The role of B cells in rheumatoid arthritis

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A dream you dream alone is only a dream

A dream you dream together is reality

The role of B cells in rheumatoid arthritis

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ABSTRACT

It has been known for a long time that B cells play a role in rheumatoid arthritis (RA). By production of autoantibodies, presentation of auto-antigens and by producing cytokines B cells may contribute to the pathogenesis of RA. In recent years it has been shown that anti-B cell therapy is a powerful tool in the treatment of RA. The aim of this thesis was to a) investigate the effect on B cell ontogeny following B cell depletion therapy, b) during B cell depletion therapy evaluate serological and humoral immune responses and finally, c) try to establish a connection between Epstein-Barr virus (EBV) infection, CD25+ B cells and outcome of B cell deletion therapy.

In paper I we could show that in bone marrow of RA patients following anti-CD20 treatment with rituximab (RTX) IgD expressing naïve cells are depleted whereas immature and memory B cells where still detectable. However, the long-term effects clearly showed a reduction of memory B cells in bone marrow. The examination of rheumatoid factor (RF) production revealed that RFs decline short after treatment but returned to baseline levels concurrently with the IgD expressing B cells when patients where subjected to an additional course.

In paper II the cellular and humoral immune responses were evaluated by immunisation of RA patients before or during RTX treatment with a protein vaccine against influenza and a pneumococcal polysaccharide vaccine. The results suggest that both cellular and humoral immune responses are affected in patients receiving RTX treatment and we therefore suggest that immunisation should be performed before RTX treatment.

In paper III we investigate the effects of EBV on selected B cell subsets and how infection may affect the clinical response to RTX treatment. The phenotypical study showed that B cells are more mature in EBV infected patients and the $CD25^+$ B cell subset was more mature as compared to the $CD25^-$ B cell population. The evaluation of clinical response to RTX treatment with regard to B cell subsets showed that non-responding EBV^+ patients had a significantly larger $CD25^+$ plasma cell population. When investigating the effects of EBV stimulation in vitro we found that the $CD25^+$ B cell population developed into antibody-producing cells to a higher extent than did the corresponding $CD25^-$ B cell population.

The results of our studies indicate that that B cells play an essential role in the pathogenesis of RA. During RTX treatment we suggest that the IgD expressing population may harbour the autoantibody producing B cells. We also claim that that there are subsets of B cells (i.e. $CD25^+$ B cells) that may have significant impact on the pathogenesis of RA, and the clinical outcome following RTX treatment.

Keywords: B cells, rheumatoid arthritis, B cell depletion therapy

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Rehnberg. M, Amu. S, Tarkowski. A, Bokarewa. M, Brisslert. M.

Short- and long-term effects of anti-CD20 treatment on B cell ontogeny in bone marrow of patients with rheumatoid arthritis
Arthritis Research and Therapy 2009; 11: R123.

II. Rehnberg. M, Brisslert. M, Amu. S, Zendjanchi. K, Håwi. G, Bokarewa. M.

Vaccination response to protein and carbohydrate antigens in patients with rheumatoid arthritis after rituximab treatment
Arthritis Research and Therapy, 2010; 12: R111

III. Rehnberg. M, Brisslert. M, Bokarewa. M.

Epstein-Barr virus persistence in patients with rheumatoid arthritis drives antibody production by the $CD25^{+}$ B cell population.
Submitted for publication.

CONTENT

ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
APC	Antigen presenting cell
BCR	B cell receptor
BM	Bone marrow
CCP	Cyclic citrullinated peptides
CRP	C-reactive protein
CD	Cluster of differentiation
DAS28	Disease activity score 28
DMARD	Disease-modifying antirheumatic drug
DC	Dendritic cell
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immonosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
ER	Endoplasmatic reticulum
ESR	Erythrocyte sedimentation rate
EULAR	European league against rheumatism
FCS	Forward scatter channel
FMO	Fluorochrome minus one
IG	Immunoglobulin

IL	Interleukin
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MNC	Mononuclear cell
MTX	Methotrexate
PB	Peripheral blood
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RTX	Rituximab
SD	Standard deviation
SFC	Spot forming cell
SLE	Systemic Lupus Erythematosus
SSC	Side scatter channel
TLR	Toll-like receptor
TNF	Tumour necrosis factor

1 INTRODUCTION

1.1 B cells

The B cell is a part of the adaptive immune system where they have three main purposes:

1. To present antigens to T cells
2. To produce antibodies
3. To produce cytokines

The normal antigen presentation involves processing and presentation of peptides and proteins. This happens via major histocompatibility complex (MHC) class II: the antigen (peptide) binds to the B cell receptor on the cell surface and is taken up by receptor mediated endocytosis into the cell and is presented on MHC class II for other cells to recognise. The B cell will probably be situated in the peripheral lymphoid organs when this happens. It will meet their specific antigen and present it to a $CD4^+$ T cell. The B cell will go through proliferation and differentiation that will give rise to antibodies (more on antibodies later). It could also be that a professional antigen presenting cell (APC) i.e. a dendritic cell (DC) present antigens to T cells followed by that the T cell presents that antigen to a B cell. The possibilities are many, but the end result is the same – the B cell gets activated and antibodies are produced.

B cells can produce many different cytokines. Usually one talks about effector B cell cytokines and B regulatory cytokines. The effector B cell cytokines are interleukin-2 (IL), IL-4, Tumour necrosis factor (TNF) alpha, IL-6, INF-gamma and IL-12 whereas the regulatory B cell cytokines are IL-10 and TGF-beta (Lund et al. 2005; Mizoguchi et al. 2006).

1.1.1 Development

B cell development starts from a hematopoietic stem cell in the bone marrow (BM) and will develop into a pro B cell. Once the cell reaches the pro B cell stage CD19 is upregulated on the surface. The pro B cell will rearrange their genes of the variable (V), diversity (D) joining (J) immunoglobulin (Ig) gene segments to form a pre B cell receptor (BCR) (Tonegawa 1983). By combining the different V, D and J gene segments an almost unlimited variability of the specificity is achieved. The BCR now consists of a heavy chain and a surrogate light chain. If the production of the heavy chain is successful a light chain will be produced. The heavy chain together with the light chain will form surface bound IgM. During the pre B cell stage, CD20 is upregulated, and the B cell is now termed "immature". A schematic figure over the development of a B cell is presented in Figure 1.

To make sure that the B cell is not self-reactive there are several control steps in the BM. Stromal cells in the BM will present self-antigens to the B cell and if the BCR binds self-antigens the receptor can be edited. The BCR that still binds self-antigen will be selected for apoptosis. This is called clonal deletion and is a control to prevent auto-reactive B cells to leave the bone marrow. (Chen et al. 1997; Pelanda et al. 1997; Melamed et al. 1998). Somewhere here IgD is also upregulated and the mature B cell now leaves the BM and migrates to peripheral lymphoid organs (Figure 1). Most mature B cells coexpress IgD and IgM and are now ready to meet their antigen.

In peripheral lymphoid organs, the B cell can meet their specific antigen and will then migrate to the T cell zone in i.e. the lymphnodes. The B cells and the T cells will then form a germinal center where the T cells and the B cell will communicate via costimulatory molecules i.e. CD40 on the B cell and CD40L on the T cell, and the T cell will give the B cell additional signals via cytokine production to proliferate and start the differentiation process (MacLennan et al. 2003). Fully activated B cells will clonally expand and at this stage the genes encoding for the variable region of Igs undergo extensive somatic point-mutations leading to increased affinity of the antigen binding sites (affinity maturation). Continuous exposure to the same antigen i.e. during repeated immune responses results in large quantities of high affinity Igs that are of great importance to prevent and fight infections.

Moreover, the B cell may also go through recombination of the constant part of the Ig resulting in a replacement of IgD and IgM with IgG, IgA, or IgE genes, known as B cell isotype switch. Which isotype the B cell is switched to is decided by the cytokine milieu in the germinal center. The switch of Ig classes indicates the formation of antigen specific memory B cells that have the ability to further differentiate into long-lived memory B cells as well as plasma cells (Figure 1) (Kosco-Vilbois et al. 1995).

The main purpose of memory B cells is to rapidly proliferate and differentiate into a plasma cell after re-stimulation with their specific antigen. This is how an infection can be "remembered". Memory B cells live for a long period of time, even as long as the host (Crotty et al. 2003), and recirculate between the peripheral lymphoid tissues via the blood and the lymph vessels on their hunt for their specific antigen (Tangye et al. 2009).

Some of the B cells formed after the germinal center reaction will differentiate into plasma cells. Plasma cells have a large endoplasmatic reticulum (ER) that will facilitate the extreme production of antibodies that is the function of a plasma cell. The plasma cells will most likely home to the lymphoid organs and the BM where they will stay for a long time and secrete antibodies (Manz et al. 1997; Slifka et al. 1998; Shapiro-Shelef et al. 2005). These resident plasma cells are termed long-lived, but there are also IgM producing short-lived plasma cells that are more likely to home to inflamed tissue and produce antibodies until the infection is conquered. Memory B cells together with plasma cells contribute to the humoral immunological memory (Sanz et al. 2008; Tangye et al. 2009).

Then there is also the matter of T dependent and T independent antigens. B cells that get T cell help and together form a germinal center will in most cases go through affinity maturation and isotype switch. The B cells can however get activated without T cell help and the antigen will then be of polysaccharide or lipid origin and activate the B cell through crosslinking of the BCR. B cells activated without T cell help will most likely not go through somatic hypermutation or class switch, but there are some exceptions (Vinuesa et al. 2003).

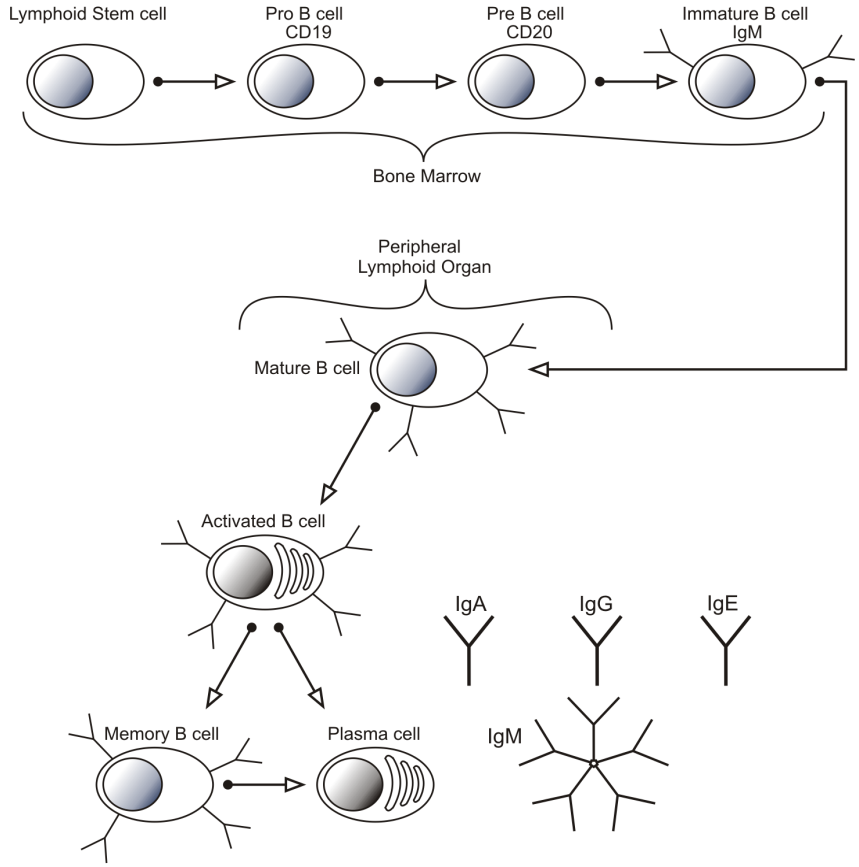

Figure 1. Schematic figure over B cell development *in the bone marrow, the activation in peripheral lymphoid organs and the differentiation into memory B cells and plasma cells. The upregulation of the earliest surface markers and immunoglobulins in the bone marrow is marked. Figure made by Jason Johnson.*

1.1.2 Immunoglobulins

There are five different isotypes of Igs. They have different effector functions and can be either soluble or surface bound.

The Ig is consisting of two identical heavy and light chains and the antigen binding site is situated at the top of the molecule where the heavy and light chain form the antigen binding site. The Fc region mediates the effector mechanism of the antibody (i.e. complement activation, phagocytic uptake etc) and is the bottom part and the Fab region is the upper part and harbours the antigen binding sites. A schematic figure of the structure of an immunoglobulin / antibody is presented in Figure 2.

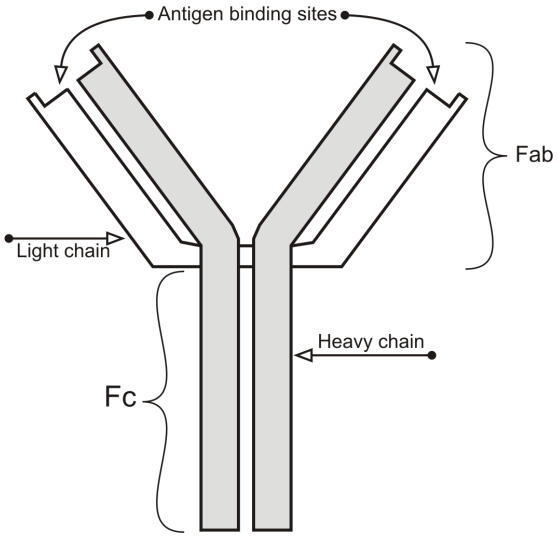

Figure 2. The structure of an immunoglobulin / antibody. *Two heavy chains and two light chains form the antibody. The upper part of the antibody is called the Fab part, and the lower part is called the Fc part. Figure made by Jason Johnson.*

The Ig classes with their effector functions will be presented here.

IgD

IgD is found membrane bound on the surface of naïve B cells where it functions as a receptor for antigen. IgD also exists in soluble form but its function is still unclear. The membrane bound form is considered as a marker for a mature B cell ready to meet its antigen.

IgM

IgM is the first Ig to be upregulated on the surface of B cells in the BM. When IgM is secreted it is in pentameric form but when attached to the cell surface it is as a monomer. IgM is the Ig that is best at complement activation (the classical pathway). IgM is also the Ig that is first secreted during an immune response.

IgG

IgG is a high affinity antibody and the B cell needs to go through affinity maturation for this Ig to be produced. IgG is involved in Fc dependent phagocyte responses. It is very efficient at opsonisation of antigens for phagocytosis, complement activation of the classical pathway and feedback inhibition of B cell activation. The production of IgG is a sign of a late immune response where the B cell has gone through Ig switch or a restimulation.

There are four IgG subclasses, IgG1-4, named after their serum concentration level (IgG1 60-65 %, IgG2 20-25 %, IgG3 5-10 %, IgG4 3-6 %) and they all have different functions (French et al. 1984; French 1986b; a).

IgA

IgA is a high affinity antibody and the B cell needs to go through affinity maturation for this Ig to be produced. There is a distinct role for IgA in mucosal immunity i.e. the gastro intestinal tract and the respiratory tract. IgA has two subtypes, IgA1 and IgA2. The IgA can be a monomer, dimer or trimer. The production of IgA is a sign of a late immune response where the B cell has gone through Ig switch or a restimulation.

IgE

IgE is involved in immediate hypersensitivity reactions and is important for immunity against helminths and mast cell degranulation.

Kappa and Lambda light chains

The Ig light chain could either be of kappa or lambda type. It is suggested that lambda rearrangement only occur if the kappa rearrangement is inaccurate or non-functional (Korsmeyer et al. 1981; Tonegawa 1983; Rolink et al. 1993).

In healthy persons the kappa/lambda ratio is supposed to be approximately 2:1, but could vary some in different diseases (Yount et al. 1970; Skvaril F 1975).

1.1.3 B cell markers

The B cell has many different molecules present on the surface of the cell depending on their developmental stage, maturity and activation. Many of them are termed according to their cluster of differentiation (CD) number. Here I will present the most commonly used markers and those that I find important for the understanding of this thesis.

CD10

CD10 is expressed on immature pre B cells and of germinal center B cells and functions as a metalloprotease (Braun et al. 1983; Greaves et al. 1983).

CD19

CD19 is the first definitive B cell marker that is expressed on the surface. It appears on the B cell surface in the pro B cell phase. CD19 is also a part of the BCR (Poe et al. 2001).

CD20

CD20 is a calcium channel in the cell membrane and is also a part of the BCR (Deans et al. 1995; Li et al. 2003).

CD24

CD24 is usually expressed on almost all B cells and promotes antigen dependent proliferation but prevents differentiation into plasma cells (Ling 1987; Pezzutto 1989).

CD25

The alpha part of the IL-2 receptor present both as a surface marker and in soluble form. Expressed on a subset of immunomodulatory B cells (Brisslert et al. 2006).

CD27

CD27 belongs to the TNF-receptor family and is the memory B cell marker. It is also present on activated T cells (Agematsu et al. 1997; Klein et al. 1998; Agematsu et al. 2000).

CD38

CD38 have bi-polar expression with high expression on early cells, lower on mature cells followed by an increase on plasma blast/cells.

CD40

CD40 is present on many cell types and is a costimulatory marker for CD40L expressed on T cells.

CD80

Is also called B7.1 and is a costimulatory molecule that has CD28 on the T cell surface as a ligand.

CD86

Costimulatory molecule that is present on the B cell. It is also called B7.2 and works together with CD80 (June et al. 1987; Linsley et al. 1991).

CD138

CD138 is the plasma cell marker and is also called Syndecan-1 (Sanderson et al. 1989; Calame 2001; Edwards et al. 2006; Mei et al. 2007).

1.1.4 Characterisation of B cell subsets

Here I will present the most commonly used B cell subsets and classifications of B cells.

Classifications including IgD

In the literature today there are two "main" ways to characterise B cells. We have used both ways to try to cover as much ground as possible.

Most common is to characterise the B cells after their expression of CD27 and IgD. Combining the expression of CD27 and IgD rendered four different populations:

$IgD^{-}CD27^{-}$ (immature B cells)

$IgD^{+}CD27^{-}$ (naïve B cells)

$IgD^{+}CD27^{+}$ (unswitched memory B cells)

$IgD^{-}CD27^{+}$ (switched memory B cells)

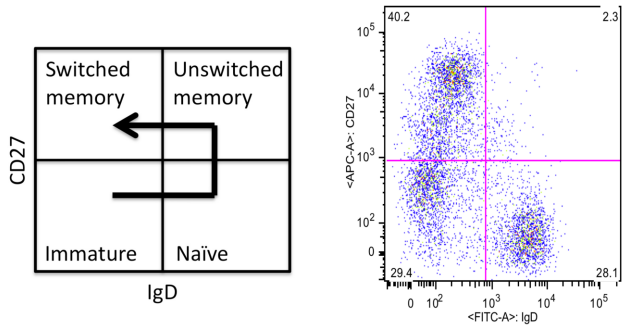

***Figure 3. Combining CD27 and IgD renders four different CD19+ populations**: Immature B cells (IgD^{-} $CD27^{-}$), naïve B cells ($IgD^{+}CD27^{-}$), unswitched B cells ($IgD^{+}CD27^{+}$) and switched B cells ($IgD^{-}CD27^{+}$).*

This classification has been used by us and others for some years (Roll et al. 2008; Sanz et al. 2008; Rehnberg et al. 2009). What is good with this classification is that all B cells are classified in a simple and useful way where it is easy to follow the B cell through their maturation (Figure 3). A limitation is that plasma blasts may be included in the $CD27^+$ populations, but probably not plasma cells since they should have dropped their expression of CD19.

The other method is described by Bohnhorst et al. and includes the marker CD38 in combination with IgD (Bohnhorst et al. 2001). Adding the marker CD10, CD24, CD27 and IgM will render even more detailed information about both pre germinal center and post germinal center populations.

$CD38^{++}CD24^{++}IgD^{+/-}$ (immature, transitional, T1)

$CD38^{+}IgD^{+}IgM^{++}CD24^{+}CD27^{-}$ (mature naive Bm2)

$CD38^{+}IgD^{-}CD24^{-}CD27^{+}$ (mature Bm5)

$CD38^{+++}IgD^{-}D27^{+}$ (plasma blasts)

We and others have used this classification or parts of it (Pascual et al. 1994; Bohnhorst et al. 2001; Vugmeyster et al. 2004; Sims et al. 2005; Pers et al. 2007; Binard et al. 2008; Rehnberg et al. 2009).

This classification is more complicated since there is a need for more markers but it also gives extensive information about many different B cell subsets. The tricky part can be to use markers with both low, intermediate and high expression since each level represents different maturation stages.

Comparing these ways of classification one can see that the mature B cell population (Bm2) is phenotypically close to the naïve B cell population expressing $CD27^{-}IgD^{+}$.

Plasma cells

The identification of plasma cells has varied during the years. The only identified marker that is increased on plasma cells is CD138 (Sanderson et al. 1989; Calame 2001). During the differentiation to a plasma cell the B cell will lose its expression of MHC II, CD19, CD20 and IgD. The

plasma cells are characterised as $CD138^{+}IgD^{-}$ and plasma blasts as $CD138^{+}IgD^{+}$ (Edwards et al. 2006; Mei et al. 2007). They should also have a high expression of CD38 and CD27.

The CD25 expressing B cell subset

We have previously identified a unique subpopulation of B cells that express CD25. Approximately one third of all circulating B cells express CD25 that is a part of the IL-2 receptor. The whole IL-2 receptor consists of CD-122 and CD-132 in combination with CD25 which makes it a high affinity receptor that can respond to IL-2.

B cells that express this surface marker display a different phenotype as compared to B cells that do not. They have less surface IgD and IgM, more IgG and IgA and the majority of them express the B cell memory marker CD27 as well as the costimulatory marker CD80 (Brisslert et al. 2006). We have also characterised $CD25^{+}$ B cells in RA where they display an even more mature and activated phenotype (Amu et al. 2007a; Amu et al. 2007b).

The functional properties of $CD25^{+}$ B cells have also been characterised. We showed that $CD25^{+}$ B cells produce less Igs than do the CD25- B cell population (Brisslert et al. 2006). In a mixed lymphocyte reaction (MLR), the allogenic $CD4^{+}$ T cells proliferated more to exposure to $CD25^{+}$ B cells as compared to $CD25^{-}$ B cells (Brisslert et al. 2006). When the MLR was performed with autologous $CD4^{+}$ T cells instead the proliferation increased further. When blocking of the CD25 surface antigen on the $CD25^{+}$ B cells was performed the proliferation in the MLR was almost totally abolished. The $CD25^{+}$ B cell population was also shown to produce significantly more IL-10 as compared to the $CD25^{-}$ B cell population after stimulation with CpG (Amu et al. 2007b).

Follicular B cells

Follicular B cells are mature naïve cells that have not yet met their antigen. They usually reside in secondary lymphoid organs were they lay in the follicles and communicate with the follicular DCs. In the spleen they are situated in the follicles and in the lymph nodes where they are present in the white pulp. These cells recirculate between the secondary lymphoid organs on the hunt for their antigen.

Marginal zone B cells

Marginal zone B cells are a little bit controversial. They are described as almost innate cells that lay in the marginal zone in the spleen where they communicate with other cell types like macrophages and DCs. They mostly give rise to T-independent responses and are therefore important in primary responses to antigens where they rapidly can proliferate and produce cytokines.

B1 cells

B1 cells have been described in mice as CD5 expressing B cells originating from fetal liver. However, their existence and classification in humans have been more controversial. B1 cells are supposed to be self-renewing and long-lived and are believed to produce natural antibodies of IgM class. They do not go through class switch and affinity maturation.

Germinal center B cells

Germinal centre B cells are formed after the B cells have met their antigen. They will then migrate to the T cell zone and form a germinal center together with the T cells. Here the T cell will give the B cell signals to go through affinity maturation.

B regulatory cells

It has been known for some years now that there exists a subset of T cells that have regulatory properties which can dampen immune responses. They have been characterised by the expression of CD25 (Sakaguchi 2000; Bennett et al. 2001). The knowledge about the corresponding B cell population is not as well established but several research groups have shown that IL-10 producing B cells are of great importance in autoimmune models (Wolf et al. 1996; Mauri et al. 2003; Duddy et al. 2004; Anderton et al. 2008; Fillatreau et al. 2008; Lemoine et al. 2009). This population of B cells have been characterised by its IL-10 production, CD1d, TIM-1 or CD25 expression (Brisslert et al. 2006; Amu et al. 2007a; Amu et al. 2007b; Amu et al. 2010; Eriksson et al. 2010; Ding et al. 2011; Iwata et al. 2011).

1.1.5 Vaccination

Vaccination has improved life for many humans during the years. Diseases like polio and smallpox are next to extinct due to vaccination. The aim of vaccination is to induce specific immunity to an antigen before it can cause its host any harm. Vaccines can be either T-dependent or T-independent. The most common type is the T-dependent and gives the strongest response.

When you get immunised, APCs in the tissue; most likely a DC, a monocyte or a neutrophil that patrol the body for foreign antigens, will recognise the antigen and if they elicit danger signals by binding to the toll-like receptors (TLRs), the cell will get activated. The activation will cause the cell to produce cytokines and chemokines that will attract other cells and a local inflammation will take place. The activated cells will start to migrate to the lymph nodes via the lymph vessels and here it will present antigen to T and B cells which in turn will get activated.

Depending on the antigen that activated the B cell, if it was a protein or a polysaccharide vaccination, there will be different IgG subclasses produced. Protein vaccination mainly gives rise to IgG1 and IgG3 antibodies, while polysaccharide vaccines give rise to IgG2 response (Siber et al. 1980; Yount WJ 1980; Stevens et al. 1983; Umetsu et al. 1985; Hammarstrom et al. 1986; Skvaril 1986). IgG4 only seems to be associated with chronic exposure to antigens as well as result of exposure to parasites (Umetsu et al. 1985; Boctor et al. 1990).

1.2 Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a chronic inflammatory disease that effects approximately 0,5- 1 % of the population with dominance in females. The characteristics of the disease are inflammation and destruction of the joints and often systemic features like fever and elevated erythrocyte sedimentation rate (ESR).

Migration of inflammatory cells like T cells, macrophages and antibody producing cells to the joints will cause an inflammation. The infiltration can be very extensive and many immune cells are recruited to the joint which makes it an ongoing process. The formation of pannus (thickened synovial tissue) causes destruction of the cartilage and bone which reduces the function of the joint.

1.2.1 Autoantibodies

In 80 % of patients with RA, B cells produce autoantibodies that are specific for the constant region of IgG. These antibodies are called Rheumatoid factors (RFs) and the most common isotype is IgM, but IgG and IgA also exist. Patients with RA also often have antibodies against cyclic citrullinated proteins (CCP) like type II collagen, heat shock proteins, proteoglycans, cartilage link proteins and heavy chain binding proteins. RF and aCCP are considered as a predictor for increased joint destruction (Drossaers-Bakker et al. 1999; Tak et al. 2000). These autoantibodies form immune complexes that contribute to increased inflammation as well as activation of the complement system. RA was for a long time considered as a T cell driven disease but clearly B cells play an important role in the pathogenesis of RA (Takemura et al. 2001; Wipke et al. 2004).

1.2.2 Diagnosis

The diagnosis of RA includes a list of criteria that needs to be fulfilled to receive the diagnosis of RA (Aletaha et al. 2010). It includes both joint swelling of large and small joints, serology (presence of autoantibodies) and inflammatory markers present in the blood like the ESR.

As of the year 2010 there is a collaborative classification criteria formed by the American College for Rheumatology (ACR) and the European League Against Rheumatism (EULAR). The classification criteria is enclosed in Table 1.

Disease activity score

One method to measure the patients' current state is the disease activity score-28 (DAS28). The DAS28 is an index calculated after the examination of 28 swollen and tender joints involving both hands, arms and knees; ESR and an assessment of the patient's general health (Fransen et al. 2001; Fransen et al. 2005). The DAS28 ranges from 1 to 9 where a low score indicates a low disease activity and a high score indicates high disease activity.

Table 1. Classification criteria for RA (score-based algorithm: add score of categories A–D; a score of ≥6/10 is needed for classification of a patient as having definite RA). The number in parenthesis is the score for each category.

A) **Joint involvement** 1 large joint (0) 2-10 large joints (1) 1-3 small joints (with or without involvement of large joints) (2) 4-10 small joints (with or without involvement of large joints) (3) >10 joints (at least 1 small joint) (5)
B) **Serology (at least 1 test result is needed for classification)** Negative RF *and* negative ACPA (0) Low-positive RF *or* low-positive ACPA (2) High-positive RF *or* high-positive ACPA (3)
C) **Acute-phase reactants (at least 1 test result is needed for classification)** Normal CRP *and* normal ESR (0) Abnormal CRP *or* abnormal ESR (1)
D) **Duration of symptoms** <6 weeks (0) ≥6 weeks (1)

CRP: C-reactive protein, ACPA: anti-citrullinated protein antibody

1.2.3 Treatment strategies

RA is commonly treated with disease-modifying antirheumatic drugs (DMARDs). Since the immune system is hyperactive most treatment strategies are immunosuppressive. The standard treatment is methotrexate (MTX) which is a folate metabolism inhibitor and will mainly interact during the mitosis of rapidly dividing cells i.e. in chronic inflammation or in a tumour.

In recent years biological treatments have been more common. The most common biological treatments used in RA today are TNF inhibitors and B cell depletion therapy. However, new biological drugs are introduced rapidly.

TNF inhibitors

The most common of the biological treatments is the TNF inhibitors. There are five different TNF inhibitors in use for the treatment of RA and they are divided into the first-generation agents; containing etanercept, infliximab and adalimumab and the second-generation agents; containing certolizumab and golimumab. They are all different monoclonal antibodies and they all result in either neutralisation of soluble TNF or membrane bound TNF, preventing TNF to bind and interact with its receptor. TNF-alpha is a proinflammatory cytokine and the end result with these drugs is less inflammation and less recruitment of macrophages and neutrophils to the joint.

Anti-B cell therapy

Rituximab (RTX) is a monoclonal chimeric mouse/human antibody targeting the B cell specific antigen CD20. The antibody has a Fc region of IgG1 type. It was approved for treatment of non-Hodgkins lymphoma 1998 and for RA 2006.

The use of RTX causes a depletion of CD20+ B cells in peripheral blood leading to an alleviation of symptoms (Edwards et al. 2004). The mechanism of action of RTX is not fully understood but may depend on three mechanisms such as: complement dependent cytotoxicity, antibody dependent cell mediated cytotoxicity and initiation of apoptosis. It has been shown to be a very effective treatment strategy in patients that are non-responsive to conventional DMARDs and anti-TNF-alpha therapy (Edwards et al. 2004; Brulhart et al. 2006; Emery et al. 2006; Bokarewa et al. 2007).

A lot of studies have been trying to clarify how deep into the tissue RTX reaches and if certain B cell populations are less sensitive to depletion. Depletion of B cells in circulation occurs in all patients, however, all patients do not respond clinically (Cambridge et al. 2006). It has been shown that patients with a higher proportion of memory B cells are relapsing earlier (week 24-40) and non responders have a larger IgD^+ memory B cell population (Roll et al. 2008). The same group showed that the first subpopulation of B cells to repopulate were the immature B cells ($CD38^+IgD^+$) followed by naïve B cells (Roll et al. 2008).

During RTX treatment, autoantibody levels decline but once the B cells have repopulated, autoantibodies reach the same levels as before treatment (Cambridge et al. 2003). Prolonged treatment with RTX may affect the plasma cell population due to a decrease of memory cells that can differentiate into plasma cells (van der Kolk et al. 2002).

A summary of some extensive studies including BM and synovia are presented in Table 2.

New B cell therapies

There are also new B cell directed biological therapies being developed directed against CD20, CD19, CD22 and BLys (reviewed in (Engel et al. 2011). However, they are still less efficient that today's anti-CD20 treatment.

Table 2. Summary of some well-done studies on the effects of RTX in PB including BM and synovia.

Study population	Time points	Compartment	Main findings	Reference
RA, 25 pts	Day 0 and 12 weeks	PB, BM, Syn	Incomplete depletion of CD19+ cells in BM in most of pts (68%). Expression of CD79a in synovial B cells were suggested to predict clinical outcome after rituximab treatment.	Teng et al, 2007
RA, 17 pts	Day 0 and 4 weeks	PB, Syn	B cell depletion in synovia was achieved in 18% of pts. B cell count was not related to DAS28 score. No changes in T cell counts in PB and synovia were found after rituximab treatment.	Vos et al, 2007
RA, 24 pts	Day 0, 4, 16 and 24 weeks	PB, Syn	Reduction of B cells in synovia observed by week 4 was further reduced by week 16.	Thurlings et al, 2008
RA, 13 pts	Day 0 and 8 weeks	PB, Syn	B cell number decreased in synovia in 80% of pts. No changes in the number of CD3+, CD138+, and CD68+ cells were observed.	Kavanaugh et al, 2008
RA, 6 pts (treated with rituximab before)	12 weeks	PB, BM	Similar degree of CD19+ cell depletion in BM and PB. An attempt to evaluate B cell subsets in BM following treatment was inconclusive.	Leandro et al, 2007
RA, 17 pts	Day 0 and every 3d mnth for 25 mnths	PB	Repopulation of B cells in PB occured between 6-10 m following rituximab treatment and consisted mainly of naive B cells. Long time (>25m) depletion of IgD+CD27+ memory B cells was observed.	Roll et al, 2006
Lymphoma, 11 pts	Unclear	PB	Repopulation of B cells to PB with cells having immature transitional phenotype (CD27-IgD+CD38high). Delayed recovery of CD27+ B cells.	Anolik et al, 2007

1.2.4 Immunisation responses in RA-patients

RA-patients are because of their immunosuppressive treatment more sensitive to infections and are therefore advised to vaccinate against influenza and pneumococci (CDC 1997; 2002). The treatment may also affect the immunisation response negatively and it is therefore of importance to perform studies in this area. However, few studies include both immunisation with T-dependent and T-independent antigens. Some studies performed regarding influenza and pneumococci immunisation are presented here.

A pneumococcal vaccination study showed that 31% of patients receiving TNF inhibitors, infliximab or etanercept, were poor responders vs 18% for MTX patients (Elkayam et al. 2004). By contrary, others did not confirm this, but showed that MTX alone can have an effect on immunisation response (Mease et al. 2004; Kapetanovic et al. 2006). Another study with RA and Systemic Lupus Erythematosus (SLE) patients medicating different DMARDs (not TNF antagonists) showed that after pneumococcal vaccination 33.3% of RA patients and 20.8% of SLE patients responded to none or only one out of the seven antigens tested, whereas all healthy controls responded well (Elkayam et al. 2002).

For immunisation with influenza, there seem to be a clearer picture on how the medications affect the vaccination response. Recent results in a study with 149 RA patients showed that patients receiving a TNF antagonist in combination with MTX or other DMARDs had poorer serological responses than did patients receiving MTX alone (Kapetanovic et al. 2007). However, most of the patients had adequate antibody levels, as seen by others (Kaine et al. 2007; Kapetanovic et al. 2007). Other medications like glucocorticosteroids, gold, azatioprine and MTX did not seem to affect the levels of protective antibodies (Malleson et al. 1993; Chalmers et al. 1994; Kanakoudi-Tsakalidou et al. 2001; Fomin et al. 2006).

Concerning the effect on immunisation in patients receiving RTX there are few studies performed. Preliminary data have shown that the response after vaccination against influenza in patients receiving anti-CD20 treatment was decreased when compared to healthy controls (Gelinck et al. 2007). Others have similar data showing that RTX treated patients have a diminished response to one of three antigens tested against influenza, whereas the other two antigens gave the same response as in controls (Oren et al. 2008).

Another study showed that after treatment with RTX in lymphoma patients 77% responded to influenza immunisation, whereas 41% responded to pneumococcal immunisation (Horwitz et al. 2004). It where though discussed whether it was really RTX that contributed to the decreased response, and not the fact that pneumococcal vaccination is a T-independent polysaccharide vaccine, and that influenza vaccination is a T-dependent mechanism. A more recent study showed that the cellular response to influenza vaccination in RTX treated patients was as good as the cellular immune response in healthy subjects and in RA-patients treated with DMARDs (Arad et al. 2011). However, the humoral immune response was severely impaired in RTX treated patients (Arad et al. 2011).

1.2.5 Epstein-Barr Virus

It has been known for many years that Eptein-Barr virus (EBV) is present in 90% of the world's population (W Henle 1979). Infection mostly occurs in early age and is asymptomatic in most humans (De-The 1982; W Henle 1982). However, if infection occurs during puberty or later, EBV may cause infectious mononucleosis (Joncas et al. 1974). EBV mainly infects B cells but may also infect epithelial cells and some lymphocytes (Sixbey et al. 1987; Jones et al. 1988; Baumforth et al. 1999; Kobayashi et al. 1999; Takada 2001).

EBV is widely known for its effects on B cells in vitro, where it immortalises B cells and turns them into activated, proliferating blasts (Aman et al. 1984; Thorley-Lawson et al. 1985a). The virus also transforms them into antibody secreting plasma cells (Rosen et al. 1977; Pender 2003) and induces upregulation of surface proteins such as CD5, CD23, CD39, CD40, CD44 and CD10 are upregulated (Kintner et al. 1981; Thorley-Lawson et al. 1985b; Wang et al. 1990; Clark et al. 1991; Klein et al. 1999).

In vivo, EBV infects resting memory B cells circulating the blood (Miyashita et al. 1997; Babcock et al. 1998; Hochberg et al. 2004) and naïve B cells that are located in the lymph nodes are transformed into long-lived memory B cells via the germinal center reaction (Babcock et al. 2000; Joseph et al. 2000a). This way, EBV secures life-long infection of the host simply because memory B cells live for a long period of time. Most of the memory B cells express no viral proteins that will alert the

immune system of infection (Qu et al. 1992; Tierney et al. 1994; Chen et al. 1995).

In immunosuppressed patients, the number of EBV-infected cells in the circulation can be up to 50 times higher (Babcock et al. 1999). There is an association between EBV and autoimmune diseases like rheumatoid arthritis, SLE and multiple sclerosis (James et al. 2001; Poole et al. 2006; Ascherio et al. 2007; Toussirot et al. 2008). In RA, higher levels of anti-EBV antibodies have been found, prevalence of higher levels of circulating EBV infected cells as well as higher viral load in these cells and impaired T cell responses against EBV proteins as compared to healthy subjects (Alspaugh et al. 1981; Depper et al. 1981; Tosato et al. 1984; Yao et al. 1986; Babcock et al. 1999; Balandraud et al. 2003).

So even though a causative role for EBV in RA is less likely, the autoimmune disease and the immunosuppressive treatment alters the immune response against EBV.

2 AIM

The aim of this thesis is to:

1. **Study the effects of anti-CD20 treatment on B cell ontogeny**

   - Shortly after treatment

   - Long after treatment

2. **Evaluate immunisation response in RA-patients treated with RTX**

   - 6 days before treatment

   - 6 months after treatment

3. **Examine the effects of EBV in RA-patients**

   - On the $CD25^+$ B cell population

   - With respect to clinical response

3 PATIENTS AND METHODS

In this section the patients and methods used in this thesis will be briefly presented. For a more detailed presentation of reagents used please be referred to the papers in this thesis.

3.1 Patients (I, II, III)

All RA patients included in the work of this thesis have been diagnosed according to the ACR criteria (Arnett et al. 1988). They visited the rheumatology clinic at Sahlgrenska University Hospital in Gothenburg between 2007 and 2008 and they did all give informed consent. Patient characteristics are presented in Table 3.

3.1.1 RA-patients treated with RTX (I, II, III)

The patients that are chosen for RTX treatment at Sahlgrenska University Hospital are all non-responsive to conventional DMARDs and most of them have failed to respond to TNF-alpha inhibitor therapy.

Since RTX is a fairly new drug there are no information about how it may affect the immune system i.e. after twenty years. This is the reason for that we have divided the RA patients into RTX-naïve patients (never treated before) and RTX-treated patients (treated one or several times before). This information is also included in Table 3. When differences were found between RTX-naïve and RTX-treated patients this was stated in the papers, we did however check for differences between RTX-naïve and RTX-treated patients in this way in all three papers.

The response to RTX treatment was evaluated on the basis of the EULAR response criteria (van Gestel et al. 1999). The reduction of DAS28 > 1.3 was considered as clinical response. The evaluation of DAS28 was made both three and six months after treatment and if the patient was a responder on any of these two occasions they were considered as a responder to RTX treatment.

Table 3. A summary of RA-patient characteristics for patients included in paper I, II and III.

	Paper I	**Paper II**			**Paper III**	
	RA patients n=37	Post-RTX n=11	Pre-RTX n=8	Controls n=10	EBV positive, n=15	EBV negative n=21
Age, years (range)	53±10 (28-76)	60.4±7.8 (45-70)	65.4±11.5 (55-82)	63.6±12.9 (48-95)	61.1 (44-81)	60.2 (33-82)
Sex, male/female	7/30	1/10	1/7	3/7	14/1	16/5
Radiological data, erosive/non-erosive	35/2	10/1	7/1	9/1		
Rheumatoid factor, +/-	33/4	11/0	8/0	10/0	13/2	19/2
Duration of the disease, years±SD	8±6	17±13	9±6	7±5	17.8 (6-35)	15.6 (6-37)
Treatment						
MTX/other	35/2	10/1	7/1	10/0	14/2	19/3
Previous anti-TNF, yes/no	37/0	10/1	5/3	2/8	15/0	21/0
Previous anti-CD20, yes/no	13/24	4/7	1/7	0/10	4/11	8/13
Time after previous anti-CD20, month	22±11 (6-61months)	30 months (14-48)	19 months (14 and 24)	0	-	-

3.1.2 RA-patients with conventional MTX treatment (II)

In paper II we have used RA-patients treated with MTX as controls and not healthy individuals. The reasons for this is that we wanted to compare differences between RTX treated RA-patients and RA-patients treated with conventional DMARDs like MTX. There are many studies analysing immunisation response in RA-patients on different treatments as compared to healthy individuals. We were more interested in studying if RTX treated RA-patients had a more impaired immune response after immunisation as compared to RA-patients treated with MTX.

3.2 Study Design

3.2.1 BM phenotype after depletion (I)

Blood and BM were drawn from RA-patients (n=37) right before their RTX infusion (day 0). The patients were then selected for a follow-up visit either one month (n=10) or three months (n=14) after their RTX infusion. One patient fulfilled both follow-ups and is included in both groups. Some patients had one or several previous courses of RTX (n=13) and were also analysed separately. Patients were flow cytometrically analysed at each time point as described in paper I. Schematic picture of the study setup is shown in Figure 4.

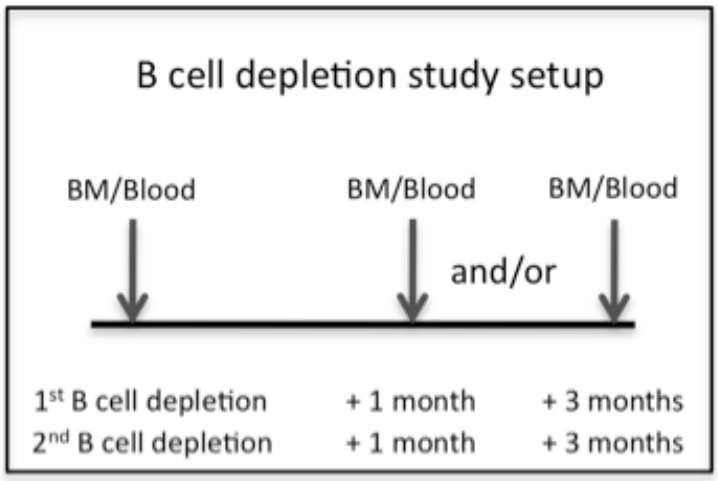

Figure 4. B cell depletion setup used in paper I.

3.2.2 Immunisation during RTX treatment (II)

Patients received immunisation with influenza vaccine or pneumococcal vaccine either six days before RTX treatment (Pre-RTX group n=8) or six months after RTX treatment (Post-RTX group n=11) Figure 5).

Blood was drawn at day 0, day 6 and day 21. The blood from day 6 was used to do an vaccine specific enzyme-linked immunosorbent spot (ELISPOT) to measure cellular response to immunisation and the blood from day 0 and 21 was used to measure humoral response with an enzyme-linked immunosorbent assay (ELISA) to detect vaccine specific antibodies.

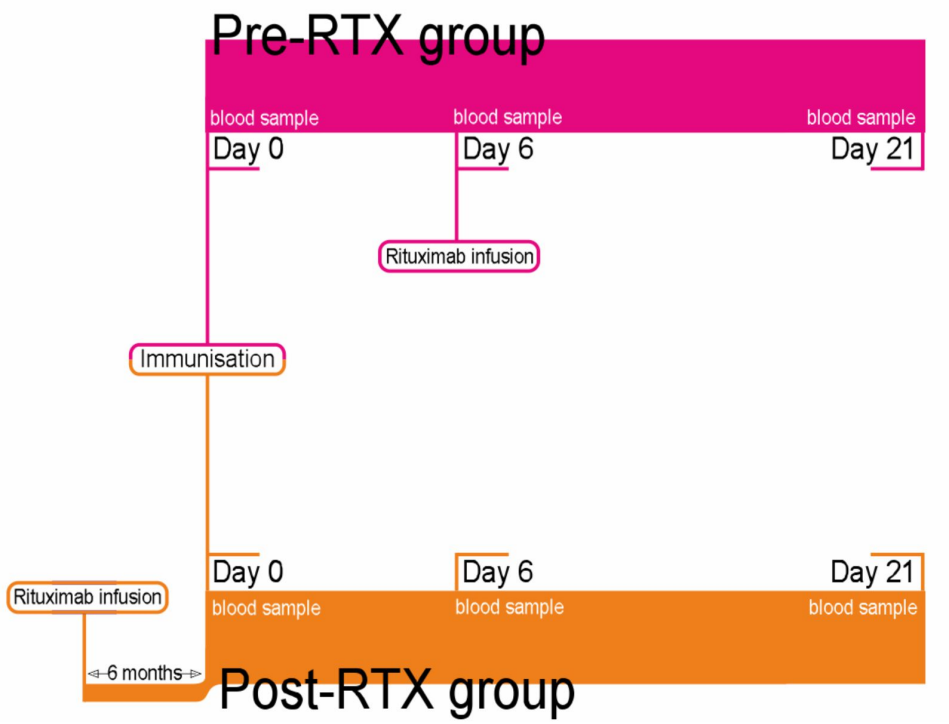

Figure 5. Study setup for paper II. *Figure made by Jason Johnson.*

3.2.3 The impact of EBV infection on B cell subsets (III)

Blood and BM were drawn from 36 RA-patients at the day of RTX treatment. EBV replication was measured in blood and BM at this time. Patients with active EBV replication in their BM were termed EBV+ patients (n=15). Patients that did not have active replication were termed EBV- patients (n=21). When we evaluated clinical response in the patient groups we found nine responders and four non-responders in the EBV+ group and thirteen responders and seven non-responders in the EBV- group. All patients were flow cytometrically analysed. Nine MTX treated patients also donated synovial fluid from the knee joint.

For functional analysis eighteen MTX treated RA-patients were selected and four healthy controls. Blood from these subjects were prepared and cell sorting into $CD25^+$ and $CD25^-$ B cell subsets was performed. ELISPOT was performed to measure Ig secreting cells and RF ELISA was performed to measure presence of RF. A study setup of the functional analysis of this study is shown in Figure 6.

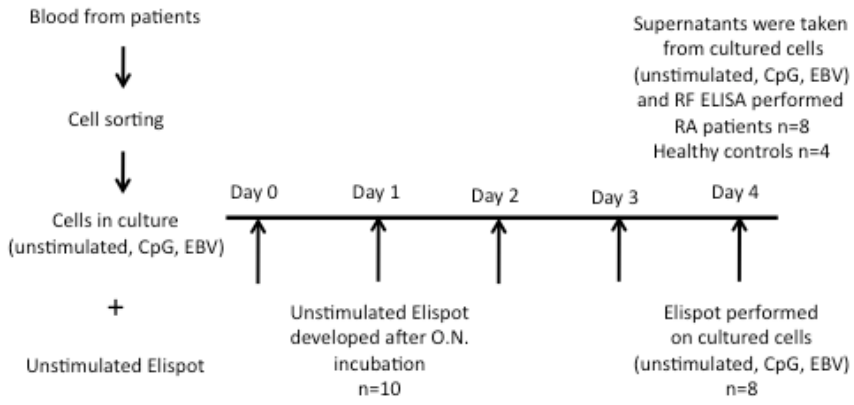

Figure 6. Study setup of the functional analysis performed in paper III.

3.2.4 Ethical considerations

The recruitment of patients and healthy subjects always involved careful explanation of what the purpose of the study was and that there were no future commitment expected of them. They could decide to leave the study whenever they pleased. Most patients were however very glad to have the opportunity to be involved in research and to help the progress of science.

All studies were approved by The Ethical Committee at Sahlgrenska Academy at the University of Gothenburg (no 34306).

3.3 Methods

3.3.1 Flow cytometry (I, II, III)

The ability to measure the properties of particles is elementary in flow cytometry. To achieve this, the suspension of particles that are injected into the FACS are ordered into a stream of single particles by the fluidics system of the machine. The sample enters via a central core that is surrounded of faster flowing sheath fluid. As the sheath fluid runs it produces a drag, hydrodynamic focusing, that makes the particles go one by one into the sheath fluid whilst the particles remaining along the walls in the central core are not moving. After hydrodynamic focusing, the particles will pass through a laser (or lasers), which will make them scatter the light, or if the particles are labelled with a fluorochrome it will emit light of a certain wavelength. Light that is scattered will go in two different directions and will be collected by a lens, the forward scatter channel (FSC) or the side scatter channel (SSC). FCS will provide information about the size of the particle and the SSC about the granular content (Shapiro 2003).

The light that is emitted after the laser has met the particle with a fluorochrome, will pass through different filters until it hits the filter that forward light of that specific wavelength to a detector (Shapiro 2003).

Cell sorting (III)

An application of flow cytometry is cell sorting. After the particles are hydrodynamically focused, each particle is hit with the laser and the scatter and fluorescence signal is compared to the sort criteria set on the

instrument. If the particle is a match to the sort criteria, it will be electrostatically charged the moment it leaves the stream. The particle will then enter an electrostatic field and depending on the charge it will decline left or right (Shapiro 2003).

In our experiments, cells were sorted according to the CD19 expression in combination with either CD25 expression or lack of CD25 expression rendering highly purified populations with the following phenotypes: $CD19^{+}25^{+}$ and $CD19^{+}25^{-}$. Gates for sorting are presented in Figure 7.

After sorting the cells were centrifuged and resuspended in an appropriate volume complete medium and counted in a Bürker chamber.

Gating (I, II, III)

For all phenotypic analysis we have used the same gating strategy.

A generous first gate was always placed to make sure that all mononuclear cells (MNC) were selected. Since the $CD3^{+}$ T cell and the $CD19^{+}$ B cell population co express many molecules we wanted to eliminate these first. A "non-CD3" gate was placed and then $CD19^{+}$ cells were selected for further analysis (Figure 8).

When analysing i.e. percentages of $CD19^{+}$ cells these were always made on MNC and not on the "non-CD3" gate. The gating of the cells was performed using Flow Jo software.

Flow cytometry considerations

FACS is an excellent method to use when you want to characterise cells phenotypically. There are however things to consider when you are setting up your experiment.

The antibodies used for FACS have different concentrations and should be titrated to the optimal concentration to avoid that one fluorochrome in your panel "takes over".

When choosing fluorochromes for your panel one should also consider that the fluorochromes have different brightness/intensity. A population with many receptors on its surface can be stained with a

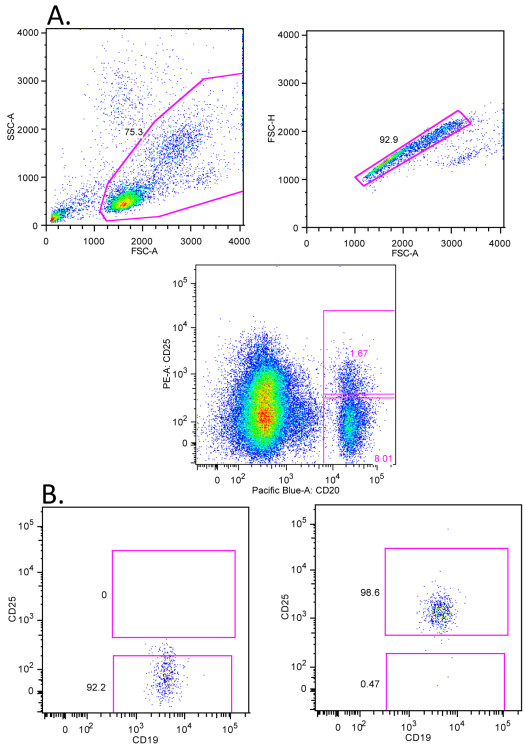

Figure 7. Gating used when sorting cells using FACS. *In* ***A)*** *mononuclear cells are selected and then single cells are gated for. CD19 and CD25 are plotten against each other and CD19^{+}CD25^{+} and CD19^{+}CD25^{-} B cells are selected for sorting. The CD25 gate is set according to the T cell expressing CD25.* ***B)*** *Sorted B cells are ran trough the FACS to assure purity of the cells.*

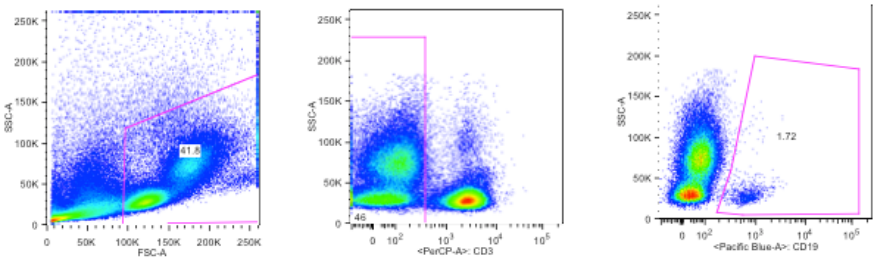

Figure 8. Gating strategy when doing phenotypical FACS analysis*. First mononuclear cells are selected. A non-CD3 gate is selected and then a CD19 gate is put in place.*

fluorochrome with low intensity but a population that only have a few receptors on its surface should be stained with a high intensity fluorochrome. If a low intensity fluorochrome is used, there is a risk that you will "lose" your population and get misleading results.

Many cells have Fc receptors that can bind antibodies unspecifically. Because of this, it is important to block the FC receptors before staining. We use a anti-human polyclonal rabbit $F(ab')_2$ immunoglobulin with irrelevant specificity.

Regardless if you stain in a tube or in a well, you should have the "right" amount of FACS antibody in relation to the number of cells. If you put too many cells in the tube or well your cells will not get stained properly and your result will be incorrect. We use 10 µl of diluted antibody / $1x10^5$-$1.5x10^6$ cells.

The analysis of the FACS data is also a crucial thing. FACS is a good method, but there is a need to be careful with the gating. If one is using a panel with many fluorochromes you always need to use compensation controls. The fluorochromes can have spectral overlaps and there could thus exist a leakage in between channels.

The use of fluorochrome minus one settings (FMO) is also of great importance when analysing the FACS data (Perfetto et al. 2004). As mentioned, there can be a leakage in between the different channels in the FACS. To avoid this, one can leave one channel empty (not stained for a specific fluorochrome) and then analyse the leakage in this channel. The leakage will be the cells that show up positive for the fluorochrome used in the sample. Since that specific fluorochrome is not added to the sample, this is falsely positive results. Our rule is that if this leakage is more than 1%, one should subtract that percentage from all samples stained with that fluorochrome.

3.3.2 ELISPOT (I, II, III)

ELISPOT is used to analyse Ig secreting cells. It is a sensitive method where one can count each cell that produces Igs. The cells are always seeded in duplicates or triplicates in concentrations 1 x 10^5, 2 x $10^{4,}$, 4 x 10^3 and 8 x 10^2 lymphocytes per well.

Spot forming cells (sfc) were counted in a microscope and wells with approximately 50 sfc per well were considered the most accurate concentration to count.

In paper II we used a vaccine specific ELISPOT to find antigen specific sfc. The plates were then pre-coated with poly-L-lysine to make the vaccine adhere better to the plate surface. More than 50 sfc / 10^6 lymphocytes were considered as response to vaccination.

3.3.3 ELISA (II, III)

ELISA is used to detect antibodies in sera or supernatants. In paper II we used an in-house ELISA where plates were coated with vaccine to detect vaccine specific antibodies from sera. The absorbance detected from the day 0 sample was used to define each patient's individual baseline level and the day 21 absorbance level were then recalculated relative to the baseline level. The cut off level for response was set according to the mean increase of vaccine specific antibodies in the MTX treated control group which were 110%.

In paper III we detected RF using an in-house ELISA that were coated with rabbit gamma globulin. Basically it is the Fc part of an Ig that is used as coating, which is what RF interacts with. Levels of RF in the EBV and CpG stimulated cultures were normalised to RF in unstimulated cultures of the same subject.

3.4 Statistics

In the work of this thesis we have used both parametrical and non-parametrical methods. When comparing values from the same patient i.e. the number of $CD19^+$ B cells at day 0 and three months later, we have used the paired T test. When comparing i.e. patient groups with each

other, regardless the parameter analysed, we have used the Mann-Whitney test.

Data in tables is mostly presented as mean and standard deviation (SD) with 95% confidence interval. When presenting graphs in figures as box and whiskers they are always based on median and range.

P values <0.05 were considered as significant.

All statistical evaluations were preformed using Prism software, Graphpad.

4 RESULTS

4.1 Short and long-term effects of anti-CD20 treatment on B cell ontogeny in bone marrow of patients with rheumatoid arthritis

4.1.1 Short-term changes in BM after RTX treatment

The short-term changes (after one and three months) in BM of RA-patients treated with RTX were characterised by a reduction of IgD expressing B cells.

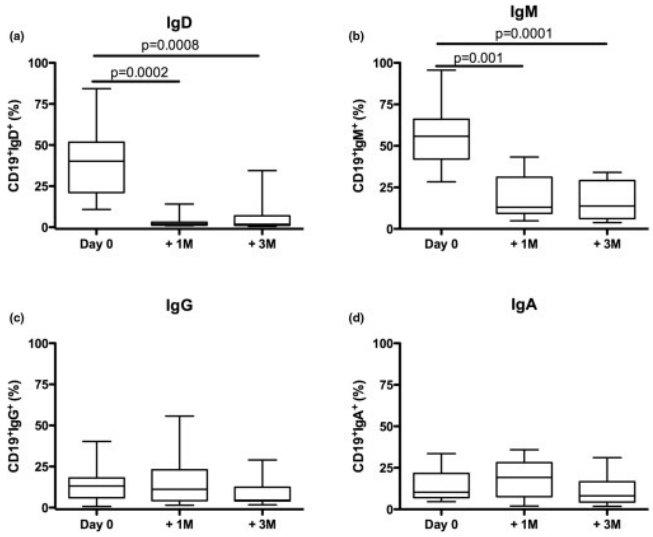

Figure 9. Short-term changes in the immunoglobulin expression of B cells following rituximab treatment. *Isolated bone marrow MNC were stained for Ig expression at day 0, 1 and 3 months after rituximab treatment. In* ***A)*** *$CD19^+IgD^+$* ***B)*** *$CD19^+IgM^+$* ***C)*** *$CD19^+IgG^+$* ***D)*** *$CD19^+IgA^+$ is shown. Box represents 25-75 percentile, line indicates median, whereas error bars represent range. Statistical evaluation was performed using paired t-test. Figure is previously published in paper I and published with the permission by Arthritis Research and Therapy.*

Analysing the surface expression of Igs on BM B cells we found a depletion of the more immature IgD and IgM expressing B cells both after one month (p=0.0002, p=0.001) and after three months (p=0.0008, p=0.0001) (Figure 9). The B cells expressing switched Ig classes (IgG and IgA) remained unchanged when analysing percentages (Figure 9). The combination of CD27 and IgD was used to analyse the maturity of the cells. We found a depletion of naïve B cells ($CD27^-IgD^+$) after one and three months (p=0.0007 and p<0.0001) (Figure 10). Immature B cells ($CD27^-IgD^-$) and unswitched B cells ($CD27^+IgD^+$) were reduced after three months (p=0.005, p=0.02), and switched memory B cells ($CD27^+IgD^-$) (p=0.01) after one month (Figure 10).

Important to remember is that in reality, all B cell subsets expressing CD20 were reduced, which was also found when analysing absolute numbers. Most of the surviving B cells did not express IgD and were hence found within the more mature B cell populations.

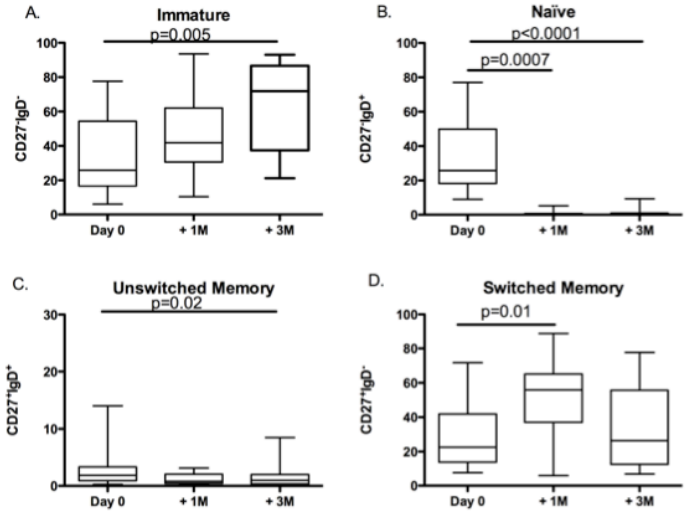

Figure 10. Analysis of short-term changes in bone marrow regarding the expression of IgD in combination with CD27. A) *Memory phenotype; immature ($CD27^-IgD^-$),* ***B)*** *naïve ($CD27^-IgD^+$),* ***C)*** *unswitched memory ($CD27^+IgD^+$) and* ***D)*** *switched memory ($CD27^+IgD^-$) B cells of rituximab naïve and treated patients at day 0. Box represents 25-75 percentile, line indicates median, whereas error bars represent range. Statistical evaluation was performed using paired t-test.*

4.1.2 Long-term changes in BM after RTX treatment

When talking about long-term changes it refers to the fact that some of the patients (n=13) had had one or several previous courses of RTX. The mean time is 22 months. Patients treated for the first time are termed naïve (n=24).

The surface expression of Igs was totally the opposite from the short-term changes. IgD and IgM remained unchanged whereas the expression of IgG (p=0.003) and IgA (p=0.001) were reduced in treated patients (Figure 11).

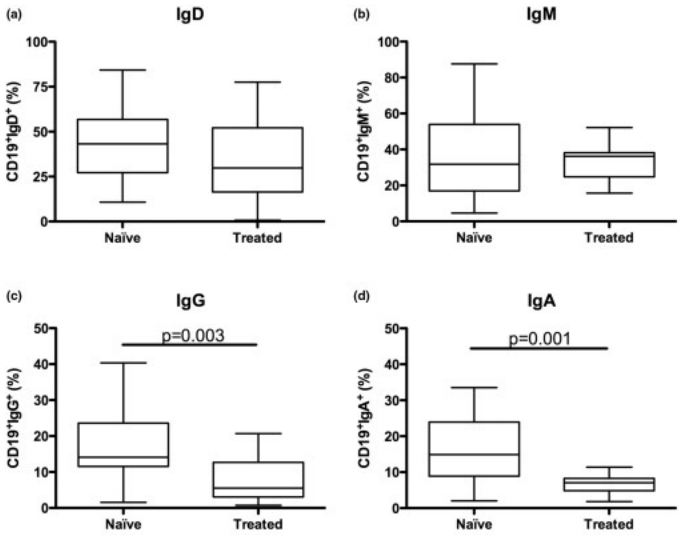

Figure 11. Long-term changes in the immunoglobulin expression of B cells following rituximab treatment. *Isolated bone marrow MNC were stained for Ig expression comparing rituximab-naïve and treated patients. In* ***A)*** *$CD19^+IgD^+$* ***B)*** *$CD19^+IgM^+$* ***C)*** *$CD19^+IgG^+$* ***D)*** *$CD19^+IgA^+$ is shown. Box represents 25-75 percentile, line indicates median, whereas error bars represent range. Statistical evaluation was performed using the Mann-Whitney t-test. Figure is previously published in paper I and published with the permission by Arthritis Research and Therapy.*

The same pattern is seen for unswitched ($CD27^+IgD^+$) and switched memory B cells ($CD27^+IgD^-$) in treated patients; they were both reduced ($p<0.0001$, $p=0.004$) (Figure 12). Immature B cells ($CD27^-IgD^-$) were relatively increased ($p=0.01$) (Figure 13), but when analysing absolute numbers both immature B cells ($CD27^-IgD^-$) and naïve B cells ($CD27^-IgD^+$) were unaffected in treated patients.

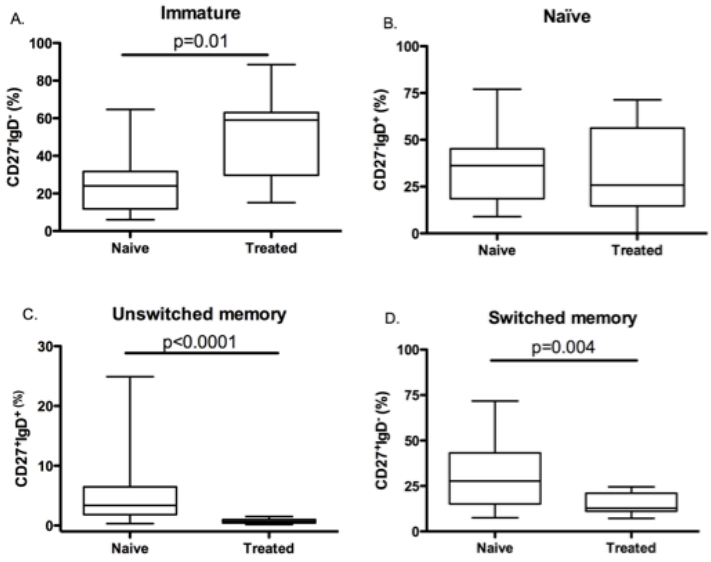

Figure 12. Analysis of long-term changes in bone marrow regarding the expression of IgD in combination with CD27. A) *Memory phenotype; immature ($CD27^-IgD^-$),* ***B)*** *naïve ($CD27^-IgD^+$),* ***C)*** *unswitched memory ($CD27^+IgD^+$) and* ***D)*** *switched memory ($CD27^+IgD^-$) B cells of rituximab naïve and treated patients at day 0. Box represents 25-75 percentile, line indicates median, whereas error bars represent range. Statistical evaluation was performed using Mann-Whitney t-test.*

4.1.3 Serological changes after RTX treatment

The production of RFs of all classes (IgG, IgM and IgA) was reduced after three and six months (Figure 13A). When comparing these RFs between naïve and treated patients one could see that when RA-patients get back for the next course of RTX the levels are back to starting level (Figure 13B).

In contrast, the total levels of Igs were unchanged short after RTX treatment (three and six months) as well as long time after treatment (mean 22 months).

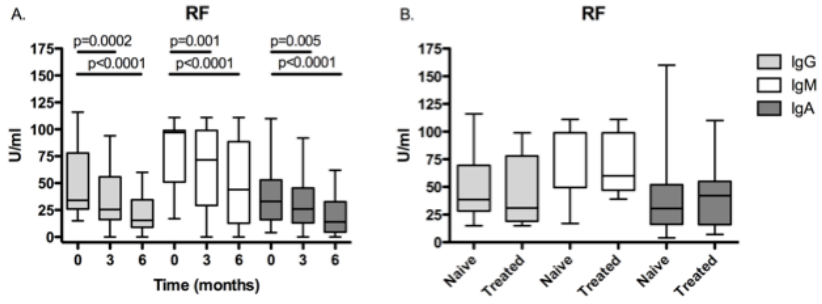

Figure 13. Short- and long-term changes of rheumatoid factor (RF) levels in PB after rituximab treatment. A) *RF-levels in PB at day 0, 3 and 6 months after rituximab treatment* ***B)*** *RF-levels in PB comparing rituximab-naïve and treated patients. Box represents 25-75 percentile, line indicates median, whereas error bars represent range. Statistical evaluation was performed using paired t-test (short term changes) and Mann-Whitney t-test (long-term changes). Figure is previously published in paper I and published with the permission by Arthritis Research and Therapy.*

4.2 Vaccination response to protein and carbohydrate antigens in patients with rheumatoid arthritis after rituximab treatment

4.2.1 Cellular response to immunisation

Cellular response to immunisation was measured by ELISPOT where vaccine specific Ig-secreting cells were enumerated in a microscope. The influenza specific IgM producing cells were present in the control group (30sfc/ 10^6 lymphocytes) and in the pre-RTX group (55 sfc/ 10^6 lymphocytes) but less in the post-RTX group (3 sfc/ 10^6 lymphocytes) ($p=0.046$ as compared to the control group and $p=0.044$ as compared to the pre-RTX group). No differences were found regarding the influenza specific IgG or IgA between the groups.

The pneumococcal vaccine specific Ig producing cells were similar between all patient groups regardless of isotype.

4.2.2 Production of IgM after immunisation

The production of IgM after immunisation with influenza vaccine only gave rise to specific antibodies in the control group (Figure 14). For RTX treated groups the result were similar for both influenza and pneumococcal vaccine – no significance were detected for vaccine specific IgM.

4.2.3 Production of IgG after immunisation

Vaccine specific antibodies of IgG isotype were measured after 21 days and each patient was compared with their baseline level.

The controls achieved a significant increase of vaccine specific IgG after immunisation with both influenza ($p=0.05$) and pneumococci ($p=0.02$) (Figure 15). The pre-RTX group only had elevated levels of specific IgG towards pneumococci ($p=0.03$) and the post-RTX group only had elevated levels of specific IgG towards influenza ($p=0.03$) (Figure 15).

Since protein and polysaccharide vaccination may give rise to different IgG subclasses we were also interested in analysing this.

The immunisation with influenza did only give rise to vaccine specific IgG1 and IgG4 in the control group whereas the pre-RTX group and the post-RTX group did not give rise to an increase in any subclass (Figure 16).

When looking at each patient's level, all patients in the control group responded to at least one of the IgG subclasses. In the pre-RTX group one patient did not respond and in the post-RTX group there were four patients that displayed a total lack of IgG subclasses.

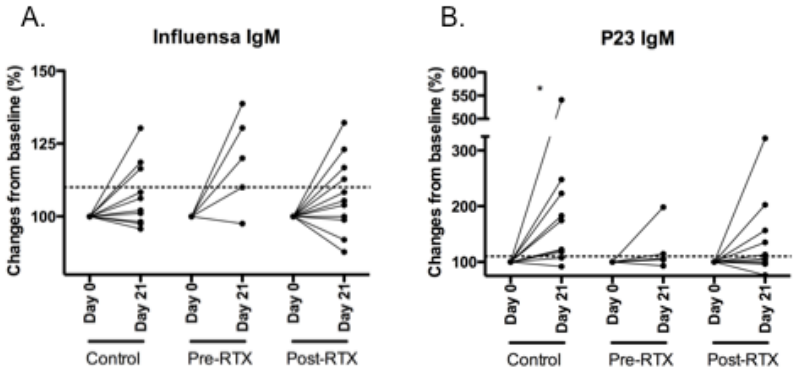

Figure 14. Vaccine specific IgM after immunisation with influenza and pneumococci*. In* ***A)*** *influenza,* ***B)*** *pneumococci. In all figures, day 0 levels are set at 100% and day 21 indicate changes relative to day 0. Dotted line indicates cut off set at 110%. Each dot corresponds to one patient, and statistical evaluation was performed using the paired T-test and statistical significance is set as p-value < 0.05. * p< 0.05, ** p<0.01, *** p<0.0001.*

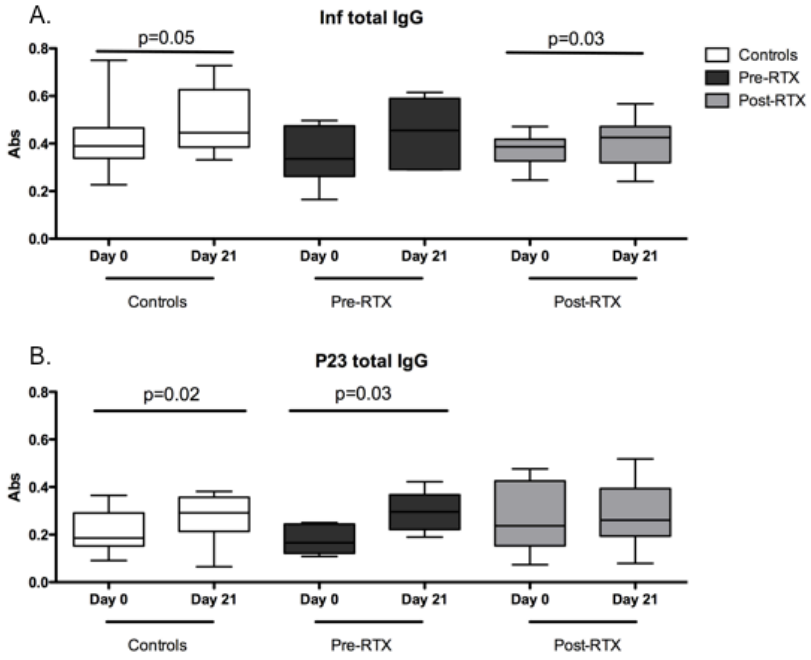

Figure 15. Vaccine specific total IgG after immunisation with influenza and pneumococci*. Values are presented as absorbance.*

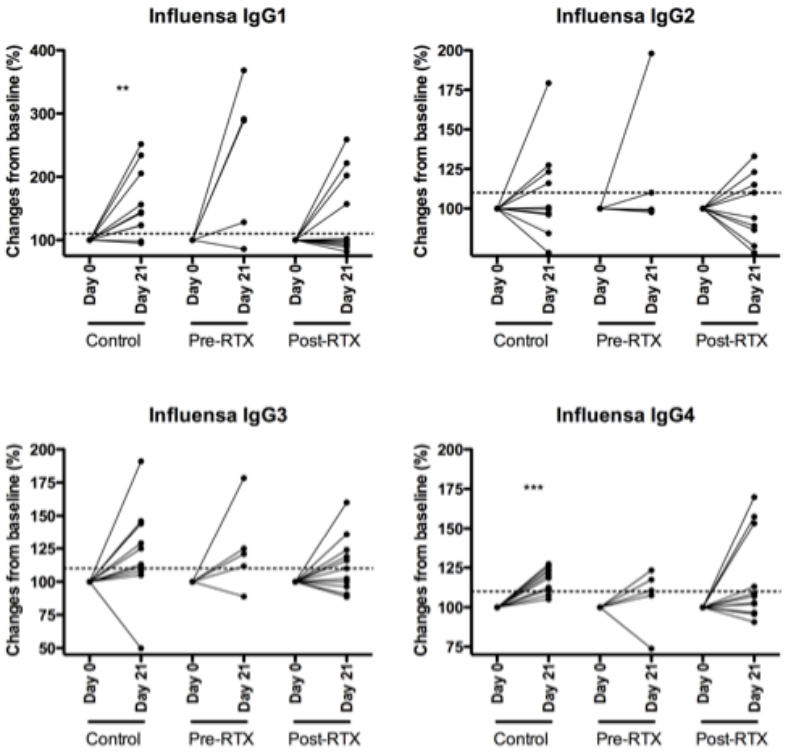

Figure 16. Changes in influenza specific IgG subclasse pattern *in RA-patients before and after RTX treatment. Changes at day 21 as compared to day 0 in IgG 1-4 are shown following influenza vaccination. In all figures, day 0 levels are set at 100% and day 21 indicate changes relative to day 0. Dotted line indicates cut off set at 110%. Each dot corresponds to one patient, and statistical evaluation was performed using the paired T-test and statistical significance is set as p-value < 0.05. * p< 0.05, ** p<0.01, *** p<0.0001. Figure is previously published in paper II and is published with the permission by Arthritis Research and Therapy.*

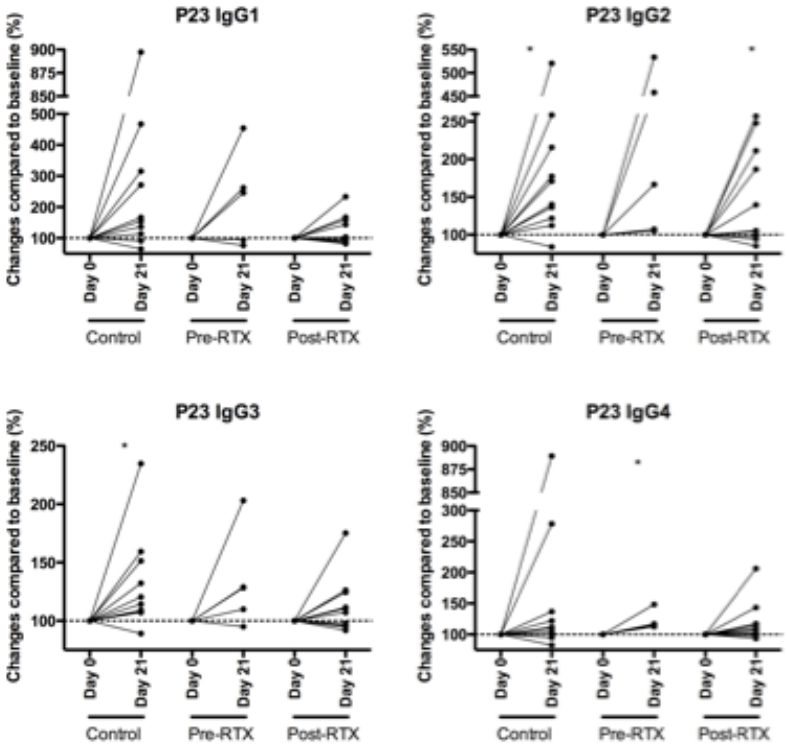

Figure 17. Changes in pneumococcal specific IgG subclasse pattern *in RA-patients before and after RTX treatment. Changes at day 21 as compared to day 0 in IgG 1-4 are shown following pneumococcal vaccination. In all figures, day 0 levels are set at 100% and day 21 indicate changes relative to day 0. Dotted line indicates cut off set at 110%. Each dot corresponds to one patient, and statistical evaluation was performed using the paired T-test and statistical significance is set as p-value < 0.05. * p< 0.05, ** p<0.01, *** p<0.0001. Figure is previously published in paper II and is published with the permission by Arthritis Research and Therapy.*

The immunisation with pneumococci gave rise to vaccine specific IgG2 and IgG3 whereas the pre-RTX group showed an increase of IgG4 and the post-RTX group of IgG2 (Figure 17).

Analysing each patient's individual response we found that all patients in the control group and the pre-RTX group responded to at least one of the IgG subclasses. However, four patients in the post-RTX group did not respond to any of the IgG subclasses. A summary of the IgG subclass pattern after immunisation with influenza and pneumococci is presented in Table 4.

Table 4. The IgG subclass pattern after immunisation with influenza and pneumococcal vaccine. X indicates a significant increase in the group.

	Influenza			Pneumo 23		
	Controls	Pre-RTX	Post-RTX	Controls	Pre-RTX	Post-RTX
IgG1	x					
IgG2				x		x
IgG3				x		
IgG4	x				x	

4.2.4 Production of kappa and lambda light chains after immunisation

We measured the production of vaccine specific kappa and lambda light chains after immunisation. All three patient groups showed elevated levels of vaccine specific lambda light chains after immunisation with both influenza and pneumococci. In contrast, only controls showed elevated levels of vaccine specific kappa light chains for both vaccines ($p=0.001$ for influenza and $p=0.001$ for pneumococci). The pre-RTX group did also show an increase of vaccine specific kappa light chains after immunisation with pneumococci ($p=0.05$) whereas the post-RTX group did not show any increase for either of the vaccines used. A summary of the kappa and lambda light chain production during immunisation is presented in table 5.

Table 5. Summary of the kappa and lambda light chain production after immunisation with influenza and pneumococci in RA-patients after RTX treatment. X indicates a significant increase in the group.

	Influenza			Pneumo 23		
	Controls	Pre-RTX	Post-RTX	Controls	Pre-RTX	Post-RTX
Kappa	x			x	x	
Lambda	x	x	x	x	x	x

4.3 Epstein-Barr virus persistence in patients with rheumatoid arthritis drives antibody production via the CD25$^+$ B cell population

4.3.1 B cell phenotype in EBV infected RA-patients

B cell populations in peripheral blood (PB) and BM of EBV$^+$ and EBV$^-$ RA-patients were phenotypically analysed. We found that CD27$^+$ memory B cells were increased in PB of EBV$^+$ patients as compared to EBV$^-$ patients (p=0.04). To support this we also found an extended IgG$^+$ population (0.003) in PB as well as a smaller IgD$^+$ population (p=0.004) in EBV$^+$ patients. The BM of EBV$^+$ and EBV$^-$ patients were though similar regarding these markers.

Combining the markers IgD and CD27 to get more detailed information about the memory B cell populations showed that in BM of EBV$^+$ patients there was an enrichment of switched memory B cells (CD27$^+$IgD$^-$) (p=0.03). In contrast, EBV$^-$ patients had an enrichment of unswitched memory B cells (CD27$^+$IgD$^+$) (p=0.05).

4.3.2 $CD25^+$ B cells in EBV infected RA-patients

Analysing the expression of CD25 on B cells isolated from RA-patients showed that the BM has lowest level of $CD25^+$ B cells with 10%±11. In PB we found 15%±11 CD25+ B cells and in synovial fluid 35%±27.

$CD25^+$ B cells reached the same levels in EBV^+ patients (17%±11 in PB, 12%±13 in BM) as in EBV^- patients (14%±11 in PB, 9%±10 in BM).

It is known that $CD25^+$ B cell population is more mature than the $CD25^-$ B cell population. We confirmed that this is also the case in EBV^+ patients. Analysing the surface expression of Igs we found that the $CD25^+$ B cells had larger populations of switched B cells (IgG^+ and IgA^+) as compared to $CD25^-$ B cells both in PB and BM (Table 6). The IgD^+ population was smallest in $CD25^+EBV^+$ patients and the IgG^+ population was smallest in $CD25^-EBV^-$ patients (Table 6).

The expression of CD25 was also associated with memory B cells, present on both switched ($CD27^+IgD^-$) and unswitched ($CD27^+IgD^+$) B cells. Memory B cells expressed CD25 to a similar degree in EBV^+ (52%±24) and EBV^- (58%±20) patients.

The normally immature $CD25^-$ B cell population was also more matured in EBV^+ patients as compared to EBV^- patients with a larger population of switched memory cells in PB ($p=0.02$) and in BM ($p=0.04$).

Table 6. Differences in the surface Ig expression on the $CD25^+$ and $CD25^-$ B cell populations of EBV^+ (n=15) and EBV^- (n=21) RA-patients in PB and BM. Values are presented as the mean±SD of the CD19+ population, and P-values <0.05 are considered as significant.

a: $CD25^+$ vs. $CD25^-$ B cell populations in the EBV^+ group.

b: $CD25^+$ vs. $CD25^-$ B cell populations in the EBV^- group.

	EBV+		EBV-		P-value
	CD25+	CD25-	CD25+	CD25-	
Peripheral blood					
IgD	45.04±21.76	66.96±23.86	59.19±22.92 **p=0.03**	80.30±17.04	<0.0001*a* <0.0001*b*
IgM	69.95±18.56	72.74±19.41	74.00±20.68	80.68±14.91	
IgG	41.03±23.38	18.41±12.05	34.31±12.64 **p=0.03**	10.42±7.54	<0.0001*a* <0.0001*b*
IgA	24.39±13.37	12.06±8.76	25.29±15.55	11.05±10.79	0.01*a* 0.0003*b*
Bone marrow					
IgD	33.71±19.39	35.83±24.07 **p=0.03**	46.57±18.20	45.10±22.05	
IgM	69.09±20.51	53.17±23.90	71.19±14.84	53.31±19.44	0.0004*a* 0.003*b*
IgG	41.17±25.68	14.34±9.53	39.00±21.53	11.17±8.70	<0.0001*a* 0.002*b*
IgA	37.00±17.77	18.13±22.67	33.80±15.64	9.85±9.41	0.0002*a* 0.01*b*

4.3.3 EBV infection affects the clinical outcome of RTX treatment

The clinical outcome of RTX treatment was defined as a decrease of DAS28 > 1.3 three and six months after treatment. When dividing the patient groups into responders and non-responders we have nine responders and four non-responders in the EBV^+ group and thirteen responders and seven non-responders in the EBV^- group.

When analysing the levels of $CD25^+$ memory B cells it looks like the non-responding patients have higher levels of these cells both in the EBV^+ and EBV^- patient group as compared to the responding patients (Table 7). Combining IgD and CD27 and analysing the responders and non-responders we found that responding patients in the EBV^+ patient group had a larger CD25 expressing switched memory B cell population in PB ($p=0.03$) and a smaller naïve B cell population ($p=0.05$) as compared to the EBV^- responders. The EBV^- responding patients also had larger naïve B cell population ($p=0.04$) as compared to non-responding EBV^- patients.

Table 7. The percentage of $CD25^+CD27^+$ PB and BM B cells isolated from RA-patients treated with RTX. Patients are divided into EBV^+ and EBV^- groups and are compared with respect to clinical response where R represents responding patients and NR represents non-responding patients. Values are presented as mean±SD of the $CD19^+$population.

	EBV+		EBV-	
	R	NR	R	NR
PB	55%±23	61%±9	49%±21	65%±28
BM	45%±29	68%±11	52%±21	66%±15

We also analysed the CD25 expression on the plasma cell population ($CD138^+CD19^-$) as well as on the plasma blast population ($CD138^+CD19^+$) in BM. We found that 17%±24 of plasma blasts and 19%±20 of plasma cells expresses CD25. Analysing these populations with respect to EBV infection showed no differences. However, when adding clinical response we found that EBV^+ non-responders had a larger population of CD25 expressing plasma cells (p=0.0008) as compared to the responding patients. In EBV^- patients CD25 expressing plasma cells and plasma blasts were similar.

Total plasma blast and plasma cell numbers in EBV^+ and EBV^- RA-patients were also analysed. Data are presented in table 8.

Table 8. $CD138^+$ populations are shown in BM of EBV^+ and EBV^- RA patients. CD138 values are presented as the mean±SD of the mononuclear cell population, plasma blast and plasma cell values are presented as the mean±SD of the $CD138^+$ population, the mature memory B cell population as the mean±SD of the $CD19^+$ population.

Cell population	EBV+	EBV-
$CD138^+$	0.88±0.29	0.76±0.55
Plasma blasts, $CD138^+CD19^+$	70.11±10.42	67.33±11.76
Plasma cells, $CD138^+CD19^-$	29.27±10.75	32.19±11.58
Mature memory B cells, $CD19^+CD27^+CD138^-$	33.57±22.15	23.48±16.85

4.3.4 In vitro infection with EBV on $CD25^+$ B cells

After phenotypical studies we also wanted to perform some functional experiments. We sorted PB MNC $CD19^+CD25^+$ and $CD19^+CD25^-$ from eighteen RA-patients and performed an ELISPOT to study the number of Ig producing cells. Ten of the patients were selected for a direct ELISPOT whereas the eight remaining patients were cultured for 96 hours in EBV containing media and then an ELISPOT was performed.

The EBV stimulation resulted in an increase of Ig producing $CD25^+$ B cells whereas it rather downregulated Ig production by the $CD25^-$ B cell population.

After induction of Ig production by EBV we were also interested in studying RF producing B cell clones in $CD25^+$ and $CD25^-$ B cell populations. In addition to the eight patients that were used for sorting, four healthy subjects were also included and their sorted cells were also cultured as described. Induction of RF production could only be seen after stimulation with CpG where both patients and controls had elevated levels ($p=0.04$ for the $CD25^-$ B cell population). The results after stimulation with EBV rather showed a downregulation of RF production ($p=0.02$ for the $CD25^+$ B cell population). We could only detect RF of lambda light chains.

5 DISCUSSION

5.1 Why doesn't RTX cure RA?

RA is a complex disease that involves many cells of the immune system. Inflammatory cells are recruited to the joint and different cell types play different roles. There are many different treatment strategies today, many different kind of immunosuppressants. One thing they all have in common is that they dampen the inflammation by blocking, inhibiting, neutralising or destroying factors involved in the RA pathogenesis making the patients feel better, at least for a short while. For some patients MTX treatment is sufficient to dampen the disease activity, whereas some patients need stronger medications such as TNF inhibitors or anti-CD20 treatment, or combinations of several treatment strategies to achieve clinical improvement. However, if the treatments are removed the disease activity increases and clinical symptoms aggravate. So the treatment do not cure the disease or reverse the damages already caused by the disease (i.e. joint destruction).

After RTX treatment, B cells return in all patients. Usually B cells start repopulating the blood after six months, but the return of B cells to the circulation does not automatically mean that patients relapse clinically (Roll et al. 2006; Bokarewa et al. 2007). Clinical relapse can occur whenever after B cell repopulation and this is not understood properly. Some patients have been shown to relapse as late as two years after RTX treatment even though the B cells are back to normal levels (Bokarewa et al. 2007). To maintain the clinical improvement that patients experience during RTX treatment there is a need for retreatment.

The fact that clinical signs return some time after RTX treatment is a well-established argument for that RTX does not cure RA. The removal of the effector functions of B cells (production of autoantibodies, interaction with and presentation of antigens to T cells, contribution to the inflammatory milieu in the joints and elsewhere) by depletion is not enough to abrogate autoreactivity. The mechanism responsible for the recognition of self-antigens is not destroyed. Whether it is the failure of receptor editing in the BM, wrong apoptosis signals or something else - the autoreactivity is still there.

5.2 The return of autoantibodies

One thing that is discussed a lot in RA is the autoantibodies. They are used as a prognostic marker for a more severe and erosive disease. During RTX treatment, autoantibody levels decline but once the B cells have repopulated, autoantibodies reach the same levels as before treatment (Cambridge et al. 2003). We also show this in paper I where naïve and treated patients have the same levels of RFs. This is an indication that RTX does not cure RA, but only halts it. And it also indicates that autoantibodies are only a symptom, not the cause of the disease.

Another interesting thing with this is the fact that RFs decline after RTX treatment. It tells us that the RF producing cells are depleted by RTX but when the patients come back for the next course of RTX, and the RF levels are increased again, the cells responsible for this production are also back. This gives us an indication that the B cells that are depleted during RTX treatment must be producing these autoantibodies. When we analysed this in paper I it was obvious that it was the naïve IgD expressing B cell population that was depleted by RTX and that returned after depletion. This suggests that the autoreactive Ig producing cells are harboured in this IgD expressing B cell population and not in the memory B cell population or in the plasma cell population which are supposed to be the "classical" Ig producing populations. However, this needs more investigations to find the "responsible" B cell population.

This also suggests that RTX depletes the autoreactive clones but not the autoreactive precursors. So what is it that drives the B cell population to start producing autoantibodies after RTX treatment even though the autoreactive autoantibody producing B cell clones were temporarily gone? It may be that the antigen presentation is once more ongoing between T cells and B cells, that they present autoantigens to eachother that give rise to the start of autoantibody production again.

5.3 Why do the short-term and long-term effects of RTX differ?

In paper I we show that the short-term changes in BM are characterised by a decrease of immature IgD expressing B cells and that the long-term changes are characterised by a decrease of the more mature IgG and IgA expressing memory B cells.

Three months after RTX treatment the switched IgG and IgA expressing cells as well as the memory B cells seemed to be harder to deplete than the more immature IgD expressing cells. It has been observed that the B cells that are not depleted by RTX may be memory B cells, regardless of their expression of CD20 (Uchida et al. 2004; Gong et al. 2005; Hamaguchi et al. 2005). It has also been suggested that more mature B cells, i.e. memory B cells can escape depletion even though they express CD20 (Schroder et al. 2003; Vugmeyster et al. 2005; Martin et al. 2006).

The mechanisms for this is currently unknown but it may be that memory B cells can mask their CD20 receptor somehow, or maybe the expression of the CD20 molecule on the surface is expressed in low concentrations that makes it less sensitive to the depletion mechanisms that RTX exercises i.e. induction of apoptosis or lysis. Several other studies also observed that CD38 expressing B cells that had a simultaneous lack of surface IgD (i.e. memory B cells) might have less or no CD20 present on the cell surface and therefore would be more protected from depletion by RTX (Reff et al. 1994; Lucio et al. 1999; McKenna et al. 2001).

The reason for that the short-term changes and the long-term changes are different is that if you deplete the immature B cell subsets over and over again there are fewer B cells left that can differentiate into memory B cells and class switch to IgG and IgA. This has also been observed by Roll et al. They saw that memory B cells are decreased long after RTX treatment which is in accordance with our own findings (Roll et al. 2006).

5.4 If the renewal of memory B cells is disrupted…

What happens then to our immunological memory?

In paper I we showed that short-term effects of RTX treatment are depletion of the IgD expressing B cell population. The long-term effects were that memory B cells were decreased over time. It is quite logical that if you deplete the immature and naïve B cells over and over again there will be fewer cells that can differentiate into memory B cells.

During the encounter of an antigen and the formation of memory B cells, plasma cells are also formed. Plasma cells do not express CD20 and should therefore not be depleted by RTX, which we also show in paper I.

We also have memory T cells that are not directly affected by RTX since they do not express CD20. However, the communication between B cells and T cells is affected (i.e. antigen presentation). Even if the renewal of memory B cells is affected, we still have immunological memory left. In support of this, several studies have shown that repeated courses of RTX treatment did not increase the number of infections (Cohen et al. 2006; Emery et al. 2006). It has also been shown that the total levels of Igs directed against immunogens such as tetanus toxoid and pneumococcal polysaccharides did not decrease during or after RTX treatment (Cambridge et al. 2006). This suggests that the already acquired long-term immunological memory is not affected during RTX treatment.

The vaccinations that are performed during RTX treatment may however give rise to an impaired immunological response which may affect i.e. the yearly influenza vaccination that RA-patients are advised to take. It could be a good idea to vaccinate before the start of RTX treatment, or in between treatments so the immunological memory and vaccine specific memory B cells have time to form.

To conclude this section: even though B cells are depleted and the memory B cells are repopulating with a delay, the immune system can still conquer infections and a long-term immunological memory can be acquired.

5.5 RTX and vaccination in RA patients

In paper II we examined if it is possible to get a cellular and a humoral response after immunisation with protein and polysaccharide vaccines in RA patients during RTX treatment.

Many studies have been performed during all sorts of treatments with DMARDs and TNF inhibitors. And already with ”normal” immunosuppressive treatments like MTX and with TNF inhibitors we do see an impaired immune response after immunisation (Elkayam et al. 2004; Kaine et al. 2007; Kapetanovic et al. 2007).

RTX depletes B cells in the circulation and even though there have been studies that show incomplete depletion in synovial tissue and BM, the B cell numbers are still decreased over all. With less B cells there are of course less B cells that can be activated in the secondary lymphoid tissues upon immune activation. It is not that strange that the immunisation response is impaired. What is important to remember is that there are still many other cell types that can respond to pathogens.

The whole immune system is built on communication between different cell types and during RTX treatment this communication is decreased since B and T cells cannot present antigens to each other. This is the mechanism that is important in vaccination and the whole purpose of it is to induce vaccine specific antibody responses. But even though antibody responses are decreased according to many studies as well as our own, other cell types may still be activated by the antigen and some kind of protection may be acquired even though the amount of protecting and neutralising antibodies may not be adequate.

5.6 B cell subclasses and response to immunisation in RTX treated RA-patients

B cells activated with the help of T cells will go through the germinal center reaction and the antibodies formed will most probably be switched. For a T cell to be able to communicate with the B cell, the antigen needs to be a peptide. For a non-peptide antigen the T cell help is not needed and the antibodies formed will most probably be of IgM class and switched responses are more rare.

This is also the case for immunisation with protein and polysaccharide antigens. In the event of a polysaccharide antigen giving rise to the switched isotype IgG, the production will most likely be of another IgG subclass than if the immunisation had been performed with a protein antigen (French 1986b).

The question raised here is: Does RTX affect the IgG subclass pattern in RA-patients?

It is interesting that the MTX-treated patients in paper II develop an adequate response to both protein and polysaccharide vaccines of the

"correct" isotypes: IgG1 for protein immunisation and IgG2 for polysaccharide immunisation (Siber et al. 1980; Stevens et al. 1983). The pre-RTX and the post-RTX group did not develop humoral response of any subclass after influenza immunisation (protein) which is a bit puzzling since the pneumococcal immunisation gave rise to increased vaccine specific IgG subclasses in both groups.

The immunisation with pneumococci could also have given rise to a restimulation of memory B cells and what we see could be a secondary immune response. That could also explain why polysaccharide immunisation gave rise to an increase of IgG subclasses and not the influenza immunisation. One explanation to this could be that infection with pneumococci is something that most people will experience during their lifetime. The influenza virus however, changes every year and that is the reason why we have a different vaccine every year. The immunisation with pneumococcal vaccine would therefore more likely give rise to a restimulation and the immunisation with influenza would most likely be a primary response.

This might be an explanation as to why the IgG subclass pattern is more or less absent after the influenza vaccination in RTX-treated groups. RTX does affect the immunisation response and a primary response would be harder to mount with fewer B cells whereas reactivation of already existing memory B cells would still be possible.

5.7 EBV and CD25$^+$ B cells in RA

We have previously shown that active EBV replication in RA-patients gives a better response to RTX treatment (Magnusson et al. 2010). However, we did not find any clear-cut data of why they responded better to RTX treatment. In paper III we wanted to analyse if selected B cell subclasses in EBV$^+$ and EBV$^-$ patients were involved in the clinical outcome of RTX treatment.

It is known that EBV drives B cells into memory B cells to hide from the immune system. The already more mature CD25$^+$ B cell population was shown to be even more mature in EBV$^+$ patients as compared to EBV$^-$ RA-patients. The CD25$^-$ population was also more mature in EBV$^+$ patients which I believe is a sign of the virus affecting the B cell population (Miyashita et al. 1997; Babcock et al. 1998; Joseph et al.

2000a; b; Hochberg et al. 2004). One of EBV's mechanisms to avoid being detected by the immune system is to drive the naïve B cells in peripheral lymphoid organs into memory B cells as well as infecting resting memory B cells (Miyashita et al. 1997; Babcock et al. 1998; Babcock et al. 2000; Joseph et al. 2000a; Hochberg et al. 2004). The reason for this is that memory B cells seldom divide and they seldom interact with other immune cells or express viral proteins (Qu et al. 1992; Tierney et al. 1994; Chen et al. 1995). EBV is also known to downregulate receptors that are used to interact with other cell types.

Analysing the response to RTX in EBV^+ and EBV^- RA-patients we could see that the EBV^+ responders had a larger $CD25^+$ switched memory B cell population as compared to the EBV^- patients. It is interesting since the memory B cells have been shown to be more resistant to RTX during the first course of treatment.

Plasma cells are not supposed to be affected during RTX treatment due to the lack of CD20 expression. It was therefore of great interest when we found that EBV^+ non-responding patients had a larger $CD25^+$ plasma cell population. However, these cells are probably not producing the RFs, since the RF levels decline after RTX treatment but are back at the time of retreatment. Trying to find out the role of the $CD25^+$ B cell population in RA-patients we also performed ELISPOT with and without EBV stimulation in paper III. After stimulation with EBV the $CD25^+$ B cell population develops into antibody-secreting cells to a higher extent than the $CD25^-$ B cell population. Taken together with the fact that non-responding EBV^+ RA-patients have an increased $CD25^+$ plasma cell population… – I find this is a very interesting finding!

I would be very interested to find out more about the role of $CD25^+$ plasma cells in RA-patients and to examine the influence of EBV on this cell population. Maybe the presence of a larger $CD25^+$ plasma cell population can even be used as a prognostic marker to predict response to RTX treatment?

5.8 Ig light chains

In paper II and III we evaluated the kappa and lambda light chain production in different ways. In paper II we examine the vaccine specific kappa and lambda light chains after immunisation and in paper III we

checked for RF production after stimulation with CpG and EBV on sorted B cell populations.

After immunisation of RA-patients, vaccine specific kappa and lambda light chains were examined and interestingly, we could only detect both kappa and lambda light chains for both vaccines in the MTX-treated controls and not in the RTX-treated groups. All RTX-treated patients had though presence of lambda light chains specific for both vaccines. So even though vaccine specific kappa chains were missing in RTX-treated patients it still suggests that there were vaccine specific antibodies in all patients regardless of treatment (of lambda type). The normal kappa and lambda ratio in healthy individuals is shown to be 2:1, but there have been reports that it can differ in some diseases (Yount et al. 1970; Skvaril F 1975). Maybe RTX affects this ratio?

In paper III, we sorted B cells into $CD25^+$ and $CD25^-$ B cell populations and stimulated these cells in culture for 96 hours. We then wanted to check for RF after stimulation with CpG and EBV. Interestingly we could not detect increased levels of RFs with kappa chains but only for lambda light chains and only after stimulation with CpG.

One possible explanation as to why we could not detect kappa chains to the extent that is supposed to be "normal" could be that it has been suggested in studies on mice that antigenic challenge could disrupt the kappa and lambda pattern with a shift towards lambda production instead of kappa (Rolink et al. 1993; Zou et al. 1993). Both immunisation and stimulation with CpG are antigenic challenge and this may be the reason why we only see increase of lambda light chains in both paper II and paper III. This would however need further studies.

6 CONCLUSION

The conclusions of this thesis are:

*

RTX induces a short-term depletion of IgD expressing B cells in BM of RA-patients whereas the renewal of memory B cells is delayed.

When RA-patients come back for the next course of RTX - RFs are also back to high levels suggesting that RF producing B cell clones have repopulated after RTX treatment.

**

Both the MTX-treated patients and the pre-RTX group could mount a cellular response after influenza vaccination whereas the cellular response for pneumococcal vaccination was somewhat lower in all groups.

The humoral response was adequate in the MTX-treated control group but the RTX-treated groups displayed an impaired humoral response. The humoral response was least pronounced in the post-RTX group where the number of patients that did not respond to any IgG subclass was highest.

RTX may change the normal kappa and lambda light chain production with a shift in the kappa/lambda ratio towards lambda production.

***

B cells are more mature in EBV infected RA-patients and clinical response to RTX is better in patients with a larger $CD25^+$ switched memory B cell population.

In non-responding EBV^+ RA-patients, a larger $CD25^+$ plasma cell population was found. This may be used as a prognostic marker for clinical response to RTX treatment.

7 POPULARISED SUMMARY IN SWEDISH

En berättelse om B celler, ledgångsreumatism och rituximab

Det var en gång ett land långt härifrån som hette geléhallonlandet. Det var ett vackert land med ängar och skogar (vävnader) inneslutna av höga och starka berg (skelett) som kantades av många floder (blodådror). Under bergen fanns det underjordiska källor (benmärgen) som var helt fantastiska.

Geléhallonen (cellerna) som bodde här var alla runda och använde sin sockriga yta (receptorer) till att prata med andra geléhallon. De vandrade över skog och mark för att hitta elaka inkräktare (bakterier) och sluga små banditer (virus) som kom vallfärdandes från avlägsna land för att invadera landet. Med gemensam styrka motades dem alla bort och lugnet återgick. För att förhindra framtida invasioner vandrade de gamla och erfarna B geléhallonen (minnesceller) till bergen och de underjordiska källorna (benmärgen) för att hålla utkik och spana efter återkommande banditer. Varje dag gick geléhallonen till den stora marknaden (tarmen) i mitten av landet. Väl där passade de på att gå på ”Lymfnodscaféerna” som kantade marknaden. Där skvallrades det och tisslades och tasslades om vad som hänt och de for sedan västerut för att fortsätta skvallret på det världsberömda hotellet ”Mjälten”. På ”Mjälten” var det en dag uppståndelse då det visade sig att ett av B geléhallonen hade fått för sig att det skulle bränna ner en bro (led). Detta var fel men B geléhallonet var så starkt och manipulativt (autoreaktivt) så det fick med sig många olika geléhallon av alla färger och typer. Fler och fler anhängare slöt upp och begav sig ut för att bränna fler broar (leder) och förstöra fler skogar (vävnader). Det blev ett inbördeskrig och bron kunde inte längre användas och ängarna saknade blommor och gräs (leden förstördes). Det smärtade! Uppploppet blev känt som **ledgångsreumatism**.

Vid gränsen till ett annat land som hette **Reumatologen** hittade man räddningen, **rituximab**, som var ett hemligt vapen som tog bort många B geléhallon. Landet mådde tillfälligt bättre, det kom inga manipulativa (autoreaktiva) B geléhallon på ett tag men skadorna som ledgångs-reumatismen orsakat försvann inte och de kortsiktiga förändringarna i benmärgen utmärktes av en depletion av naiva IgD-uttryckande B geléhallon. När man märkte att uppploppen startat igen skickade man expressbud till Reumatologen och begärde mer av det hemliga vapnet rituximab. På så sätt kunde man hålla ledgångsreumatismen i schack och skadorna blev inte längre

lika jobbiga och ansträngande för landet men de gamla visa och erfarna minnescellerna tycktes dock erfara svårigheter att nybildas.

Landet fick även testa vaccinering mot influensa-banditer och pneumokock-inkräktare både innan och under behandling med rituximab och resultatet blev att den grupp som vaccinerats innan behandling kunde bilda fler antikroppar än den som vaccinerats under rituximab. B geléhallonen kämpade, men de var helt enkelt inte tillräckligt många.

Inuti vissa av B geléhallonen bodde det också små banditer som kallades för EBV. Man märkte inte så mycket av EBV-banditerna men det verkade som om det var fördelaktigt när man använde det hemliga vapnet rituximab. EBV-banditerna verkade göra B geléhallonen äldre och mer erfarna och speciellt de B geléhallon som hade CD25 på sina sockerkorn. Det fanns dock några väldigt gamla och stora CD25 B geléhallon (plasmaceller) som hade EBV-banditer i sig som uppträdde annorlunda när rituximab inte gav lika bra förbättring efter behandling. Kanske kan man i framtiden använda detta som ett tecken på att rituximab inte kommer att fungera på dessa patienter? Det får framtiden utvisa.

Men för många fungerar det hemliga vapnet rituximab väldigt väl och förundran fortsatte att vara stor över vapnets framgång i geléhallonlandet.

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