of cold PBS. The samples were kept protected from light and at 4° until data analysis and collection. Data collection was carried out either on the same day or the day after sample collection and preparation. Cell viability was checked before incubation with antibodies, by trypan blue assay (as described in previous chapter) and was more than 90%. Peripheral blood samples were prepared using the same technique except that they were not diluted before red cell lysis (as described under methods on Chapter 4).

5.2.2.2 Antibodies used

Four-colour immunophenotyping of cells of B-lymphocyte lineage in bone marrow aspirate was performed using matched combinations of anti-human antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-Texas RedR (PE-TR), peridinin-chlorophyll a complex (PerCP) or allophycocyanin (APC). Antibodies to κ and λ light chains were anti-human goat polyclonal antibodies provided as $F(ab')_2$ fragments. All other antibodies used were anti-human monoclonal antibodies. Combinations of anti-κ (FITC), anti-λ (FITC), anti-CD5 (FITC), anti-CD10 (APC), anti-CD19 (PE and APC), anti-CD20 (FITC and PerCP), anti-CD27 (PE), anti-CD34 (PerCP), anti-CD38 (APC) and anti-CD138 (FITC) were used (table 5.2). For analysis of B lymphocytes in the peripheral blood, combinations of anti-IgD (FITC), anti-CD19 (PE), anti-CD20 (FITC), anti-CD27 (FITC) and anti-CD38 (PE-Cy5) were used (table 4.1). All antibodies were purchased from Pharmingen (BD biosciences, San Diego, California). More detailed characteristics of the monoclonal antibodies used are included in table A in appendix II.

Table 5.2. Antibody panel used for analysis of cells of B-lymphocyte lineage in bone marrow aspirates.

FITC	PE	PerCP	APC
sLC (κ + λ)	CD19	CD34	CD10
CD20	CD19	CD34	CD10
CD20	CD27	sIgD	CD19
CD138	CD19	CD20	CD38
CD5	CD19	CD20	CD10

Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin-chlorophyll *a* complex protein; APC, allophycocyanin; CD, cluster of differentiation antigens; sLC, surface light chain; sIgD, surface immunoglobulin D.

Characterization of the CD antigens used for study of B lymphocyte lineage in the bone marrow aspirates is included in table C in appendix II. Absortion and emission spectra for the fluorochromes used are included in table B in appendix II.

5.2.2.3 Data acquisition / collection

Data was acquired on a FACSCalibur (BD Biosciences Immunocytometry Systems) flow cytometer. Cellquest software was used (BD Biosciences Immunocytometry Systems). The same principles and techniques described in chapter 4 were used in this study. In bone marrow samples a region of interest was drawn around the lymphoid population defined by a low to moderate forward-angle and low right-angle light-scattering properties (figure 5.1A). As can be seen in the figure separation of lymphoid cells in two different subpopulations was seen. It is possible that this separation reflected different cell sizes but an artefact caused by handling of the sample could not be excluded. Data was acquired until 40,000 events were collected in the lymphoid gate. Data on all cell populations was stored. Peripheral blood samples were acquired as in chapter 4.

Lymphoid and plasma cell gates for bone marrow analysis

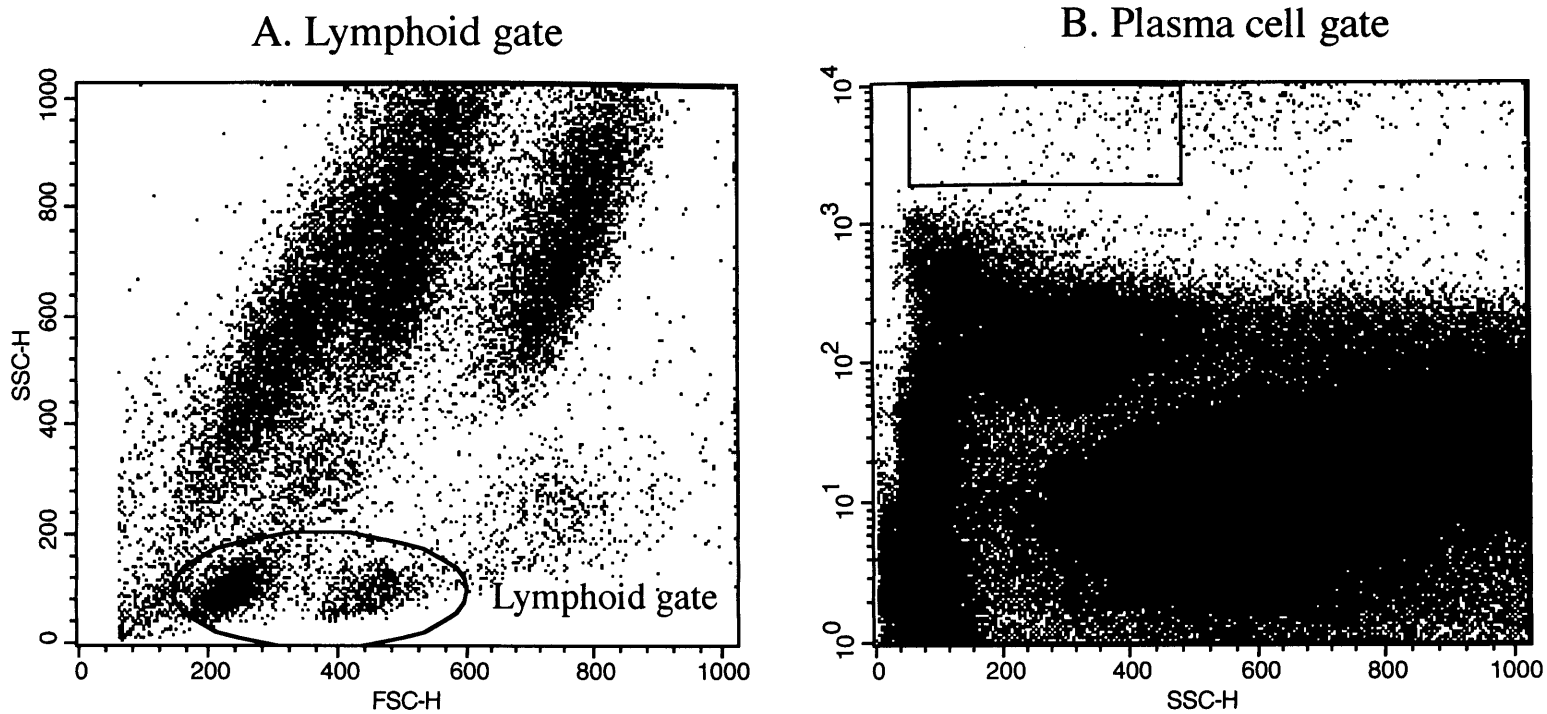


Figure 5.1 Lymphoid and plasma cell gates used in the analysis of bone marrow samples. A) Lymphoid gate. B) plasma cell gate.

5.2.2.4 Data analysis

Data analysis was performed using Cellquest software (BD Biosciences Immunocytometry Systems) starting with manual gating around the lymphoid population for all subpopulations except for plasma cells (figure 5.1A). For plasma cells a different gate was used to include all cells that expressed high levels of CD38 and had the expected right-angle light scattering properties (figure 5.1B) (Pellat-Deceunynck et al., 2004, Schneider et al., 1997). Results were expressed both as proportion of positive cells in total events and, where relevant, proportion of positive cells in the lymphoid gate and total number of positive cells per 40,000 events in the lymphoid gate.

The same principles applied in the analysis of the peripheral blood data were applied in the analysis of the bone marrow data.

Pro-B lymphocytes were defined as CD19+CD10+CD34+λL/κL- cells, pre-B lymphocytes as CD19+CD10+CD34-λL/κL- cells, immature B lymphocytes as CD19+CD10+CD34-λL/κL+ cells and mature B lymphocytes as CD19+CD10-CD34-λL/κL+ cells, all within the lymphoid gate (Pontvert-Delucq et al., 1993, Ghia et al., 1996, Ghia et al., 1998, Loken et al., 1987, Dworzak et al., 1997, McKenna et al., 2001). Plasma cells were defined as CD19+/-CD138+CD38+++ cells gated for the expected right-angle light scatter properties (Pellat-Deceunynck et al., 2004, Schneider et al., 1997).

Peripheral samples were collected and analysed and results expressed as described under methods in chapter 4.

5.2.2.5 Quality control in flow cytometry

Calibration of the flow cytometer used was carried out by the personal responsible every 3 weeks during the time the data was being collected. As described before, a sample with unstained cells was used as a negative control to compensate for autofluorescence of cells and samples incubated with only one directly conjugated monoclonal antibody were used to compensate for overlap between the different fluorochromes spectra.

During the period of B-lymphocyte depletion each peripheral blood patient sample was tested in parallel with another sample where B lymphocytes were present to allow for compensation between the different antibodies used.

Inter-observer variation was nonexistent as only Maria Leandro collected and prepared the samples, collected and analysed the data. Subjective items like compensation and region of interest for analysis drawing were kept to the minimum possible.

5.2.2.6 Statistical analysis

Limited descriptive statistics of results (median and range) were used given the small number of samples. Correlations were determined by calculating Spearman rank correlation coefficient.

5.3 Results

5.3.1 B-lymphocyte depletion in peripheral blood

Total numbers of peripheral blood B lymphocytes decreased a median of 97% (range 84.7 to 99.5) at 1 month and a median of 99% (range 98.6 to 99.8) at 3 months following treatment with rituximab. At three months, peripheral blood samples from all patients remained depleted of B lymphocytes, showing a median of 0.75 x 10^6/L CD19+ cells. More than 75% of the very small number of cells observed expressed a memory or plasma cell precursor phenotype (CD27 +/++) (table 5.3).

5.3.2 Presence of cells of B-lymphocyte lineage in bone marrow aspirates three months after treatment with rituximab

Despite similar degrees of depletion in the peripheral blood at 3 months, the frequency of cells of B-lymphocyte lineage in bone marrow aspirates varied between patients (table 5.3, figure 5.2A). Frequency of CD19+ cells in the lymphoid gate varied from 0.1 to 3.3% (median 1.0). Patient 2 showed almost total absence of CD19+ cells (0.1%) while patients 1 and 3 to 6 showed signs of B-lymphocyte development to different

Table 5.3. Detailed findings on bone marrow sample and peripheral blood analysis 3 months after rituximab therapy.

Patient		1	2‡	3	4	5	6
Bone marrow							
CD34+ cells							
	N/40,000*	899	642	493	638	769	1943
	%*	2.74	1.61	1.23	1.59	1.92	4.86
	%**	0.22	0.07	0.09	0.23	0.15	0.41
CD19+							
	N/40,000*	914	42	304	530	199	1299
	%*	2.28	0.1	0.76	1.32	0.5	3.25
	%**	0.20	0.003	0.05	0.19	0.04	0.26
Pro-B lymphocytes							
	N/40,000*	297	0	89	122	130	780
	%*	0.67	0	0.22	0.30	0.25	2.02
	%**	0.07	0	0.002	0.04	0.02	0.16
	%***	29.6	0	29.4	22.9	50.43	62.01
Pre-B lymphocytes							
	N/40,000*	178	7	192	372	52	292
	%*	0.40	0.03	0.48	0.92	0.1	0.75
	%**	0.04	0.0009	0.03	0.13	0.08	0.06
	%***	17.74	29	63.5	69.84	20.09	23.22
Immature B lymphocytes							
	N/40,000*	444	3	15	42	23	160
	%*	1.01	0.01	0.04	0.10	0.04	0.41
	%**	0.1	0.0004	0.003	0.01	0.004	0.03
	%***	44.27	14	5.04	7.89	8.97	12.68
Mature B lymphocytes							
	N/40,000*	147	9	0	37	10	30
	%*	0.34	0.04	0	0.09	0.02	0.09

%**	0.03	0.001	0	0.01	0.002	0.006
%***	14.7	38	0	6.88	3.85	2.35
Plasma cells						
Number of cells**	1850	218	60	153	64	109
%**	0.36	0.02	0.01	0.05	0.01	0.02
Total of events collected (mean)	521067	816865	554867	231419	629643	627238
% of total events in lymphoid gate (mean)	8.46	4.19	8.05	14.48	7.90	7.95
CD34+% in total of events collected	0.22	0.07	0.09	0.23	0.15	0.41
CD34+% in lymphoid gate	2.74	1.61	1.23	1.59	1.92	4.86
CD19+ % in total of events collected	0.2	0.003	0.05	0.19	0.04	0.26
CD19+% in lymphoid gate	2.28	0.1	0.76	1.32	0.5	3.25
CD20/3/56						
Total events	324120	243300	260115	126285	358605	280785
% in lymphoid gate	8.69	4.26	8.46	16.81	7.64	8.93
						(25070)
CD3+% in lymphoid gate	47.72	75.03	53.64	55.06	72.24	78.68
CD56+% in lymphoid gate	38.52	16.31	23.69	31.14	10.52	8.41
Peripheral blood						
CD19+ cells						
Number of cells x 10^6/L	1.1	0.56	0.7	0.8	0.25	1.41
%****	0.2	0.06	0.06	0.06	0.02	0.07
CD27+CD19+/CD19+ cells (%)	76	90	77	94	83	100

*in the lymphoid gate; **in the total of events collected; ***of CD19 positive cells in lymphoid gate; **** in the lymphocyte gate; ‡Percentages of different B lineage subpopulations less reliable as based on a very small number of cells.

Frequency of cells of B-lymphocyte lineage in bone marrow samples following Rituximab

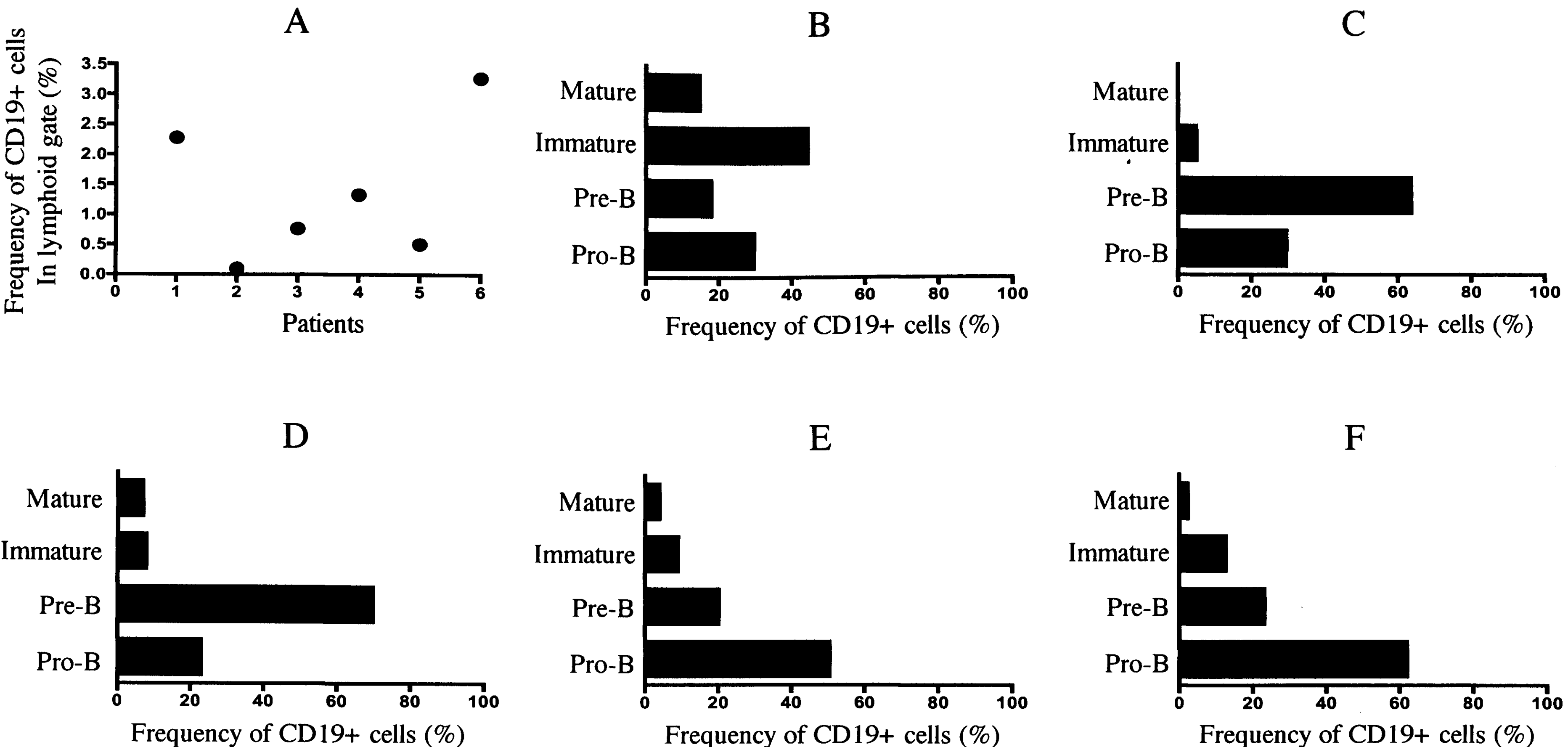


Figure 5.2 Proportion of B lineage cells in the lymphoid gate and phenotype of B lymphocytes and B-lymphocytel precursors in different samples. A) Proportion of CD19 positive cells in the lymphoid gate in patients 1 to 6 (%). B), C), D), E) and F) Relative proportions of different B-lineage cell subpopulations expressed as a percentage of the total of CD19 positive cells in the lymphoid gate in patients 1 and 3, 4, 5 and 6, respectively.

degrees. There was no correlation between total B-lymphocyte numbers in the peripheral blood at baseline and frequency of CD19+ cells in bone marrow aspirate samples at 3 months (r = 0.26, P = 0.66, Spearman rank correlation coefficient). However, the frequency of CD19+ cells in the bone marrow samples did correlate with total numbers of CD19+ cells in the peripheral blood at 3 months (r = 0.94, P = 0.02, Spearman rank correlation coefficient; figure 5.3).

CD19+ cells in the peripheral blood and in the bone marrow
3 months following Rituximab

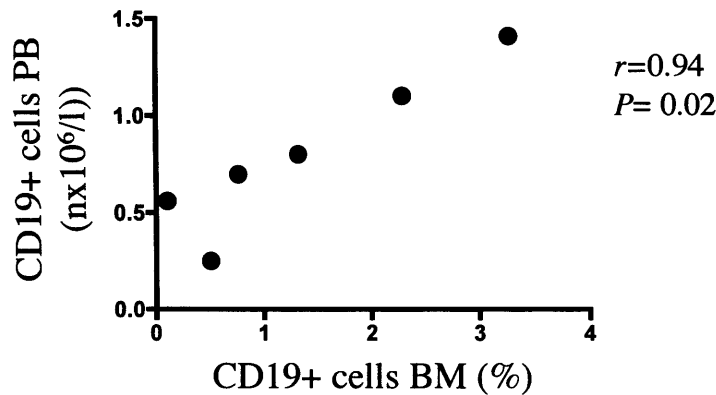


Figure 5.3 Relationship between the frequency of CD19+ cells in bone marrow (BM) aspirate samples and total numbers of CD19+ cells in the peripheral blood (PB) samples collected at the same time.

Frequency of CD34+ cells (marker of stem cells and early precursors) in the lymphoid gate varied from 1.2 to 4.9% (median 1.8) (table 5.3). The proportion of CD34+ cells committed to the B-lymphocyte lineage (expressing CD19) varied between 0% and 42.19% of the total number of CD34 positive cells in the lymphoid gate (median 15.08%; figure 5.4). Frequency of CD19+ cells did not correlate with frequency of CD34+ cells in the bone marrow samples (r = 0.54, P = 0.30, Spearman rank correlation coefficient).

Proportion of CD34+ cells committed to B-lymphocyte lineage

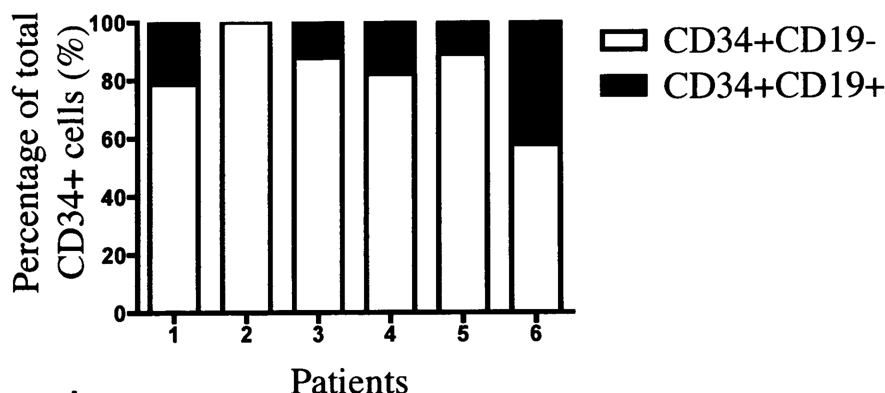


Figure 5.4 Proportion of all CD34+ cells in the lymphoid gate expressing CD19 (B-lymphocyte lineage).

5.3.3 Different B-lymphocyte precursor subpopulations predominate in different patients

Pro-B lymphocytes were undetectable in patient 2. The majority of B-lymphocyte precursors were pro-B lymphocytes in patients 5 and 6 (50 and 62% of CD19+ cells, respectively), pre-B lymphocytes in patients 3 and 4 (64 and 70%) and immature B lymphocytes in patient 1 (44%) (table 5.3, figures 5.2 B to F and figure 5.5). In patients 1 and 3 to 6, the ratio between the frequencies of more mature B lymphocyte precursors (pre-B lymphocytes and immature B lymphocytes) and more immature B lymphocyte precursors (pro-B lymphocytes) was 2.09, 2.33, 3.39, 0.58 and 0.58, respectively.

In the patients where mature B lymphocytes could be found based on their expression of CD19 and immunoglobulin light chains and absence of expression of CD34 and CD10, CD27+ B lymphocytes could be detected (figure 5.6) (patients 1, 4, 5 and 6).

CD19 positive cells in the bone marrow samples were generally CD5 negative (figure 5.7).

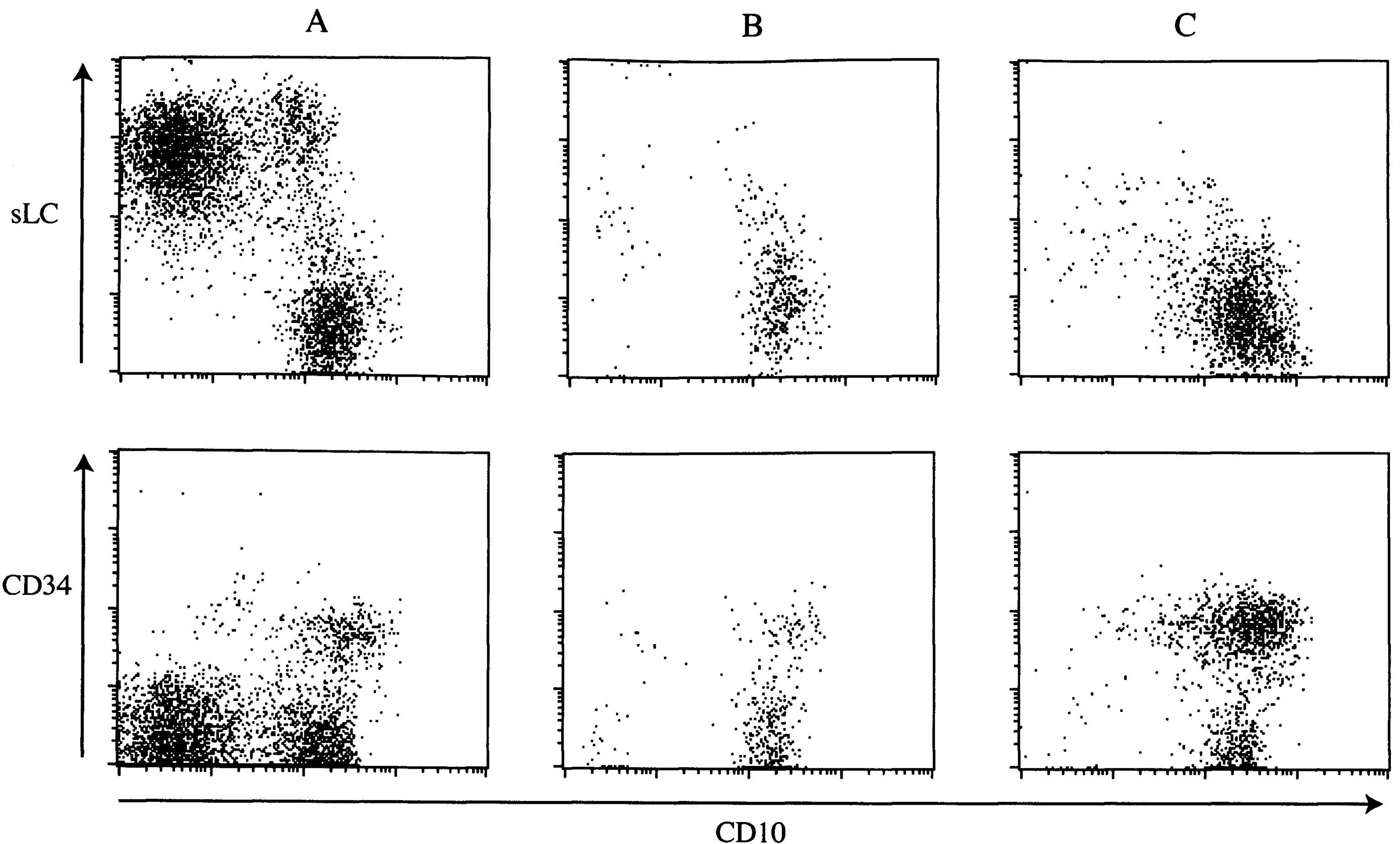


Figure 5.5 Expression of surface immunoglobulin light chains (sLC) and of CD34 versus CD10 on CD19 positive cells in the lymphoid gate. A) lymphoma patient after peripheral blood B-lymphocyte repopulation showing the expected B-lineage cells subpopulations; B) patient 4, showing a predominance of pre-B lymphocytes (CD19+CD10+CD34-sLC- cells); C) patient 6, showing a predominance of pro-B lymphocytes (CD19+CD10+CD34+sLC- cells).

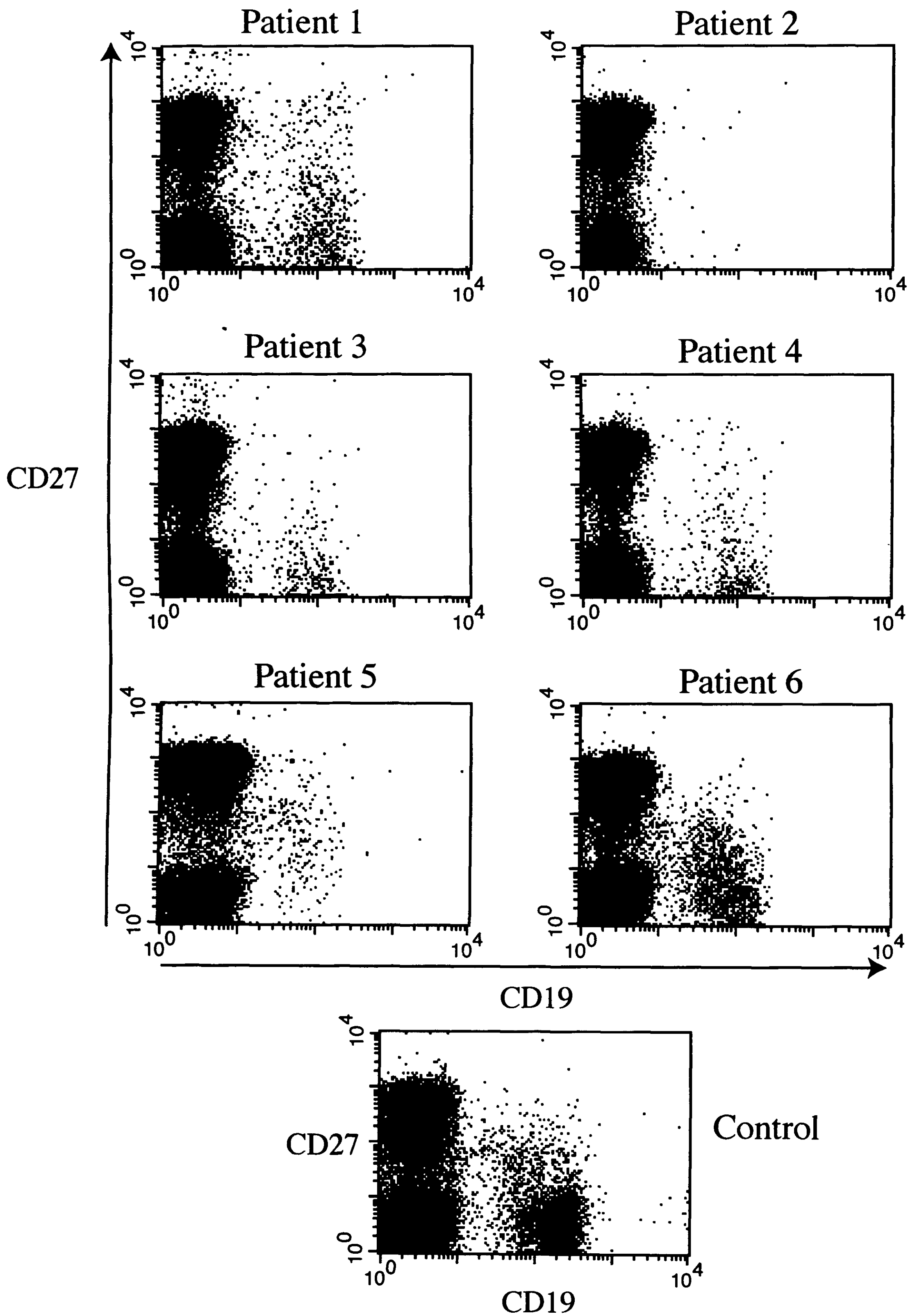


Figure 5.6 CD27+CD19+ B lymphocytes (recirculating memory B lymphocytes) were observed in the bone marrow samples of patients 1, 4, 5 and 6.

CD5 expression was absent on the majority of CD19+ cells detected in the bone marrow

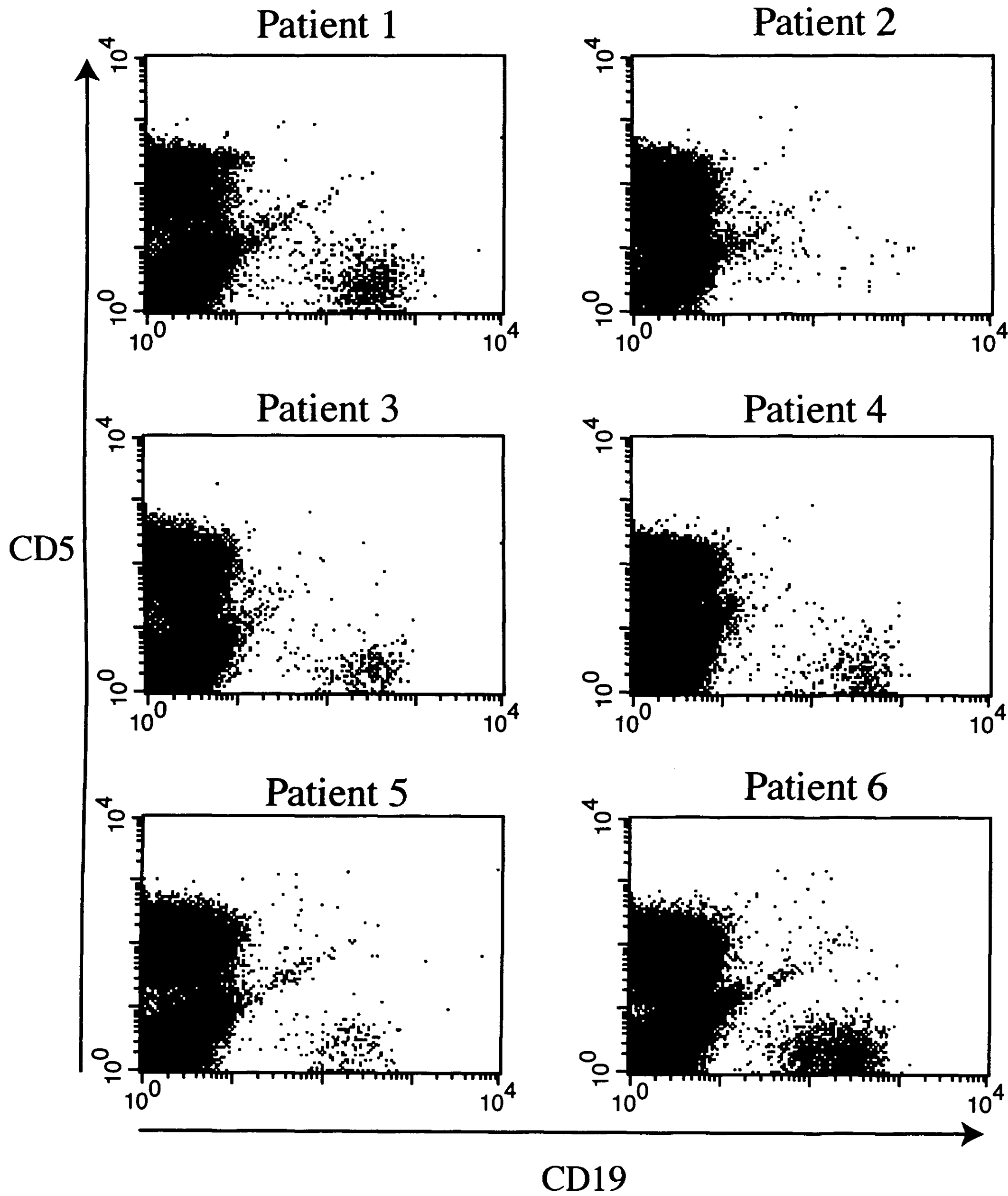


Figure 5.7 The majority of CD19+ cells found in the bone marrow samples did not express CD5.

Detectable expression of CD20 on B lymphocytes and B-lymphocyte precursors in the bone marrow is low or absent

CD20 expression on CD19+ cells, including cells with an immature and mature B-lymphocyte phenotype, was either low or absent (figure 5.8).

CD20 on pro-B lymphocytes

In the BM sample obtained from a lymphoma patient at a time that peripheral blood repopulation had occurred, it is clear that while expression of CD34 defines two separate subsets of cells (positive and negative subsets), CD20 expression is continuous. A small number of CD19+CD34+ cells (pro-B cells) are seen to express low levels of CD20) (figure 5.9).

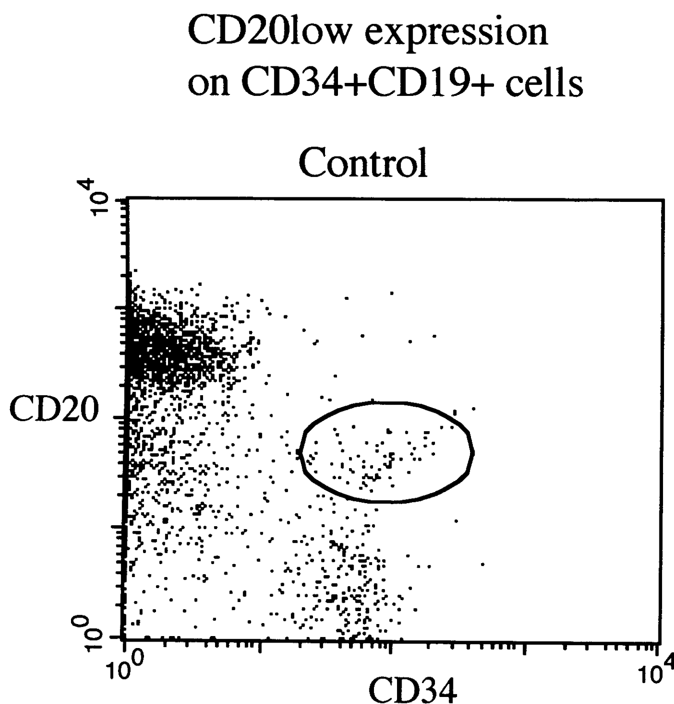


Figure 5.9 In the sample used as control (lymphoma patient after repopulation) a small number of CD34+CD19+ cells express low levels of CD20.

5.3.4 Plasma cells

The proportion of plasma cells in the total of events collected varied from 0.01 to 0.36% (median 0.02) (table 5.3, figures 5.10 and 5.11). In the lymphoid gate the percentage of

CD20 expression on CD19+ cells was either absent or low

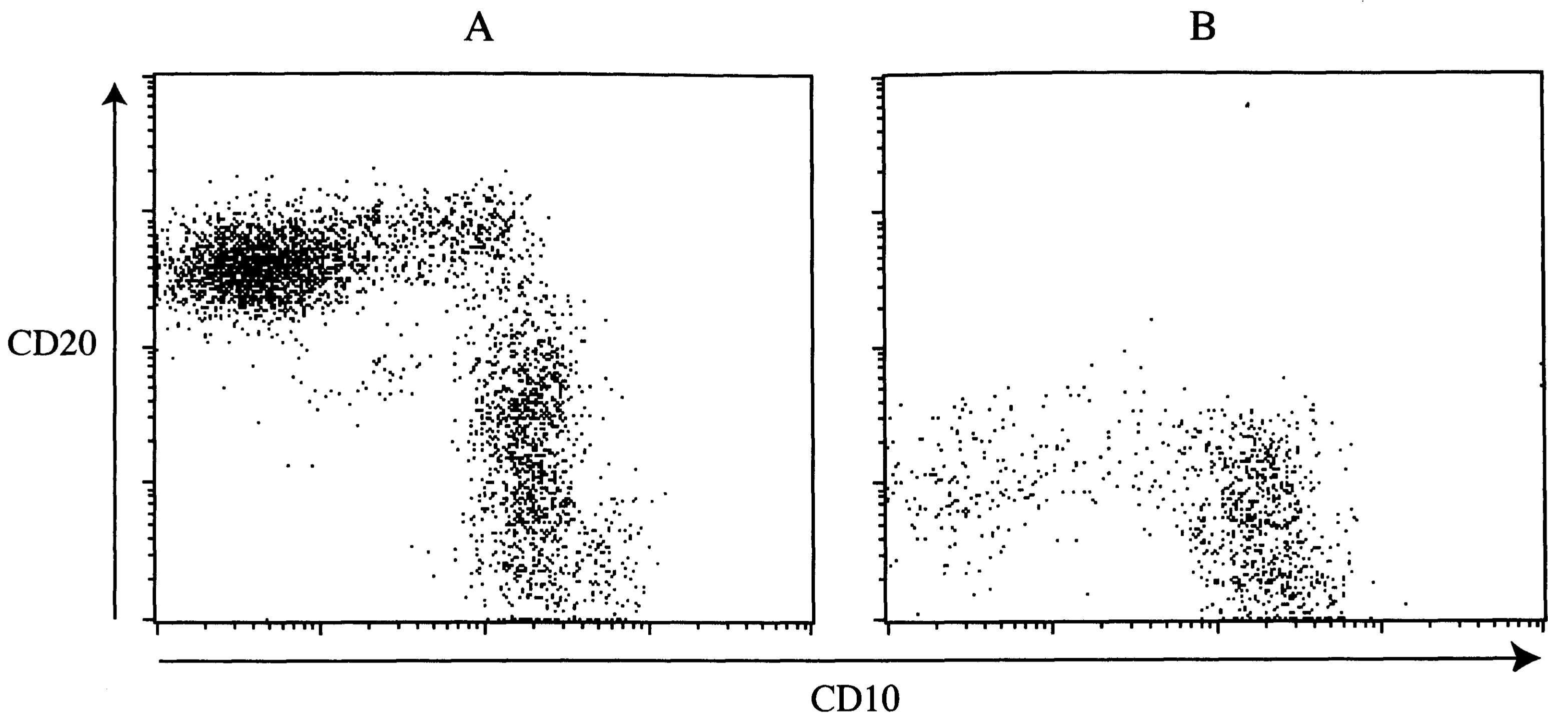


Figure 5.8 Expression of CD20 versus CD10 on CD19 positive cells in the lymphoid gate. A) Lymphoma patient after peripheral blood B-lymphocyte repopulation showing the expected B-lineage cell subpopulations; B) Patient 1, showing decreased CD20 expression.

CD19+ cells that were CD138+ varied between 3.05% and 57.14% (median 9.82%). No correlation was found between the proportion of CD19+ cells in the lymphoid gate or in the total of events collected and the proportion of plasma cells in the total of events collected in these bone marrow samples. The proportion of plasma cells in the total of events collected did not correlate significantly with serum total immunoglobulin levels at the time of bone marrow sampling (IgA $r = 0.12$, $P = 0.80$; IgG $r = 0.65$, $P = 0.18$; IgM $r = 0.38$, $P = 0.50$, Spearman rank correlation coefficient).

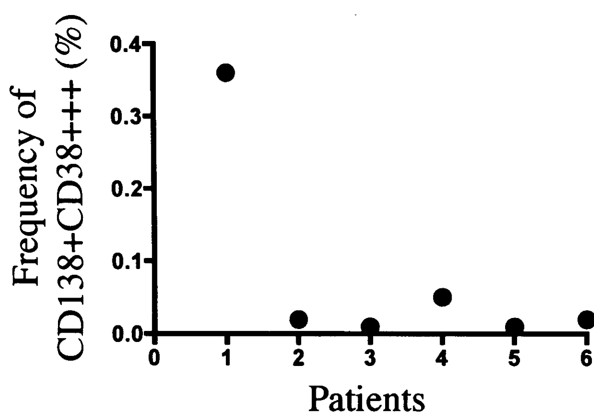


Figure 5.10 Frequency of CD138+CD38+++ cells in the bone marrow samples of patients 1 to 6, 3 months following BLDT.

Two subpopulations of plasma cells could be distinguished based on their expression of CD19. One population expressed levels of CD19 similar to other B lymphocyte precursors, the other population expressed low levels of CD19 or no CD19 (figure 5.12). Relative proportions of the two subpopulations varied between patients with no particular pattern (figure 5.12).

Plasma cells in bone marrow samples 3 months after Rituximab

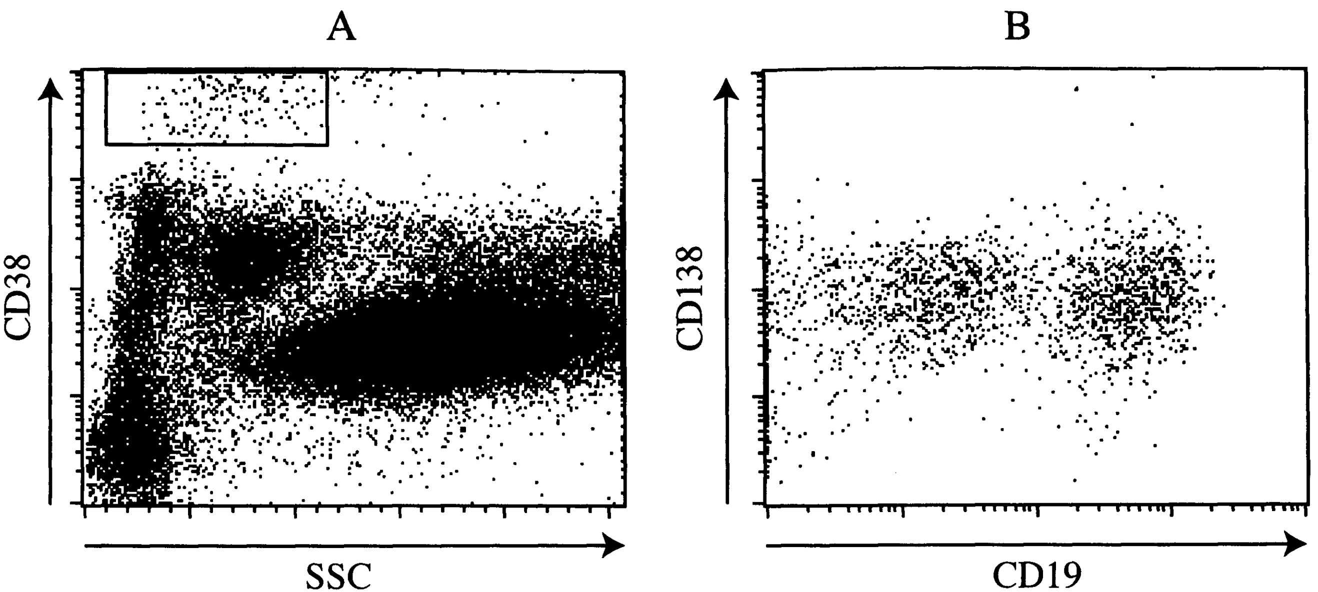


Figure 5.11 Gating for plasma cell analysis and phenotype of plasma cells in one of the samples. A) expected right-angle scatter profile for plasma cells in the bone marrow set around cells expressin high levels of CD38; B, expression of CD138 versus CD19 on CD38+++ cells with the expected right-angle scatter profile of plasma cells (patient 1).

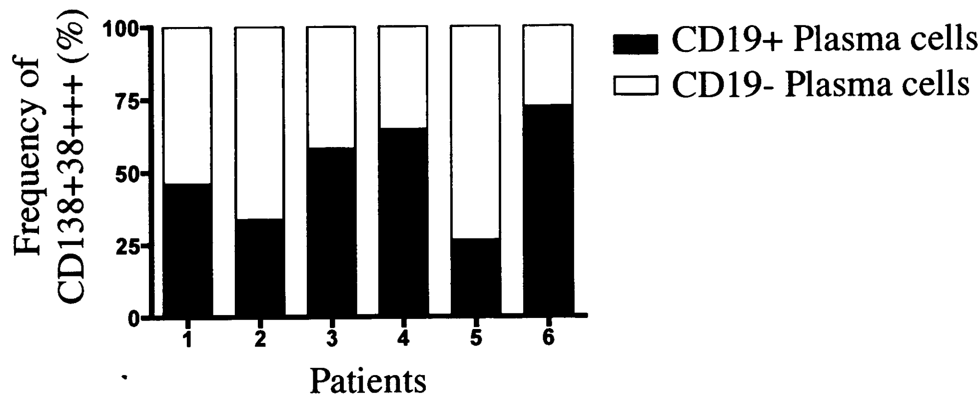


Figure 5.12 Presence of both CD19+ and CD19- plasma cells in the bone marrow samples of patients 1 to 6, 3 months following BLDT.

5.3.5 Clinical correlations

On further follow up, patients who showed a higher relative proportion of more mature precursors in their bone marrow (patients 1, 3 and 4) relapsed clinically at B lymphocyte return to the peripheral blood (at 5, 11 and 11 months respectively) (table 5.1). The proportion of plasma cells in the samples was higher in patient 1 who relapsed earlier than the other patients. The other three patients did not relapse at B-lymphocyte return. Patient 2, who showed almost complete depletion, repopulated at 8 months and relapsed clinically at 18 months. Patients 5 and 6 repopulated at 11 and 6 months, respectively, and had not yet relapsed at 15 and 13 months of follow up, respectively.

5.4 Summary

Six bone marrow samples from six patients with rheumatoid arthritis were analysed three months after treatment with rituximab to look for the presence or absence of CD20 positive and CD20 negative B lymphocyte precursors, mature recirculating B lymphocytes and plasma cells, using four-colour flow cytometry.

In one patient there was an almost total absence of B-lymphocyte precursors but all other samples showed evidence of repopulation to different degrees. Despite this fact, there were no signs of repopulation in the peripheral blood. The relative proportions of the different B-lymphocyte precursor subpopulations varied between patients. CD5 was not expressed by the B-lymphocyte precursors. In patients with B lymphocytes with a mature phenotype some CD19 positive cells expressed normal levels of CD27 (a phenotype of recirculating memory B lymphocytes).

Plasma cells could be detected in all patients and these included cells expressing normal levels of CD19 and low or no CD19.

Patients who showed a higher relative proportion of more mature precursors in their bone marrow relapsed clinically at B-lymphocyte return to the peripheral blood.

There are potential problems with bone marrow immunophenotyping studies using flow cytometry that involve sampling and dilution of samples with fat and contaminating peripheral blood. For this reason results were expressed both as percentage of total events collected (nucleated cells) and as percentage of cells in lymphoid gate. These results should be comparable but may be influenced by low proportion of lymphoid cells in patient 2 and high proportion of lymphoid cells in patient 4 (table II). In this study, significant contamination of bone marrow aspirate samples by peripheral blood influencing findings for B-lineage cell populations can be excluded as peripheral blood samples collected at the same time showed almost complete B-lymphocyte depletion.

CHAPTER 6 DISCUSSION OF RESULTS

6.1 Introduction

BLDT based on rituximab was first undertaken in patients with RA at UCL based on an hypothesis that postulated a primary role for RhF-specific B-lymphocyte clones and certain species of RhF in the initiation and perpetuation of the disease process. This hypothesis included pathogenic roles for B lymphocytes and autoantibodies in both afferent and efferent pathways of the disease (Edwards and Cambridge 1998, Edwards et al., 1999). The process was also postulated to be T-lymphocyte dependent in the sense that T-lymphocyte help needed to be provided to autoreactive B-lymphocyte clones but that there was no need for loss of T-lymphocyte tolerance to self. The proposed effector mechanism for activation of macrophages in the joints was based on the interaction of small immune complexes and FcγRIIIa on these cells similar to the mechanism known to be the basis of type III hypersensitivity reactions (Abrahams et al., 2000). This mechanism was compatible with hystopathological observations in synovial tissue and provided a unifying mechanism to explain both articular and extra-articular disease (Bhatia et al., 1998).

Based on this hypothesis, it was hoped that BLDT would deplete pathogenic B-lymphocyte clones and stop the formation of new plasma cells and the production of pathogenic autoantibodies. Patients should therefore go into remission following the decline of autoantibodies. If reconstitution of the B-lymphocyte population occurred in the absence of autoantibodies, the risk of relapse should be similar to the individual risk of initial development of RA.

Effective, specific depletion of B lymphocytes became a possibility with the availability of rituximab. In 1998, at the start of the initial open label trial in RA, available data from animal studies and from human lymphoma studies showed that, with the doses used, the B-lymphocyte depletion induced by rituximab was major but incomplete, particularly in the lymph nodes, and that repopulation occurred starting 6 to 9 months after treatment (Reff et al., 1994, Maloney et al., 1994, Maloney et al., 1997a, Maloney et al., 1997b, McLaughlin et al., 1998). The lymphoma studies also showed that during the period of depletion serum total immunoglobulin levels showed some decrease but usually remained within the normal range which was thought to be due to the fact that

plasma cells do not express the CD20 antigen and therefore are not specifically depleted by rituximab (Maloney DG 1994, Maloney DG J clin Oncol 1997, Maloney DG 1997, McLaughlin P J Clin Oncol 1998). Information was also available that rituximab and several chemotherapeutic agents showed synergistic effects in vitro and that combination trials of rituximab and the standard chemotherapy regimen in B-cell NHL (CHOP) showed much better results than rituximab monotherapy (higher rates of response and longer duration of response) (Czuczman et al., 1999).

In the initial open label trial 5 patients with active, refractory seropositive rheumatoid arthritis were treated with a combination of rituximab, cyclophosphamide and corticosteroids (Edwards and Cambridge, 2001). All five patients showed a major improvement in clinical signs and symptoms of active disease, achieving at least an ACR50 grade of improvement sustained at 6 months following a single course of treatment. None of the patients relapsed before B-lymphocyte return to the peripheral blood but while 2 of the 5 patients relapsed at the time of B-lymphocyte repopulation the other 3 patients remained well for a variable period of time. The 2 patients who relapsed earlier showed higher serum titres of RhF as measured by RAPA (a semi-quantitative agglutination test), at the time of B-lymphocyte repopulation. These two patients improved following re-treatment but relapsed again at the time of B-lymphocyte repopulation.

The primary aims of this thesis were to investigate why patients with RA relapse after a good initial response to BLDT and whether it is possible to prevent relapse and improve the long-term results of this therapeutic approach. The working hypothesis was that relapse was due to persistence of pathogenic B-lymphocyte clones, to the presence of long-lived plasma cells producing pathogenic autoantibodies or to a mixture of both.

The experimental approach taken in this thesis was therefore to perform serial studies of the clinical response of patients with RA to BLDT in a larger group of patients treated with different protocols, in parallel with changes in humoral parameters and peripheral blood B lymphocytes phenotype following treatment. Bone marrow aspirates were also examined in a limited number of patients, 3 to 4 months after BLDT.

6.2 BLDT is effective in RA and patients do not relapse before B-lymphocyte repopulation

6.2.1 Clinical response to treatment and use of different protocols

The extended clinical study reinforced the suggestion that BLDT based on rituximab could lead to major improvement in disease manifestations in seropositive RA with a good safety profile. It served as the basis for designing the protocol for the randomised, double blind, placebo-controlled phase IIa trial undertaken by Roche. This phase IIa rituximab trial has proved that rituximab is effective in treating active refractory RA removing any remaining doubt that the positive results in the UCL open label trials could have been due merely to the effects of cyclophosphamide or corticosteroids (Edwards et al., 2004). The design of this trial did not allow definite conclusions, in particular on the extent of the benefit of rituximab monotherapy, as all patients included were on baseline methotrexate treatment and therefore probably methotrexate partial responders, with some arms stopping methotrexate and others continuing this medication. What was clear was that in patients who stopped methotrexate at baseline, treatment with rituximab and cyclophosphamide was more effective than rituximab alone and that at the 6-months time point, treatment with rituximab and cyclophosphamide after stopping methotrexate was as effective as continuing methotrexate and adding rituximab. On further follow-up, this later protocol was shown to produce longer responses and so it was the protocol chosen for further trials (Strand et al., 2006).

The extended open-label trial presented in this thesis suggested a dose-response to rituximab and also an additive or synergistic effect of cyclophosphamide, at least when half-dose rituximab was used. These findings suggested that higher degrees of depletion increased the chance of response to treatment, presumably by being associated with major depletion of pathogenic B-lymphocyte clones. This study also showed that a prolonged course of oral corticosteroids was not necessary for a response to treatment but did not exclude that it might contribute to a longer duration of response. Further treatments at UCL showed that rituximab monotherapy was also associated with significant improvement in RA and the use of cyclophosphamide was abandoned.

Many of the observations referred to above in the cohort of 40 patients described in this thesis were later reproduced and extended in the phase IIa and phase IIb randomised controlled trials of rituximab in RA as well as in other small cohorts by other groups of investigators (De Vita et al., 2002, Moore et al., 2004, Edwards et al., 2004, Higashida et al., 2005, Emery et al., 2006, Cohen et al., 2006).

A dose response to rituximab was also suggested in the randomised phase IIb trial (DANCER) that compared treatment with 2x500mg of rituximab against 2x1000mg in patients who continued methotrexate, as patients treated with the higher dose showed a tendency to respond better (higher incidence of ACR70 and EULAR Good Response) (P Emery et al., 2006). However, contrary to what results from cohort II in the extended open-label trial presented in this thesis seemed to suggest, treatment with the lower dose of rituximab (2x500mg) on patients on baseline methotrexate, without cyclophosphamide, led to significant improvement in disease manifestations sustained at 6 months, with similar frequencies of patients achieving ACR20 and ACR50 in the two treatment groups. No further follow up data has been published and so it is not known whether the two groups will exhibit differences in duration of response.

The DANCER trial also showed that treatment with corticosteroids did not influence the clinical response at 6 months (P Emery etal., 2006). Corticosteroids proved to be useful in decreasing the risk of infusion-reactions to rituximab and one 100mg iv infusion of methylprednisolone before each rituximab infusion is recommended. Again, no follow up data on duration of response have yet been published and therefore no definite conclusions can be drawn as to whether corticosteroids influence duration of response.

6.2.2 Host factors affecting the efficacy of rituximab killing

Some reports have suggested that the potency of rituximab in B-lymphocyte cytolysis is dependent on the immunoglobulin Fc receptor genotype of the subject (Cartron et al., 2002, Anolik et al., 2003, Treon et al., 2005). In the cohort presented in this thesis no apparent relationship was found between FcγRIIIa genotype and clinical response (maximal response and duration of benefit) or duration of the period of peripheral B-lymphocyte depletion following BLDT in RA patients. Comparison of these results with those published in the literature, suggests that the influence of FcγRIIIa genotype may

depend on protocol intensity and the underlying pathology. When lower doses of rituximab are used or in the presence of cells more resistant to rituximab-induced killing, the affinity of the FcγRIIIa allotype of the subject may influence rituximab effectiveness in a clinically significant way.

6.2.3 Clinical response was only seen in seropositive patients

It was found that in the cohort of 40 patients presented here, seronegative patients did not respond clinically to BLDT. Two of the 4 seronegative patients were treated with full dose rituximab monotherapy under oral steroid cover and this was not clinically effective. However, the other 2 seronegative cases were treated with protocols that even in seropositive patients were not generally associated with a significant response sustained at six months (cohorts II and V of the extended open-label trial).

De Vita et al also described a seronegative patient who did not respond to therapy (De Vita et al., 2002). Although in the phase IIb (DANCER) and phase III (REFLEX) rituximab trials in RA some "seronegative" patients were seen to respond to treatment there is insufficient data on these patients to allow for definite conclusions (Emery et al., 2006, Cohen et al., 2006). In particular, there is no available data on exactly how many seronegative patients have responded to rituximab and how well did these patients respond (for example, if only reaching ACR20 or higher), which assays were used to detect "total" RhF and RhF isotypes and what were the cut-off levels, and whether the classification of patients as seronegative was based only on "total" RhF or also on the absence of RhF isotypes. It is also not known whether these patients were anti-CCP positive.

6.2.4 Clinical relapse

Eventually all patients included in this thesis relapsed following BLDT. It was found that no patient relapsed before B-lymphocyte repopulation. In addition, B-lymphocyte return was not always associated with disease relapse. This suggests that the presence of new B-lymphocytes is necessary but not sufficient for clinical relapse to occur.

The studies included in this thesis confirmed the two patterns of relapse following BLDT previously observed in the initial open label trial. Patients either relapsed at the time of B-lymphocyte repopulation or only later, at a variable time after B-lymphocyte return (up to a maximum of 32 months). Interestingly, patients tended to conform to the same pattern of relapse following re-treatment. A number of explanations for the two patterns of relapse can be proposed such as: the relative degree of depletion of pathogenic B-lymphocyte clones; differences in the levels or characteristics of autoantibodies present at the time of B-lymphocyte repopulation; differences in the total numbers or maturity of repopulating B lymphocytes; or in the mechanisms underlying induction of pathogenic B-lymphocyte clones.

6.2.5 Re-treatment was effective

Re-treatment with BLDT was effective in up to three courses in all patients who had responded to their initial course of treatment. Re-treatment of patients first treated with protocols that were judged as not effective showed mixed results, with 1 out of 5 seropositive patients not responding and another one remaining relatively resistant when these patients were re-treated with regimens that were found to be effective in other patients.

The results of re-treatment with BLDT also showed that one of the patients treated initially with half-dose rituximab without cyclophosphamide failed to deplete efficiently on her second treatment. This suggested that this patient may have developed specific HACA to rituximab and raised the possibility that treatment with lower doses of rituximab might increase this risk. This has been confirmed in the phase IIb rituximab trial (DANCER) (Emery et al., 2006). In this trial, a higher incidence of HACA in the low-dose rituximab group was observed (4.9% compared to 2.7% in the high-dose treatment group) but there is no data yet whether this had any influence on re-treatment, in particular on the rate of infusion-reactions or on the efficiency of B-lymphocyte depletion. Even though, in lymphoma trials, no association between the presence of HACA and the risk of side effects or secondary resistance to treatment has been described, failure to deplete on re-treatment due to a specific HACA response to rituximab and rapid clearance of the drug has been documented at UCL in a patient with SLE (Grillo-Lopez et al., 1999, Leandro et al., 2005, Tahir et al., 2005).

6.3 Changes in IgA-, IgG- and IgM-RhF, anti-CCP and anti-microbial antibodies following B-lymphocyte depletion

Serum levels of IgA-, IgG- and IgM-RhF and of anti-CCP antibodies decreased in all patients following BLDT. Total serum immunoglobulin levels decreased significantly following treatment but median levels remained within the normal limits. A selective effect of BLDT on autoantibody production was suggested as decreases in RhF of IgA and IgG isotypes and of anti-CCP antibodies levels were proportionally greater than decreases in the respective Ig class. IgM-RhF decreased to the same amount as total IgM with total IgM being the immunoglobulin class that showed the larger decrease. In contrast to the disease-associated autoantibodies measured, antibodies to microbial antigens either did not decrease (IgG anti-PCP antibodies) or decreased to the same extent as total IgG (IgG anti-TT antibodies).

These observations suggested that production of the measured autoantibodies was more susceptible to BLDT when compared with production of the anti-microbial antibodies studied. It was possible that, as there is an ongoing autoimmune response, a higher proportion of the autoantibodies were produced by plasma cells with a shorter half-life and were therefore more dependent on constant formation of new plasma cells for their sustained production when compared with anti-microbial antibodies. However, only rarely did patients became seronegative or anti-CCP antibody negative suggesting that at least some autoantibody-producing plasma cells are long-lived.

Interestingly, the two anti-microbial antibodies analysed behaved differently. Anti-TT antibodies, antibodies to a classical TD antigen, decreased significantly 3 months after treatment (to the same extent as total IgG) and then showed some recovery at the time of B-lymphocyte repopulation but anti-PCP antibodies, antibodies to a TI-2 antigen, remained reasonably constant following treatment and could even increase while the peripheral blood was still depleted of B lymphocytes. This suggested that anti-PCP antibodies might be produced mainly by long-lived plasma cells but also raised the possibility that PCP-specific B lymphocytes may be resistant to killing by rituximab. Response to TI-2 antigens such as PCP, is thought to be the responsibility of marginal zone B lymphocytes residing predominantly in the spleen and a study in human CD20-

transgenic mice showed that the marginal zone cells in these animals were indeed relatively resistant to depletion by anti-human CD20 antibodies (Gong et al., 2005).

Anti-TT antibodies decreased significantly following BLDT. In several patients, levels increased at the time of B-lymphocyte repopulation in the absence of specific immunisation, with the differences being no longer statistically significant when the levels where compared to pre-treatment ones. This increase in anti-TT may reflect recruitment of new naïve B lymphocytes in the presence of specific T lymphocytes and antigen persistence in the form of immune complexes but it may also reflect re-expansion of specific memory clones not completely depleted by rituximab. It does suggest that persistence in serum of specific antibodies to TT is due to the existence of long-lived plasma cells but also to continuous or recurrent proliferation and expansion of TT-specific B-lymphocyte clones with differentiation into plasma cells.

Changes in disease-associated autoantibodies were not only associated with treatment but also with clinical response as serum levels of the 3 RhF isotypes and of anti-CCP antibodies decreased more following treatment in the group of patients who responded to treatment when compared to the group of patients who did not respond to treatment. Also, in the responder group, a gradual decline in CRP and autoantibody levels was observed, while in the non-responder group the course of CRP (as expected) and autoantibody decline was more erratic. In addition, relapse was associated or preceded by a detectable increase in autoantibody serum levels, more frequently, IgM-RhF, in all patients except one (1 out of 22). Increases in circulating autoantibody levels were rarely seen without relapse and, in these circumstances, were transient and involved mostly IgM-RhF.

As previously discussed, the use of rituximab in the treatment of lymphoma, either as a monotherapy or in association with CHOP, is not associated with a major decrease in serum total immunoglobulin levels (McLaughlin et al., 1998, Czuczman et al., 1999). This is thought to be due to the fact that plasma cells do not express the CD20 antigen and will therefore continue producing immunoglobulins during the period of B-lymphocyte depletion.

Rituximab in autoantibody-associated autoimmune diseases

The first publications on the use of rituximab to treat autoimmune diseases were of patients with cold agglutinin disease and IgM-associated polyneuropathy (Lee et al., 1998, Levine et al., 1999). In both diseases, clinical manifestations are a consequence of the pathogenicity of a monoclonal IgM however the rational for using rituximab was different. In cold agglutinin disease, the pathogenic IgM paraprotein is frequently associated with the presence in the bone marrow of by a CD20-expressing lymphoplasmacytic clone. Therefore, rituximab was used with the objective of depleting the cells producing the pathogenic autoantibody. In IgM-associated polyneuropathy, the kinetics of serum autoantibody levels and disease manifestations suggested that the plasma cells producing the autoantibodies might have short half-lives and needed to be replaced frequently for sustained autoantibody production. Rituximab was therefore used with the objective of depleting the precursors of the autoantibody-producing cells, therefore preventing their renewal and consequently decreasing autoantibody production. In both cases, rituximab therapy was associated with a decrease in autoantibody serum levels and clinical improvement.

The use of rituximab in other autoimmune diseases has sometimes produced apparently conflicting results regarding its effect on autoantibody production and serum levels and its clinical efficacy (Edwards et al., 2002, Anolik et al., 2004, Looney et al., 2004, Eisenberg and Looney, 2005, Edwards and Cambridge, 2006). In immune thrombocytopenic purpura, clinical response was not always associated with significant decreases in measured titres of anti-platelet antibodies in the group of patients who responded to treatment (Stasi et al., 2001, Zaja et al., 2002). Similar observations have been made in patients with SLE (Anolik et al., 2004, Looney et al., 2004). Despite this observation, detailed analysis of individual patients who responded to treatment frequently showed a major decrease in anti-platelet antibody levels. Technical problems associated with detecting and quantifying anti-platelet antibodies may contribute to these discrepancies. In addition, it is often not clear in the different studies, whether serial samples have been tested simultaneously (when possible) to allow the detection of true variations in serum levels over time. Also, a recent study at UCL in patients with SLE has shown interesting differences in the response of different autoantibodies serum levels to BLDT (Cambridge et al., 2006). Antibodies to native DNA and to histones decreased significantly following BLDT but antibodies to extractable nuclear antigens

remained largely unchanged. These observations show that production of different autoantibodies have different kinetics and show different sensitivities to BLDT.

Changes in disease-associated autoantibodies in RA

Several studies have examined changes in RhF and less frequently, in anti-CCP serum levels in RA following different immunosupressive/immunomodulatory therapies and looked at any association of observed changes with treatment itself or a positive response to treatment and disease activity. These studies were mainly carried out either in the 1970s and 1980s and included patients treated with penicillamine, parenteric gold or methotrexate or, more recently, in patients treated with the anti-TNF agent infliximab in combination with methotrexate.

The earlier studies examining mainly IgM-RhF levels as detected by agglutination assays and less frequently IgM-, IgG- or IgA-RhF by RIA or ELISA assays, showed varied results. However, in several of these studies, treatment of patients with active RA with either penicillamine, parenteric gold or methotrexate resulted in progressive decreases in circulating RhF levels, with decreases in IgM-RhF levels often being relatively greater than those observed in total serum IgM (Bluestone and Goldberg, 1973, Huskisson and Berry, 1974, Lorber et al., 1978, Dixon et al., 1980, Wernick et al., 1983, Hanly et al., 1986, Pope et al., 1986, Alarcon et al., 1990). In some studies, a significant correlation with disease activity and response to treatment was found as well as an association with specific types of therapy, in particular, parenteric gold. Patients who responded to methotrexate were also found to show decreased spontaneous production of IgM-RhF by peripheral blood mononuclear cells in vitro, in an assay which had been previously found to have a good correlation with disease activity (Alarcon et al., 1985, Olsen et al., 1987, Olsen et al., 1988). In one of the patients studied, disease flare while on stable treatment was associated with increased spontaneous production of IgM-RhF in vitro. It was noted that when clinical response to treatment was not so marked, changes in serum IgM-RhF levels following treatment were more varied. Changes in circulating RhF levels described with methotrexate were said to occur early while with parenteric gold and penicillamine they were progressive with a suggestion of a dose response (Olsen and Jasin, 1984, Olsen et al., 1984).

In the more recent studies treatment of RA patients with infliximab combined with methotrexate resulted in a decrease in serum IgM-RhF levels, particularly in patients who responded to therapy (Alessandri et al., 2004, Bobbio-Pallavicini et al., 2004, De Rycke et al., 2004, Mikuls et al., 2004, Nissinen et al., 2004, Caramaschi et al., 2005). Several studies did not show significant changes in anti-CCP levels following treatment (De Rycke et al., 2004, Nissinen et al., 2004, Caramaschi et al., 2005). When a significant decrease was observed it was either not sustained or detected only in patients with disease of shorter duration (less than 1 year) (Bobbio-Pallavicini et al., 2004, Mikuls et al., 2004). Only in one study, a decrease in anti-CCP antibodies following treatment correlated with clinical response (Alessandri et al., 2004). Treatment with infiliximab was not significantly associated with development of RhF or anti-CCP antibodies on patients seronegative for these antibodies at baseline while the well known association of treatment with infliximab and development of autoantibodies such as ANA and more rarely anti-dsDNA was confirmed (Bobbio-Pallavicini et al., 2004).

In a recent five-year follow up study, in patients with early RA (less than 1 year duration) treated with different DMARDs, anti-CCP antibodies decreased within the first year (Ronnelid et al. 2004). This decrease was associated with treatment with sulphasalazine but not with any other DMARD. Anti-CCP serum levels increased after 1 year independent of drug treatment and disease activity. Differences in the changes observed in some of these studies for serum RhF and anti-CCP antibody levels following treatment suggest that these two autoantibody systems in RA are independently regulated.

Changes in serum immunoglobulins

In the cohort of 40 patients included in this thesis, total serum immunoglobulin levels decreased significantly but remained within the normal limits following the first course of BLDT. Exceptions were a small number of patients who had baseline levels close to the lower limit of normal. However, repeated courses of treatment were associated with more frequent hypogammaglobulinaemia, involving IgM or IgG and only rarely IgA. Absolute as well as percentage drops in total Ig levels were similar following repeated courses of treatment. Hypogammaglobulinemia was observed in a small number of patients in two different settings: in patients who had relapsed at the time of B-lymphocyte repopulation and who had been treated repeatedly in rapid succession; and

in a few patients who relapsed only later but failed to increase their immunoglobulin levels following B-lymphocyte repopulation and before re-treatment.

B-lymphocyte repopulation of the peripheral blood was associated with an increase in total Ig levels in many patients but not in all. In some patients Ig levels tended to stay stable for a variable period of time. Changes in Ig levels did not always correlate with changes in serum autoantibody levels or with clinical relapse. The similarities found between repopulating naïve B lymphocytes following BLDT based on rituximab and BMT raise interesting possibilities, including, that the absence of an increase in Ig levels following B-lymphocyte repopulation may reflect the lack of maturity of the repopulating B lymphocytes in these patients. Following BMT, Ig levels return to the serum following a pattern that resembles normal ontogeny, and in many patients, antibody production in in vitro assays remains deficient for a prolonged period of time (Witherspoon et al., 1981, Pahwa et al, 1982, Kagan et al., 1989, Glas et al., 2000).

In summary, the results of the studies presented in this thesis showed that autoantibody levels decreased following BLDT and were closely associated with the clinical outcome. Decreases in serum autoantibody levels were only found to be statistically significant in patients who responded to treatment. Further, rises in autoantibodies were associated with clinical relapse. This indicated that decreases in autoantibody serum levels were a specific effect of the treatment as would be expected for an agent which targets B lymphocytes, and suggested that decreases in autoantibodies contribute to clinical response.

6.4 Changes in soluble CD23 antigen and BLyS following BLDT

6.4.1 Soluble CD23

Soluble CD23 antigen in serum was studied in the hope of finding a possible surrogate for the degree of B-lymphocyte depletion in solid lymphoid tissues or of B-lymphocyte activation and possible association with relapse. The CD23 antigen is expressed by naïve mature B lymphocytes and the soluble molecule is shed from the cellular surface when these cells are activated and enter a germinal centre reaction. Soluble CD23 levels

correlated positively at baseline with peripheral B-lymphocyte counts, decreased significantly following BLDT and increased before (less frequently) or at the time of B-lymphocyte return to the peripheral blood suggesting that B lymphocytes are major contributors to serum sCD23 levels.

Percentage decreases in sCD23 levels in patients who responded to treatment tended to be greater than in those who did not respond to treatment. Most of the patients who did not respond to treatment had been treated with less intensive, potentially less depleting, protocols, suggesting that percentage decreases in sCD23 levels might reflect the degree of B-lymphocyte depletion in particular in solid lymphoid tissues (as degrees of depletion in peripheral blood between patients were similar within the sensitivity of the method used by the Central Pathology Laboratory).

Soluble CD23 levels increased at the time of B-lymphocyte repopulation but not in all patients. One can raise the possibility that there might be a difference in the maturity of the repopulating B lymphocytes between patients in whom sCD23 increases at repopulation and those in whom it does not increase.

No clear association between changes in sCD23 levels and clinical relapse were found. It remains, however, to be seen whether sCD23 levels may reflect germinal centre experience and thus give an indication of the extent of repopulation of peripheral lymphoid tissues that takes place in individual patients before relapse can occur.

In summary, sCD23 levels correlated positively with B-lymphocyte depletion and repopulation in the peripheral blood following BLDT. Tendency for smaller percentage decreases in the group of patients who did not respond to treatment suggested that these patients may have depleted less that patients who have responded to treatment. No correlation between changes in sCD23 levels and clinical relapse was noted.

6.4.2 BLyS

BLyS was raised in 6 out of the 15 RA patients studied at baseline but this was not associated with differences in the levels of total Ig, of the specific antibodies measured

(autoantibodies and anti-microbial antibodies) or circulating lymphocyte counts when compared to patients with undetectable BLyS baseline values.

BLyS levels rose markedly following BLDT and decreased with B-lymphocyte repopulation. The mechanism that leads to an increase in serum BLyS levels after BLDT and to a decrease following B-lymphocyte repopulation is not yet understood. Two main mechanisms are possible which are not mutually exclusive. Either BLyS levels increase because there are no B lymphocytes to adsorb free circulating BLyS or there is an increased stimulus for its production as a consequence of the major absence of B lymphocytes. However, the relationship between changes in BLyS levels and changes in peripheral blood B-lymphocyte counts was complex. At baseline, serum BLyS levels did not correlate with peripheral blood B-lymphocyte counts. Correlation between serum BLyS levels and peripheral blood B-lymphocyte counts was found in some patients but not in others. These observations suggested that circulating BLyS levels might reflect more the degree of depletion in solid lymphoid tissues or possibly the numbers of particular B-lymphocyte subpopulations (BLyS has a preferential effect, due to differential expression of its receptors, at certain stages of B-lymphocyte development).

BLyS is a relatively recently identified molecule and there are no published studies in the literature studying changes in serum BLyS levels following other B-lymphocyte depleting therapies such as BMT.

The group of patients who relapsed earlier (i.e. at B-lymphocyte return) showed significantly lower levels of BLyS at repopulation when compared to the group of patients who relapsed only later (5 to 32 months following B-lymphocyte return). This might possibly reflect a greater expansion of B lymphocytes in solid lymphoid tissues at this time point in this group of patients. Whether this could also be related to different initial degrees of depletion is not known. No differences were detected when BLyS levels pre-treatment, 1-2 months or 3-4 months following BLDT were compared between the two groups of patients.

At the time of B-lymphocyte repopulation following BLDT peripheral B-lymphocyte counts may not reflect the state of repopulation of solid lymphoid tissues. B-

lymphocytes continuously migrate through the body, but little is known about the rate of B lymphocyte traffic from blood to extravascular compartments in humans. Studies of the kinetics of B lymphocytes infused into humans in the context of peripheral blood stem cell transplantation supports the notion that the rate of lymphocyte migration from blood to tissues is very rapid with total lymphocyte counts less than 15% and 3% 30 and 60 minutes following transfusion of 10^{10} cells. Only 1.5% and 0.3% of the infused B lymphocytes were present in the peripheral blood at 2 and 24 hours, respectively, after graft administration (a median of 3.7 x 109 B lymphocytes were infused) (Storek et al., 2002).

In summary, BLyS levels correlated negatively with B-lymphocyte depletion and repopulation in the peripheral blood following BLDT. Patients who relapsed earlier, at the time of B-lymphocyte repopulation showed significantly lower BLyS levels than patients who relapsed only later, suggesting that the level of repopulation or re-expansion of B lymphocytes in solid lymphoid tissues in these patients may have been greater in this group of patients despite no significant differences in peripheral B-lymphocyte counts.

6.5 B-lymphocyte depletion and reconstitution in the peripheral blood following BLDT

Treatment with rituximab induced a profound depletion of all peripheral blood B-lymphocyte subpopulations for at least five months in all patients except in patient 8 following the second course of BLDT. In this patient, repopulation was detected 2 months after re-treatment with BLDT and the patient did not improve clinically. This suggested that a quantitative threshold for B-lymphocyte removal may have to be reached for BLDT to be clinically effective in RA. The same patient had been treated with half-dose rituximab 40 months before and it is possible that she had developed specific HACA to rituximab leading to rapid clearance and decreased efficacy. Rapid clearance of rituximab in this patient is also suggested by the unusual presence of detectable CD20 expression on circulating B lymphocytes at 1 month after treatment.

During the period of B-lymphocyte depletion a small number of cells were found circulating, the majority showing a memory or plasma cell precursor phenotype. This

confirms that depletion of normal B lymphocytes, although major, is not complete, as previously shown in animal studies in primates and one lymphoma study (Reff et al., 1994, Maloney et al., 1994, Alwayn et al., 2001, Schroder et al., 2003, Vugmeyster et al., 2003a). It also suggests that memory B lymphocytes may be more resistant to depletion by rituximab than naïve cells. The presence of circulating plasma cell precursors suggests that some activation and differentiation of residual B lymphocytes into plasma cells still takes place in solid lymphoid tissues during the period of depletion.

Repopulation occurred mainly from naïve B lymphocytes

Repopulation of the peripheral blood following BLDT occurred mainly with naïve B lymphocytes with an increased proportion of cells showing a phenotype of immature B lymphocytes. These phenotypic characteristics, suggesting a second round of ontogeny, were similar to those described on repopulation after BMT in patients without chronic graft versus host disease (Small et al., 1990, Storek et al, 1993). The pattern of qualitative B-lymphocyte reconstitution observed, with a high proportion of naïve B lymphocytes expressing IgD and overexpressing CD38 and CD5 and a decreased frequency of memory cells, is characteristically seen after bone marrow transplantation in patients without chronic graft versus host disease and is also similar to that found in cord blood and very young children (Small et al., 1989, Small et al., 1990, Hannet et al., 1992, Storek et al., 1993).

These findings showed that repopulation of the peripheral blood did not occur from memory B lymphocytes that escaped depletion. In all patients, repopulation occurred predominantly with naïve B cells with increased number of cells expressing an immature phenotype, indicating that repopulation was dependent on resumption of production of naïve B lymphocytes in the bone marrow. Residual mature B lymphocytes did not therefore appear to be able to expand and repopulate the peripheral blood. This may be for one of several reasons. It is possible that prolonged availability of rituximab could interfere with the capacity of these cells to expand and fill the space freed by rituximab (Berinstein et al., 1998, Ng et al., 2005). Binding of CD20 to mature B lymphocytes is known to interfere with cellular activation and proliferation (Tedder et al., 1985, Golay et al., 1985, Clark et al., 1985, Tedder et al., 1986). It is also possible that residual mature B lymphocytes were themselves unable to expand in sufficient

numbers to repopulate the peripheral blood. BMT studies would support this as repopulation following this procedure never occurs from transferred mature B lymphocytes, independent of the type of graft used (Small et al., 1990, Koehne et al., 1997).

Circulating immature B lymphocytes identified at repopulation

At repopulation, an increased frequency of IgD+CD38++ B lymphocytes was found as previously described. Phenotypic studies presented in this thesis show that these cells were most likely young naïve cells exiting the bone marrow and not germinal centre founder cells as they had previously been classified and described by several authors based on similarities between these cells and tonsil germinal centre founder cells (Arce et al., 2001, Bohnhorst et al., 2001, Odendahl et al., 2003). These cells expressed low levels of CD10 but high levels of CD24. CD24 expression decreases when B lymphocytes enter the germinal centre and is absent in the majority of germinal centre B lymphocytes (Galibert et al., 1996, Ingvarsson et al., 1999, Suzuki et al., 2001). If circulating IgD+CD38high B lymphocytes were germinal centre founder cells, they would be expected to express lower or at least normal levels of CD24 when compared to mature naïve circulating B lymphocytes but not higher. This pattern of increased expression of CD38 by IgD+ B lymphocytes was seen even in the patient who did not deplete well and could be wrongly interpreted as an increase in circulating germinal centre founder cells (Anolik et al., 2004). In SLE, where cells with these phenotype are found at increased frequencies in patients with active disease, increased frequency at repopulation following rituximab therapy can be wrongly interpreted as a sign of relapse.

Further supporting the interpretation that circulating B lymphocytes with the IgD+CD38high phenotype are immature cells and not germinal centre founder cells, circulating B lymphocytes with this phenotype have recently been described in normal adults with functional studies confirming their immaturity (Carsetti et al., 2004, Sims et al., 2005). It is possible that this is actually the phenotype of all "young" B lymphocytes when they exit the bone marrow. In normal adults these cells are probably not easily detected as they are diluted in the much bigger naïve mature recirculating B-lymphocyte population that is IgD positive but CD38 low. This would imply that B lymphocytes need to mature in solid lymphoid tissues other than the bone marrow before becoming

naïve mature recirculating B lymphocytes, a phenomenon previously not described in man but well known in mice (Chung et al., 2003).

Repopulation was also associated with increased expression of CD5 by circulating B lymphocytes. Interestingly, this increase seemed to be in some way independent of the increased expression of CD38 on naïve B lymphocytes as return to pre-treatment frequencies of IgD+CD38++ expression preceded the return of CD5 expression to baseline levels. Similarly, after BMT, not all patients who show increased numbers of IgD+CD38++ B cells show increased expression of CD5 (Drexler et al., 1987, Bengtsson et al., 1989, Kagan et al., 1989, Small et al., 1990).

It has been suggested that expression of CD5 occurs at the immature B-lymphocyte stage and that its expression may be related to the surface immunoglobulin specificity. This would help to explain why there is not a perfect concordance between expression of IgD+CD38++ pattern and expression of CD5. In B lymphocytes with polyreactive, low affinity BCR, potentially self-reactive, it has been suggested that CD5 expression would be induced and this molecule would inhibit B-lymphocyte activation and autoreactivity (Hippen et al., 2000). Nevertheless, while in cord blood the majority of CD5 positive B lymphocytes have polyreactive BCR, in adults, this percentage decreases to around 40% (Chen et al., 1998). Studies of B-lymphocyte and Ig repertoires following BMT have described transient preferential expression of smaller VH families similar to ontogeny (Berman et al., 1991, Fumoux et al., 1993). These rearrangements may be more polyreactive and be useful in recognising common pathogens. It would make sense from the evolutionary point of view, that when the bone marrow is regenerating, to first use VH rearrangements that will be useful for fighting common pathogens and then diversify. These cells may be more prone to be self-reactive and so CD5 expression would be induced (Sanz et al., 1989, Pascual and Capra, 1991). Whether this happens or not and the underlying mechanism is not known. The frequency of polyreactive BCR following BMT or rituximab is not known.

At repopulation a variable proportion of memory B lymphocytes could be detected. Patients who relapsed at the time of B-lymphocyte repopulation showed a tendency to have a higher frequency and particularly higher numbers of memory B lymphocytes at this time point when compared with patients who relapsed only later. This suggested

that depletion of memory B lymphocytes in solid lymphoid tissues may have been less complete in those patients who relapsed earlier. This therefore raised the possibility that the response to treatment may be prolonged, by inducing a more extensive B-lymphocyte depletion. However, it is also possible that the memory B lymphocytes circulating at the time of B-lymphocyte repopulation were "young" B lymphocytes that had already differentiated into memory cells and were not related to residual memory B lymphocytes. Their increased numbers in patients who relapsed earlier would therefore merely be a reflection of accelerated B-lymphocyte maturation associated, in these patients, with relapse.

Patterns of repopulation

B-lymphocyte repopulation occurred either rapidly with normal B-lymphocyte counts within 4 weeks, or more frequently, more slowly, over a period of several months. The speed of B-lymphocyte repopulation did not correlate with the pattern of relapse with patients with slow and rapid patterns of repopulation relapsing both at the time of B-lymphocyte return or later. The speed at which B-lymphocyte counts reach the normal range also varies between patients following BMT (Storek and Saxon, 1992, Storek et al., 1997a). This may reflect different regenerative capacities of the individual's bone marrow. Another key finding in the study described in this thesis was that in patients who repopulated slowly, B-lymphocyte repopulation could initially be missed if the less sensitive techniques used routinely in UCL Central Pathology Laboratory, were used to detect peripheral B-lymphocyte counts.

Changes in other cell types following BLDT

CD20 is usually described as an antigen specific for B-lymphocyte lineage cells but a small number of previous reports had described the presence of T cells expressing low levels of CD20 in the peripheral blood and in the bone marrow (Hultin et al., 1993, Quintanilla-Martinez et al., 1994, Algino et al., 1996). In the study presented in this thesis, a small population of circulating T cells but also of NK cells, expressing low levels of CD20 was detected. These cells disappeared from the circulation for a mean of 5 months following treatment with rituximab. Flow cytometry data on a few individual samples collected in the study, comparing expression of CD20 on T and B lymphocytes suggested that CD20low T and NK cells were actually depleted and not just masked by circulating rituximab. The fact that in most patients these CD20 low T and NK cells

started being detected again around 5 months suggested that this may be the time point at which rituximab had already been cleared from circulation thus allowing repopulation (or detection) of these cells.

No other significant changes in the total number or frequency of the other peripheral blood T cell populations studied except for a transitory decrease at one month in the frequency of CD4+CD25++ T cells (regulatory T cells) was observed. In patients with lymphoma or with cold agglutinin disease treated with rituximab no significant changes on peripheral blood T lymphocyte populations have been described (Lee and Kuech, 1998, Maloney et al., 1994). In one report on SLE patients, decreased expression of activation markers on CD4+ circulating T lymphocytes following rituximab therapy has, however, been reported (Sfikakis et al., 2005).

In summary, BLDT induced a profound depletion of all peripheral blood B-lymphocyte subpopulations in patients with RA. Repopulation occurred mainly with naïve mature and immature B lymphocytes. The group of patients who relapsed earlier, at the time of B-lymphocyte repopulation, tended to show repopulation with a higher frequency and higher numbers of memory B lymphocytes.

6.6 Depletion of B-lymphocyte lineage cells in the bone marrow and possible association with clinical response

As shown in chapter 5, in the 6 patients studied, the relative proportions of different B-lymphocyte precursor cells in bone marrow samples 3 to 4 months after BLDT varied between patients, despite patients showing similar levels of depletion in peripheral blood. It was possible that this might in some way reflect the degree of depletion achieved initially in the bone marrow in individual patients.

In children treated for acute lymphoblastic leukemia several authors have found differences in the pattern of regeneration of B-lymphocyte precursors that seemed to be related to the intensity of the preceding treatment and presumably to the degree of cell killing induced (Dworzak et al., 1997, van Lochem et al., 2000, van Wering et al., 2000). Van Lochem and colleagues found that the ratio between 'more mature' and 'immature' B-lymphocyte precursors was less than 1.0 in regeneration after more

aggressive therapy, between 1.2 and 2.8 after less aggressive therapy and 6 to 8 after cessation of all treatment. In normal bone marrow samples the more immature subsets are usually present in relatively small numbers (Dworzak eet al., 1997). In the 6 patients with RA studied, those who relapsed clinically earlier (at the time of B-lymphocyte repopulation), showed higher proportions of more mature B-lymphocyte precursors than those patients who relapsed only five or more months after B-lymphocyte return. This suggested that patients who relapsed earlier might have depleted less well in the bone marrow and possibly in other solid lymphoid tissues. Animal studies have shown that progressively higher rituximab doses are needed to deplete B lymphocytes in peripheral blood, bone marrow and lymph nodes in this order (Reff et al., 1994, Alwayn et al., 2001, Schroder et al., 2003). Different individuals treated with the same rituximab dose show different degrees of depletion but consistent levels of depletion within solid lymphoid tissues in each individual were found (Reff et al., 1994, Schroder et al., 2003).

It was also of interest that despite the presence of B-lymphocyte precursors in the bone marrow in 5 of the 6 samples, B-lymphocyte repopulation of the peripheral blood was only seen 2 to 8 months later. In addition, the observation was made that CD20 expression by cells of B-lineage in the bone marrow samples was either low or absent. This could be explained by the presence of bound rituximab, which would mask full detection of the CD20 antigen by monoclonal antibodies. Rituximab has a long half-life and rituximab could be able to prevent full regeneration of the bone marrow until its complete clearance (Berinstein et al., 1998, Ng et al., 2005). Similar results (absent or low CD20 expression) had been found in the small number of B lymphocytes found circulating in the peripheral blood samples. Other authors have also reported that the CD20 antigen cannot be detected by flow cytometry on the surface of B lymphocytes following treatment with rituximab suggesting that this is due to the masking of CD20 by rituximab and results reported by Jilani I and colleagues strongly suggest that this is the case (Jilani et al., 2003). Rituximab was shown to have unusual pharmacokinetic characteristics with antibody levels detected in the serum for more than 6 months in patients with lymphoma despite an half-life of 9 days (Berinstein et al., 1998, Grillo-Lopez, 2000). Another possibility was that anti-CD20 caused downregulation of the CD20 antigen. Jilani and colleagues have shown that this can occur in some CLL patients treated with rituximab but there is no suggestion that this is a major mechanism for resistance to rituximab treatment in lymphoma (Jilani et al., 2003, Maloney et al.,

2002). There is no information available on whether this can happen in normal B lymphocytes.

Some authors have found variable expression of low or intermediate levels of CD20 by pro-B lymphocytes, but the majority of these cells do not express CD20 (Dworzak et al., 1997, McKenna et al., 2001, Lucio et al., 1999). Therefore, pro-B lymphocytes should not be depleted by rituximab and as B-lymphocyte formation occurs throughout life in humans, they were expected to have been present at least in normal numbers and proportionally increased in samples of patients treated with rituximab. Unexpectedly, a consistent proportional increase on pro-B lymphocytes was not observed in 5 of the 6 patients and in 1 no pro-B lymphocytes were detected. The fact that no pro-B lymphocytes were detected in one of the samples may be due to the patient's bone marrow only containing very small number of B-lymphocyte precursors and below the sensitivity of the method used. Following previous treatments with BLDT, the same patient had only started repopulating at 12 months or later and usually repopulated slowly starting with small numbers of cells.

The absence of a proportional increase in pro-B lymphocytes in all patients could also suggest that pro-B lymphocyte survival might be affected following treatment with rituximab. This would help explain why B-lymphocyte repopulation after rituximab treatment takes longer than that after bone marrow transplantation following myeloablative therapy. In patients with B-cell NHL treated with rituximab, recovery of B-lymphocyte counts in the peripheral blood started at 6 to 9 months, and normal numbers were observed after 9 to 12 months (McLaughlin et al., 1998). In the phase II RA study, B-lymphocyte depletion lasted more than 6 months in all patients (Edwards et al., 2004). In the RA cohort that is the focus of this thesis, B-lymphocyte repopulation usually occurred between 6 and 9 months after treatment and occasionally later (up to 21 months). Time of B-lymphocyte repopulation after bone marrow transplantation varies between the studies but the period of profound B-lymphocyte depletion in the peripheral blood lasts usually only 1 to 4 months (Zintl et al., 1989, Storek et al., 1993, Witherspoon et al., 1981). Studies in mice suggest that interactions between B-lymphocyte precursors may play an important role in promoting their own development but no similar data exists in humans (Stoddart et al., 2001). Also, in patients with X-linked agammaglobulinaemia, where there is a maturation defect occurring after the

pro-B lymphocyte stage, pro-B lymphocytes develop normally and accumulate in the bone marrow frequently in the absence of significant numbers of other B-lymphocyte precursors (Campana et al., 1990).

It is therefore likely that both prolonged availability of rituximab and some depletion of pro-B lymphocytes explain the differences in time to B-lymphocyte repopulation described above. It is possible that a proportion of human pro-B lymphocytes do express very low levels of CD20 and that rituximab is available for prolonged periods of time and mediates specific death signals to developing B-lymphocyte precursors, including pro-B lymphocytes. Each subpopulation of B cell precursors defined on a certain combination of markers will contain cells that are only beginning their maturation stage and also cells that are almost finishing their maturation stage and therefore heterogeneity on their expression of some of the markers is expected. In the BM sample obtained from a lymphoma patient at a time when peripheral blood repopulation had already occurred, it was clear that while expression of CD34 defined two separate subsets of cells (positive and negative subsets), CD20 expression was continuous. A small number of CD19+CD34+ cells (pro-B lymphocytes) were seen to express low levels of CD20.

In conclusion, in this small cohort of 6 RA patients treated with BLDT, differences found in total numbers and relative frequencies of different B-lymphocyte precursor cells are probably due to different regenerative capacities of the bone marrow in individual patients together with the time gap between treatment and sampling and possibly different degrees of depletion achieved initially. There was a trend to longer duration of clinical response to treatment in patients whose bone marrow findings suggested a greater degree of depletion. Time to full generation of new B lymphocytes following treatment with rituximab might be determined mainly by the regenerative capacity of the bone marrow and clearance of the drug.

CHAPTER 7 FINAL DISCUSSION

7.1 What does BLDT tells us about primary roles of B lymphocytes and T lymphocytes in the pathogenesis of rheumatoid arthritis – Mechanisms of Relapse

BLDT based on rituximab in RA provides a unique opportunity to study disease pathogenesis, as reflected in why patients respond to treatment and particularly why they relapse. The major improvement in manifestations of active disease seen in a significant number of patients and the fact that no patient relapsed before B-lymphocyte repopulation of the peripheral blood showed that disease perpetuation in these patients was B-lymphocyte dependent.

The hypothesis that led to the use of BLDT in the treatment of RA postulated that a clinical response should occur if both depletion of pathogenic B-lymphocyte clones and concomitant decrease in pathogenic autoantibody levels was achieved. It also suggested that long-term remission might be achievable if B-lymphocyte return occurred in the absence of pathogenic autoantibodies. A natural extension of this hypothesis postulated that relapse could be due to insufficient depletion of pathogenic B-lymphocyte clones, to existence of pathogenic autoantibody-producing long-lived plasma cells or to a combination of both. This was the working hypothesis for this thesis.

Relapse following BLDT could also be due to a primary abnormality of B-lymphocyte tolerance that would lead to a new but again autoreactive B-lymphocyte repertoire on B-lymphocyte reconstitution (Samuels et al., 2005).

It is also important to discuss whether the pathogenic B-lymphocyte clones reside mainly in the synovial tissue and, therefore, whether effectiveness of BLDT is mainly due to local depletion of cells, or whether they reside predominantly in solid lymphoid tissues with plasma cells localised both in the bone marrow and in the synovial tissue.

7.1.1 Relapse due to incomplete depletion of pathogenic B-lymphocyte clones

Several observations described in this thesis suggest that relapse following BLDT may be due to the persistence of pathogenic B-lymphocyte clones that escape depletion by rituximab:

(1) The results suggested a dose-response to rituximab and the existence of a threshold of depletion for patients with RA to respond clinically to BLDT;

(2) Earlier relapse, at the time of B-lymphocyte repopulation, was associated with a tendency to repopulate with higher frequency and higher numbers of memory B lymphocytes possibly representing residual cells;

(3) Earlier relapse was also seen in patients in whom bone marrow sample analysis suggested less significant initial depletion;

(4) BLyS serum levels at repopulation were lower in patients who relapsed earlier, possibly reflecting a greater expansion of B lymphocytes in solid lymphoid tissues.

Rituximab is known to induce an almost complete depletion of B lymphocytes in the peripheral blood for a period that usually lasts between 6 to 9 months but as previously discussed, little is known about the exact degree of depletion of normal B lymphocytes in solid lymphoid and extra-lymphoid tissues in humans following treatment with rituximab. Studies in patients with lymphoma suggest that standard doses of rituximab are below the plateau in the dose-response curve for the drug. The standard protocol with 4 infusions of 375mg/m^2 was chosen to undergo phase II lymphoma trials because it had been shown to have a major biological effect in dose-escalating trials and did not exceed the company's manufacturing capacity at the time.

In mice rendered transgenic for human CD20, different B-lymphocyte subpopulations had different sensitivities to killing by anti-human CD20 monoclonal antibodies. This is probably related to innate and acquired survival characteristics of the cells as well as to the characteristics of their microenvironment and recirculation patterns (Gong et al., 2005). Follicular B lymphocytes were most susceptible to killing by anti-CD20 compared with marginal B lymphocytes, which were relatively resistant, and with B lymphocytes undergoing germinal centre reactions at the time of anti-CD20 administration, which were the most resistant to killing. Depletion of B-lymphocyte

subpopulations correlated closely with recirculating patterns (Gong et al., 2005). Additional factors that will certainly influence B-lymphocyte killing by rituximab relate to anti-CD20 pharmacokinetics and pharmacodynamics such as concentration achieved in different tissues, drug clearance, availability and effectiveness of effector mechanisms recruited by the monoclonal antibody.

The UCL extended open-label trial suggested a dose-response to rituximab. In the peripheral blood immunophenotyping study, the patient who did not deplete well in the peripheral blood did not respond to treatment. Autoantibodies decreased in all patients but only significantly in the group of patients that responded to treatment. Also, percentage decreases in sCD23 levels in patients who responded to treatment tended to be greater than in those who did not respond to treatment. Altogether these data suggested that there was a quantitative threshold that needed to be achieved for BLDT to be effective in the treatment of RA. They suggested that a critical mass of pathogenic B-lymphocyte clones had to be depleted to achieve a detectable effect on inflammation and disease activity.

During the period of B-lymphocyte depletion in the peripheral blood that follows rituximab therapy, a very small number of circulating B lymphocytes were always detected. The majority of these cells expressed a memory or plasmablast phenotype. This observation proves that depletion is not complete and suggests that the majority of B lymphocytes remaining are memory cells.

Patients either relapsed at the time of B-lymphocyte return or at a variable time after. In the immunophenotyping study patients who relapsed earlier, at the time of B-lymphocyte return, showed a tendency to have a higher frequency of memory B lymphocytes at repopulation when compared to patients who relapsed only later. This suggested that in the former group of patients a higher number of surviving memory B lymphocytes including possibly, pathogenic B-lymphocytes, was present. Also, the small bone marrow study showed differences in ratios between more immature and more mature B-lymphocyte precursors and suggested that patients who relapsed earlier had depleted less well.

B lymphocytes with a memory phenotype that were detected circulating at the time of B-lymphocyte repopulation may be memory B lymphocytes that have escaped depletion by rituximab but they may also be newly formed B lymphocytes that have already undergone differentiation into memory cells. In this later case, the tendency for these memory cells to be present at higher frequencies and higher numbers in patients who relapsed at the time of repopulation could be just a sign of relapse. However, increased numbers of plasma cell precursors, which were found circulating in the initial stages of B-lymphocyte repopulation in several patients, did not correlate with time of relapse. Plasma cell precursors should possibly be a better marker for active disease and relapse than memory B lymphocytes themselves.

Although, the data presented above, seem to draw a suggestive and relatively simple picture of the existence of a threshold for B-lymphocyte depletion to be effective and of relapse being due to persistent B-lymphocyte clones as earlier relapse seemed to be associated with less effective depletion, the reality is probably much more complex. The group of patients who relapsed earlier (at B-lymphocyte return) showed lower levels of BLyS at repopulation when compared to the group of patients who relapsed only later. This might possibly reflect a greater expansion of B lymphocytes in solid lymphoid tissues at this time point in this group of patients. This might suggest that B-lymphocyte depletion was less pronounced in this group of patients, but no differences were detected when BLyS levels during the period of depletion were compared between the two groups of patients. It is not known whether BLyS serum levels depend more on the physical presence of certain B-lymphocyte subpopulations or on BLyS uptake.

If pathogenic memory B-lymphocyte clones persist in solid lymphoid tissues during depletion with rituximab why does relapse not occur before B-lymphocyte repopulation? One possible explanation is that the capacity for expansion of memory B lymphocytes is limited and that recruitment of naïve B lymphocytes is needed to allow reconstitution of a critical mass of pathogenic B-lymphocyte clones. A second possible explanation is that until rituximab levels decrease below a critical threshold, B lymphocytes that have not been killed are susceptible to its potential anti-proliferative effects, not being able to proliferate or differentiate into plasma cells.

Studies on B-lymphocyte reconstitution after BMT, showed that repopulation of the peripheral blood does not derive from mature B lymphocytes but only from newly formed naïve B lymphocytes. This suggests that these are two separate cellular compartments, which are differentially regulated or that the capacity for expansion of mature B lymphocytes is limited. Competition between naïve immature and mature B lymphocytes for survival signals probably provided in lymphoid follicles is thought to play a major role in the death of the majority of naïve immature B lymphocytes formed daily. What the data from BMT patients suggest is that this competition does not seem to play a significant role in the capacity for expansion of memory B lymphocytes which is most likely dependant on specific antigenic stimulation and competition for survival signals within the memory B lymphocyte subpopulation. But in the case of autoimmune diseases, the antigen is always present and therefore, if pathogenic B-lymphocyte clones persist, either rituximab plays a role in preventing the expansion of autoreactive B lymphocytes that have escaped depletion or new naïve B lymphocytes somehow need to be recruited for the immune process to restart.

However, very small number of cells can be detected during depletion that have a plasma cell precursor phenotype suggesting that some B lymphocytes are still undergoing maturation i.e. activation and differentiation into plasma cells. The significance and importance of these phenomena is not known. Two of the patients reported in this thesis were immunised with TT and PCP during the period of depletion and did not respond to the vaccines (no increase in measured specific antibody levels). Treatment with rituximab is known to interfere with immune responses to primary and secondary antigenic challenges (Gonzalez-Stawinski et al., 2001).

Interestingly, individual patients showed similar dynamics of relapse following each of repeated courses of treatment, i.e. always either relapsed at B-lymphocyte return or at a variable time after B-lymphocyte return to the peripheral blood. This suggests that there are parameters of disease dynamics which vary between patients but which remain relatively constant for a given patient. Differences between patients may be due to:

(i) Differences in the load of pathogenic B-lymphocyte clones or autoantibody-producing long-lived plasma cells;

(ii) Differential pathogenic potential of autoantibodies associated with B-lymphocyte clones;

(iii) Differential presence of B-lymphocyte clones that are more resistant to killing by rituximab (due to their maturation status or the characteristics of their microenvironment);

(iv) Differences in host effector mechanims recruited by rituximab to kill B lymphocytes (antibody-dependent cellular cytotoxicity and complement-induced cytotoxicity).

It is also possible that patients differ at repopulation in the maturity status of the repopulating naïve B lymphocytes and that this influences their capacity to influence relapse.

7.1.2 Relapse due to presence of long-lived plasma cells producing pathogenic species of autoantibodies

Relapse may be due to the presence of autoantibody-producing long-lived plasma cells. Serum levels of autoantibodies decreased significantly following BLDT in patients who responded to treatment but patients only rarely became seronegative. Relapse was preceded or associated with a rise in autoantibodies in almost all patients.

Normal plasma cells do not express CD20 and are therefore not depleted by rituximab. Following B-lymphocyte depletion with rituximab, plasma cells with a short half-life will die and will not be replaced as their precursors will have been depleted by the anti-CD20 antibody. In contrast, plasma cells with a relatively longer life will continue to be present and to produce antibodies. If plasma cells producing certain species of RhF survive for longer than 6 to 9 months, the usual period of B-lymphocyte depletion following treatment with rituximab, the availability of the autoantibodies they produce could contribute to the aberrant survival of newly formed naïve RhF-specific B lymphocytes by providing their own antigen in the form of small immune complexes associated with C3d, leading to re-instigation of a vicious cycle and potentially to clinical relapse.

The results of the serologic studies suggested a selective effect of BLDT on autoantibodies possibly due to the fact that a significant proportion of the disease-associated autoantibodies were produced by relatively short-lived plasma cells and

therefore more susceptible to BLDT. IgA-, IgG- and IgM-RhF and IgG anti-CCP antibodies decreased in all patients following BLDT and for IgA and IgG autoantibodies, the decrease was proportionally more than the decrease observed in the respective serum total immunoglobulin levels. IgM-RhF decreased to the same extent as total IgM. Anti-microbial antibodies behaved differently. IgG anti-TT antibodies decreased significantly but transiently and to the same extent as total IgG. IgG anti-PCP antibodies did not decrease significantly.

These serologic studies also showed that only rarely did IgA-, IgG- or IgM-RhF or anti-CCP antibodies decreased to within the normal range or became undetectable suggesting that a proportion of autoantibody-producing plasma cells have a longer half-life. Plasma cells were detected in all bone marrow samples three months after treatment. Animal studies have showed that some anti-dsDNA antibody producing plasma cells can be long-lived (Hoyer et al., 2004). This means that autoantibodies will be available at the time of repopulation.

In this cohort of patients only those seropositive for RhF responded to BLDT. The kinetics of the clinical response followed the kinetics of autoantibody serum levels more closely than the presence or absence of B lymphocytes per se. Disease-associated autoantibodies decreased more in patients who responded to treatment than in patients who did not, suggesting that autoantibodies might need to decrease below a critical level for patients to respond to BLDT. B-lymphocyte repopulation was not always associated with clinical relapse but relapse was almost always preceded or associated with a rise in autoantibody serum levels.

In addition, patients frequently took two to three months to respond to treatment while B-lymphocyte depletion in the peripheral blood is known to occur very quickly and the bulk of the depletion in the lymphoid solid tissues is thought to occur within the first few weeks. Primate studies have suggested that there is no progression of the degree of depletion of normal B lymphocytes from 2 to 4 weeks on repeated biopsies of lymph nodes (Reff et al., 1994). However, in the first phase II study of rituximab in lymphoma, median onset of clinical response was 50 days, ranging from 7 to 112 days, with maximum response occurring 3 to 4 months after therapy (Maloney et al., 1997b). In lymphoma, response to rituximab will certainly reflect the amount of killing of tumour

cells, although the kinetics of killing of tumour cells may be different from killing of normal human B lymphocytes. The presence of increased numbers of cells targeted by rituximab and differences in cell cycling rates may increase the time taken to have an impact on tumour reduction.

In the KBxN mouse, an animal model of spontaneous chronic destructive arthritis in which the initiating event is the presence of autoreactive T lymphocytes, an essential effector role for B lymphocytes through their production of polyclonal autoantibodies to an ubiquitous antigen, GPI, has been shown (Matsumoto et al., 1999). Inflammation is caused by anti-GPI antibodies and mediated by their interactions with Fcγ receptors and complement (JiH et al., 2002). As expected in a model involving Fcγ receptors and the necessity of cross-linking these receptors for intracellular signalling to be triggered, monoclonal antibodies were not able to induce disease (Maccioni et al., 2002). This raises the general point that pathogenic pathways may depend not simply on the presence of antibody but also on the co-availability of several antibody species with synergistic roles.

7.1.3 Relapse is due to the fact that the memory for the disease resides in other cells, such as autoreactive T lymphocytes

It has been proposed in this thesis that clinical relapse could be due to one or both of the mechanisms proposed above (7.1.1.1 and 7.1.1.2). Altogether, the results suggested that the kinetics of clinical response and clinical relapse reflected more the kinetics of the autoantibody response than the presence or absence of B lymphocytes per se. This suggests that the central role that B lymphocytes play in the perpetuation of inflammation in at least a subgroup of patients with RA, is mediated through their production of autoantibodies. Autoantibodies may have direct roles in inducing inflammation but may also facilitate aberrant interactions between B and T lymphocytes.

However, the results can also be interpreted in terms of other hypothesis for the pathogenesis of RA. The main alternative hypothesis is based on the premise that autoreactive T lymphocytes are the effector cells driving macrophage activation in rheumatoid synovium. The suggested roles for B lymphocytes to explain the B-

lymphocyte dependency of T-lymphocyte activation include antigen presentation and cytokine production. BLDT efficacy would therefore be mainly mediated through removal of such B lymphocytes from the synovial tissue itself. In this scenario, relapse would be due to the persistence of pathogenic autoreactive T-lymphocyte clones which would be able to re-initiate the process once B lymphocytes with the appropriate specificity and maturity status became available.

Only seropositive patients in the cohort described in this thesis responded to BLDT. Similar results have been described in other small cohorts. However, as discussed earlier, in the recently published phase IIb and III rituximab trials in RA, seronegative patients are said to have responded to BLDT. As discussed earlier, no detailed data is yet available on these patients and therefore, no definite conclusions can be drawn. If seronegative RA patients do respond to BLDT, this raises the possibility that B lymphocytes may also contribute to inflammation by supporting autoreactive T lymphocytes with inflammatory effector functions, through presentation of antigen and eventually cytokine production.

The puzzle in seronegative RA is that if the pathogenic role of B lymphocytes is to present antigen to T lymphocytes, these B lymphocytes would still be expected to secrete autoantibodies, as has been observed in the NOD mice model for diabetes mellitus type 1 (Wong et al., 2004, Tian et al., 2006). It seems very unlikely that a truly "seronegative" autoimmune process might be B-lymphocyte dependent. However, it cannot be excluded that, in seronegative RA patients, autoantibodies of different specificities, for which detection systems are currently unavailable, may be involved in disease pathogenesis.

In addition, an essential role for B lymphocytes in driving the inflammation through production of inflammatory cytokines seems unlikely on the basis of the numbers and location of B lymphocytes in the patients. In the majority of synovial samples from rheumatoid samples B lymphocytes are present in only small numbers (Edwards and Wilkinson, 1995). It cannot be excluded that they may stimulate systemic inflammation in secondary lymphoid tissues, where autoimmune responses are generated but in in vitro studies B lymphocytes always secrete much smaller amounts of cytokines when

compared with other cells and it seems unlikely that these would play a more important role than specific B-T lymphocyte interactions.

If the importance of B lymphocytes in the perpetuation of inflammation was to activate autoreactive T lymphocytes either by presenting antigen or by secreting pro-inflammatory cytokines and chemokines, patients would be expected to always respond relatively quickly to BLDT as animal studies suggested that depletion of normal B lymphocytes by rituximab occurs quickly. However, maximal responses in patients with lymphoma can take 3 to 4 months indicating that continuing B-lymphocyte depletion can occur. Whether this also applies to normal B lymphocytes is not known.

Many studies have focused on the role of B lymphocytes on the pathogenesis of animal models of autoimmune diseases, including animal models of arthritis, with several reporting the effects of absence of B lymphocytes, presence of B lymphocytes unable to secrete immunoglobulin and with restricted immunoglobulin repertoire or, more recently, effects of B-lymphocyte targeted therapies, on disease expression.

For example, in proteoglycan-induced arthritis, experiments suggested that B lymphocytes play an essential role in disease pathogenesis both in priming autoreactive T lymphocytes as well as producing autoantibodies that may have direct effector roles in causing inflammation (O'Neill et al., 2005). Optimal priming of autoreactive T lymphocytes required both cognate B-T lymphocyte interactions and therefore specific autoreactive B lymphocytes and the presence of secreted autoantibodies.

In collagen-induced arthritis, B-lymphocyte depletion therapy inhibited the development of clinical and histological features of arthritis (Dunussi-Joannopoulos et al., 2005). This effect was said to be independent of its possible effect on autoantibody production as elimination of antibodies to collagen type II was not necessary for the prevention of arthritis in these animals. However, the possibility that the efficacy of B-lymphocyte depletion could also reflect its effect on preventing the maturation of the antibody response and consequent development of potentially pathogenic species of antibodies to collagen type II was not addressed or discussed. Several studies have shown that the capacity of antibodies to collagen type II to cause disease on serum-transfer experiments is dependent on the susceptibility of the recipients but also on the

characteristics of the antibodies transferred. Not all combinations of antibodies to collagen type II are arthritogenic (Stuart and Dixon, 1983, Reife et al., 1991, Brand et al., 1996).

In the SCID mouse/RA synovium chimaera model, activation of T lymphocytes extracted from follicular structures in RA synovium samples was shown to be B-lymphocyte dependent (Takemura et al., 2001b). In this setting, T-lymphocyte activation was HLA-DR restricted and presence of oligoclonal proliferation suggested that it was antigen specific. The results suggested that either B-lymphocytes in these follicular structures were essential APC to the T lymphocytes or they contained the relevant antigen. However, even if this mechanism might contribute to synovial inflammation in some patients with RA, it is very unlikely that it could justify B-lymphocyte dependency of inflammation and disease activity in a significant number of RA patients. Germinal centre-like structures are seen in only approximately 20 percent of RA synovium samples while in synovium samples from many patients with RA, B lymphocytes are absent or present in only small numbers (Takemura et al., 2001a) (Edwards and Wilkinson, 2005). In contrast, up to 70% of patients with RA in trials have been reported to respond significantly to treatment (Edwards et al., 2004).

In the MRL/lpr mouse, an animal model of lupus, absence of B lymphocytes prevented autoimmune manifestations (Shlomchik et al., 1994). However, reconstitution with B lymphocytes capable of expressing surface membrane immunoglobulin but not of secreting antibodies was associated with the development of interstitial nephritis and perivascular inflammation but not, as predicted glomerulonephritis and immune deposits which are antibody mediated (Chan et al., 1999a). Interstitial nephritis is thought to be T-lymphocyte mediated in this model and these results showed that presence of B lymphocytes was essential for its expression. How much these results reflect pathogenic mechanisms in human lupus (where the most common manifestation is glomerulonephritis which is most likely immune complex mediated) or in other autoimmune diseases is not known. In support of a possible role for B lymphocytes in T-lymphocyte activation, a recent report, described a decrease in the expression of activation markers (CD40 ligand, CD69 and HLA-DR) on circulating CD4+ T cells in patients with lupus nephritis who responded to rituximab (Sfikakis et al., 2005).

Interestingly, in the NOD mouse model for diabetes mellitus type 1 in which an essential role for specific autoreactive B lymphocytes in the activation of effector autoreactive T lymphocytes has been demonstrated, there are still doubts whether this role depends only on the surface immunoglobulins expressed by these B lymphocytes or also on secreted autoantibodies. Also, studies in this mouse model suggested that these specific autoreactive B lymphocytes were not sufficient to break T-lymphocyte tolerance to islet cell antigens but were essential for the expression and possibly diversification of the T-lymphocyte autoreactivity (Moore et al., 2005, Tian et al., 2006). B lymphocytes seemed to activate autoreactive T lymphocytes only if T lymphocyte tolerance had not been established (Moore et al., 2005).

In many of these animal models of autoimmune diseases, breakage of T-lymphocyte tolerance is known to be primary to disease pathogenesis. Underlying the discussion on a primary role for B lymphocytes in the pathogenesis of several of these diseases is whether B lymphocytes can have an essential role in priming naïve T lymphocytes and consequently breaking tolerance. However, even though animal models of disease can be very useful to understand elements of the disease process, their role in leading to an understanding of primary events in human disease initiation and perpetuation is much more limited.

In summary, a primary pathogenic role for B lymphocytes in activating autoreactive T lymphocytes in RA is not supported by the data presented in this thesis but cannot be totally excluded.

7.1.4 Effects of B-lymphocyte depletion on B lymphocytes residing in the synovial tissue

Since the effectiveness of BLDT has been proven in RA there has been a tendency to assume that its effects are a direct reflection of depletion of B lymphocytes localised in the synovial tissue. Although local depletion and particularly the lack of replacement of local short-lived plasma cells, will certainly contribute to a reduction in inflammation, it is unlikely that this is the main reason for clinical improvement in such a significant number of patients.

Firstly, B lymphocytes are usually present in only small numbers in rheumatoid synovium. In contrast to T lymphocytes, B lymphocytes do not normally accumulate in sites of chronic inflammation. Synovial biopsy samples from patients with recent onset RA show mainly macrophage activation. Also, in only approximately 20% of patients with RA, are structures similar to lymphoid follicles present in synovium samples. In the phase II rituximab trial in RA, BLDT combination protocols were effective in around 70% of patients and it is unlikely that all these patients posessed significant numbers of B lymphocytes in their synovium. As mentioned in the previous section, in the SCID-rheumatoid synovium chimera mouse model, T-lymphocyte activation was B-lymphocyte dependent only in samples containing increased numbers of B lymphocytes.

Secondly, it is very unlikely that production of the more prevalent autoantibodies in RA, RhF and anti-CCP antibodies, is a local phenomenon occurring in involved joints or draining lymph nodes. RhF and anti-CCP antibodies, are directed to ubiquitous antigens and not to antigens preferentially expressed in synovium (or diarthrodial joints). In individuals who later developed RA, RhF and anti-CCP have been detected in the serum up to 14 years before development of clinical disease. It is very unlikely that initiation of the production of these autoantibodies at these time points is just a local epiphenomenon, secondary to subclinical inflammation in the joints. The fact that their detection in serum can precede clinical disease by years suggests that they may be involved in disease initiation events and that these events take place probably in secondary lymphoid tissues and do not start in the joints.

Plasma cells are present in increased numbers in most biopsy samples of rheumatoid synovium from patients with established disease. These plasma cells have been shown to produce RhF among other antibodies. Autoantibodies produced by these plasma cells localised in the synovium are thought to contribute to local inflammation but their contribution to serum autoantibody levels is not known. Plasma cells in the synovium are thought to be formed in solid lymphoid tissues and then to migrate to the synovium as part of the chronic inflammatory response, as they are often present in the absence of significant numbers of local B lymphocytes and follicle-like structures. In addition, production of RhF in RA has been identified not only in the synovium but also in the

bone marrow and in the peripheral blood. It is not known for how long plasma cells survive in the synovium.

Thirdly, even though diarthrodial joints are the main target in RA, this is a systemic disease and it is very unlikely that extra-articular manifestations (which are occasionally more evident than joint disease) other than systemic symptoms could be a consequence of synovitis.

7.2 What do the consequences of BLDT in RA tell us about B-lymphocyte biology

The study of B-lymphocyte depletion and repopulation, of changes in different antibodies and BLyS serum levels following BLDT provided a unique opportunity to study a number of aspects of B-lymphocyte homeostasis and humoral memory in humans.

7.2.1 Longevity of plasma cell populations and humoral memory

The results suggested that although a proportion of circulating antibodies are produced by short-lived plasmablasts or plasma cells, the majority are produced by longer-lived plasma cells. These later cells can survive at least 12 months or longer as serum immunoglobulin levels remain within the normal range for the whole period of peripheral blood B-lymphocyte depletion following BLDT in the majority of patients. Interestingly, in a patient with SLE treated at UCLH, B-lymphocyte depletion in the peripheral blood persisted for more than 4 years (Leandro et al., 2005). Serum immunoglobulin levels remained within the normal range at time of writing but have shown a slow, progressive decrease.

Plasma cells were detected in all bone marrow samples 3 to 4 months after treatment with BLDT, with both CD19 positive and CD19 negative populations being present. It has been suggested that CD19 negative plasma cells may be more mature and have longer half-lives than CD19 positive cells. The study reported in this thesis suggests that both types of plasma cells survive for at least 3 to 4 months.

Serum levels of IgA and IgG autoantibodies decreased more than their respective immunoglobulin class suggesting that a higher proportion of plasma cells secreting autoantibodies were short-lived when compared to plasma cells secreting antimicrobial antibodies. The results confirmed this to be true for IgG anti-TT and anti-PCP antibodies. Whether this is a consequence of antigen persistence in antigen excess conditions (such as in the presence of abundant autoantigen) or of abnormalities in regulation and location of the autoimmune reactions is not known. In animal models, autoimmune cells have been shown to proliferate and undergo somatic hypermutation outside the germinal centre (William et al., 2002, Shlomchik et al., 2003).

The fact that anti-TT decreased to the same extent as total IgG suggested that serum anti-TT antibody levels were maintained by a mixture of cells with the majority of anti-TT secreting plasma cells being long-lived but a fraction being short-lived. Frequently, anti-TT serum levels increased following B-lymphocyte repopulation without any immunisation. This suggested that some anti-TT memory B lymphocytes have survived rituximab and that they undergo either activation by antigen persisting in immune complexes on follicular dendritic cells or "non-specific" activation (possibly through Toll-like receptors) and differentiation into plasma cells. As T lymphocytes are not targeted by rituximab, specific T-lymphocyte help would be available for both naïve and memory B lymphocytes. Two patients immunised during depletion did not respond to TT but two other patients immunised following repopulation did respond.

As far as anti-PCP antibodies were concerned, the fact that their levels remained reasonably constant following BLDT and could even increase while the peripheral blood was still depleted of B lymphocytes, suggested that anti-PCP antibodies might be produced by long-lived plasma cells but also that the PCP-specific B lymphocytes might be resistant to killing by rituximab. However, 2 patients did not respond to immunisation during the period of B-lymphocyte depletion suggesting that depletion of PCP-specific B lymphocytes may vary. PCP are TI-2 antigens. IgG anti-PCP is predominantly IgG2. TI-2 antigens are not efficient inducers of memory, that is, there is no anamnestic response on rechallenge, which is thought to reflect the absence of an expanded specific memory B (and T lymphocyte) population. However, following immunisation and natural infection, anti-PCP can be detected in serum up to 5 years afterwards suggesting that their production is probably the responsibility of long-lived

plasma cells. Response to this type of antigens is thought to be the responsibility of marginal zone B lymphocytes residing predominantly in the spleen. In mice, these cells are particularly dependent on survival signals provided by BLyS and it is possible that increased serum BLyS levels during the period of depletion contributed to sustained anti-PCP antibodies.

Following BMT, some studies showed that patients only recovered the capacity to mount normal antibody responses to PCP after 2 years (Avanzini et al., 1995). This is thought to be due to a recapitulation of ontogeny with the B-lymphocyte subpopulations responsible for responding to TI-2 antigens and secretion of IgG2 taking longer to reconstitute than populations responding to TD antigens. Interestingly, the patient immunised with PCP shortly after B-lymphocyte repopulation (3 months after) did not respond while the patient immunised 15 months after repopulation did. This suggested that the same phenomenon described following BMT may occur after rituximab therapy and again suggested that PCP-specific B lymphocytes are susceptible to depletion by rituximab.

7.2.2 Biology of residual B lymphocytes in the presence of rituximab and mechanisms of repopulation

During the period of depletion following rituximab therapy, a very small number of B lymphocytes were always seen circulating. The majority of these cells showed a memory or plasma cell precursor phenotype. This suggested that some immunological activity involving B lymphocytes that have not been depleted by rituximab was taking place through the period of depletion in the secondary lymphoid organs but the importance of this phenomenon is not known. As previously described, two patients immunised with TT and PCP during the period of depletion did not respond to the vaccines and rituximab is known to interfere with immune responses to primary and secondary antigenic challenges (Gonzalez-Stawinski et al, 2001). In addition, this phenomenon is probably of very low quantitative importance as total immunoglobulin serum levels only rarely increase in patients with RA during the period of depletion and usually only very close to the time of repopulation.

It is not known whether the B lymphocytes that circulate during the period of depletion are eventually killed once they start circulating, at least in an earlier phase when rituximab levels are still significant. Flow cytometry data presented in this thesis suggested that surface CD20 on these cells was masked by bound rituximab. Pharmacokinetic studies have shown that rituximab remains available in the circulation for months. Animal studies suggest that following rituximab administration B-lymphocyte depletion in the peripheral blood occurs rather rapidly and ADCC and the reticuloendothelial system in the liver and less in the spleen seem to be the main effector mechanisms. ADCC probably also occurs in solid tissues as suggested by biopsies of lymph nodes in primates where increased numbers of activated macrophages were found and also evidence from the response in patients with lymphoma who have extra-nodular disease. Studies in mice have shown that depletion by rituximab of certain subpopulations, B-1a and marginal zone, correlated with their re-circulation patterns. Whether the same phenomenon occurs in humans and whether continued B-lymphocyte depletion occurs is not known. In humans, there is no evidence that B lymphocyte subpopulations located in the serosal cavities equivalent to B-1 populations in mice exist and there is some evidence that B lymphocyte subpopulations equivalent to mouse marginal zone B lymphocytes re-circulate.

Whether expansion of these remaining cells that have escaped depletion occurs in solid lymphoid tissues particularly once rituximab levels decrease is not known. The bone marrow study shows that proliferation of B-lymphocyte precursors occurs, including of cells that express CD20, in the absence of repopulation of the peripheral blood. It is very interesting to note that repopulation of the peripheral blood does not occur from remaining mature B cells, not even in the presence of increased serum levels of BLyS. Repopulation is only seen when the formation of new B cells is resumed, presumably when rituximab has been cleared and the normal development of late precursors which express CD20 is able to proceed as normal. After BMT, repopulation also always occurs from newly formed naïve B lymphocytes and not from mature cells present in the graft.

Together, immunophenotyping data from the peripheral blood and the bone marrow studies presented in this thesis suggested that the time of B-lymphocyte repopulation of the peripheral blood was determined by two main factors: 1. the clearance of rituximab

which seems to be essential to allow full development of B-lymphocyte precursors in the bone marrow; 2. the regenerative capacity of the individual's bone marrow.

7.3 The optimal B-lymphocyte depletion protocol in the treatment of rheumatoid arthritis

The optimal Blymphocyte depletion protocol for patients with RA has yet to be established. The extended open-label trial in 22 patients presented in this thesis and the most recent dose-ranging controlled trial suggested a dose-response to rituximab (Emeery et al., 2006). The èvidence that currently used regimes do not induce a complete B-lymphocyte depletion suggest that these doses of rituximab are not at the top of the dose response curve, although it is unclear whether additional major clinical benefit should be expected at higher dosages. In contrast to lymphoma, where response to treatment is directly related to the extent of malignant cell killing, the exact mechanism by which BLDT is effective in RA is not known.

A better understanding of the mechanisms of relapse in RA following response to BLDT will eventually help to design potentially more effective B-lymphocyte targeting therapies. If the main mechanism for relapse is incomplete B-lymphocyte depletion then higher doses (with or without different administration regimens) of rituximab, more effective B-lymphocyte depleting agents or combination therapy may lead to better results. Fully humanised anti-CD20 antibodies may prove more efficient in depleting B lymphocytes than chimaeric antibodies like rituximab. Anti-CD20 antibodies with different ligation kinetics to the CD20 antigen (more prolonged binding) and different capabilities in activating complement or leading to ADCC may have advantages over rituximab and may prove to be more efficacious.

Combination therapy may be especially important to target subpopulations of B lymphocytes which may have innate or acquired survival mechanisms or reside in a protective microenvironment. Ideally, one would hope that pathogenic B-lymphocyte clones would be more dependent on certain mechanisms for their development, expansion and survival allowing the development of more specific therapies with potentially higher efficacy and less side effects. Agents that neutralize BLyS and anti-CD22 monoclonal antibodies may prove to be useful B-lymphocyte targeting therapies

that may be used alone or in combination with anti-CD20 antibodies. Inhibition of IL-21 may block the generation of new plasma cells.

Several agents have been developed to target BLyS, including anti-BLyS antibodies and immunoglobulin Fc fusion proteins with each of the three receptors that are known to bind this cytokine. Some of these agents (TACI-Ig and BCMA-Ig) also neutralize a sister cytokine APRIL. APRIL's precise role in B-lymphocyte biology is still not completely understood. An antibody to BLyS, Lymphostat B, has been tested in phase I and II trials in patients with RA, and has shown significant effects on B-lymphocyte numbers and antibody profiles (Stohl et al., 2005). However, clinical efficacy appears to be modest for monotherapy. For the receptor-based agents (BR3-Ig, TACI-Ig and BCMA-Ig) there is some early evidence of biological effects but, again, it is still unclear whether these agents alone will have major clinical efficacy. Nevertheless, studies in human CD20 transgenic mice suggest that, in combination with anti-CD20 antibodies, BLyS blockade may allow a much more complete depletion of solid tissue B lymphocytes (Gong et al., 2005).

CD22 antigen is a protein found on the surface of B lymphocytes with negative signalling properties. Anti-CD22 antibodies with agonistic effects may prove to be useful B-lymphocyte targeting therapies alone or in combination with anti-CD20 antibodies. Positive results have been described in an open label trial in patients with SLE (Dorner et al., 2006)

A further possible strategy under investigation is the blockade of IL-21, which is involved in the generation of new plasma cells from naïve and memory B lymphocytes, particularly for TD antigens (Ozaki et al., 2002).

If relapse is due to the presence of long-lived plasma cells producing pathogenic species of autoantibodies, combination therapy with agents targeting both B lymphocytes and plasma cells might be more effective. This approach will be limited by the availability of an effective agent to selectively kill fully differentiated plasma cells and the potentially higher risk of infections associated with such a strategy. Fully mature plasma cells are thought to be resistant to radiotherapy and most chemotherapy agents. As previously referred to, inhibition of IL-21 may block the generation of plasma cells but

may not affect existing plasma cells. Targeting one of the receptors for BLyS and APRIL, BCMA, may be useful, as BCMA is selectively expressed by plasma cells.

If the memory for the disease resides in other types of cells, such as autoreactive T lymphocytes, then combination therapies with agents targeting B lymphocytes and agents targeting other cells or their interaction with B lymphocytes like CTLA4-Ig (abatacept) may be able to induce long-term remission.

In summary, strategies to prevent relapse or at least increase the duration of clinical response to BLDT in RA may include use of higher rituximab doses, different anti-CD20 antibodies or combination therapies with other biological agents, in particular, other B-lymphocyte or plasma cell targeting drugs.

7.4 Conclusion

B-lymphocyte depletion based on rituximab was first greeted with suspicion but has now been proved to be an effective therapy for patients with active RA refractory to other therapies. This indicates that in a significant number of patients perpetuation of synovial inflammation is B-lymphocyte dependent. The fact that only seropositive patients responded to treatment suggested that RhF or RhF-specific B-lymphocyte clones play a primarily role in the perpetuation of inflammation but this may have to be reviewed once more detailed data from phase III trials is available. The specificity of this therapeutic intervention provides a wonderful opportunity to study the mechanisms of drug action and disease relapse. Studies on dose-response, changes in autoantibody serum levels and peripheral blood and bone marrow B-lineage cell populations that occur following treatment suggested that a quantitative threshold has to be reached for patients with RA to respond to BLDT and that differences on duration of response may be related to the efficiency of the initial depletion. This suggests that more efficient B-lymphocyte depletion protocols may be more effective in treating RA.

7.5 Future work

The findings of this thesis suggest that future clinical focus should be on development of a more effective B-lymphocyte depleting treatment protocol and possibly a more selective one (for autoreactive cells).

Research on mechanisms of relapse following BLDT should focus on whether clinical relapse is related to the maturity of the repopulating B lymphocytes or more to the repertoire of the repopulating or re-expanding B-lymphocyte subpopulations or the specificity of persistent or newly synthesised autoantibodies.

An improved B-lymphocyte depletion protocol should be effective in inducing a major clinical response in a higher number of patients and also in prolonging the duration of response to treatment in these patients while keeping a relatively similar safety profile. It should involve increased initial depletion of B lymphocytes, targeting of plasma cells or prevention of re-emergence of pathogenic B lymphocytes clones. This may be achievable with rituximab, using higher doses or different administration regimes, with other anti-CD20 antibodies, with combination therapies of rituximab and other B-lymphocyte targeting agents such as agents targeting BLyS or B-T lymphocyte interactions.

New trials should be accompanied by research into surrogate markers particularly for the extent of B-lymphocyte depletion in solid tissues but also for the degree of B-lymphocyte activation, so that comparison between different protocols can be made.

Continuing research into the mechanisms of relapse should include comparative studies of B lymphocytes and immunoglobulin characteristics (including repertoires of both and functional status of cells) before treatment, at repopulation and at relapse, to try to establish which cells or antibodies are associated with relapse (residual or new) and to direct further therapeutic protocol development.

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APPENDIX I: REAGENTS, BUFFERS AND SUPPLIERS

Reagents and Buffers

ELISA

- *Phosphate buffered saline (PBS)*
  10x concentrate of Dulbecco's phosphate buffered saline solution without calcium chloride and magnesium chloride (Sigma). Diluted to a 1x concentration in distilled water.

- *Washing buffer (PBS/tween)*
  0.05% tween-20 (polyoxyethylene sorbitan monolaurate) (Sigma P-1379) in PBS.

Flow Cytometry

- *Red cell lysing reagent*
  10x concentrate of ammonium chloride-based lysing reagent (PharM Lyse, BD PharMingen). Diluted to a 1x concentration in distilled water. 1x solution warmed to room temperature prior to use.

- *Heat-inactivated foetal calf serum* (Life Technologies Ltd)
  2% (for peripheral blood) and 20% (for bone marrow) solutions diluted in PBS.

- *Trypan blue* (0.4% stock from Sigma)
  Diluted to 0.1% trypan blue solution in PBS.
  Cells diluted 1/2 to 1/8 for cell and viability counts.

- *Fixing buffer*
  2% paraformaldehyde diluted in PBS (heated to 60°C for 2-3 hours).
  Stored at 4°C protected from light for 1 week.

Suppliers

BD Biosciences
21 Between Towns Road
Cowley, Oxford, UK
Tel: 01865 781688

Life Technologies (Life Technologies, Paisley, UK)
3 Fountain Drive
Inchinnan Business Park
Paisley, UK
Tel: 0141 814 6100

Sigma
Fancy Road
Poole, Dorset, UK
Tel: 0800 717181

Appendix II: Antibodies used in flow cytometry and characterization of CD antigens

Table A. Antibodies used in flow cytometry analysis of peripheral blood lymphocytes and of cells in B-lymphoic lineage in the bone marrow.

Monoclonal antibody specificity	Conjugate	Species	Clone	Isotype
CD3	PE	Mouse	HIT3a	IgG2a, κ
CD4	FITC	Mouse	RPA-T4	IgG1, κ
CD5	FITC	Mouse	UCHT2	IgG1, κ
	PE-Cy5	Mouse	UCHT2	IgG1, κ
CD8	FITC	Mouse	RPA-T8	IgG1, κ
CD10	APC	Mouse	HI10a	IgG1, κ
CD19	PE	Mouse	HIB19	IgG1, κ
	APC	Mouse	HIB19	IgG1, κ
CD20	FITC	Mouse	2H7	IgG2b, κ
	PE-Cy5	Mouse	2H7	IgG2b, κ
	PerCP	Mouse	L27	IgG1, κ
CD24	PE	Mouse	ML5	IgG2a, κ
CD25	PE-Cy5	Mouse	M-A251	IgG1, κ
CD27	FITC	Mouse	M-T271	IgG1, κ
	PE	Mouse	M-T271	IgG1, κ
CD34	PerCP	Mouse	8G12	IgG1, κ
CD38	FITC	Mouse	HIT2	IgG1, κ
	PE-Cy5	Mouse	HIT2	IgG1, κ
	APC	Mouse	HIT2	IgG1, κ
CD45RA	PE-Cy5	Mouse	HI100	IgG2b, κ
CD45RO	PE-Cy5	Mouse	UCHL1	IgG2a, κ
CD56	PE-Cy5	Mouse	B159	IgG1, κ
CD138	FITC	Mouse	Mi15	IgG1, κ
IgD	FITC	Mouse	IA6-2	IgG2a, κ
κ light chain	FITC	Goat	Polyclonal	$F(ab')_2$
λ light chain	FITC	Goat	Polyclonal	$F(ab')_2$
Isotype control	PE-Cy5	Mouse	G155-178	IgG2a, κ

Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; PE-Cy5, phycoerythrin-indodicarbocyanine; PerCP, peridinin-chlorophyll *a* complex protein; APC, allophycocyanin; CD, cluster of differentiation antigens; sIgD, surface immunoglobulin D.

Table B. Fluorochromes conjugated to monoclonal antibodies used in three- and four-colour fluorescence in a FACS Calibur™ (equipped with 488 and 633 nm excitation) in the flow cytometry studies.

Channel	Filter peak transmission (typical wavelengths; nm)	Fluorochrome	Wavelength (nm) Excitation	Wavelength (nm) Emission
FL1 (green)	530 ± 30	FITC	492	516-525
FL2 (orange)	585 ± 42	PE	480	575
FL3 (red)	660 ± 30	PE-Cy™5	480, 565, 650	667
		PerCP	490	675
		PE-TR	480, 565	613
FL4 (long red)	> 670	APC	650, 755	767

Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; PE-CY™5, phycoerythrin-indodicarbocyanine, also called Cy-chrome; PE-TR, phycoerythrin-Texas Red; PerCP, peridinin-chlorophyll *a* complex; APC, allophycocyanin.

Table C. Characterization of CD antigens identified by the monoclonal antibodies used in the immunophenotyping studies.

Antigen	Other names	Molecular mass (kDa)	Molecular structure (family relationships)	Cellular distribution	Function
CD3	T3, CD3 complex	γ: 25-28 δ: 20 ε: 20	Immunoglobulin superfamily	Thymocytes upon maturation; all peripheral T lymphocytes	Associated with the TCR. Required for cell-surface expression of and signal transduction by the TCR.
CD4	T4, L3T4	55	Monomeric molecule containing four immunoglobulin-fold domains (immunoglobulin superfamily)	Thymocytes subsets; peripheral T-lymphocyte subsets (helper T lymphocytes); monocytes; macrophages	Co-receptor for MHC class II molecules. Signal transduction in T-lymphocyte activation and maturation.
CD5	T1, Ly1	67	Extracellular region has three SRCR domains (SRCR family)	Thymocytes; peripheral T lymphocytes; subset of B lymphocytes	Modulates signalling through the antigen-specific receptor (TCR and BCR)
CD8	T8, Lyt2,3, T-cell co-receptor	α: 32-34 β: 32-34	Disulphide-linked type I transmembrane glycoprotein composed of an α and β chain	Thymocytes subsets; peripheral T-lymphocyte subsets (cytotoxic T lymphocytes)	Co-receptor for MHC class I molecules. Signal transduction in T-lymphocyte activation and maturation.
CD10	CALLA, neutral endopeptidase	100	Type II integral membrane protein	Lymphoid precursors (B and T), bone	Regulation of B-lymphocyte growth

Antigen	Other names	Molecular mass (kDa)	Molecular structure (family relationships)	Cellular distribution	Function
			(zinc metalloproteinase family)	marrow stromal cells, germinal centre B lymphocytes, mature neutrophils	and proliferation. Acts as a zinc metalloproteinase.
CD19	B4	95	Transmembrane protein with two extracellular C2-type immunoglobulin-like domains (immunoglobulin superfamily)	B lymphocytes	Forms complex with CD21 (CR2) and CD81 (TAPA-1). Critical signal transduction molecule that regulates B-lymphocyte development, activation and differentiation.
CD20	B1	33-37	Transmembrane protein (4 transmembrane segments)	B lymphocytes	Oligomers of CD20 my form a Ca^{2+} channel. Possible role in egulating of B-lymphocyte activation and proliferation.
CD24	Possible human homologue of mouse HAS	35-45	GPI-anchored glycoprotein	B lymphocytes, granulocytes	Unknown
CD25	Tac, IL-2 receptor α chain	55	Il-2 receptor α chain	Activated T lymphocytes, B	Il-2 receptor α chain

Antigen	Other names	Molecular mass (kDa)	Molecular structure (family relationships)	Cellular distribution	Function
				lymphocytes and monocytes, regulator T lymphocytes	
CD27	T14, S152	55	Type I transmembrane protein (TNF receptor family)	Medullary thymocytes, T lymphocytes, NK cells, memory B lymphocytes	Binds CD70. Can function as a co-stimulator for T and B lymphocytes
CD34	GP105-120	105-120	Heavily glycosylated type I transmembrane protein (sialomucin family)	Early lympho-haematopoietic stem and progenitor cells, capillary endothelium	Ligand for CD62L (L-selectin). Cell-cell adhesion.
CD38	T10	45	Single-chain type II transmembrane protein	Expressed on B and T lymphocytes, particularly during early differentiation and activation, plasma cells	NAD glycohydrolase. Regulator of cell activation and proliferation, involved in adhesion between lymphocytes and endothelium.
CD45	LCA, T200, B220	180-240 (multiple isoforms)	Long single-chain type I molecule	All haematopoietic cells	Tyrosine phosphatase. Augments signalling through TCR and BCR. Multiple isoforms result from alternative splicing.

APPENDIX III: PUBLICATIONS

Original papers

Leandro MJ, Edwards JC, Cambridge G.
Clinical outcome in 22 patients with rheumatoid arthritis treated with B lymphocyte depletion.
Annals of Rheumatic Diseases 2002; 61(10): 883-8.

Cambridge G, Leandro MJ, Edwards JCW, Ehrenstein MR, Salden M, Bodman-Smith M, Webster ADB.
Serological changes following B lymphocyte depletion therapy for rheumatoid arthritis.
Arthritis & Rheumatism 2003; 48(8):2146-2154.

Leandro MJ, Cambridge G, Ehrenstein MR, Edwards JC.
Reconstitution of peripheral blood B cells after depletion with rituximab in patients with rheumatoid arthritis.
Arthritis & Rheumatism 2005; 54(2): 613-20.

Cambridge G, Stohl W, Leandro MJ, Migone TS, Hilbert DM, Edwards JCW.
Circulating levels of B lymphocyte stimulator (BlyS)in patients with rheumatoid arthritis following rituximab treatment: relationships with B cell depletion, circulating antibodies, and clinical relapse.
Arthritis & Rheumatism 2005; 54(3):723-732.

Leandro MJ, Cooper N, Cambridge G, Ehrenstein MR, Edwards JC.
Bone marrow B-lineage cells in patients with rheumatoid arthritis following rituximab therapy.
Rheumatology 2006; May 30 (Epub ahead of print).

Leandro MJ, Edwards JC, Cambridge G, Ehrenstein MR, Isenberg DA.
An open study of B lymphocyte depletion in systemic lupus erythematosus.
Arthritis & Rheumatism 2002; 46(10):2673-7.

Leandro MJ, Cambridge G, Edwards JC, Ehrenstein MR, Isenberg DA.
B cell depletion in the treatment of patients with systemic lupus erythematosus: a longitudinal analysis of 24 patients.
Rheumatology 2005; 44(12):1542-5.

Ng KP, Leandro MJ, Edwards JC, Ehrenstein M, Cambridge G, Isenberg DA. Repeated B cell depletion in treatment of refractory systemic lupus erythematosus. Annals of Rheumatic Diseases 2005; 65(7):942-945.

Cambridge G, Leandro MJ, Teodorescu M, Manson J, Rahman A, Isenberg DA, Edwards JC.
B cell depletion therapy in systemic lupus erythematosus: effect on autoantibody and antimicrobial antibody profiles.
Arthritis & Rheumatism 2006; 54(11): 3612-3622.

Reviews

Edwards JC, Leandro MJ, Cambridge G.
B-lymphocyte depletion therapy in rheumatoid arthritis and other autoimmune disorders.
Biochemical Society Transactions 2002;30(4): 824-8.

Edwards JCW, Leandro MJ, Cambridge G.
B lymphocyte depletion with rituximab in rheumatoid arthritis.
Rheumatic Disease Clinics of North America 2004;30(2):393-404.

Edwards JCW, Leandro MJ, Cambridge G.
B lymphocyte depletion in rheumatoid arthritis: targeting of CD20.
Current Directions in Autoimmunity 2005;8:175-192.

Leandro MJ, Cambridge G, Edwards JCW
B-cell depletion in rheumatoid arthritis: the prospect of long term benefit.
Future Rheumatology 2006; 1(4): 493-499.

Edwards JC, Cambridge G, Leandro MJ.
B cell depletion therapy in rheumatic disease.
Best Practice & Research Clinical Rheumatology 2006; 20(5): 915-928.

Gorman C, Leandro M, Isenberg D.
Does B cell depletion have a role to play in the treatment of SLE?
Lupus 2004; 13(5):312-316.

Gorman C, Leandro M, Isenberg D.
B cell depletion in autoimmune disease.
Arthritis Research & Therapy 2004; 5(supplement 4):S17-S21. Review.

Leandro MJ, Ehrenstein MR.
B cell depletion in systemic lupus erythematosus.
Clinical Medicine 2007 (*in press*).