From The Rheumatology Unit
Department Of Medicine
Karolinska Institutet, Stockholm, Sweden

Immune Monitoring in Humans after Manipulation by B cell Depletion and Immunization

Therese Vallerskog

Karolinska Institutet

Stockholm 2007

Published by Karolinska Institutet. Printed by Larserics Digital Print AB, Sundbyberg

ISBN 91-7357-061-3

With Blood, Sweat, and Tears
And with Joy and Laughter!

ABSTRACT

The overall goal of this thesis was to study the influence of immunomodulatory drugs on the human immune system. The approach was bi-directional; *ex vivo* studies were performed after B cell depletion with rituximab treatment in patients with SLE and RA, and an *in vivo* assay was established to study effects of drugs on immune responsiveness.

To study immunological consequences of B cell depletion by rituximab in treatment resistant patients with SLE and RA we collected blood samples before and after treatment. Our analysis was focused on time points related to absence and return of B cells after depletion. In patients with SLE, changes of cellular phenotypes were analyzed by flow cytometry and changes in antibody levels in serum by ELISA. We observed an increase of activated T cells as well as an increase of regulatory T cells after B cell depletion. There was a selective decrease of antibodies in serum. IgM and IgE decreased rapidly after B cells depletion as well as the disease activity associated autoantibodies against dsDNA and C1q. BAFF and APRIL levels were measured by ELISA in serum of both SLE and RA patients. Levels of BAFF increased in all patients after rituximab therapy. In patients with SLE the levels of APRIL decreased after B cell depletion, and in patients with RA we observed heterogeneous changes.

To set up an assay to measure individual immune responsiveness, we used immunizations as a tool to trigger an immune response. Healthy volunteers were immunized with four weeks interval with either influenza vaccine, three or four times (n=11) or with tetanus toxoid four times (n=7). Blood samples were collected before and at four occasions between days 4 to 10 after each immunization. Humoral immunity to the influenza vaccine and tetanus toxoid was measured by ELISPOT (circulating B cells) and ELISA (antibodies). Cellular reactivity to the influenza vaccine was measured by FASCIA (proliferation) and Bio-Plex suspension array system (cytokines). Comparing the response to influenza and tetanus, the first immunization with influenza vaccine resulted in ten times more B cells secreting antigen-specific antibodies compared to immunization with tetanus toxoid. The second and third influenza immunizations induced comparable B cell immune responses, while there was a very low or no response to tetanus toxoid. Antibody titers and T cell responses only increased significantly after the first immunization. Thus measuring B cell responses by ELISPOT to repeated immunizations with influenza vaccine provide a method to evaluate immune responsiveness. Tetanus toxoid was not a suitable antigen trigger for such studies.

In conclusion, we observed an effect on both the humoral and the cellular immune systems after rituximab-induced B cell depletion in patients with SLE. Further on, levels of BAFF increased after the therapy in patients with SLE and RA, while APRIL decreased in SLE only.
We have set up an assay in which reproducible immune responses can be measured after immunization 2 and 3 with influenza vaccine. Such an assay allows us in the future to estimate the influence of immunomodulatory drugs on the immune responsiveness in single patients.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numeral (I-IV).

I. Vallerskog T., Gunnarsson I., Widhe M., Risselada A, Klareskog L., van Vollenhoven R., Malmström V., Trollmo C.
Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE
Clinical Immunology, 2007, 122 (1):62-74

II. Vallerskog T., Heimbürger M., Gunnarsson I., Zhou W., Wahren-Herlenius M., Trollmo C., Malmström V.
Differential effects on BAFF and APRIL levels in rituximab treated patients with systemic lupus erythematosus and rheumatoid arthritis
Arthritis Research and Therapy, 2006, Nov 8; 8 (6): R167

III. Vallerskog T., Gaines H., Feldman A., Culbert E., Klareskog L., Malmström V., Trollmo C.
Serial re-challenge with influenza vaccine as a tool to study individual immune responses
Manuscript to be published

IV. Vallerskog T., Klareskog L., Culbert E., Trollmo C.
Multiple immunizations with tetanus toxoid are not suitable to measure reproducible immune responsiveness
Manuscript to be published

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen Presenting Cells
NK Cells	Natural Killer Cells
MHC	Major Histocompatibility Complex
IFN	Interferon
TNF	Tumor Necrosis Factor
TGF	Transforming Growth Factor
IL	Interleukin
CD	Cluster of Differentiation
HIV	Human Immunodeficiency Virus
CD40L	CD40 Ligand
TCR	T Cell Receptor
BCR	B Cell Receptor
FasL	Fas Ligand (CD178)
PBMC	Peripheral Blood Mononuclear Cells
ELISA	Enzyme Linked Immunosorbent Assay
CBA	Cytometric Bead Assay
PCR	Polymerase Chain Reaction
cDNA	complementary DNA
Ig	Immunoglobulin
SLE	Systemic Lupus Erythematosus
RA	Rheumatoid Arthritis
NSAID	Non Steroidal Anti-Inflammatory Drug
RF	Rheumatoid Factor
HACA	Human Anti Chimeric Antibodies
SHM	Somatic HyperMutation
BAFF	B Cell Activation Factor belonging to the TNF family
FCS	Fetal Calf Serum
HS	Human Serum
DC	Dendritic Cells

PREFACE

This thesis is written to present and discuss the results from my studies after manipulation of the human immune system.

I have chosen to write the general introduction as a "general introduction" which if not otherwise stated is referring to Immunobiology by Janeway et al ((Janeway et al. 2005)) to give a basic overview of my subject for everyone reading this thesis. Also, I wanted to give some background information of the diseases I have studied and the main therapies these patients commonly are taking to relieve the symptoms. By this I wanted to weave together the complex nature of the human immune system and immune responses with two autoimmune rheumatic diseases.
In the section which follows the general introduction I am presenting my more in-depth scientific thoughts and discussions regarding my projects.
It is my hope I have fulfilled the scientific view of the results from the studies within this thesis.

Enjoy your reading.

1 GENERAL BACKGROUND

1.1 THE HUMAN IMMUNE SYSTEM

To stay healthy it is important to have a functional and alert immune system protecting us from pathogens in the environment. A dysfunctional immune system can cause acute and/or latent infections from bacteria and virus, malignancies, and autoimmunity. Several parts of the body contribute to our immune defense: the skin and the epithelial lining of the respiratory- and gastro-intestinal tract form the outer barrier protecting against pathogens. The lymphoid organs are anatomical sites important for the maturation, activation and communication of the cells participating in immune responses. The bone marrow is one of the primary lymphoid organs where lymphopoesis in an ongoing process and is also the site where B cells develop and mature (see section 1.3.1). The thymus is also a primary lymphoid organ where T cells mature. It is an important site where T cells are selected for tolerance and ensuring an immune system not reacting to self. The secondary lymphoid organs (spleen, lymph nodes, mucosal-associated lymphoid tissue [MALT]) are sites which filter blood and lymph in search for antigens and where the antigen presenting cells activate the lymphocytes following infection.

1.1.1 The innate- and adaptive immune system

The immune system can be divided in two co-dependent parts, the innate- and the adaptive immune system.
The innate immune system consists of the skin, mucosal lining and cells that recognize specific molecular patterns of the invading pathogen. Immediately after infection, a strong response is elicited by cellular phagocytosis and secretion of proteins to disseminate the pathogens. Tissue residing macrophages engulf the infecting pathogen efficiently and secrete cytokines (see section 1.1.2) alarming and activating other cells of the defense. Neutrophils respond quickly to the signals from macrophages and invade the inflamed site followed shortly by the monocytes. By releasing proteins which are toxic to the invading organisms they efficiently kill the pathogens. Neutrophils have a short life span and they die themselves after the battle and are making up the major part of the pus seen after an infection. Other cells participating in the innate response are NK cells, mast cells, eosinophils and basophils. Dendritic cells (DC) have an important role in the innate immune system by secreting activating cytokines (IFN-α, IL-12) in response to infections. Immature DC reside in peripheral tissue and phagocytose the pathogens which have bound to their receptors. They also "scan" the surroundings by taking up extracellular material (which can contain invading organisms) by micropinocytosis. The DC becomes activated after ingestion of pathogens and begin migrating to nearest lymph node. During migration, DCs matures and processes the ingested antigen to present it's peptides in MHC class I or class II molecules on the cell surface. In the lymph node they function as an antigen presenting cell (APC) and present antigen to T cells. And with this, we have now traveled from the innate- to the adaptive immune system.

The adaptive immune system is characterized by receptor specificity for the antigen and development of immunologic memory for encountered antigens. The T cells become activated when they recognize antigen-loaded MHC and receive co-stimulatory signals from the APC. T cells, depending on their subpopulation (see section 1.2.1) either kill the invaders or provide help to and activate B cells (see section 1.4.1 for more details). They also efficiently secrete cytokines further stimulating cells in the immune response. The activated T- and B cells migrate from the lymph nodes or spleens to the site of inflammation to help clear out the infection. The B cells produce antibodies which neutralize and/or tag the pathogens for destruction (see section 1.4.2 for further details). Also, like dendritic cells they take up pathogen, process it, and present it to T cells in MHC molecules, amplifying the immune response.

1.1.2 Cytokines, chemokines and complement

Not only cells but also soluble proteins are important for a functional immune system.
Cytokines are soluble proteins which are secreted from cells to deliver important signals to other cells in the immune system. Different cytokines have different properties, and they can be pro-inflammatory or anti-inflammatory. Common proinflammatory cytokines released in response to an infection are IFN-γ, TNF-α, IL-1 and IL-2, which all induce cell activation and proliferation. TGFβ and IL-10 are seen as typical anti-inflammatory cytokines, primarily produced to stop the immune response when the pathogen is cleared.
Chemokines are important proteins in the immune defense with chemoattractant properties directing cells to their proper location. They attract effector cells from the blood stream to the site of infection, but also direct cells for migration to and within lymphoid tissue. Both chemokines and cytokines are parts of the innate immune system.
The complement system is a well preserved and important part of the innate immune system. It consists of a range of plasma proteins activated in a cascade. Several of the complement proteins are enzymes that become activated when they are cleaved by an "earlier" enzyme in the cascade. The complement proteins have three functions; (1) either directly bind to molecules on the surface of the pathogen, building a membrane attack complex which destroys the surface and thereby killing the pathogen; (2) bind directly to the pathogen, opsonizing it for phagocytosis; and (3) bind to antibodies which have opsonized the pathogen for destruction, again building the membrane attack complex destroying the surface and killing the pathogen. Also, the cleaved parts from the activated enzymes act as mediators of inflammation. A tight control of the complement cascade is necessary to avoid unspecific activation which could destroy tissues that are not infected.

1.2 T CELLS

T cells constitute one part of the adaptive immunity. Their development starts in the bone marrow and ends in the thymus where they have matured into two populations; CD8+ cytolytic T cells and CD4+ T helper cells. In the thymus the important process of central tolerance occurs which negatively selects the T cells not reacting to self. Without central tolerance, severe autoimmunity with morbidity and even mortality

develops due to self reactive cells. T cells are important for a well functioning immune system; T cell deficiency (which can be caused by HIV infections and AIDS) increase risks for tumor development, opportunistic infections such as pneumonia (caused by *Pneumocytstis carinii)*, and activation of latent disease such as cytomegalovirus infections (Chaisson et al. 1998; Goedert 2000; Bower et al. 2006). T cells improperly activated or regulated can cause autoimmune disease and tissue damage (Davidson and Diamond 2001).
During the immune response T cells are activated by the professional antigen presenting cells (APC): dendritic cells, macrophages and B cells. When the T cell receptors (TCR) bind to matching antigen-loaded MHC and receive proper co-stimulatory signals, the cells become activated. In turn, the activated T helper cells activate and provide help to antigen specific B cells, macrophages, and cytolytic T cells. Also, by secreting cytokines like IL-2, TNF-α, IFN-γ, and IL-4, the T cells further activate other cells in the immune response.

1.2.1 Mature T cell subsets

Below is a phenotypic and functional description of the T cell populations I have focused on in our studies.

Naïve mature T cells: CD3+CD45RA+CD28+ CD4+ or CD8+
Resting mature T cells which have not encountered antigen.
Activated T cells: CD3+CD25+ CD4+ or CD8+
Mature T cells which are activated by recognition of antigen:MHC complex and proper co-stimulatory signals.
Regulatory T cells: CD3+CD4+CD25^{bright}FOXP3+
The described population of regulatory T cells is seen as natural, i.e. not induced, and develops in the thymus. Apart from CD25 and FOXP3 most of them also express CTLA-4 which is a receptor with high affinity for CD80/CD86, which out-competes CD28. Upon ligation it thereby inhibits activating co-stimulatory signals to other T cells. The exact mechanism of how the regulatory T cells regulate the activity of other cells is yet not fully known, it is likely to involve both contact dependent mechanisms and soluble factors such as cytokines. (Sakaguchi et al. 1995; Sakaguchi and Sakaguchi 2005; Wan and Flavell 2006)
Effector memory T cells: CD3+CD4+CD45RO+CCR7-
These memory T cells are specialized for quickly entering inflamed tissues. They are rapidly secreting cytokines (IFN-γ, TNF-α) after re-stimulation (Sallusto et al. 1999; Sallusto et al. 2004).
Central memory T cells: CD3+CD4+CD45RO+CCR7+
This population of memory T cells is very sensitive to cross-linking of their receptor whereby they rapidly express CD40L for co-stimulation of APC (Sallusto et al. 1999; Sallusto et al. 2004).
CD28^{null} cells: CD3+CD28- CD4+ or CD8+
These T cells are oligoclonally expanded and have a restricted TCR repertoire. They are hyperresponsive and rapidly secrete both TNF-α, IFN-γ, and also directly cytotoxic mediators such as perforin and granzyme B. CD28^{null} cells are often found with

increased frequencies in a number of autoimmune and other chronic inflammatory disorders as well as in infections by CMV (Fasth et al. 2004; Vallejo 2005).

The T cell response to any antigen is thus a sum of the participating subpopulations, and alterations and/or dysregulation in any of them can contribute to the settings I have studied.

1.3 B CELLS

B cells are the second important part of the adaptive immune system. They start their development in the bone marrow and migrate to the peripheral secondary lymphoid organs to differentiate into mature naïve B cells. They constantly circulate in blood and lymphoid tissue until they encounter an antigen. Upon binding of antigen to the BCR, the BCR:antigen complex is internalized, processed, and presented as antigen-MHC class II complex to the T cells. The antigen specific T cell thereafter co-stimulates the B cell which now further develops into a memory B cell or a plasma cell.

1.3.1 Ontogeny

It is a long and hard way for the B cell precursor to become a mature naïve B cell. Similar to the T cells in the thymus, only a small percentage of the B cell precursors survive the maturation process.
The earliest stage of B cells is the pro-B cell, and the process of assembling a correct BCR occurs in the bone marrow during the development to the pre-B cell stage. The stromal cells in the bone marrow which are crucial for the development of pro- to pre-B cells provide adhesive contacts and soluble factors like stem cell factor, CXCL12 and the cytokine BAFF. RAG-1 and RAG-2 (recombination-activating genes) are enzymes which are also crucial for the development of the BCR, and without these enzymes no lymphocytes can develop. If the B cell fails to express the receptor the cell is forced to undergo apoptosis. However, one way of rescue is to perform gene rearrangement of the BCR and if this is successful the B cell can continue its development to become an immature B cell. The receptor on the cell surface of the immature B cell is a complete IgM molecule and this is important for the negative selection which now takes place. An immature B cell that expresses an IgM which strongly binds self molecules is destined to die by apoptosis. Alternatively, the cell gets a second chance through the process of receptor editing of the IgM molecule, and if this is successful the cell survives. Depending on the nature of the self antigen the immature B cell has two other fates than apoptosis and gene rearrangement: (1) a soluble self molecule induces anergy in the cell which eventually dies, (2) low-affinity non-crosslinking self molecules induce a clonally ignorant cell (which means that the antigen is present but it will not be able to activate the cell).
The B cell that does not bind self molecules continues its journey as a transitional B cell for development into a naïve mature B cell. The transitional B cell expresses both IgM and IgD on its cell surface and it migrates through the blood to peripheral secondary lymphoid organs where the final maturation occurs. Once again, the B cell is tested for self-reactivity: (1) if it strongly binds self-antigen and the receptor is cross-linked the cell dies by apoptosis. This time there is no second chance, the deletion is

inescapable. (2) Again, soluble self antigen which do not cross link the BCR induces anergy in the B cell which eventually dies. If the B cell survives this final test, it has become a mature naïve B cell and is now ready to patrol for pathogens.
Encounter with an antigen activates the B cell through the BCR and after a subsequent co-stimulatory signal by T helper cells it becomes a memory B cell or a plasma cell. The conditions induced by the immune response; cytokines, chemokines, signal strength etc, determines the fate of the B cell.

1.3.2 Somatic hypermutation, affinity maturation, and class switch

Antigen specific B cells have a unique ability to fine tune their receptor after an encounter with antigen in order to improve the binding to the antigen. This is the process of somatic hypermutation which if the outcome is successful leads to affinity maturation. Somatic hypermutation occurs in germinal centers after activation of B cells and subsequent help from T cells. The enzyme Activation Induced cytidine Deaminase (AID) is crucial for this process which deaminates cytosine at hotspots within the variable (V) region of the immunoglobulin DNA. Point mutations and possible permanent base changes of the DNA are induced after the lesions are repaired. The consequences of the mutation can either be (1) better binding of antigen (affinity maturation) and those B cells are the one selected for further maturation, or (2) a non productive BCR or a non translational protein which both leads to apoptosis of the B cell (reviewed by Longo and Lipsky (Longo and Lipsky 2006)).
Successful affinity maturation is one of the mechanisms enabling a faster and more efficient secondary humoral immune response compared to the primary response. Also, B cells with high-affinity receptors are preferentially selected for differentiation to memory B cells or plasma cells.
Another process in which AID is involved is isotype, or class, switching, of the BCR (reviewed by Chaudhuri and Alt, (Chaudhuri and Alt 2004)). All mature B cells start out as IgM+ IgD+ and after antigen encounter and CD40-CD40L interaction they can exchange the IgM and IgD for a receptor of a different isotype; IgG, IgA or IgE. Class switching is an irreversible recombination of DNA and a process that exchanges the constant region of the heavy chain but leaves the variable region intact. Figure 1 illustrates the DNA regions where the switching process occurs. The DNA breaks at the switch (S) regions which are located upstream of the genes for each of the heavy chain isotypes except the delta gene which does not have an S-region. The DNA between the S-region of choice is looped out and forms an episomal circle/excised intervening DNA. Cytokines induced during an immune response determine to what isotype the receptor is switched. The change of isotype provides the secreted antibodies with a different effector function while the antigen specificity remains as before class switching.

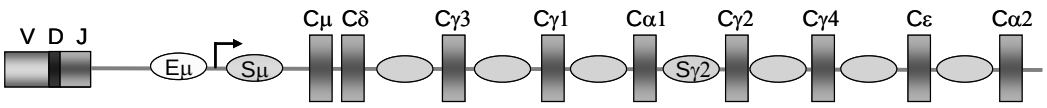

Figure 1: DNA regions for class switch of immunoglobulin receptor.

1.3.3 Mature B cell subsets

Below is a phenotypic and functional description of the B cell populations I have focused on in our studies.

Naïve mature B cells: CD19+CD20+CD27-IgD+IgM+
Resting mature B cells which have not encountered antigen.
Memory B cells: CD19+CD20+CD27+ IgG+ or IgA+, IgE+ or IgM+
Mature B cells which have encountered antigen.
Plasmablasts: $CD19^{low}CD20^{low/-}CD27++$
Plasmablasts are activated antibody-secreting B cells which are in a maturation stage towards becoming fully mature plasma cells. They can be found in increased frequency in patients with SLE. (Jacobi et al. 2003)
Plasma cells: CD19-CD20-CD27+CD38++CD138+
Plasma cells are the producers of soluble antibodies. They can be both short lived (days) and long lived (months). Plasma cells reside in so called survival niches in the bone marrow, secondary lymphoid organs, and also at inflammatory sites as reviewed by Radbruch and colleagues (Radbruch et al. 2006).
CD5+ B cells (B-1 B cells?): CD19+CD5+
These cells are regarded as the human equivalent to the mouse B-1 B cell, however this is debated (Vernino et al. 1992; Youinou et al. 1999; Gagro et al. 2000). B-1 B cells are regarded as a B cell lineage of its own which develops already during the fetal stage. They are self-renewing cells mainly residing in the peritoneal and pleural cavities. Natural IgM antibodies with low affinities are produced by these cells which therefore are seen to bridge innate and adaptive immunity.

1.3.4 Cytokines important for B cells

Below is a short overview of cytokines which are important for B cell maturation and function. These cytokines are not the only ones affecting B cells, however they are of central importance.

BAFF
B cell activating factor belonging to the TNF family, BAFF, is crucial for the maturation of immature B cells to mature B cells. Mice that are not expressing BAFF do not have any mature B cells in the periphery. This recently discovered cytokine is produced constitutively by stromal cells in lymphoid organs, and by cells of myeloid origin (dendritic cells, macrophages, neutrophils, osteoclasts) after induction by cytokines (mainly IFN-α, IFN-γ, and IL-10). In mice, over-expression of BAFF leads to a massive B cell proliferation and production of autoantibodies resulting in SLE-like autoimmune disease. BAFF is found to be increased in many autoimmune diseases (Stohl et al. 2003; Stohl et al. 2004; Koyama et al. 2005; Pers et al. 2005; Seyler et al. 2005).
IL-4
This cytokine was initially termed B cell growth factor, B cell differentiation factor γ, and B cell stimulatory factor 1. The names reveal the main effect on B cells; growth

factor, increase of survival, and class switch towards IgG4 and IgE in humans. IL-4 is mainly produced by T cells (Keegan 2000).

IL-6

IL-6 is a pleiotropic cytokine and has other alternative names such as B cell differentiation factor and B cell stimulatory factor 2. This cytokine stimulates B cells to proliferate, differentiate, and to secrete immunoglobulin. However, it does not influence isotype switch. The main sources for production of IL-6 are macrophages, fibroblasts and endothelial cells. B cells and T cells can also produce IL-6 (Matsuda and Hirano 2000).

IL-10

This cytokine is very potent on human B cells. It enhances survival, proliferation and differentiation of B cells. IL-10 also drives the isotype switch to IgG1-3 and IgA, and together with IL-4 to IgG4 and IgE. IL-10 is produced by T cells, NK cells, B cells, and monocytes (de Waal Malefyt 2000).

IL-21

This cytokine provides co-stimulation to B cells, induces plasma cell differentiation, immunoglobulin secretion and class switch. IL-21 is produced by activated peripheral T cells and spontaneously from CXCR5+ follicular helper T cells (Parrish-Novak et al. 2000; Chtanova et al. 2004; Ettinger et al. 2005).

IL-2

Activated B cells express CD25 (IL-2Rα) and IL-2 is involved in the differentiation of B cells but it does not influence isotype switch. IL-2 cytokine is solely produced by activated T cells (Smith 2000).

1.4 IMMUNE RESPONSES

All parts of the immune system (skin, mucosal linings, cells, and proteins) interact and co-operate with the aim to eliminate and control invading pathogens. The innate immune system is the player in the early phase of an infection. However, it is not always the winner of the battle; pathogens have developed sophisticated ways to evade the immune defense in order to survive, replicate and spread the infection. Meanwhile fighting the infection, the innate immune system have prepared and induced the adaptive immune system to take over the combat in case it fails. Some time is required for the adaptive immune system to become activated, it takes several days for the clonal expansion and differentiation of naïve T cells and B cells to occur. The adaptive immune system is now ready to continue the fight against the infection, and it is divided in two arms; the cellular- and the humoral immunity.

1.4.1 Cellular immunity

The cellular immunity consists of cytotoxic CD8+ T cells and CD4+ T helper cells. They constantly re-circulate between the blood circulation and the lymphoid organs as naïve T cells until they become activated. The innate immune response induce increased drainage of the site of inflammation which augments the transportation of pathogens and their products to the lymphoid organs, as well as migration of dendritic cells presenting pathogen-derived peptides in their MHC molecules. The re-circulating naïve T cell enters the lymphoid organs through high endothelial venules to the T cell

compartment where they meet antigen presenting cells (APC). It has been shown that there is one naïve T cell in 10^4-10^6 T cells being specific for a particular antigen. The T cells "scan" the APC they encounter within the T cell compartment, and if they find an APC with a peptide-loaded MHC matching their T cell receptor (TCR) they bind to the cell via the receptor and the CD4/CD8. Co-stimulatory signals, most important CD80/86 binding to CD28, presented on the APC induce the proper activation signals in the T cell which thereafter expresses CD40L. Binding of CD40L to CD40 on APC further transmit activation signals to the T cells and increased up-regulation of CD80/86 on APC. Thus, the process amplifies the stimulation for T cell proliferation. Moreover, activated T cells secrete IL-2 which is essential for proliferation and differentiation of T cells. IL-2 binds to a receptor complex built of three subunits, $\alpha, \beta,$ and γ on the cell surface. The beta and gamma subunits are constitutively expressed on the T cells making low affinity binding of IL-2 possible (reviewed by Susan Gaffen, (Gaffen 2001)). Addition of the alpha subunit, CD25, changes the affinity from low to high, rendering the T cell to respond to very low concentration of IL-2. Binding of IL-2 triggers the cell cycle to proceed and proliferation to occur. The activated T cell is capable to divide for several days, thus efficiently driving clonal expansion of antigen-specific T cells. After four to five days of rapid expansion, the activated T cell now has differentiated into an armed effector T cell. The T cells can now migrate to site of inflammation guided by chemotactic signals and adhesion molecules. As activated effector cells, they do not longer need co-stimulatory signal and thereby act rapidly and efficiently after binding of TCR to peptide-MHC complex on APC.

1.4.1.1 CD8+ cytotoxic T cells

Many viruses and some bacteria are preferentially located intracellularly, for example cytomegalovirus and *Mycobacterium Tuberculosis*. The infected cell loads their MHC class I molecule with peptides derived from the infecting intracellular pathogen, and thereafter expresses the peptide-MHC complex on their cell surface. The killing ensemble of the CD8+ T cells is comprised of cytotoxic proteins which are produced and loaded in granules during activation of the CD8+ T cell in the lymphoid organs. Special conditions within the granules prevent the proteins from being activated and thereby harmful to the CD8+ T cell itself.

1.4.1.2 CD4+ T helper cells

One of the main tasks for activated CD4+ T cells is to provide help to other cells of the immune system. One of them is to help infected macrophages to conquer against intracellular pathogens (for example *Mycobacterium Tuberculosis*) which have means to evade the immune system and thereby silencing the macrophage. The CD4+ T cells are needed to help infected macrophages to become activated and overcome the pathogen. This can be done by secretion of IFN-γ by activated CD4+ T cells which re-activates the macrophage. Further on, binding of CD40L on the activated CD4+ T cells to CD40 on macrophages sensitizes the macrophage for responding to IFN-γ. All together, activation of macrophages by CD4+ T cells converts them into a highly potent effector cells with antimicrobial properties.

As for activated CD8+ T cells, activated CD4+ T cells express FasL which by binding to Fas on infected cells induce apoptosis, providing yet another contribution to the immune defense.
CD4+ T cells are also important for a well functioning humoral immune system. By providing co-stimulatory signals via CD40L-CD40 ligation they are crucial for the B cell proliferation, differentiation, and antibody switching. Further on, in a later stage of the immune response the up-regulated CD70 on CD4+ T cells binds to CD27 on memory B cells, driving the B cell differentiation towards plasma cells. Moreover, the T cells secrete cytokines important for the process in B cell proliferation and differentiation (see section 1.3.4 above).

1.4.2 Humoral immunity

B cells and their products, mainly antibodies, constitute the backbone of the humoral immunity. It is an important part of the immune defense, protecting extracellular spaces from invading pathogens.
Like naïve T cells, the naïve mature B cell circulates between blood and lymphoid organs, patrolling until encounter of invading pathogens or binding of antigens to the B cell receptor (BCR). Binding of antigen to the BCR induce the first activation signal, but as for T cells also a secondary signal is necessary to fully activate the B cell. This secondary signal can be provided by either T cell independent responses or T cell dependent responses. T cell independent responses (TI) are induced when the antigen itself either binds to Toll like receptors (TLR) on the B cell or cross-links the B cell receptors. Antigens with structures of long polysaccharide chains often induce TI responses. On the contrary to TI responses, T cells are needed to induce T cell dependent responses (TD). The B cell receives the first activating signal by binding of antigen to the BCR. When the signal is induced, the antigen:BCR complex is internalized and an endosomal processing results in display of antigen-derived peptides in MHC II on the B cell surface. T cells recognizing the MHC:antigen complex are activated and upregulate CD40L which in turn co-stimulates the activated B cells. This signal is crucial for the B cell to proceed with the immune response by affinity maturation and isotype switching. The cytokine milieu determines the pathway for the B cell to take; the one leading to become a memory B cell or to differentiate to the final state of a plasma cell. The pathway of the humoral immune response is complex with multiple factors involved for direction of the response. Cytokines, co-stimulatory signals and also the type of antigen drives the direction of B cell differentiation.

1.4.2.1 Antibodies

The main product of B cell immune responses are the antibodies, immunoglobulins (Ig). Their structure has been known for about 30 years and is one of the most studied molecules in biology. The antibody is based on four protein chains; two light chains and two heavy chains. Each chain has a constant part which determines the isotype and a variable part which contains the antigen binding structure.
Antibodies have three main functions: (1) neutralization, where the antibodies bind to the surface of the pathogen, preventing it and it's product from adhering to or entering the cells, (2) opsonization, where the antibodies coat the surface of the antigen, and induce/enhance phagocytosis by binding of the constant part to receptors on the

phagocytic cells, and (3) activation of the complement system and thereby inducing killing of the pathogen.
There are five isotypes of the immunoglobulins; IgD, IgM, IgG, IgA, and IgE. The naive B cells are expressing both IgD and IgM on their surface. After activation, they undergo somatic hypermutation (as described in section 1.3.2) and depending on the conditions for the immune response they switch their IgD and IgM to either IgG, IgA, or IgE. As described in Table 1, the isotypes have different functions and life time in plasma. Further on, IgA and IgG can be divided in to subclasses (IgA1, IgA2, IgG1, IgG2, IgG3, and IgG4 in humans), which again the milieu from the immune response determines.

Isotype	Half-life (days)	Some properties and effector functions
IgM	10	Complement fixation (classical pathway) Predominates in the primary response
IgG	21 IgG3: 7	Complement activation, IgG3>IgG1>IgG2 Transports across placenta Activation of cellular Fc receptors (macrophages and phagocyte)
IgA	6	Protection of mucosal surfaces Activation of cellular Fc receptors (macrophages and phagocyte) Complement activation (alternative pathway)
IgE	2	High affinity binding to mast cells and basophils Activation of cellular Fc receptors (macrophages and phagocyte)
IgD	3	Unknown

Table 1: Immunoglobulin isotypes, half-life in serum and main effector function.

1.5 IMMUNOLOGIC MEMORY

Immunologic memory is developed from the adaptive immune responses. It provides a long-term protection after encounter with some pathogens. Also, the immune system responds faster and more efficiently against a pathogen to which there is an established memory. This can be reflected by measuring antibodies in serum and time of response after challenge with antigen. It takes around 14 days for the antibodies to reach maximum level after the primary response and only six to eight days after the secondary response.
The immunological memory consists of long-lived antigen specific B- and T cells that has been clonally expanded after the original (primary) challenge of the pathogen.

1.5.1 T cell memory

After immunization antigen specific T cells proliferate vigorously and give rise to effector T cells. The number of the antigen-specific memory T cells is increased 100 to 1000 times above the initial frequency and the numbers remain there for the rest of the life. Memory T cell survival is dependent on cytokines like IL-7 and IL-15. Since memory cells are in a resting state, they need to be reactivated to become effector cells. However, they are more sensitive to antigenic restimulation than naïve cells and are also faster and more efficient to produce effector cytokines like IFN-γ and IL-4.

Memory T cells can be detected *ex vivo* and *in vitro* by tetramers (MHC class I for CD8+ and MHC class II for CD4+) and flow cytometry.

1.5.2 B cell memory

Antigen specific antibodies secreted from plasma cells provide the memory of humoral immunity, and so does long lived memory B cells. Memory B cells have through the process of somatic hypermutation a high affinity of their BCR which is also (mostly) class switched. Moreover, they express higher levels of MHC class II and CD80/86 on their surface compared to naïve B cells. In a secondary immune response the memory B cell responds to lower doses of the antigen which facilitates a rapid uptake and presentation. Today, there is extensive research to elucidate the existence of long lived plasma cells and how they differ from short lived plasma cells. A plasma cell is seen as the end stage of B cell development and it does not proliferate. Then, how can a life long memory for certain pathogens like measles be sustained, and how do the plasma cells survive and for how long? The life span of plasma cells has been suggested to be from days to years. There are several theories to explain the maintenance of life-long memory (Radbruch et al. 2006). The first is based upon constant stimulation and differentiation of memory B cells to plasma cells by persisting antigen captured on follicular dendritic cells in germinal centers (Zinkernagel et al. 1996). The second is suggesting constant renewal of plasma cells from memory B cells by bystander activation during any immune response (Bernasconi et al. 2002; Traggiai et al. 2003). Lastly, the third provides the theory of competition of survival niches in bone marrow and secondary lymphoid organs by competent plasma cells (Tokoyoda et al. 2004) (also reviewed by Moser et al, (Moser et al. 2006)).
Memory B cells and plasma cells are easily detected by specific cell surface markers like CD27 and CD138 respectively. The specificity of the antibodies, hence their memory, is analyzed by ELISA and ELISPOT.

1.6 AUTOIMMUNITY

The immune system has a fantastic capability to distinguish self from non-self which is crucial in order to fight off infections and to avoid by-stander destruction of tissue. The immune system is schooled for tolerance to self antigens during lymphocyte maturation, where cells which strongly recognize and react to self undergo programmed cell death, apoptosis. Despite the strict regulation, immune tolerance can be broken and lead to inflammatory reactions against the body's own tissue and development autoimmune disease.
However, it has been postulated that it is normal with continuous low levels of autoreactivity and that naïve B and T cells need exposure to self antigen for survival (reviewed by Anne Davidson and Betty Diamond, (Davidson and Diamond 2001)).
Despite extensive research in the field, it remains to be determined when and how self reactivity becomes pathogenic with following autoimmune disease.

1.6.1 Autoimmune rheumatic disease

Rheumatology derives from the greek word "*rheuma*" which means "that which flows as a river". Rheumatic disease is a definition for diseases which constitute a

combination of "musculoskeletal diseases" and "systemic inflammatory diseases" (Lars Klareskog 2005). To be more specific, one can define rheumatic disease as a disorder of connective tissue, especially the joints and related structures, which are characterized by inflammation, degeneration, or disturbed metabolism. In addition, an autoimmune component is involved in many of the rheumatic diseases. The direct causes for the development of the autoimmune rheumatic diseases are unknown, most of them are complex diseases with a mixture of many contributing components being both hereditary and environmental (reviewed by (Davidson and Diamond 2001; Marrack et al. 2001)). Also, the patients often experience different combinations of disease manifestations which classify the rheumatic diseases as syndromes. There are theories of involvement of infectious agents as being one trigger for development of disease. The autoimmune component involved in many of the rheumatic diseases might derive from a cross reaction, molecular mimicry, between the antigenic epitopes and self epitopes. One example of that is rheumatic fever, where an infection with group A streptococcus induces an autoimmune response against various tissues, including heart valves.

1.6.2 Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a chronic systemic rheumatic disease, mainly affecting women (6:1) with onset of disease in their fertile age. The disease etiology is unknown, but it is known that hereditary and environmental factors are of importance for development of the disease (Fauci 2006). SLE is a highly heterogeneous disease with symptoms ranging from the skin to the brain. The severity of the manifestations varies from mild to severe, and the disease can even be mortal. Most patients experience periods when the disease is relative quiescent, however systemic symptoms

Classification criteria for the diagnosis of SLE	
Malar rash	Fixed erythema, flat or raised, over the malar eminences
Discoid rash	Erythematous circular raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur
Photosensitivity	Exposure to ultraviolet light causes rash
Oral ulcers	Includes oral and nasopharyngeal ulcers, ovserved by physician
Arthritis	Nonerosive arthritis of two or more peripheral joints, with tenderness, swelling, or effusion
Serositis	Pleuritis or pericarditis documented by ECG or rub or evidence of effusion
Renal disorder	Proteinuria >0.5 g/d or ≥3+, or cellular casts
Neurologic disorder	Seizures or psychosis without other causes
Hematologic disorder	Hemolytic anemia or leukopenia (<4000/µl) or lymphpenia (<1500/µl) or thrombocytopenia (<100,000/µl) in the absence of offending drugs
Immunologic disorder	Anti-dsDNA, anti-Sm, and/or anti-phospholipid
Antinuclear antibodies	An abnormal titer of ANA by immunofluorescence or an equivalent assay at any point in time in the absence of drugs known to induce ANAs

Table 2: Classification criteria for the diagnosis of SLE. If ≥4 of these criteria, well documented, are present at any time in a patient's history, the diagnosis is likely to be SLE. Specificity is ~95%: sensitivity is ~75% (Tan et al. 1982). ECG = electrocardiography; dsDNA = double stranded DNA; ANA = antinuclear antibodies

like fatigue and myalgias/arthralgias are present most of the times. The diagnosis is based on classification criteria based on clinical features and laboratory findings, see Table 2. Like for many other rheumatic diseases there is today no curative therapy and the drugs available control acute severe flares and suppress the symptoms to prevent organ damage (Fauci 2006). Common treatments for patients with SLE are analgesics, anti-inflammatory, antimalarials, glucocorticoids, and cytotoxic drugs. One way to measure disease activity is by the Systemic Lupus Activity Measurement, SLAM. It is a measurement which includes variables as clinical manifestations as well as laboratory measurements (Ward et al. 2000).

1.6.2.1 Pathogenesis

One of the main feature in patients with SLE is presence of an array of autoantibodies mainly against nuclear antigens (Lipsky 2001). These autoantibodies are found in more than 95% of the patients. Presence of autoantibodies has been detected years before onset of the disease (Arbuckle et al. 2003). Several autoantibodies from patients with SLE are clearly involved in tissue damage; anti-double stranded DNA (dsDNA) in glomerulonephritis, anti-cardiolipin in thrombosis, and anti-Ro in congenital heart block (Lipsky 2001). Autoantibodies bound to antigen forms circulating immune complexes which eventually deposits in the small vessel walls (Fauci 2006). This often leads to inflammation of kidneys, blood vessels, skin, and other organs. Table 3 presents the some of the autoantibodies and their estimated frequency in SLE.

Antibody	**Prevalence, %**	**Antigen recognized**
Antinuclear antibodies	98	Multiple nuclear
anti-dsDNA	70	Double stranded DNA
Antihistone	70	Histones associated with DNA
Antiphospholipid	50	Phospholipids, β_2 glycoprotein 1 cofactor, prothrombin
anti-RNP	40	Protein complexed to U1 RNAγ
anti-Ro (SS-A)	30	Protein complexed to hY RNA, primarily 60kDa and 52 kDa
anti-Sm	25	Protein complexed to 6 species of nuclear U1 RNA
Antiribosomal P	20	Protein in ribosomes
anti-La (SS-B)	10	47-kDa protein complexed to hY RNA

Table 3: Examples of autoantibodies present in SLE. (Fauci 2006)

The lymphocytes in SLE are hyperactivated with defective regulation (Fauci 2006). The presence of affinity matured and class switched B cells indicates that T cells are involved in the pathogenesis (reviewed by Robert Hoffman, (Hoffman 2004)). T cells are found in biopsies of cellular infiltrates of inflamed kidneys, and the T cells are clonally expanded which indicates that they are restricted to a few antigens. There are studies showing that T cells from patients recognize epitopes on several lupus autoantigens (Holyst et al. 1997; Lu et al. 1999; Talken et al. 2001). Moreover, SLE lymphocytes have been shown to have lowered thresholds for activation and also hyperexpression of co-stimulatory molecules like CD40 and CD40L (Kammer and Tsokos 2002; Fauci 2006). There are also studies that have shown that patients with SLE have a decreased clearance of apoptotic bodies by phagocytic cells and also an

increased apoptotic load (reviewed by M.Kaplan) (Kaplan 2004). This may lead to increased exposure of self antigens from the apoptotic cells. Further on, an abnormal antigen processing can expose cryptic epitopes on self antigens that are not seen as self but foreign to the immune system (reviewed by Liang and Mamula, (Liang and Mamula 2000)). In addition to hyperreactivity of lymphocytes in SLE, an altered cytokine balance and increased levels of cytokines important for B cells such as IL-6, IL-10, and BAFF have been observed (Handwerger 1999; Stohl et al. 2003). Figure 2 provides hypothetical points which can lead to loss of immunological tolerance in SLE.

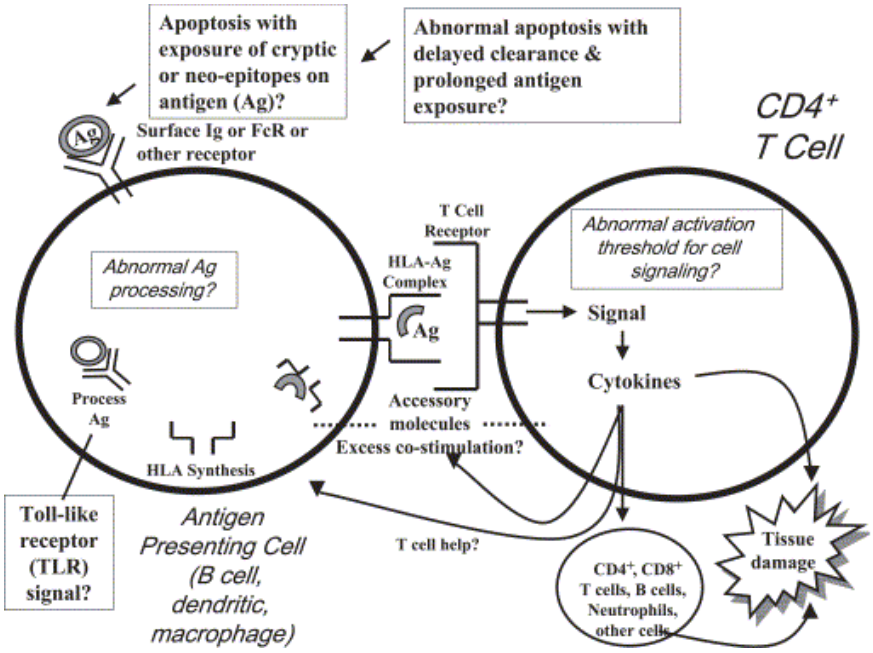

Figure 2: This figure illustrates hypothetical points where abnormalities of T cell–antigen-presenting cell interaction could lead to loss of immunological tolerance in systemic lupus erythematosus (SLE) . In SLE, it appears that there may be multiple immunological abnormalities that can lead to breaking immunological self-tolerance and several of these may be simultaneously operative. In this model, self-antigen (Ag) is taken up by an antigen-presenting cell, processed and presented to T cells. T cell receptor signaling events lead to T cell activation and the production of soluble factors, such as cytokines, that may in turn provide help to autoantibody-producing B cells, assist in the recruitment of other cells to sites of inflammation, or in some instances directly mediate tissue damage. Structural modification of antigen, abnormalities of antigen processing, excess costimulation via accessory molecules, adjuvant-like signals through Toll-like receptors, or abnormal activation threshold of T cell receptor could each contribute to breaking immunological tolerance and be important in the pathogenesis of SLE. *Reprinted from Clinical Immunology, 113 (1), Hoffman R.W., T cells in the pathogenesis of systemic lupus erythematosus, page 4-13, 2004, with permission from Elsevier.*

1.6.3 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic multisystemic disease with unknown etiology. As for most autoimmune diseases a combination of genetic and environmental factors

predisposes for disease. The genetic factor is evident in studies of monozygotic twins, in which 15-20% are concordant for RA (Fauci 2006). The strongest genetic risk factor is presence of HLA-DRB1 where the shared epitope HLA-DR4 and HLA-DR1 are the most important. Recently another gene, PTPN22, has been found to be linked to the risk of developing RA (Begovich et al. 2004). Today, smoking is the only environmental risk factor verified by epidemiological studies (Stolt et al. 2003). RA mainly affects women (3:1) with onset of disease later than for SLE, around 50-60 years (Fauci 2006). The hallmark of the disease is a persistent synovial inflammation with cartilage destruction and bone erosion. The severity of the disease varies in the range from mild with minimal joint damage to progressive polyarthritis with functional impairment. As in SLE, the diagnosis for RA is set after classification criteria based on clinical manifestations and laboratory findings which are presented in Table 4. There are several standardized ways to measure disease activity, where some are based more on laboratory findings and other more on the assessment of the physician. One of the measurements is DAS28 (disease activity score using 28 joint counts) which is commonly used in clinical trials for evaluation of response to therapy (www.das-score.nl). The variables which are included in DAS28 are the number of swollen and tender joints, the erythrocyte sedimentation rate (ESR), together with the patient's general health (GH) or global disease activity measured on a visual analogue scale (VAS). DAS28 above 5.1 means high disease activity, below 3.2 indicates low disease activity, and DAS28 lower than 2.6 is seen as disease remission.

ACR criterion for classification of RA	
1	Morning stiffness > 1 hour
2	Arthritis of three or more joint areas
3	Arthritis of hand joints
4	Symmetric arthritis
5	Rheumatic nodules
6	Serum rheumatoid factor
7	Radiographic changes

Table 4: The American College of Rheumatology (ACR) criteria for classification of RA. Four of these seven criteria have to be fulfilled in order for the diagnosis of RA, and criteria 1-4 must have been present for at least six weeks. (Arnett et al. 1988)

1.6.3.1 Pathogenesis

As mentioned above, the hallmark of RA is a persistent synovial inflammation with cartilage destruction and bone erosion. It is believed that the first events leading to the synovial inflammation are injury to the microvasculature and an increase in the number of synovial lining cells, followed by infiltration of cell of myeloid origin such as macrophages and neutrophils (Fauci 2006). Hyperplasia and hypertrophy of the synovial lining cells are characteristic changes including neovascularization and edema. Cytokines induce upregulation of adhesion molecules on endothelial cells which facilitates entry of immune cells into tissue. The dominating infiltrating cell population is T cells with a predominance of activated CD4+ T helper cells over CD8+ T cells. Moreover, also present in the synovium are B cells and antibody producing plasma cells which can form ectopic germinal centers with T cells. Local production of antibodies and especially rheumatoid factor (RF, antibodies mostly of IgM isotype which binds the Fc part of IgG) which forms immune complex contributes to further inflammation by activating complement and subsequent release of anaphylatoxins. It

has also been shown that antibodies against citrullinated proteins are produced locally in the rheumatic joint (Masson-Bessiere et al. 2000). Antibodies against citrullinated proteins are recent discovered autoantibodies which are highly specific (>90%) for RA and they can be found during the early stage of disease (Rantapaa-Dahlqvist et al. 2003). Production of chemokines and pro-inflammatory cytokines seem to account for many of the pathological and clinical manifestations of RA. They drive the inflammatory process into a negative spiral, with further cellular activation of mainly T cells, myeloid cells, fibroblasts and endothelial cells. This activation increases the production of more cytokines and chemokines advancing the disease.

1.7 THERAPIES

The therapies described below are used in treatment of SLE and RA, and are the main therapies of the patients within our studies. None of the drugs can cure the disease, but they offer means to control flares, suppress symptoms, and prevent to some extent tissue damage. Unfortunately, as a long intake of the medications is needed more or less adverse effects occurs due to the drugs. The type of treatment the patient is receiving depends on the severity of the disease manifestations, and also, what is suitable for one patient might not be the most appropriate for the other patient.

1.7.1 Anti-malarials

Common drugs: chloroquine, quinacrine, hydroxychloroquine
It may seem strange that drugs used to treat malaria, which is a condition caused by a parasite, also are used for treatment of autoimmune diseases (H.P. Rang 1999; Fauci 2006). However, anti-malarials are commonly used in SLE and RA and often reduce dermatitis, arthritis and fatigue. The pharmacological effect is not immediate and may take one month or even longer. In SLE around 50% of the patients treated with anti-malarials respond to the therapy.
The mechanisms by which the drugs are working are not fully understood. However, it is known that anti-malarials inhibits mitogen-induced lymphocyte proliferation. Further on, it also decreases leukocyte chemotaxis, lysosmal enzyme release, generation of toxic oxygen metabolites, as well as a reduction of IL-1 production. Also, due to inhibition of phospholipase A2 a reduction in formation of eicosanoids occurs. Finally, it is believed that anti-malarials possibly intercalates with DNA and inhibits synthesis of DNA and RNA in the same manner as it does for the parasite causing malaria.
Apart from common unwanted effects from drugs as nausea, dizziness, and headache, there is a potential risk for toxic effects on the retina. Therefore, patients treated with anti-malarials should get yearly examinations of the eye.

1.7.2 Glucocorticoids

Common drugs: prednisolone, dexamethasone, hydrocortisone
Glucocorticoid is a powerful drug with potency of suppressing both inflammation and immune responses (H.P. Rang 1999). It affects all types of inflammatory reactions regardless of the pathological cause. The overall effects of glucocorticoids is a reduction of chronic inflammation and autoimmune reactions, but also a decreased

healing as well as reduced protection of the inflammatory response to pathogens occurs.
The mechanism of action is inhibition of various transcription factors, whereof the most important are NF-κB and AP-1. These transcription factors normally switches on genes for COX-2, a range of cytokines, and the inducible form of nitric oxide synthase.
The consequences due to this inhibition on transcription factors are regulatory actions on cellular events such as; (1) reduced influx and activity of leukocytes to site of inflammation; (2) decreased activity of mononuclear cells, reduced proliferation of blood vessels, as well as decreased fibrosis in the areas of chronic inflammation; (3) decreased clonal expansion of T- and B cells, and (4) decreased action of cytokine secreting cells.
A long term use of glucocorticoids induce unwanted effects which the most common include; (1) suppressed immune responses to infections; (2) osteoporosis; (3) decreased production of endogenenous glucocorticoids; (4) iatrogenic Cushing's syndrome (increased abdominal fat, moon face, muscle wasting of legs and arms, easy bruising, thinning of skin and more).

1.7.3 Cytotoxic drugs

Common drugs: cyclophosphamide, methotrexate, azathioprine, mycophenolate mofetil
Cytotoxic drugs were originally developed to treat cancer. Observations were made that they were cytotoxic to dividing lymphocytes and hence immunosuppressive as well. In rheumatic diseases they are often used in lower doses in combination with glucocorticoids to reduce unwanted immune responses (H.P. Rang 1999).
Cyclophosphamide is a cytotoxic agent commonly used in systemic lupus erythematosus. It is an alkylating agent with its main action in cellular replication during the S-phase which blocks the G2 phase directing the cells into apoptotic death.
In rheumatoid arthritis, the use of methotrexate has been very successful and it has a rapid onset of action. It is a folate antagonist, hence an antimetabolite disturbing the DNA synthesis and cell division.
Being a drug affecting proliferating cells the unwanted effects derive from systems which are dependent on constant cellular dividing. The patients may experience leukopenia, thrombocytopenia, anemia, hair loss, damage of intestinal epithelium, as well as decreased immune function.

1.7.4 Biological agents

Available drugs: infliximab (anti-TNF), etanercept (anti-TNF), adalimumab (anti-TNF), anakinra (IL-1 blocker)
Today we are in the biotech era with large possibilities to design biological drugs with specific targets and effects. The biological agents are modified from natural proteins such as antibodies, cytokines, and cellular receptors, or they can be synthetically produced peptides. Also bone marrow derived cells used for treatment (transplantation) are classified as a biologic agent. There is a range of biological agents approved for treatment of disease and more biological drugs are in clinical trials. The development of new drugs targeting specific pathways of immune responses is fast, for example in the treatment of SLE: inhibitors of B cell survival (anti-BAFF), anergy-inducing agents

(synthetic molecules crosslinking BCR), complement inactivation (anti-C5 antibody), and anti-IL-10 antibodies (Vasoo and Hughes 2005).
The high risk for infections and an over-suppressed immune system is of major concern for all biological agents (Prete et al. 2005). Also, they are costly and need often to be administered by infusions which need clinical supervision.
The most known biological agent used today for treatment of rheumatoid arthritis and other autoimmune diseases (Chron's disease, psoriatic arthritis, ankylosing spondylitisis) is the anti-TNF alpha therapy (Present et al. 1999; Brandt et al. 2000; Mease et al. 2000). In Sweden it has been used in the clinics since the end of 1990's with very good results. However, despite the remarkable response in patients there are 1/3 of the patients who does not respond to the therapy (Feldmann 2002). The reasons for the unresponsiveness are not known and extensive research is focusing on finding the answer.

1.7.4.1 Anti-TNF therapy

There are three anti-TNF alpha drugs: Infliximab is a chimeric antibody which is of human origin except parts of the variable region which derives from mouse, while adalimumab is fully human. Etanercept is a human recombinant fusion protein of the TNFR p75 fused to a human IgG Fc (Feldmann 2002). TNF alpha binds to the drug whereby blocking of activity is induced, thus reducing the inflammatory process induced by TNF-α. The observed effects of the therapy on biological processes in RA has lead to: reduction of RF levels, reduced levels of pro-inflammatory cytokines in joints and serum, diminished damage to cartilage and bone, reduced angiogenesis, decrease in platelets and fibrinogen, and a restoration of heamoglobin levels (Feldmann 2002). There is an associated risk for activation of intracellular infectious pathogens like *Mycobacterium Tuberculosis* where the immune system is dependent on TNF alpha for controlling the infection (Keane et al. 2001; Rychly and DiPiro 2005). Also, due to the immunosuppressant effect there is a risk for opportunistic infections, bacterial infections, and development of lymphoma (Brown et al. 2002; Kamath et al. 2002; Nakelchik and Mangino 2002; Rychly and DiPiro 2005). There is also a risk for development of drug-induced lupus and adverse reactions induced by antibodies against the mouse-derived parts of the drug (HACA) .

1.7.5 Rituximab

Rituximab is a chimeric monoclonal IgG1 antibody and was originally developed to treat non-Hodgkin's B cell lymphoma (Maloney et al. 1997; Grillo-Lopez et al. 1999). Lately, it has also been successfully used mainly in RA and SLE, but also in other autoimmune diseases (reviewed by (Edwards et al. 2002; Shaw et al. 2003; Looney et al. 2004; Eisenberg 2005; Sfikakis et al. 2005; Thatayatikom and White 2006)).
Rituximab targets CD20 which is expressed on all B cells except early pro-B cells and plasma cells (Riley and Sliwkowski 2000). Since B cell progenitors are unaffected, the B cells can continue to develop to mature peripheral B cells in 6-12 months on average. Surprisingly few cases of severe infections after treatment have been observed and this is believed to be due to the remaining plasma cells secreting protecting antibodies.
Only the variable parts on the epitope binding site on rituximab are derived from mouse which decreases the risk for the patients to develop antibodies (HACA) against

rituximab. The mechanism of how the B cell depletion of rituximab occurs is not completely elucidated. It is believed that the main effector function by which B cells are depleted is ligation of the IgG1 Fc part to Fc receptors on effector cells, inducing antibody dependent killing (ADCC) (Golay et al. 2002; Anolik et al. 2003; Uchida et al. 2004). Additionally, activation of the complement cascade via the Fc part (CDC) is also seen as an important effector function of rituximab-induced B cell killing (Di Gaetano et al. 2003; Farag et al. 2004). Apoptosis is probably the minor consequence of ligation of the antibody to B cells. What mechanism is the most important also seems to be different in different diseases and most studies done in this area is performed on samples from patients with non-Hodgkin's lymphoma and not in autoimmune diseases (Cartron et al. 2002; Anolik et al. 2003; Farag et al. 2004).

The basic treatment protocol today differs in RA and SLE. Patients with SLE receive four infusions with one week interval and often in combination with cyclophosphamide (SLE) and glucocorticoids. After this, most often glucocorticoids is the only treatment given until repopulation of B cells or recurrence of disease manifestations. Patients with RA often receive two infusions of rituximab with an interval of two weeks, often in combination with methotrexate and glucocorticoids. The peripheral B cells are in most cases below measurable values already after the first infusion, and as mentioned above does not reappear until after 6 to 12 months. Some patients might repopulate the B cells sooner, probably depending on the B cell load pre-treatment or possibly the distribution of subpopulations in the B cell pool. It has been shown in animal studies that peripheral B cells are depleted while B cells in lymph nodes and bone marrow are not completely depleted (Reff et al. 1994; Schroder et al. 2003). Gong et al showed in a study where they used a mice model positive for human CD20 that germinal center B cells and particularly marginal zone B cells were more resistant to killing by rituximab (Gong et al. 2005).

Interestingly, there are also patients which have been B cell depleted for up to two years or even longer (Cambridge et al. 2003; Leandro et al. 2005; Vallerskog et al. 2007). No explanations to this prolonged depletion have so far been published.

The clinical efficacy of rituximab in patients with RA and SLE is strikingly good with decreasing disease activity score as well as decreasing levels of autoantibodies (Shaw et al. 2003; Eisenberg 2005; Sfikakis et al. 2005; Vasoo and Hughes 2005; Thatayatikom and White 2006). There are few adverse events, though cases of infusion reactions and respiratory tract problems have been reported.

2 THE AIMS OF THIS THESIS

The overall goal of this thesis has been to study influence of drugs on the human immune system. Our approach has been bi-directional; first we have performed *ex vivo* studies after therapy in patients, second we have set up an *in vivo* assay which can be used to study effects of drugs on immune responsiveness.

Specifically, we set out to do the following:

- To study immunological consequences of B cell depletion by rituximab on cellular phenotypes and antibody levels in patients with systemic lupus erythematosus (paper I)

- To study the effects of B cell depletion by rituximab on cytokines specifically important for B cells in patients with systemic lupus erythematosus and rheumatoid arthritis (paper II)

- To set up an assay which induce reproducible immune responses after multiple immunizations in healthy individuals (paper III and IV)

3 METHODOLOGICAL CONSIDERATIONS

The following section describes the main features of the methods used in the studies. Detailed protocols are described in respective papers. All studies were approved by the ethical committee at the Karolinska Institutet, Stockholm, Sweden.

3.1 HEALTHY VOLUNTEERS (PAPER III AND IV)

Paper III: Eleven individuals (seven women and four men, median age 28 years, range 22-48 years) volunteered to participate in the study after replying to advertisement at Karolinska Institutet and Karolinska University Hospital. Five of the same individuals (four women and one man, median age 28 years, range 22-39 years) volunteered to participate in the extension study of a fourth immunization.
Paper IV: Seven healthy adult volunteers (6 women and 1 man, median age 45 years, range 25-57 years) volunteered to participate in the study. Five of the individuals were recruited from the Rheumatology Clinic at Karolinska University Hospital and two of the individuals had participated in the previous study with influenza immunizations (paper III).

3.2 IMMUNIZATION PROTOCOLS (PAPER III AND IV)

Paper III: The volunteers were immunized subcutaneously three times at four-week intervals with 0.5 ml influenza vaccine (Fluvirine® 2001/2002, Evans Vaccine, Liverpool, Great Britain) containing 15μg purified hemagglutinine and neuraminidase from inactivated influenza virus strains; A/New Caledonia 20/99 IVR-116, A/Panama/2007/99 RESVIR-17, and B/Guangdong. None of the subjects stated to have had any influenza immunizations or the flu within the last year. Five volunteers were immunized as described above a fourth time five to ten months after the third immunization.
Paper IV: The volunteers were immunized intramuscularly four times with four-week intervals with 1 ml tetanus toxoid (Statens Serum Institut, Copenhagen, Denmark) containing ≥ 40 I.E. tetanus toxoid. None of the subjects stated any recent tetanus immunizations.

3.3 PATIENTS AND HEALTHY CONTROLS (PAPER I AND II)

Patients with SLE (paper I and II): Eleven patients (females, age 19-56 years, median age 33 years) were recruited from the Rheumatology Clinic at Karolinska University Hospital. They all had active disease and had failed conventional therapy. Disease activity was measured by SLAM. Samples were collected before treatment and at 1, 2, 3, 4, 6, 9, 12, 18, 24 and 30 months after treatment. The patients were followed for at least 6 months after treatment.
Patients with RA (paper II): Nine patients (7 females and 2 men, age 38-73 years, median age 60 years) were recruited from the Rheumatology Clinic at Karolinska University Hospital. They all had active disease and were non-responders (did not reach ACR20) to or intolerant to (adverse reactions) anti-TNFα therapy. Samples were collected before treatment and at 3 and 6 months after treatment.

Healthy controls (paper I): Five age-matched non-treated healthy individuals were used as controls for the serology. Also, samples from four non-treated healthy donors were collected at three time points to control for normal variations of leukocyte populations.
Healthy controls (paper II): Thirteen non-treated healthy individuals with a median age of 60 years (range 20-85 years) were used as controls.

3.4 RITUXIMAB-TREATMENT PROTOCOLS (PAPER I AND II)

Patients with SLE: The patients received four weekly infusions of 375 mg/m^2 rituximab (rituxan, mabthera; Roche, Basel, Switzerland). Cyclophosphamide, 0.5g/m^2, was given at the first and fourth infusion of rituximab. Corticosteroids were given throughout the treatment, and were the only therapy given after the fourth infusion until B cell repopulation occurred.
Patients with RA: The patients received two infusions of 1000 mg rituximab/infusion with an interval of fourteen days together with oral methotrexate (10-20 mg/week). Corticosteroids were given throughout the treatment.

3.5 B CELL RELATED TIME POINTS

Since our aim was to correlate changes in the immune system to the absence and presence of B cells and it was known that the B cells repopulate at different time points in the patients, we decided to study the samples at following B cell related time points:

1. **Baseline**: before treatment
2. **Depletion**: B-cells <0.5% of lymphocytes and <0.01x10^9/L blood
3. **Repopulation**: when B-cells constitute a significant number of lymphocytes, >1.0%
4. **Recovery***:* the next following sample (2-6 months) after repopulation

3.6 SAMPLE PREPARATION

Serum: Serum was prepared by centrifugation of whole blood with no additives. The serum was stored in -70°C until analysis (paper I and II).
Heparinized plasma: The plasma was prepared by mild centrifugation of heparinized whole blood and collection of 1 ml plasma from the top layer in the tube. The plasma was stored in -70°C until analysis (paper III and IV).
Lymphocytes: Venous blood was collected in heparinized vacutainer tubes. Mononuclear cells (PBMC) were isolated by centrifugation of a Ficoll-Hypaque™ PLUS (Amersham Bioscences, Sweden), washed and resuspended in RPMI 1640 medium supplemented with 5% heat inactivated fetal calf serum (FCS), 1% Penicillin and Streptomycin, 1% Glutamine, and 1% Hepes buffer for the ELISPOT assay, or in PBS supplemented with 5% heat inactivated human serum (HS) for flow cytometry.
For cryopreservation, the cells were frozen in freezing media (50% RPMI 1640 supplemented with 1% Penicillin and Streptomycin, 1% Glutamine, and 1% Hepes buffer; 40% heat inactivated fetal calf serum; 10% DMSO) in 20x10^6 PBMC/ml.

3.7 FLOW CYTOMETRY (PAPER I)

As described in the sections above for T- and B- cells, different cellular subsets can be identified by specific combinations of proteins (markers) on their surface. During an immune response, the cells up- or down regulate several of the proteins on the membrane and/or intracellularly. This phenomenon can be used to monitor cellular reactions of immunological events, for example immunizations, infections, or drug therapies. With help of antibodies conjugated with fluorochromes, expressions of cell surface markers/intracellular proteins are analyzed with flow cytometry. In addition to analyzing, also the density of the markers as well as cellular size and granularity can be determined by this method.
Pros: This method is easy to use and detects singular cells. It is a good method to use for screening of *ex vivo* samples.
Cons: It is important to include controls to discriminate the positive staining from the negative. Also, to avoid false positive/negative cells it is crucial to have correct compensations between the spectral overlap of fluorescence emission.

$5x10^5$ PBMC were used per sample if possible, and $2.0x10^5$ as the minimum number. The cells were stained with appropriate antibodies in 5% HS-PBS for 20 minutes in 4°C, and washed twice in 1% HS-PBS. Finally, the cells were re-suspended in 1% HS-PBS and kept in 4°C and in darkness until analysis. 100,000 events in the lymphocyte gate (based on FSC and SSC properties) were collected if possible.
The first step of the data analysis was always to gate live cells through FSC and SSC before subsequent analysis.

3.8 ELISPOT (PAPER III AND IV)

The enzyme linked immunospot assay, ELISPOT, is a development of the ELISA assay (see below). For detection of antigen-specific antibodies, the antigen of interest (here the vaccines) are bound to a membrane in a 96-well plate. The cells (here PBMC) in suspension are seeded in the wells and washed away before addition of a biotinylated secondary antibody (here IgG, IgA, and IgM). The streptavidine-conjugated enzyme is added to the secondary antibody, and addition of the enzymatic substrate forms precipitate which visualizes the spots. One spot illustrates/equals one antibody secreting cell. The result is presented as antibody-secreting cells/x no. cells (ex. 2500 influenza-IgG secreting cells/10^6 PBMC).
Pros: The method is very sensitive detecting a low number of spot forming cells. It also quantifies the number of antibody secreting cells for a specific antigen.
Cons: It is laborious and time consuming. High backgrounds and non-specific spots can cause problems.

Thorough titration of cell numbers, kinetics, amount of vaccine and antibodies were performed before start of the studies. We also investigated if it was possible to use the method on frozen PBMC, and compared the results from fresh and frozen cells from five donors taken at one occasion. The results were similar but with fewer spots from the frozen cells. However, we still decided to use fresh cells in the study to avoid any potential bias caused by the cryopreservation. To cover high versus low production of antibodies from the cells we decided to seed the cells in three different concentrations

and in triplicates. The wells that had less than 100 spots were clear and most similar within the triplicates were chosen to be included in the results. The mean value of the triplicates was then recalculated into spots/10^6 PBMC. The intra-assay variation (CV%) was for influenza IgG mean 16% (range 2-38%), influenza IgA mean 18% (range 3-68%), and influenza IgM mean 27% (range 6-57%). The coefficient of variation is a measure of relative variability, therefore the lower numbers, the higher CV% even if the difference it self is not high (compare 1 versus 2 spots [47 CV%] with 268 versus 232 spots [10 CV%]). Thus, the high percentages of CV derive from wells with few spots/10^6 PBMC.
Wells coated with antibodies which catch all isotypes of secreted antibodies were used as positive control for function of the assay. Several negative controls were used for detection of unspecific binding: (1) wells treated with PBS only and (2) wells coated with the protein keyhole limpet hemocyanin (which is a respiratory protein from Keyhole Limpet, a mollusk living in the oceans), and (3) coated wells without addition of cells.

3.9 ELISA FOR DETECTION OF CYTOKINES AND ANTIBODIES (PAPER I-IV)

Antigen specific cells and other cells participating in the immune response secrete cytokines which are important for regulation of the response. There is a wide array of cytokines produced under certain conditions and time points for the battle. By investigating the cytokines it is possible to draw conclusions regarding the cellular reaction on the conditions of the *in vitro* culture. However, it is also possible to do *ex vivo* analyses of soluble cytokines in the serum/plasma of blood and other body fluids.
Unique for the humoral immune system is the expression and secretion of antibodies. They are produced after a response to a pathogen, both *in vivo* and *in vitro*, and are therefore an easy accessible protein to detect humoral immune responses.

Enzyme Linked Immunosorbent Assay, ELISA, is a common method to measure presence of proteins in fluid. As such, it is used for detection of secreted cytokines/antibodies in cell culture medium of *in vitro* cultures or *ex vivo* from body fluids (here serum and plasma). The ELISA can be set up in two ways; (1) direct where the wells are coated with the antigen (here the vaccines), or (2) indirect (sandwich ELISA), where the wells are coated with an antibody specific for the protein of interest (here cytokines). The cytokine/antibody present in the fluid binds to the antibodies/antigen which the wells are coated with. A secondary antibody which detects the cytokine/antibody from the fluid is added after washing. Finally, an enzymatic process visualizes the antibody/cytokine complex. The result is often presented as (1) concentration of the cytokine, which is calculated by a standard curve produced from known amounts of cytokine added to the analysis, or (2) arbitrary units calculated by comparison of the investigated sample to a standard sample which is present in all analyses.
Pros: The method is easy and cheap to use and provides the possibility to quantify the amount of protein.
Cons: It requires careful titration, pipetting, and timing for reliable results.

ELISA's performed with commercial kits were done according to instructions from the manufacturer. The samples were all analyzed in duplicates. Following ELISA's were performed with kits:
Anti-IgE in serum, Alpha Diagnostic International, USA (paper I)
Anti-Measles IgG in serum; Dade Behring, Germany (paper I)
Anti-Tetanus IgG in serum; Institut Virion, Germany (paper I)
BAFF in serum; R&D Systems, USA (paper II)
APRIL in serum; BenderMed Systems, Austria (paper II)

The protocols for the in house ELISA's were tested thoroughly for reliable results, and careful titrations of dilutions of samples, antibody levels, coating and kinetics was performed. The samples were run in duplicates. Following ELISA's were performed after in-house protocols:
Anti-Ro52 IgG in serum (paper I)
Anti-Ro60 IgG in serum (paper I)
Anti-La44 IgG in serum (paper I)
Anti-influenza IgG, IgA, and IgM in plasma (paper III)
Anti-influenza IgG1, IgG2, IgG3, and IgG4 in plasma (paper III)
Avidity of anti-influenza IgG in plasma (paper III)
Anti-tetanus toxoid IgG, IgA, and IgM in plasma (paper IV)
Anti-tetanus toxoid IgG1, IgG2, IgG3, and IgG4 in plasma (paper IV)
Avidity of anti-tetanus toxoid IgG in plasma (paper IV)

3.10 FASCIA (PAPER III)

When the cells recognize a presented antigen, they respond by proliferation to expand and differentiate the antigen-specific effector cells.

FASCIA (Flow-cytometric Assay of Specific Cell-mediated Immune response in Activated whole blood) is an assay which detects proliferating lymphocytes (lymphoblasts) in whole blood by flow cytometry. It can be used to detect lymhoblasts only, or in combination with antibodies to detect specific cell surface antigen or intracellular cytokines.
Pros: The method is easy to use and no addition of allogeneic serum is needed whereby confounding factors from the serum are avoided. Only small amount of blood is needed.
Cons: It is only possible to work with fresh samples when working on whole blood.

Cultures from whole blood were set up in duplicates and were stimulated with either dialyzed influenza vaccine, influenza vaccine, inactivated influenza virus strain A/New Caledonia 20/99 IVR-116, inactivated influenza virus strain A/Panama/2007/99 RESVIR-17, and inactivated influenza virus strain B/Guangdong. Cultures stimulated with PHA were used as a positive control and cultures in medium only were used as a negative control for the method. A cut off for a positive reaction was 10-20 antigen reactive CD4+ blasts.

Statistical analysis
The analysis was performed using Graphpad Prism 3.03 and Statistica 7.1.

In paper I, Wilcoxon-matched pairs test for non-parametric data was used. For analysis of correlations, Pearson correlation of parametric data was used after test for normal distribution. In paper II, Wilcoxon-matched pairs test for non-parametric data was used for comparison of samples before and after treatment, Mann-Whitney analysis was used for comparison between the different groups. Spearman's rank order test was used for analysis of correlation. In paper III and IV, Wilcoxon-matched pairs test for non-parametric data was used. Comparison of average size of spots in paper III was performed with Kruskal-Wallis analysis followed by Dunn's test.

4 RESULTS AND DISCUSSION

4.1 IMMUNOLOGICAL EFFECTS OF TREATMENT WITH RITUXIMAB IN PATIENTS WITH SLE AND RA (PAPER I AND II)

Rituximab was originally developed to treat patients with lymphoma and was successfully used in an open study on five patients with rheumatoid arthritis 2001 (Edwards and Cambridge 2001). The first open study on patients with SLE treated with rituximab was published 2002 (Leandro et al. 2002). The rheumatology clinic here at Karolinska University Hospital begun at the year of 2000 a study of rituximab therapy in patients with SLE which were resistant to other therapies. With our interest in immunological mechanisms of treatments, our aim was to elucidate the effects of B cell depletion on the immune system in the patients who were treated with rituximab. To investigate the consequences of removing circulating B cells we studied changes of cellular phenotypes, levels of antibodies, and cytokines at time points which were set dependent on absence and presence of B cells.

In paper I, we followed eleven patients with SLE who were treated with rituximab for up to 30 months. All patients improved clinically as measured by disease activity index, SLAM and kidney biopsies scoring the nephritis. Successful depletion of circulating B cells was observed in all patients but with varying time for repopulation (median time 7.5 months). This is in accordance to other studies performed on rituximab treated patients with SLE, who showed that repopulation occurs earlier by treatment with lower doses of rituximab or without addition of cyclophosphamide. (Leandro et al. 2002; Looney et al. 2004; Sfikakis et al. 2005; Cambridge et al. 2006; Ng et al. 2006; Smith et al. 2006) As expected, the repopulating B cells were mainly of naïve phenotype. This has also been observed by Anolik et al in their study of SLE patients (Anolik et al. 2004)and by Leandro et al in a study of patients with rheumatoid arthritis, (Leandro et al. 2006) both cohorts treated with rituximab.

In paper II, we also followed nine rituximab-treated patients with rheumatoid arthritis for six months after treatment. At that time point they had still not repopulated their B cells. Just as the patients with SLE, they all improved clinically as measured by disease activity index, DAS28.

Below are our main findings and more results from other analyses performed in our studies are reported in the papers attached to this thesis.

4.1.1 Main results in paper I (SLE) (Figure 1)

- An increase of activated (CD25+) T cells, both CD4+ and CD8+ T cells, starting already during B cell depletion and sustained during recovery (2-6 months after repopulation of B cells)
- An increase of FOXP3+ CD4+ regulatory T cells, starting during B cell depletion and lasting during recovery (2-6 months after repopulation of B cells)
- Serum levels of total IgM and IgE decreased after rituximab treatment, as well as autoantibodies (IgG isotype) against double stranded DNA and C1q which are both associated with disease activity. Autoantibodies that are not associated with disease activity remained constant.

4.1.2 Main results in paper II (SLE and RA) (Figure 2)

- Serum levels of BAFF increased after rituximab treatment, both in patients with SLE and RA
- Serum levels of APRIL decreased after rituximab treatment in patients with SLE but remained unchanged in patients with RA

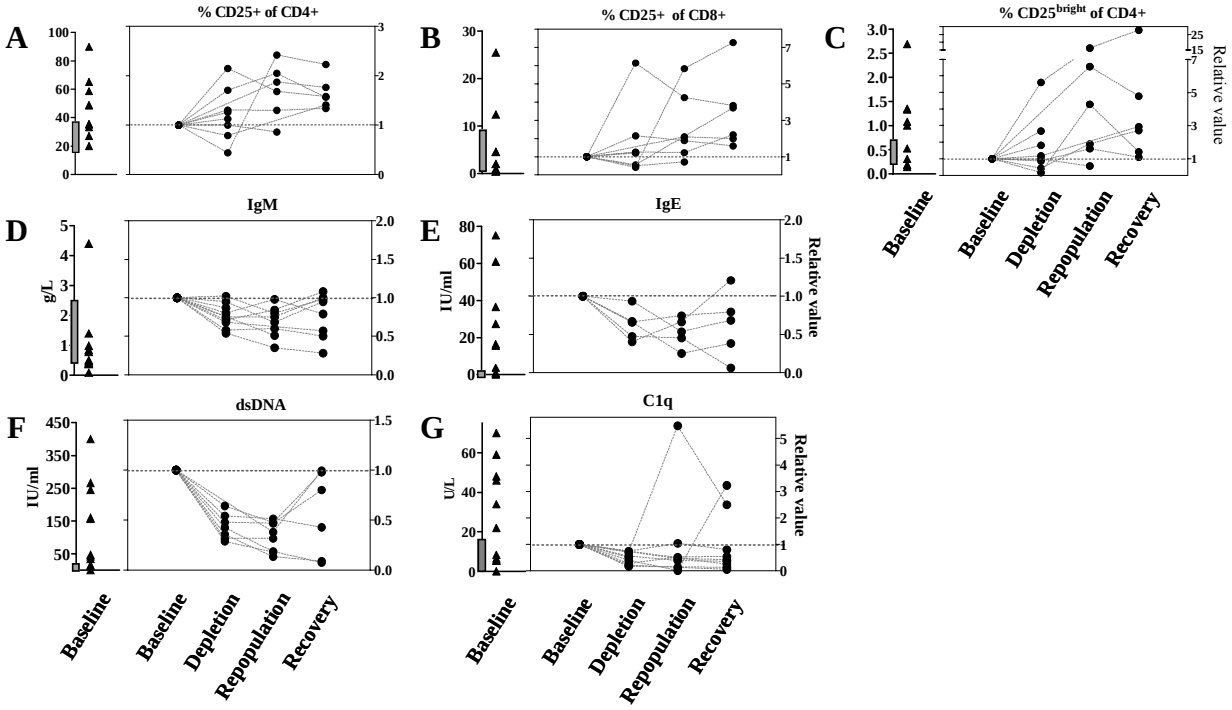

Figure 1: Main results in Paper I. To the left of each figure the normal range (cellular frequency or serum titers) is indicated as gray bars, each triangle represents one patient at baseline. To the right are the frequencies of the indicated populations at the B cell related time-points presented, where the baseline is set to 1 for each patient and following samples are compared to baseline value (relative value=sample X/baseline sample). For each figure, the analyzed parameter is indicated above the figure and the relative values to the right of the figure. (A) CD25+ cells of CD4+ T cells, (B) CD25+ cells of CD8+ T cells, (C) $CD25^{bright}$ cells of CD4+ T cells, (D) Total serum levels of IgM in serum, (E) Total serum levels of IgE, (F) Serum levels of IgG anti-dsDNA, (G) Serum levels IgG anti-C1q

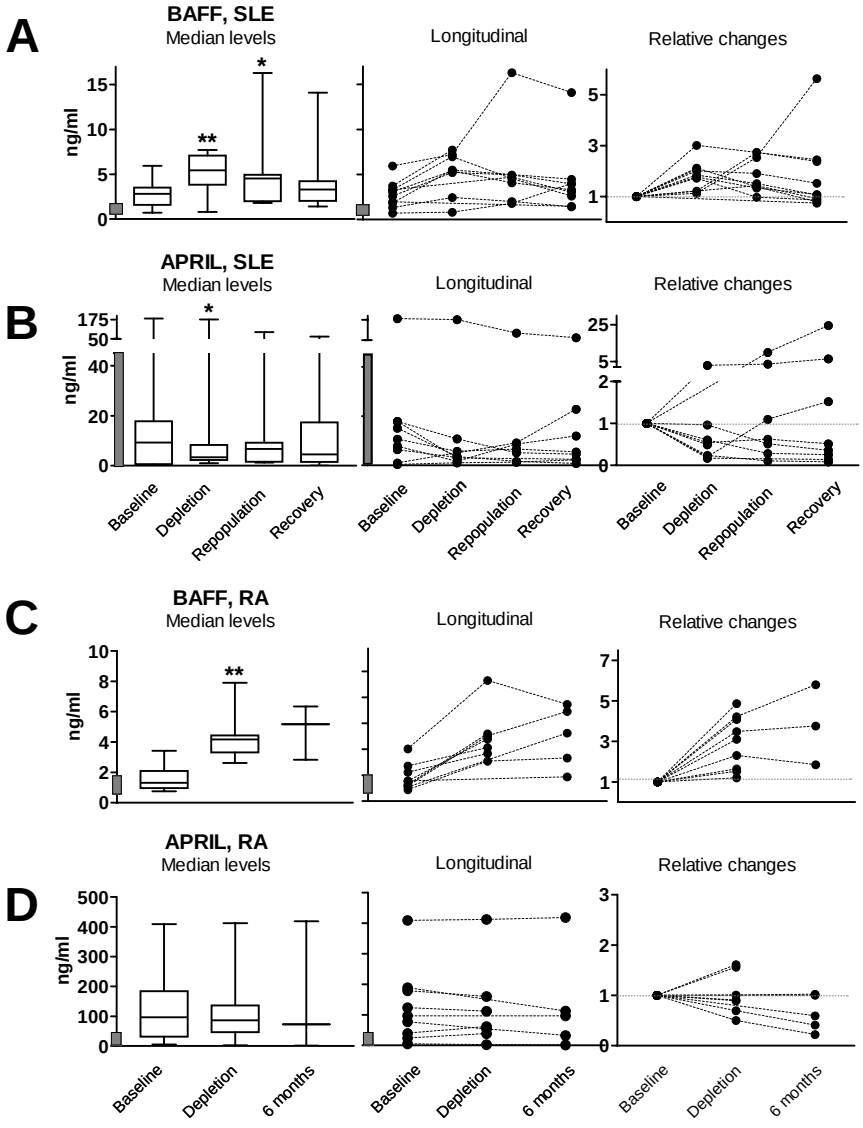

Figure 2: Main results in Paper II. Serum levels of BAFF and APRIL at B cell related time-points. Left panel: levels of (A) BAFF and (B) APRIL in SLE patients, (C) BAFF and (D) APRIL in RA patients. SLE: A significant increase compared with baseline was observed in BAFF at depletion and at repopulation. In APRIL a significant decrease occurred at depletion compared with baseline. RA: There was a significant increase in BAFF levels at depletion compared with baseline. Middle panels: longitudinal levels of BAFF and APRIL in patients with (A,B) SLE and (C,D) RA; each line corresponds to one patient. Right panels: relative changes compared with baseline of BAFF and APRIL in patients with (A,B) SLE and (C,D) RA. Relative change=sample X/baseline sample. The gray bar on the y-axis illustrates the level in healthy controls. * $p<0.05$, ** $p<0.01$

4.1.3 Discussion

4.1.3.1 Activated T cells (paper I)

CD25 (IL-2Rα subunit) expression on T cells characterizes activated T cells. In our healthy subjects 16-37% of the CD4+ T cells expressed CD25. 50% of the patients had a higher frequency of CD25+ CD4+ T cells than the healthy controls. This frequency did not correlate with the disease activity index (SLAM). Liu et al did not either find a correlation between the frequency of CD25+ CD4+ T cells and disease activity index, here SLEDAI (Liu et al. 2004). In addition to the frequency, the number of CD25+ CD4+ T cells increased after treatment with rituximab and remained elevated even after B cell repopulation. The increase was similar for CD8+CD25+ T cells. Even though the activated T cells increased, the SLAM value decreased after therapy.

4.1.3.2 Regulatory T cells (paper I)

Interestingly, also regulatory T cells increased in frequency and number after treatment. As the activated T cells, they remained increased even after B cell repopulation. Regulatory T cells which are characterized by a bright expression of CD25 ($CD25^{bright}$) have been demonstrated to be important in controlling experimental autoimmunity (Sakaguchi and Sakaguchi 2005). FOXP3 is a nuclear transcription factor which is specific for functional regulatory T cells (Valencia et al. 2006). Studies in our laboratory demonstrated that $CD25^{bright}$ cells from the joint of patients with RA express FOXP3 (Cao et al. 2006). Thus it is of importance to include analysis of FOXP3 in studies of regulatory T cells. To confirm that the increase of regulatory T cells was not contaminated by activated T cells, we analyzed FOXP3 expression on samples from two patients obtained before treatment and at repopulation of B cells. The results verify that the vast majority of FOXP3+ regulatory cells resided within the $CD25^{bright}$ gate. There are T cells which can be induced to become regulatory by secreting IL-10 (Tr1) but they do not express FOXP3 nor a bright expression of CD25, alternatively TGF-β (Th3) which can express FOXP3 but without particular surface marker (reviewed by Wan and Flavell, (Wan and Flavell 2006)). There are few studies performed on regulatory T cells in patients with SLE; Liu et al report that in their patient cohort the patients had both lower number and frequency of CD4+ $CD25^{bright}$ T cells, Crispin et al showed that patients with an active disease had lower frequency of $CD25^{bright}$ regulatory T cells, and Suarez et al state in their recent publication a raised percentage of CD25+ and $CD25^{bright}$ CD4+ T cells in patients treated with glucocorticoids (Crispin et al. 2003; Liu et al. 2004; Suarez et al. 2006). The discrepancy between the different studies is probably due to selection criteria of the patients, and also in the settings of the experiments such as the combination of cell surface antigens or the gating techniques during analysis. Vigna-Perez et al have studied a group of patients with active systemic lupus and with renal involvement that were treated with infusions of rituximab at two occasions (14 days apart) (Vigna-Perez et al. 2006). This study is relative comparable with ours with similar selection criteria, however the treatment schedule is different. Even though they do not use B cell related time points, both samples taken 60 and 90 days after treatment should correspond to our sample at "depletion". They not only show an increase of CD4+ regulatory cells (either $CD25^{bright}$, ic.CTLA-4+, or producing either TGFβ or IL-10), they also show an increased *in vitro* suppression of

PHA stimulated CD25- CD4+ T cells. This study supports our observations of a true increase of FOXP3+ regulatory T cells and our speculations of regulatory T cells controlling the increase of activated T cells after B cell depletion and thereby contributing to the amelioration of disease. The mechanism behind the increase of activated T cells and regulatory T cells is not known. There might be an induction of regulatory T cells from conventional T cells: Suarez et al suggests in their study that corticosteroids induce expansion or generation of regulatory $CD25^{bright}$ T cells (Suarez et al. 2006). Barrat et al show in a study that vitamin D3 together with dexamethasone (glucocorticoid) induce human and mouse naïve CD4+ T cells to differentiate *in vitro* into regulatory T cells secreting IL-10 (Barrat et al. 2002). Our patient cohort was already before the start of the rituximab therapy treated with glucocorticoids, hence the increase of the regulatory T cells should not be influenced by the corticosteroids.
It has also been shown by e.g. Bettelli et al that TGF-β can induce expression of FOXP3 in T cells (Bettelli et al. 2006). When we analyzed TGF-β in serum from three patients we observed a small increase (mean 1.3 fold) at depletion compared to baseline (data not shown), however only one patient had higher than normal levels at that time point. This patient had only a slight increase of the regulatory T cells while the other two more than doubled their population. Alternatively, the increase of the regulatory T cell populations in peripheral blood could be a result of a decreased migration to inflammatory sites. The infiltrating T cells at inflammatory sites in renal tissue have been shown to diminish after the therapy with rituximab (personal communication with Dr. Iva Gunnarsson). The hypothesis with decreased migration to inflammatory sites can be supported by the results from our study of patients with non-Hodgkin's lymphoma treated with rituximab, who did not demonstrate this high increase of regulatory T cells and activated T cells, Figure 3 (unpublished data). Additionally, one can speculate that the observed increase in our cohort could also be the result from an increased thymic output of regulatory T cells. However, this needs to be confirmed.

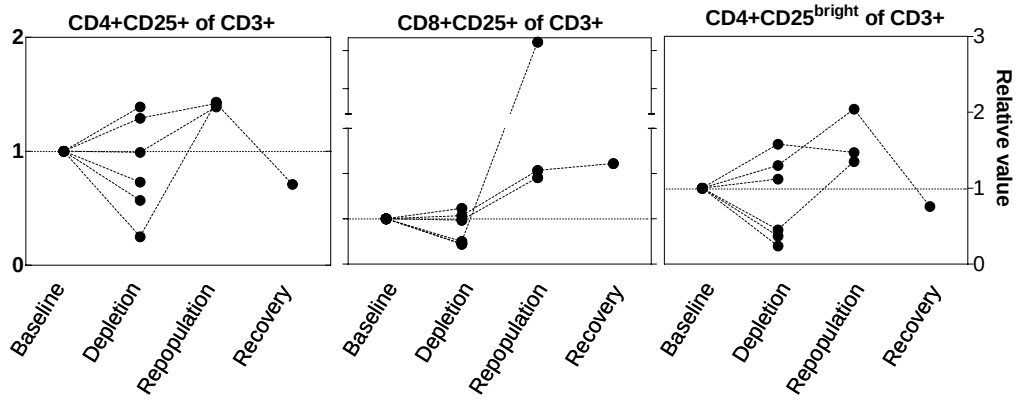

Figure 3: Changes in frequency of T cells in patients with non-Hodgkin's lymphoma treated with rituximab. (Left) CD25+ cells of CD4+ T cells, (Middle) CD25+ cells of CD8+ T cells, (Right) $CD25^{bright}$ cells of CD4+ T cells. Relative value is calculated as follows: the baseline is set to 1 for each patient and following samples are compared to baseline value (relative value=sample X/baseline sample)

4.1.3.3 Changes of antibodies in serum (paper I)

It is important to understand the events that take place within the patient after treatment with rituximab. Additionally our study can also provide a general understanding of B cell immunology and antibody production which will have bearing in a normal immune system.

There are three known subsets of B cells which have the capacity to produce and secrete antibodies; plasmablasts, short-lived plasma cells and long-lived plasma cells (reviewed by Radbruch et al, (Radbruch et al. 2006)). However, there are ongoing debates how long-term humoral immunity is sustained and if long-lived plasma cells exist.

A hallmark of SLE is the presence of a wide range of antibodies against mainly nuclear antigens. They can be present in serum several years before development of the disease with antibodies against Ro52 some time before anti-dsDNA antibodies (Arbuckle et al. 2003). Some titers of autoantibodies, e.g. anti-dsDNA and C1q, have been shown to follow disease activity, while others like anti-SSA/SSB (anti-Ro/anti-La) remain unchanged (Gunnarsson et al. 1997; Reveille 2004; Linnik et al. 2005; Marto et al. 2005). The expression of autoantibodies have been shown to be persistent also in patients in remission, however with fewer number of B cells expressing the antibodies (Yurasov et al. 2006). Patients treated with immunosuppressive therapy have persistence of autoantibodies in serum, indicating their production from long-lived plasma cells which are insensitive to such therapy (reviewed by Hoyer et al, (Hoyer et al. 2005).

Studies of serum titers of antibodies of different specificities after rituximab-induced B cell depletion offers an opportunity to indirectly investigate the matter of different antibody secreting cells. Studies of patients with rheumatoid arthritis treated with rituximab have shown a selective decrease of antibodies in serum; antibodies against citrullinated peptides (anti-CCP) and rheumatoid factor (RF) decreased while protective antibodies against tetanus toxoid and pneumococcal capsular polysaccharides (anti-PCP) remained unchanged (Edwards and Cambridge 2001; De Vita et al. 2002; Cambridge et al. 2003; Edwards et al. 2004). Therefore we chose to test (1) total levels of IgM, IgG, IgA, and IgE, (2) disease activity associated autoantibodies, (3) autoantibodies not associated to disease activity, and (4) protective antibodies.

The titers of IgG and IgA did not change after therapy. We observed a decrease of IgM and IgE shortly after B cell depletion, however they were still measurable and did not disappear. The half-life for IgG is 25 days, IgA 6 days, IgM 5 days, and for IgE only 2 days. The median time for B cell repopulation was 7.5 months (225 days). Without presence of cells secreting antibodies after B cell depletion the titers of antibodies should be below measurable levels. The stable titers of IgG and IgA and the decrease of IgM and IgE suggests the presence of long-lived plasma cells secreting mainly IgG and IgA but also some IgM and IgE.

As for the studies of rituximab-treated patients with RA, also in our SLE cohort we observed selective changes in titers of different antibodies. There was a decrease of anti-dsDNA and anti-C1q that are associated with disease activity, but we could not see a change in titers of anti-Ro or anti-La antibodies. These results are supported by Cambridge and colleagues who reported that in patients with SLE treated with a similar protocol of rituximab no change in anti-SSA (anti-Ro) titers were observed (Cambridge

et al. 2006). Further on, they report changes in anti-dsDNA and anti-nucleosome (both linked to development of tissue damage) after treatment but no changes in anti-histone and anti-RNP/Sm (less closely linked to disease pathogenesis). Also other studies on rituximab-treated SLE patients show changes in titers of anti-dsDNA (Leandro et al. 2002; Smith et al. 2006).
The decrease in titers after rituximab therapy indicates that antibodies against dsDNA and C1q are mainly produced from plasmablasts and short-lived plasma cells. Alternatively, if the antibodies were produced by long-lived plasma cells who resided in survival niches at site of inflammation (for example kidneys), they should as we observed decrease due to resolution of inflammation and thereby death of the specific plasma cells. Our results with sustaining titers of anti-Ro and -La suggest that they are mainly produced by long-lived plasma cells residing in bone marrow.
Finally, we did not observe any statistically significant changes in levels of protective antibodies against measles and tetanus toxoid. However, the data indicates a trend towards lower titers of tetanus toxoid antibodies, see Figure 4. Other rituximab studies of both SLE and RA do not report changes in protective antibodies (Cambridge et al. 2003; Edwards et al. 2004; Cambridge et al. 2006). However, it should be of importance to follow the titers of protective antibodies especially in patients who are suggested additional treatments with rituximab.

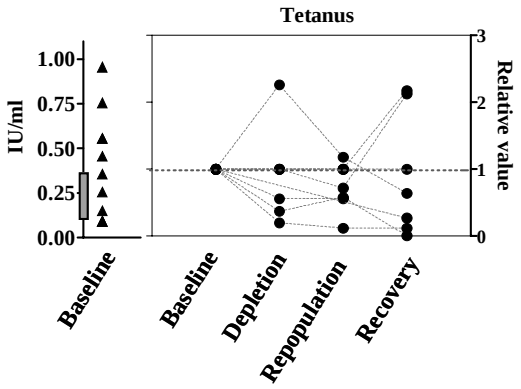

Figure 4: Changes in serum levels of IgG antibodies against tetanus toxoid after treatment with rituximab in patients with SLE. To the left of each figure the normal range (cellular frequency or serum titers) is indicated as gray bars, each triangle represents one patient at baseline. To the right are the frequencies of the indicated populations at the B cell related time-points presented, where the baseline is set to 1 for each patient and following samples are compared to baseline value (relative value=sample X/baseline sample)

4.1.3.4 Effects on BAFF and APRIL serum levels (paper II)

Here, we followed serum levels of BAFF and APRIL before and after B cell depletion with rituximab in patients with SLE and RA.
BAFF and APRIL are two cytokines belonging to the TNF family which have been shown to be indispensable for B cell maturation and survival (reviewed by Mackay et al, Dillon et al, and Treml et al (Mackay et al. 2003; Dillon et al. 2006; Treml et al. 2006). NZBW/F_1 and MRL-*lpr* are two mouse strains which spontaneously develop lupus and which display increased serum levels of BAFF (Gross et al. 2000). Increased

levels of BAFF have also been found in serum of patients with SLE, Sjögren's syndrome, and RA (Stohl 2003; Jonsson et al. 2005; Pers et al. 2005; Seyler et al. 2005; Szodoray and Jonsson 2005).

4.1.3.4.1 BAFF before treatment

In agreement with the published data, we could detect increased levels of BAFF in the majority of the patients with SLE and in 30% of the patients with RA.
The mechanisms behind the raised levels of BAFF in these patients are not known. However, it is known that high levels of BAFF up-regulates anti-apoptotic proteins in B cells thereby disturbing the peripheral tolerance and thus the deletion of autoreactive B cells (Mackay et al. 2003; Lesley et al. 2004; Thien et al. 2004). Patients with SLE have an aberrant peripheral B cell repertoire whereof an increased frequency of plasmablasts is prominent (Odendahl et al. 2000; Jacobi et al. 2003). The abnormal levels of BAFF can be one cause to the increased plasmablasts since they express both BCMA and BAFF-R (Avery et al. 2003; O'Connor et al. 2004).

4.1.3.4.2 Changes in levels of BAFF after rituximab treatment

Upon treatment with rituximab, we observed an increase of BAFF in all patients (both with SLE and RA) except in one patient with SLE from whom we miss samples at depletion and repopulation. This observation is consistent with Cambridge and colleagues observation in rituximab-treated patients with RA, and Lavie and colleagues in patients with rheumatic disease (2 with SLE, 1 with Sjögren's syndrome, and 1 with RA) (Cambridge et al. 2006; Lavie et al. 2006).
Since B cells are the main cells expressing receptors for and thus "consuming" BAFF, we interpret our data primarily as a result from the B cell depletion per se. BAFF is mainly produced by two sources; (1) constitutively by stromal cells in lymphoid organs (Gorelik et al. 2003; Lesley et al. 2004; Schaumann et al. 2007), and (2) inducible from monocytes, macrophages, neutrophils, dendritic cells, and osteoclasts (reviewed by Ng et al and Dillon et al) (Ng et al. 2005; Chang et al. 2006; Dillon et al. 2006). The increase of BAFF after rituximab-induced B cell depletion indicates a lack of negative regulation of the production of BAFF, or alternatively (but less likely) an increased production due to the lack of B cells.

4.1.3.4.3 APRIL before treatment

In contrast to BAFF, the serum levels of APRIL in the patients with SLE were within the normal range before start of rituximab therapy. There are contradicting reports regarding APRIL in SLE, one study describes normal serum levels in a majority of the patients (Stohl et al. 2004) while another study report increased serum levels in their SLE cohort (Koyama et al. 2005). The difference between the reports can be due to the different study set ups: the study by Stohl et al had patients with an active SLE (measured by SLEDAI) and mainly of Hispanic origin, while the patients in the study by Koyama et al were Japanese and differed in their disease activity from inactive to active (measured by BILAG). Since APRIL is a recently detected cytokine not many studies have been published so far.

Our patients with RA had ten times higher levels of APRIL in serum compared to the patients with SLE and to the healthy controls. As for SLE, only a few publications are found regarding levels of APRIL in RA. Koyama et al reports normal serum levels of APRIL in their Japanese patients with RA (Koyama et al. 2005), and Tan et al show higher levels of APRIL in synovial fluid compared to serum in their cohort which were mainly of Hispanic origin (Tan et al. 2003). The median serum levels of APRIL in the study by Tan et al were around 25 ng/ml, and they used the same ELISA protocol as Stohl et al (patients with SLE) where the normal values were between 9-28 ng/ml (Tan et al. 2003; Stohl et al. 2004). There were no healthy controls included in the study by Tan et al. Seyler et al showed that APRIL mRNA correlates with inflammation severity in synovial biopsies from patients with RA (Seyler et al. 2005).
We can only speculate why we see this difference in levels of APRIL in our two patient cohorts: SLE and RA are two systemic inflammatory diseases driven by different mechanisms and cytokine expressions. (1) For instance the initiation of APRIL could have different origin, patients with SLE have increased levels of IL-10 and IFN-α, while IFN-γ is more prominent in RA. BAFF expression in monocytes, macrophages and DC is augmented by IL-10, IFN-γ and IFN-α. APRIL expression is induced by stimulation by IFN-γ and IFN-α on macrophages and DC (reviewed by Ng et al, (Ng et al. 2005). (2) One could also expect production of BAFF and APRIL from different cellular sources in SLE and RA. Osteoclasts derived from the inflamed RA joint have been shown to be good producers of APRIL (Moreaux et al. 2005). Synovial fluid from inflamed joints of RA patients contains very high levels of both BAFF and APRIL, probably locally produced by neutrophils, dendritic cells, and macrophages (Tan et al. 2003; Seyler et al. 2005). Also, fibroblast-like synoviocytes secrete BAFF after stimulation with IFN-γ and TNF-α, which are known to be effector cytokines in the inflamed joint of patients with RA (Ohata et al. 2005). Lastly, the patients in the two cohorts were at baseline treated with different immunosuppressive therapies, the patients with SLE with cyclophosphamide and the patients with RA with methotrexate. Further more, the patients with RA were all non-responders to TNF-α therapy. It is not known if and how the different therapies the patient cohorts were treated with influence production of BAFF and APRIL. It

4.1.3.4.4 Changes of APRIL after rituximab therapy

After treatment with rituximab we observed different effects on APRIL levels in patients with SLE and RA; in the patients with SLE the levels decreased while in the patients with RA the high levels in serum were sustained. Hence, we saw different effects on BAFF and APRIL after rituximab therapy.
One study on SLE reports of constant levels of APRIL but decreased levels of BAFF after treatment with high doses of corticosteroids (Stohl et al. 2004), indicating different effects on BAFF and APRIL after the same therapy. This study supports that our data is not an effect of the glucocorticoids given with the rituximab infusions since the levels of BAFF increased after treatment and APRIL decreased in the SLE patients. Also, our patient cohorts were previously treated with glucocorticoids before the rituximab infusions, hence already affecting the levels of BAFF and APRIL.

Today we can only speculate whether the increase of BAFF, even if only temporary, have any consequences for the patients. There are some concerns indicating possible risks such as the risk for an increased output of autoreactive B cells (Lesley et al. 2004; Thien et al. 2004). As mentioned above, autoreactive B cells are normally deleted during negative selection in the periphery. This negative selection can be inhibited by high availability of BAFF, which up-regulates anti-apoptotic proteins, hence overriding the selection process. Also, BAFF and APRIL have been associated with different forms of non-Hodgkin's lymphoma and with B cell chronic lymphocytic leukaemia (He et al. 2004; Novak et al. 2004; Fu et al. 2006; Haiat et al. 2006) Moreover, an increased risk for lymphoma development in patients with rheumatic diseases has been reported, however BAFF and APRIL levels have not been analyzed in these studies (Cibere et al. 2001; Zintzaras et al. 2005; Baecklund et al. 2006; Smedby et al. 2006). Further on, BAFF and APRIL enhance plasmablast survival and prolong the life of plasma cells in the bone marrow (Avery et al. 2003; O'Connor et al. 2004; Ng et al. 2005; Radbruch et al. 2006). In addition, plasma cell homing to the bone marrow is believed to be facilitated by binding of APRIL to CD138 (Ingold et al. 2005).
Not only B cells are affected by BAFF and APRIL, studies have shown that T cells co-stimulated by BAFF secrete IL-2 and IFN-γ, express CD25, and proliferate (Huard et al. 2001; Huard et al. 2004). Indeed, in our study (paper I) we observed an increase of CD25 expression on both CD4+ and CD8+ T cells. It has also been shown that BAFF stimulated T cells upregulate Bcl-2, hence survival is increased (Ng et al. 2004).
A recent publication demonstrates that BAFF strongly induces human monocytes survival, activation, and differentiation into macrophages (Chang et al. 2006). The BAFF treated monocytes produced IL-6, TNF-α, and IL-1β, as well as upregulated CD80 and CD40 on the cell surface. They also showed upregulation of TACI upon stimulation with BAFF or IL-10.
Animal studies on mice expressing human CD20 show that residual splenic B cells after treatment with anti-hCD20 alone are diminished after combination therapy of anti-hCD20 and BAFFR/BR3-Fc fusion protein (Gong et al. 2005).
Today, there are ongoing clinical studies (phase I-II) with anti-BAFF therapies which have been shown to be safe and biologically active in patients with SLE (Sabahi and Anolik 2006).
With the above discussion in mind, we agree with Cambridge and colleagues who suggest that rituximab-treated patients may benefit from complementary anti-BAFF therapy to temporarily remove excess BAFF until the B cells have been repopulated (Cambridge et al. 2006).

4.2 MULTIPLE IMMUNIZATIONS AS A TOOL TO MEASURE REPRODUCIBLE IMMUNE RESPONSIVENESS (PAPER III AND IV)

The overall aim with these studies was to set up an assay where reproducible immune responses could be measured following repeated immunizations on an individual level. This assay would provide a tool to investigate the degree of modulation of the immune system induced by drugs.
In paper III we studied the immune responses triggered by immunizations with a common influenza vaccine in healthy individuals. We followed the immune responses

by detecting changes in circulating influenza/vaccine specific B cells before and after three subsequent immunizations. To further increase our understanding of the events after multiple immunizations we also studied circulating antibodies and their subclasses and avidity, as well as proliferation of antigen specific T cells and secretion of IFN-γ and IL-13.
In paper IV we used multiple immunizations with tetanus toxoid in order to see if this also would be a useful candidate antigen for the assay. We also sought to compare the humoral immune responses triggered by tetanus toxoid to the responses induced by influenza vaccine.

4.2.1 **Main results in paper III and IV** (Figure 5 and 6)

- The number of B cells secreting antigen specific antibodies was five to ten fold higher after immunization 1 with influenza vaccine or tetanus toxoid as compared to immunization 2 and 3 as measured by ELISPOT
- Immunization 2 and 3 with influenza vaccine resulted in comparable immune responses on the B cell level as measured by ELISPOT
- Immunization with influenza vaccine resulted in more than ten times more B cells secreting antigen specific antibodies compared to tetanus toxoid when measured by ELISPOT
- Immunization 2 and 3 with tetanus toxoid did not result in any measurable B cell responses by ELISPOT

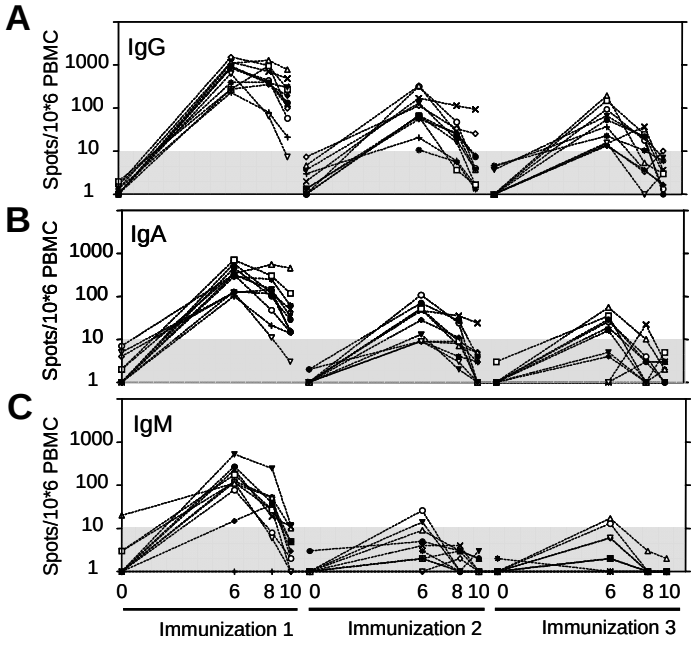

Figure 5: Main result Paper III. Kinetics of frequency of influenza specific B cells in response to three subsequent immunizations. Healthy subjects (n=11) were immunized three times with four weeks interval with influenza vaccine and the immune response was followed on cellular B cell level by ELISPOT. Number of antigen-specific B cells secreting antibodies were measured before (day 0), and 6, 8. and 10 days after each immunization. Influenza specific (A) IgG, (B) IgA, and (C) IgM. The gray fields illustrate the threshold for an antigen-specific response when 0.5 x 10^6 PBMC were seeded per well.

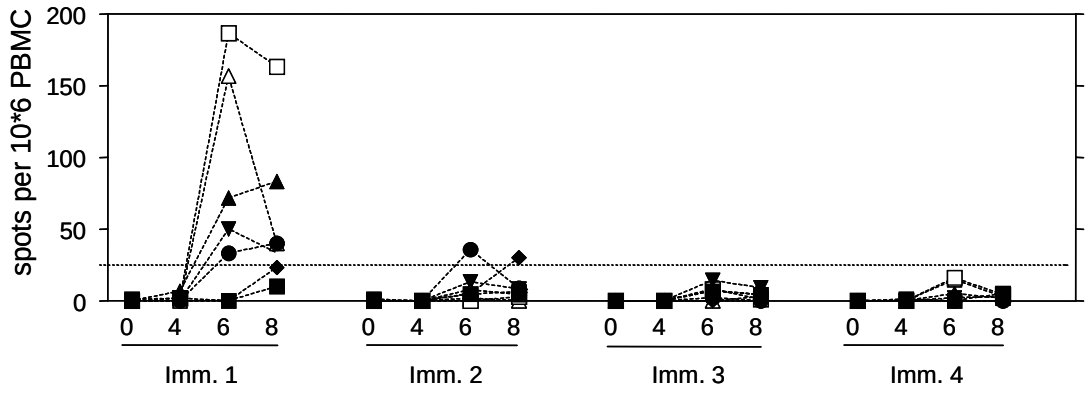

Figure 6: Main results Paper IV. Kinetics of frequency of tetanus toxoid specific B cells in response to four subsequent immunizations. Healthy individuals (n=7) were immunized four times with four weeks interval with tetanus toxoid, and the immune response was followed on cellular B cell level by ELISPOT. The number of antigen-specific B cells secreting antibodies were measured before (day 0), and 4, 6, and 8 days after each immunization. The graph illustrates the number of B cells secreting tetanus toxoid specific IgG. The line illustrates an antigen-specific response of five spots in a well seeded with 0-3 x 10^6 PBMC.

4.2.2 Discussion

4.2.2.1 Influenza immunization 1

We observed a mix of both primary (with IgM) and secondary (with IgG) immune responses by B cell analysis with ELISPOT after the first immunization with influenza vaccine. The titers of influenza specific IgM increased dramatically eight days after immunization 1 and decreased thereafter. Increased levels of influenza IgG antibodies were measured already ten days after immunization 1 and remained thereafter at the same level. Also, the avidity of the influenza IgG antibodies changed from medium (30>50%) before immunization to high (>50%) ten days after immunization 1. Studies by Hedman et al on individuals with primary rubella infections or previous infection/vaccinations shows that IgG antibodies with low avidity are found in primary infections while high avidity antibodies are found in immune individuals (Hedman et al. 1989; Hedman and Rousseau 1989). The serum analysis confirms the ELISPOT data indicating both a primary and a secondary immune response to the influenza vaccine in all but one individual. The influenza vaccine contains three influenza strains and at least one strain is exchanged from year to year depending on the type of flu for that year. Our volunteers had most probably encountered at least one of the strains of the vaccine previous to our study, thereof both the primary and secondary responses.

4.2.2.2 Tetanus toxoid immunization 1

In contrast, we could only detect a secondary (IgG) immune response by ELISPOT after immunization 1 with tetanus toxoid. As for the influenza immunization the tetanus toxoid specific IgG antibodies in serum rose early after immunization 1 indicating a secondary response. Here, the avidity started out at high (>50%) which is a sign of affinity maturation and memory for tetanus toxoid.

4.2.2.3 Immunizations 2, 3, and 4

After immunization 1 the individuals were immunized every fourth week (total three or four immunizations) with influenza vaccine or tetanus toxoid.
The magnitude of spots was five to ten times lower after immunization 2 and 3 compared to immunization 1, this was similar in both influenza and tetanus toxoid. Janeway's Immunobiology (Fig. 10.30) show that the frequency of specific B cells decrease from $1:10^4$-$1:10^5$ in the primary response to $1:10^2$-$1:10^3$ in the secondary response of an immunized donor (Janeway et al. 2005). Whether this decrease continues after subsequent immunizations is not mentioned. However, we observed this only after immunization 1 with influenza vaccine and not after immunization 2 and 3. For tetanus toxid, we could only observe this decrease after immunization 1 since we did not get any spots after immunization 2, 3, and 4. Steven and Saxton demonstrate a reduced *in vitro* production of tetanus toxoid specific antibodies after repeated immunizations (Stevens and Saxon 1979). They also show a correlation ($r=0.93$, $p<0.001$) between the *in vitro* synthesis of tetanus specific antibody with the time since prior booster immunizations. This supports our data with decreased number of antigen specific B cells after immunization 1.

There was a marked difference in the B cell response to the influenza vaccine and the tetanus toxoid. First, the number of spots was more than ten fold higher at peak of response after immunization with influenza vaccine than with the tetanus. Second, the features of the spots were very different; influenza specific spots were distinct and heterogeneous in size being both small and large, while the tetanus specific spots were mainly diffuse and huge. Third, the influenza specific spots increased in size after immunization 2 and 3 compared to immunization 1 and the tetanus specific spots looked the same after all immunizations (the few spots that were). These observations indicate an already established long-term memory for tetanus toxoid. It is known that the size of the spots correlates to the amount secreted antibodies (Hesse et al. 2001) and the tetanus spots in our analyses were huge. Plasma cells are the most efficient antibody producers. It is likely that we mainly find plasma cells which secrete large amounts of tetanus specific antibodies and no new immunization-activated naïve B cells which should produce fewer antibodies and hence smaller spots.

Why do we not see an immune response after tetanus immunizations as we do after influenza? First, there are major differences between the antigens: the influenza vaccine contains multiple immunogenic epitopes from three virus strains, the tetanus toxoid is derived from a bacterial toxin and it also contains the adjuvant alum (aluminum hydroxide). The difference of the two vaccines probably triggers different courses of immune responses. This we observe in the differences in isotypes and IgG subclasses of antibodies produced after the immunizations: influenza immunizations induced mainly the isotypes IgG and IgA and the subclasses IgG1 and IgG3; tetanus immunizations triggered IgG and the subclasses IgG1 and IgG4. Studies have shown that influenza virus/vaccinations induce primarily IgG1, IgG3 and some IgG4, while

tetanus toxoid immunization induced mainly IgG1 and some IgG4 (Devey et al. 1987; Powers 1994; Stepanova et al. 2002; Schauer et al. 2003).
The minor response to the tetanus toxoid after immunization 2, 3, and 4 might be due to the presence of high amounts of high-avidity tetanus specific antibodies in serum before immunizations, being sufficient enough to eliminate the pathogen (the vaccine) without the need for a response to occur. When we analyzed the amount of tetanus specific antibodies in serum we observed that the titers were at their peak day 0 of immunizations 2-4 and that they decreased slightly at day 4, 6, and 8, possibly due to binding free antigen.

In order to use our assay to evaluate drug-induced immunomodulation it is necessary to get a sufficient number of spots after immunizations in order to be able to compare differences with and without the drug. If there is a low number of spots secreted (<20) before introduction of the drug, the risk of getting blank wells after immunization when on the drug is high, and estimation of the effects is difficult. Therefore we consider the influenza vaccine and not the tetanus toxoid as the suitable tool to repeatedly trigger the immune system for usage of the assay to evaluate immunomodulatory effects by drugs.

5 CONCLUSIONS

- We studied immunological consequences of rituximab-induced B cell depletion in treatment resistant patients with SLE. The humoral immune system was affected by a selective decrease of certain immunoglobulin isotypes and antigen specificity. Hence, our results conclude an influence on B cell memory after B cell depletion by rituximab. In addition, the cellular immune system was affected by an increase of activated T cells and regulatory T cells after B cell depletion.

- Rituximab therapy in patients with SLE and RA is followed by changed serum levels of cytokines important for B cells. The concentrations of BAFF increase after treatment independent of disease. APRIL is differentially affected in the patient cohorts; in SLE it decreased after the therapy while it was heterogeneously affected in RA.

- We have set up an assay in which reproducible immune responses are measured after multiple immunizations with influenza vaccine. By three immunizations it is possible to study influence of drugs on immune responsiveness by counting the number of responding B cells using the ELISPOT method. By introducing a fourth immunization it would also be possible to measure the immune response after termination of the drug and a wash out period. Table x illustrates an example of a schedule for such an assay.

Day	Imm.	Sampling and Assay	Notes
0*	1	x	
42**			
0*	2	x	
5		x	
6		x	
42**			Intake of drug
0*	3	x	
5		x	
6		x	
42**			Wash out period
0*	4	x	
5		x	
6		x	
12		x	

Table 1: Schedule for immunizations, sampling, and assays for results. Four immunizations offer evaluation of drug-induced modulations before, during, and after intake of the drug.

6 CONCLUDING REMARKS

One of the most fascinating things with research is a similarity with the difficult task to kill a hydra: each achieved result generates a range of new questions. Therefore, I would like to share a few new questions evoked from the results in our studies.

Our immune system is a remarkable fantastic and complex network of cells collaborating to fight against invading pathogens and to keep our body healthy. It is amazing how well it works through our entire life time. Yet, it sometimes fails its mission and there can be many reasons to why considering the multifaceted system. Failure in the process of tolerance to self can result in autoimmunity, invading pathogens can manipulate and evade the immune system, tumors can also manipulate and evade the immune defense, or drugs can influence the function of our immune system. The big challenge in studying the immune system in humans is the limited access to the main sites of action. Therefore, we use the means we have access to (mainly blood) and base our research on methods from which we draw conclusions hopefully true to or close to the *in vivo* situation.

6.1 EFFECTS OF MULTIPLE IMMUNIZATIONS ON THE IMMUNE SYSTEM

Besides the development of an assay which can be used to assess the effects of drugs on immune responsiveness, the project with multiple immunizations evoked some questions concerning basic immunological mechanisms. Why could we not detect any or very few circulating tetanus specific B cells after immunizations 2-4? Did we induce anergy? Or was the somatic hypermutation unsuccessful, inducing apoptosis of the previously tetanus-specific memory B cells? Is there a regulatory mechanism determining the need for more antibodies? In that case, what would it be? These questions are probably difficult to answer with experiments from the human setting because it would need access to lymphoid organs.

6.2 EFFECTS OF RITUXIMAB-INDUCED B CELL DEPLETION ON THE IMMUNE SYSTEM

In our study of rituximab treated patients we were interested to see what consequences B cell depletion had on the immune system. We also looked at the treatment with rituximab as an opportunity to learn more about the function of B cells in the immunological network. The main question we asked was: what are the effects on the remaining immune cells after months of absence of B cells? In my eyes the most striking result was that the patients who were severely ill and refractory to previous treatments all responded clinically to the B cell depleting therapy! Also, the immunological results we observed were very interesting and gave rise to more questions. Why were the activated T cells increasing after B cell depletion? The patients all improved and that should indicate a lesser activated immune system. One explanation to the disease improvement could be the increase of the regulatory T cells which are potent to keep the activated cells under control.

How long can the patients be without mature B cells without greater risk for infections? Due to survival of plasma cells during rituximab therapy there should be a conservation of the humoral memory. There are limited data on the long-term effects of repetitive courses of rituximab. Studies on long-term treated lymphoma patients show no severe events despite long absence of B cells and decrease of IgM levels (Ghielmini et al. 2004; Hainsworth 2004; Ghielmini et al. 2005). However, patients with lymphoma are treated with a different protocol of cytotoxic agents in combination with rituximab compared to patients with SLE and RA. It is also a possibility that the immune system is differentially affected in lymphoma and rheumatic diseases. Ng and colleagues reported repeated B cell depletion in patients with SLE to be without infectious complications (Ng et al. 2006). However, our observations of decreased titers of IgM, which is an important first defense against pathogens, and the trend towards decreased titers of anti-tetanus toxoid antibodies indicate the need for follow ups of protective antibody titers during and after rituximab therapy especially during repeated courses.

Another interesting result that evokes immunological questions is the selective decrease of antibodies in serum. The disease activity associated antibodies decreased after B cell depletion; hence they are probably produced by antibody producing cells which are affected by the depletion such as plasmablasts and short-lived plasma cells. Alternatively, they are produced by long-lived plasma cells which are residing in survival niches located at sites of inflammation. What is it that determines what type of antibody producing cells produces a specific antibody? Is it merely competition of access to survival niches? Is it the type of antigen itself? Or are there other regulatory mechanisms? The field of maintenance of long-lived memory is currently extensively investigated and hopefully we can soon get answers to our questions.

Lastly, what are the consequences from the increased levels of BAFF after B cell depletion? Is it negative or positive with these levels of BAFF? One has to keep in mind that the patients get better from the therapy. However, there might be a risk for further skewing the B cells further towards autoreactive phenotypes, or further activating cells in the immune system, or increasing the risk for development of lymphoma.

I am looking forward to further studies of immunological effects of rituximab with great interest.

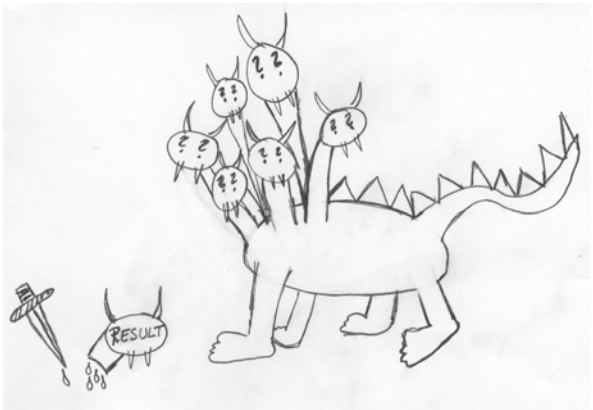

Figure 7: The Research Hydra. The definition of a hydra is a many-headed serpent or monster in Greek mythology that was slain by Hercules and each head of which when cut off was replaced by two others.

7 FUTURE PERSPECTIVES

The surprising and different results in the circulating B cell responses following multiple immunizations with influenza vaccine and tetanus toxoid raise the question to what would be the outcome of multiple immunizations with other vaccines. For example, what would be the results from immunization with a pneumococcal vaccine which is T-independent while influenza and tetanus toxoid are T-dependent? A T-independent humoral response should be less sensitive to anergy induced by a short interval between immunizations, thus the time between immunizations could be shorter than for influenza immunizations. Also, since different immunomodulatory drugs affect different parts of the immune system and in different ways, it could be an advantage to have the availability to use the assay to study the effects of drugs on different types of humoral responses.

Considering the selective changes of antibodies of rituximab treated patients and the increased levels of BAFF it would be appealing to study if the repertoire of autoreactive B cells change after repopulation of B cells. One possibility could be to study a panel of autoantibodies in serum to see if it changes after repopulation of B cells compared to pre-rituximab treatment. It would probably be informative to follow the patient at least one year after B cell repopulation in order to see long-term changes. To complement the serum levels of antibodies which mainly reflects the pool of long-lived memory and is relative insensitive to modulation, it could be of interest to study antibody production on a cellular level which reflects the circulating memory pool and is more sensitive to modulation. It would be interesting to develop an ELISPOT detecting the same panel of autoantibodies as in serum for detection of circulating autoreactive B cells to see changes before and after therapy.

The observation of the high levels of APRIL in our cohort of patients with RA, who had failed to respond to or were resistant to anti-TNF alpha therapy, would be interesting to follow up since this has not been reported previously. Is the high level of APRIL due to the anti-TNF therapy, or is it a common feature for this group of patients? To possibly answer this question it would be interesting to study three groups of patients with RA with regard to anti-TNF therapy (never treated, non-responders, and responders) comparing the levels of APRIL in the different groups with inclusion and correlation of clinical parameters.

In order to further study the effects of B cell depletion we have started to set up an *in vitro* system to mimic the rituximab-induced B cell depletion in peripheral blood. By using magnetic bead sorting we can set up cultures of whole PBMC, B cell depleted PBMC, and also reconstitute B cells in different frequencies to the B cell depleted PBMC, all from blood taken at one occasion. This set up makes it possible to study functional effects such as proliferation, cytokine secretion, and expression of cell surface marker due to presence or absence of B cells. Results from these studies will possibly help us to understand the *in vivo* effects of B cell depletion.

The question concerning immune responsiveness of patients treated with rituximab has been raised by some investigators. Van der Kolk et al performed a study in lymphoma patients who were immunized with primary antigens and recall antigens before and after rituximab treatment (van der Kolk et al. 2002). They report decreased antibody titers to recall antigen after rituximab treatment. We suggest that our assay using multiple immunizations could provide a tool to investigate the effect of rituximab on humoral immune responses. Figure X illustrates a suggestion for the layout for such a study.

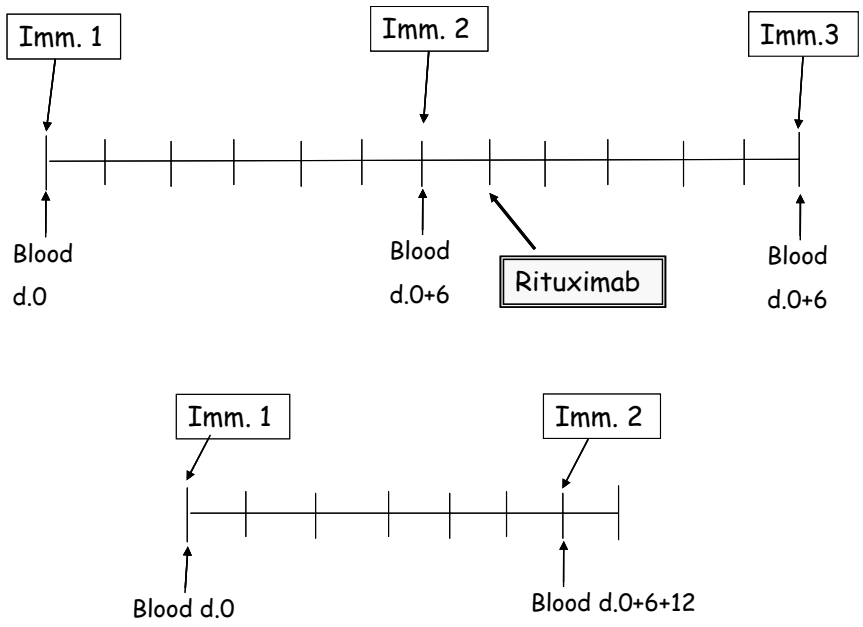

Figure 8: Experimental setup for studies of immune responsiveness after rituximab therapy. Immunization 1 with influenza vaccine would be performed nine weeks (one line on the time-line equals one week) prior to the first rituximab infusion, immunization 2 one week before rituximab, and immunization 3 two weeks after rituximab (B cells depleted). In order to determine long-term effects of B cell depletion on immune responsiveness a second round of immunizations should be performed after the B cell repopulate (the same level as before rituximab). Immunization 1 would be done with the same influenza vaccine as previous, and immunization 2 eight weeks after the first. Analysis (ELISPOT) of antibody secreting cells would be performed day 0 and 6 for each immunization, except immunizations 1 (day 0 [to see baseline response which should be 0]) and immunization 2 after B cell repopulation (day 0, 6, and 12 [to see that the response has declined to 0]). To study the effects on established long-lived memory, analysis of serum titers of protective and autoantibodies could be performed simultaneously with the ELISPOT analysis.

8 ACKNOWLEDGEMENTS

With the risk of forgetting someone I here express my gratitude to everyone in the labs on the fourth floor at CMM, Karolinska Institutet (Rheumatology Lab and Neuroimmunology Lab). I am happy for all moments of exciting scientific discussions and all other discussions about everything between heaven and earth. I have so many memories to bring with me from the years I have spent in the Rheumatology lab and I am so happy I chose to take this path in life. I doubt I could have experienced this much elsewhere. To everybody: Thank you of all my heart!

I also would like to acknowledge a number of persons, whom without, this thesis would not have happened.

Tina Trollmo and Vivi Malmström, my great supervisors: I have been so lucky to have two supervisors to go to for support and advice. You are my super heroes!
Tina: how could I have done this without you? Not at all to be honest! Thank you for taking me as your Ph.D. student and for patiently guiding me through my projects. You have taught me so much, not at least how to twist and turn things to make them better. I appreciate all your help and advice for "red lines", positive thinking, and scientific life. Thank you for always being supportive and my friend in the roller coaster of life, no matter what.
Vivi: I could not have done this without you either! I am grateful for all the time you spent with me, listening to my new (not always so) great ideas, thoughts, and questions. I hope I have learnt at least some of your exquisite way of linguistic formulations and how to polish the final touch of the manuscripts. Thanks for everything!

Lars Klareskog: Thanks for being my co-supervisor and for encouraging support. I am especially happy for the inspiring and excellent environment for translational research that the Rheumatology Karolinska provides.

Marie Wahren-Herlenius: I am truly happy for your helpful support and advice during my years in the lab. You are my idol! I hope the mentorship lasts for life….

Linn Strandberg and Liselotte Bäckdahl: I am so glad I have such great friends! We have had great times together and I will never forget our fantastic week in New York or our luxurious pampering in Tallinn (I am looking forward to our next spa-trip). Thanks for always being there!

Iva Gunnarsson: You are a great friend, supportive and encouraging. I truly appreciate all your help with my strange questions, our nice talks, and the killing of our thirsts. Let's stay in touch!

Mona Widhe: I am so happy you joined our group! Even though your signature is a moon, you spread sunshine around you. I am so grateful for your always great advice for a good table or figure, and for your contagious laughter! Good luck with your

projects, life and Bollebo. PS: you know I will send you lots of e-mails asking for advice for my tables and figures…. DS.

Eva Jemseby: Always extremely helpful with selecting and retrieving and returning and retrieving and returning and retrieving and returning samples from the bio-bank. You are always there and listening, giving good advice, and being a good friend. I will miss you!

Heidi Wähämaa: "Fru Pärlemor" with a heart of gold. I will truly miss you and all moments (in good and bad) together. Hopefully, we get to go to TV4 soon. When you the least expect things to happen….. Good luck with your projects and I do hope you won't see any more of those anti-spots!

Andreas Fasth: Always having a positive attitude and a big smile! Thanks for all fun times listening to great music. Well, except the evening at Glenn Miller then… But it was a great laugh! I wish you all luck in life and hope we can stay in touch.

The members of the human group: Sukanya – always providing good advice, interesting conversations, and laughter. *Omri* – having o good sense of humor and being smart and supportive. *Duojia* – I still miss your giggling and laughter! *Annika* – thanks for all great advice regarding flow cytometry and for fun times during "human group excursions".

Lotta Averberger: For sharing my passion to de-stress with yoga. Don't forget: Heeeeeela kroppeeennnnnnnn……

Bob Harris: For introducing the fantastic sport Kendo. I just wish I had been a better Kendoka… Arigato.

Riikka Kokkola: I still miss our weekly swimming sessions in the mornings. And after writing this thesis I now understand and share your addiction for kexchoklad and Coca-Cola!

Ingela Loell: I am so happy I found someone to discuss food obsessions and the joy of exercising with! Keep up your great spirit!

Wei Zhou: for great companion during late evening and weekend work. Thank's for all your help with tricky experiments. I wish you all luck with yours!

Margarita Diez: for the jogging tours in Haga Parken which were a boost for both body and soul. Can I consult you when you have your diploma?

Adina: a true heroine! Without you this thesis would definitely not be alive… I am so happy you chose to work with me during the summers. I wish you all luck in the world! Please stay in touch.

Therese Östberg and Alex Espinosa: Thank's for a great time and nice company in San Francisco!

Gunnel Bemerfeldt and Susanne Karlfeldt: What would I have done without you? You are the best, thank you for all help!

Caterina: För din hjälp alla år med autoklavering och diskning, det hade jag aldrig klarat själv och då hade det inte blivit något labbande! För din uppmuntran och våra pratstunder med skratt och allvar. Grazie!

Everyone at the Rheumatology Clinic: För all hjälp med provtagningar och deltagande i mina vaccinationsstudier. Ett stort tack för en fantastisk vecka och många minnen från Vintersol på Teneriffa!

Ett stort tack till mina vänner som jag aldrig hade klarat det här utan:

Sussi: Min livskompis, alltid där när man behöver. Du har gett mig så mycket inspiration och eftertanke om livet! Tack för all förståelse, stöd och uppmuntran. Inte minst för glimtarna i det "riktiga livet i verkligheten", korv och pommes frites, fika och smågodis!

Zandra: Vilken himla tur att vi läste på Komvux samtidigt och att vi kom att sitta bredvid varandra. Förresten, tack för ditt stöd under den tiden! Jag ser fram emot att få ses oftare och att få hitta på kul saker ihop.

Christina: Tack för att du har stått ut med min tystnad under den här tiden. Jag har verkligen saknat bio, fika, vin, middagar, promenader och allt annat kul vi har hittat på.

Linda: Jag har verkligen uppskattat ditt stöd under alla åren jag har varit här. Dina besök på labbet och för att du har kommit och hämtat mig för luncher och fika. Morgonpromenader, eftermiddagspromenader, kvällspromenader... Det bästa sällskapet man kan få, har aldrig tråkigt i ditt sällskap!

Gunnel: Tack för din vänskap och ditt stöd genom eld och vatten, i vått och torrt, vartsomhelst i världen!

Annica: Min kloka storasyster. Vad skulle jag gjort utan dig?!! Det finns inte ord som kan beskriva vad du betyder för mig....

Till min familj: Mamma, Pappa,Mormor, Magnus och Micke - Tack för att ni finns och för att ni tror på mig! Ni finns djupt i mitt hjärta och jag gör allt för er!

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