The expression and function of CD25 B cells in man and in mice

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Cover illustration: *Mouse CD25 expressing B cell from spleen*

ABSTRACT

B cells play an important role both in the physiology and pathology of immune responses by production of antibodies, cytokines and by presentation of antigens. It has been shown that human CD25 expressing B cells display a mature phenotype, perform better as antigen presenting cells but secrete less immunoglobulins.
The aim of this thesis was to investigate human CD25 expressing B cells phenotypically, not only in healthy individuals but also in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Further, we have also studied the phenotype and function of CD25 expressing B cells in mice.
Our results from human studies show that $CD25^+$ B cells display a highly mature and activated phenotype. These cells show high expression of IgA, IgG and CD80, and low expression of IgD and IgM both in healthy individuals and in RA patients when compared to $CD25^-$ B cells. $CD25^+$ B cells from SLE patients did not show any difference in IgA and IgG expression but did expressed higher levels of the costimulatory molecule CD80 when compared to $CD25^-$ B cells. Furthermore, we have shown that 60% of $CD27^+$ B cells in healthy controls, 51% in RA and 48% in SLE patients coexpressed CD25, suggesting that $CD25^+$ B cells belong to the memory B cell population. Finally, $CD25^+$ B cells were able to produce IL-10 in much higher levels than $CD25^-$ B cells.
In mice, CD25 expressing B cells from secondary lymphoid organs showed a mature and activated phenotype, high expression of the costimulatory molecules CD80 and CD86, in addition to IgA, IgG, and the early activation marker CD69. Functionally these B cells were efficient alloantigen presenting cells, they spontaneously secreted high levels of immunoglobulins of the IgA, IgG and IgM classes, were able to become antigen specific antibody secreting cells and produced high levels of different cytokines including IL-4, IL-6, IL-10, and IFN-γ.
In conclusion, we have shown that human CD25 expressing B cells display a highly mature and activated phenotype and belong to memory B cell subset. Also, in mice there was a clear difference both phenotypically and functionally between the $CD25^+$ versus $CD25^-$ B cells. These data suggest that CD25 expressing B cells play a major role not just in the physiology of the immune system but also may participate in the pathogenesis of autoimmunity.

Key words: *B cell, rheumatoid arthritis, systemic lupus erythematosus, CD25, mice*

ORIGINAL PAPERS

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

I. **Sylvie Amu**, **Katrina Strömberg, Maria Bokarewa, Andrej Tarkowski, and Mikael Brisslert**.
CD25-expressing B-lymphocytes in rheumatic diseases.
Scandinavian Journal of Immunology. 2007; 64:182-91

II. **Sylvie Amu, Andrej Tarkowski, Thomas Dörner, Maria Bokarewa, and Mikael Brisslert**
The human immunomodulatory $CD25^{+}$ B cell population belongs to the memory B cell pool.
Scandinavian Journal of Immunology. 2007;66:77-86

III. **Sylvie Amu, Inger Gjertsson, Andrej Tarkowski, and Mikael Brisslert**.
B cell CD25 expression in murine primary and secondary lymphoid tissue.
Scandinavian Journal of Immunology. 2006; 64:482-92

IV. **Sylvie Amu, Mikael Brisslert, and Andrej Tarkowski**
Functional characterization of CD25 expressing B cells in mice
In manuscript

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ABBREVIATIONS

Anti-CCP	Anti-cyclic citrullinated peptide antibody
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B cell activation factor of the TNF family
BCR	B cell receptor
Breg	B regulatory cells
CD	Cluster of differentiation
CpG-ODN	CpG oligodeoxynucleotides
DC	Dendritic cell
EBV	Epstein-Barr Virus
FDC	Follicular dendritic cell
FO B cell	Follicular B cell
GC	Germinal centre
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin-2 receptor
LPS	Lipopolysaccharide
LT	Lymphotoxin
MHC-II	Major histocompatibility complex class II
MLR	Mixed lymphocyte reaction
MZ	Marginal zone
Pam_3Cys	N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine × 3 hydrochloric acid
PAX5	Paired box protein 5
PBMC	Peripheral blood mononuclear cells
RA	Rheumatoid arthritis
RAG1	Recombination-activation gene 1
RF	Rheumatoid factor
RSV	Respiratory syncytial virus
S. aureus	*Staphylococcus aureus*
sCD25	Soluble CD25
SCF	Stromal cell factor
SLE	Systemic lupus erythematosus
TCR	T cell receptor
TLR	Toll like receptor
TNF	Tumour necrosis factor

INTRODUCTION

The immune system is formed of two highly overlapping parts, the innate and adaptive immunity. The innate immunity is non-antigen specific and reacts early on against, for example, invading microbes. The adaptive immunity on the other hand refers to an antigen-specific immune response, is memory driven, and leads to affinity maturation of subsequent responses. The most important task of the immune system is to defend the organism against foreign invaders and to guarantee its survival. It is also important that the immune system distinguishes between self and non-self to avoid reactions against the host's own tissues. In order to do this, the immune system has evolved different mechanisms resulting in tolerance. If tolerance is lost in the immune system this may lead to the development of autoimmunity or allergy.

B cells play an important role in both the innate and the adaptive immune system by producing antibodies, cytokines and presenting antigens to the T cells [1, 2]. B cells can also play a pathogenic role in adaptive immune responses by producing autoantibodies, proinflammatory cytokines and participate in autoantigen presentation, which give them a role as one of the major players in the development of autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [3-5]. Understanding the B cell subsets in order to investigate their role in health and disease is of great importance, especially for the development of B cell specific therapies. The aim of this thesis is to address a B cell subset of human and mice characterised by its expression of surface IL-2Rα (CD25) and to investigate its role in the immune system.

The first two papers in this thesis deal with the phenotypical characteristics of CD25 expressing B cells in patients with RA, SLE and in healthy controls. **Paper I** shows that CD25 expressing B cells display a mature and activated phenotype both in RA and SLE patients, and

healthy controls. Comparing $CD25^+$ B cells in RA and SLE patients to $CD25^+$ B cells in controls we found that $CD25^+$ B cells in patients were more mature and activated. **Paper II** shows that CD25 expressing B cells in both RA and SLE patients and in healthy controls belong to the memory B cell subset.

The next two papers deal with CD25 expressing B cells in mice. **Paper III** describes the presence of CD25 expressing B cells in primary and secondary lymphoid organs and shows that CD25 expressing B cells in secondary lymphoid organs are highly mature and activated as compared to CD25 negative B cells. Finally, **Paper IV** describes functional properties of CD25 expressing B cells in healthy mice, showing that $CD25^+$ B cells are efficient alloantigen presenting cells, they spontaneously secrete high levels of immunoglobulins, are able to become antibody producing cells upon antigenic challenge and produce high levels of various cytokines as compared to $CD25^-$ B cells.

B CELL DEVELOPMENT

B cells develop from haematopoietic stem cells located in the liver of foetus and later in the bone marrow of adults. Several transcription factors including early B cell factor, PU.1, E2A and paired box protein 5 (PAX5) are important for early development and commitment of stem cells to the B cell lineage [6]. Also, the stromal cells in bone marrow and their products like stromal cell factor (SCF) and chemokine CXCL12 are important for the B cell development. The cytokine IL-7 has been shown to be necessary in the development of B cells in mice but not in humans [7].

A successful rearrangement of immunoglobulin (Ig) heavy (H) and Ig light (L) chain loci will ensure that the developing B cells express a membrane-bound antibody as an antigen receptor, called the B cell receptor (BCR). The immunoglobulin heavy chain locus rearrangement in pro-B cells leads to formation of a precursor BCR which express the transmembrane form of the μ IgH together with two proteins, λ5 and VpreB, forming surrogate light chain and two invariable accessory chains, Igα and Igβ [8, 9]. Expression of the pre-BCR on the cell surface will signal for starting of light chain rearrangement and once this is completed, the immature B cell will express a complete IgM molecule on its surface [10, 11]. Both in humans and mice, there are two light-chain loci κ and λ, where κ tends to rearrange before the λ locus. Immature B cells go through a central tolerance checkpoint where autoreactive B cells will be killed or forced to rearrange their BCR (so called receptor editing), by reactivating their recombination-activating genes (RAG1) and (RAG2) and expressing a new Ig light chain and thus acquiring a new specificity [12, 13]. B cells that survive this selection are called mature B cells and will undergo further differentiation resulting in expression of IgD, in addition to IgM on their surface. After this step mature B cells will leave bone marrow.

Functional B cell receptors that have been formed, have high diversity that is generated in different ways. There are multiple copies of V (variable), D (diversity) and J (joining) gene segments of heavy chains and V and J gene segments of light chains that can be combined in multiple ways to give rise to B cell receptors with different specificity. Also, the addition and subtraction of nucleotides in the joints between gene segments during the process of immunoglobulin gene rearrangement results in further diversity. All rearrangement of Ig-genes occurs randomly, generating an enormous variability of the BCR [14].

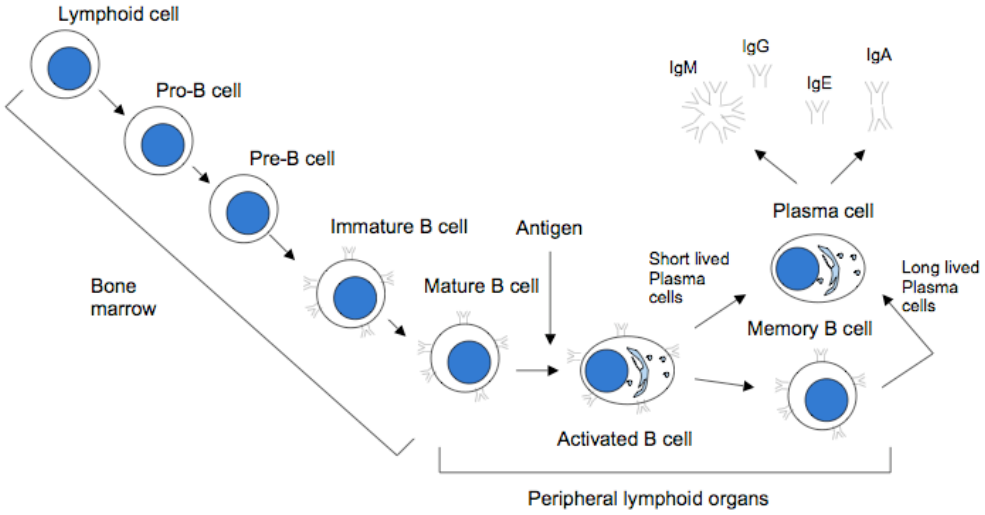

Figure 1: Stages of B cell development in bone marrow and peripheral lymphoid organs.

There are other surface markers than IgM and IgD that will be expressed during the B cell development. B220 (CD45) and CD19 which are involved in B cell receptor signalling are present on both human and mice cells while CD43, HSA and AA4.1 are expressed on very early B cells all the way to the immature B cells in mice [15]. For human the B cells makers CD10, CD24 and CD38 have been shown to be expressed on early developing B cells [16]. However, the function of these molecules for the B cell development is presently unknown.

CD25 and IL-7 receptor are expressed during the development of B cells in the bone marrow and may function as growth factor receptors in mice [7, 17]. We have recently shown that B cells expressing CD25 in bone marrow in mice are indeed of immature phenotype. $CD25^+$ B cells coexpress high levels of AA4.1 when compared to $CD25^-$ B cells and have also lower expression of surface IgM and IgD ([18] paper III). The latter finding may suggest that $CD25^+$ B cells, as they develop in the bone marrow will lose their expression of CD25 and leave this organ as $CD25^-$ B cells. Whether this also is true for human B cells is presently unknown.

The development of B cells will continue in the secondary lymphoid organs. Mature B cells go through another tolerance checkpoint in the peripheral organs where B cells that still recognise self antigens in the absence of specific T cell help will become anergic [19]. A small population of mature B cells home to the spleen and remain there as non-circulating marginal zone B cells. However, most of the mature B cells start to circulate between follicles in the spleen, the lymph nodes, and bone marrow until they meet the BCR specific antigen. There after they will undergo further maturation and become memory B cells, plasma cells, or die.

In the humoral immune response antibody production is of great importance. B cells have to become mature, have T cell help and meet their antigen in order to go trough somatic hypermutations and become antibody-producing cells. Mature B cells will then go through two genetic alterations in their immunoglobulin gene locus, a) class switch recombination (isotype switching) and b) somatic hypermutation (affinity maturation) [20-23] in order to become antibody producing cells. Somatic hypermutation introduce point mutations in the V region of heavy and light chain genes resulting in mutated BCR on mature B cells. This does not affect the C (constant) region gene of the BCR. B cells carrying immunoglobulins generated by

somatic hypermutation, will be able to bind the antigen with higher affinity than the original BCR and will be selected for to mature and eventually become antibody-secreting cells. This will give rise to high affinity maturation of the antibody population. Marginal zone (MZ) B cells and B-1 cells can become antibody producing cells without T cell help [24].

IMMUNOGLOBULINS

B cells are well known for their ability to secrete antibodies. The secreted antibodies display the same specificity as the BCR on the B cells. Antibodies are Y shaped and consist of two heavy chains and two light chains. The four chains forming an antibody are assembled in two Fab arms and the Fc region [25]. The chains are identical giving the antibody two alike antigen-binding sites. There are two types of light chain, lambda (λ) and kappa (κ), a B Cell will express only Igλ or Igκ, but not both. However, there is no known functional difference between them. The isotype of the antibodies is determined by the heavy chain portion. There are five major classes of heavy chain that give rise to five different immunoglobulin isotypes, immunoglobulin M (IgM), IgD, IgA, IgG and IgE. IgA antibodies can further be divided in to two subsets: IgA1 and IgA2. IgG may further be divided into four subclasses in humans (IgG1, IgG2, IgG3 and IgG4) and in mice (IgG1, IgG2a, IgG2b and IgG3). IgA, IgG and IgE are monomers when secreted, while IgA is a dimer in mucosal environment and IgM forms a pentamer [26]. Antibodies have multiple immunological functions including opsonization of microbes, immune complex formation, neutralisation of toxins and complement activation.

B CELLS IN THE INNATE IMMUNE SYSTEM

B cells are specialised to produce high affinity antibodies and thereby considered to be part of adaptive immune system. However, there are B cell subsets, such as B-1 cells and marginal zone (MZ) B cells that also participate in innate immune responses by producing natural low affinity antibodies (antibodies of IgM isotype that are present in the body without any apparent evidence of infection) and may play a role in the early defence against foreign antigens such as encapsulated bacteria.

The B-1 subset of B cells

The B-1 subset of B cells produce low affinity antibodies, displays a different phenotype and location in the body as compared to conventional B cell (B-2) [27, 28]. In mice the B-1 cells are phenotypically characterised as $IgM^{high}IgD^{low}CD23^{-}B220^{low}$ with Mac-1 expression on peritoneal but not on splenic B-1 cells [29]. These B cells can be further divided into B-1a, expressing CD5 and B-1b, which do not express CD5. It is unknown if the B-1a and B-1b B cells are derived from the same or different progenitor cells [30]. The existence of this B cell subset in humans is still a matter of debate.

It has been shown that surface CD19 plays an important role in B-1 cell development. Mice deficient in CD19 have impaired B-1a cell development and lower production of natural antibodies. Overexpression of CD19 results in an increased B-1a cell development but impaired B-1b development as well as an impaired adaptive immune responses to antigens [31-34]. Transgenic and gene targeted mice studies have shown strong evidence that BCR signalling is critical for B-1 cell development. Mutations that enhance BCR signal (deletion of SHP-1, CD22, CD72, Lyn) result in an expansion of the B-1 compartment while mutations that disrupt the BCR signal (deletion of

CD19, CD21/35, PI3-kinas, vav-1, BLNK/SLP-65, btk) result in a decreased B-1 compartment, as reviewed in [27, 28]. B-1 cells are responsible for the early humoral responses against different pathogens. They are primary antibody producers in response to T cell independent type 2 antigens, recognise common bacterial antigens like certain bacterial cell wall components and provide the first line of defence against these pathogens.

Antibodies produced by B-1 cells have low affinity and are usually polyreactive. B-1 cells produce mostly antibodies of IgM isotype. In fact, one study has shown that B-1a cells produce natural antibodies against capsular polysaccharides while B-1b cells produce antibodies induced by antigen specific response against the same antigen [34]. It has also been shown that peritoneal B-1 cells are main producers of T cell independent IgA in the gut [35]. We have shown that CD5 positive B cells are present in Peyer´s patches of healthy mice ([18] paper III). It may be that these cells mature in the peritoneal cavity or spleen and home to the gut keeping their expression of CD5.

Marginal zone B cells

Blood enters spleen through a structure called the marginal sinus, and flows through the marginal zone and venous sinuses before returning to the circulation [36]. Cells located in the marginal zone are constantly in contact with large amounts of blood and consequently with any antigen that may have entered the circulation. There are different cell types found in the marginal zone of a spleen. For example, two populations of macrophages (marginal metallophilic macrophages and marginal zone macrophages), fibroblasts, dendritic cells (DC) and B cells [37]. Blood born $CD11c^{low}$ immature DC has been shown to efficiently capture bacteria and transport them to the MZ of the spleen, providing bacterial antigens to MZ B cells. These DCs produce B lymphocyte stimulator (BAFF) and a proliferation-inducing ligand (APRIL) providing critical survival signals to antigen specific MZ

B cells promoting them to differentiate into IgM producing plasmablasts [38].

The location of MZ B cells permits them to respond to blood born pathogens and initiate a T cell independent antibody response to encapsulated bacteria [37]. MZ B cells do not belong to the re-circulating pool of lymphocytes in spleen and represent only about 5% of splenic B cells. These B cells can be characterised by their surface expression of $IgM^{high}IgD^{low}CD23^{low}CD21^{high}CD38^{high}CD9^{high}$ [39, 40]. CD21, a complement receptor, is highly expressed on MZ B cells. CD21 is part of the BCR and together with CD19 and CD81, it lowers the threshold for BCR activation of B cells. CD21 binds C3d coated antigen, this complex then leads to cross-linking of CD19 and CD81 and B cell activation [41]. MZ B cells also express higher levels of LFA-1, α4β7 integrin, and B7 proteins (CD80 and CD86) [24, 42]. LFA-1 and α4β7 integrin are homing receptors guiding the B cells to the site where they are needed. It has been shown that MZ B cells, compared to follicular B cells, have the ability to efficiently upregulate costimulatory molecules in response to early activation *in vitro*, proliferate much faster in response to low doses of polyclonal mitogens and differentiate into plasma cells within hours of activation [24].

We have investigated if MZ B cells in healthy mice express CD25. However, on comparison of $CD25^+$ versus $CD25^-$ B cells in spleen, the expression of IgD was high on both subpopulations making it difficult to classify them into either MZ or follicular B cells ([18] paper III).

B CELLS IN THE ADAPTIVE IMMUNE SYSTEM

B-2 cells

B-2 cells or follicular B cells (FO B cells) are important for long-term T cell dependent antigen responses. The B-2 cells may switch isotype and differentiate into high affinity memory B cells and to long-live plasma cells. The high affinity memory generation is associated with the capacity of B cells to undergo somatic hypermutation.

Regulatory B cells

Recent experimental studies have indicated the existence of a distinct B cell subset that suppresses the development and enhances the recovery from an adaptively mediated immune inflammation by production of IL-10. These B cells have now been termed regulatory B cells (Breg). IL-10 has anti-inflammatory activities that includes down regulation of costimulatory molecules on the antigen presenting cells (APCs), which are directly involved in the differentiation of Th1 response and in the inhibition of antigen-specific T cell proliferation [43, 44]. IL-10 producing B cells have been identified in the diseases models such as experimental autoimmune encephalomyelitis (EAE) [45], experimental RA [46], experimental SLE [47, 48] and in mice infected with *Schistosoma mansoni* [49]. *In vitro* studies have shown that even human B cells have the ability to produce IL-10 [50]. We have shown that human $CD25^+$ B cells produce IL-10 when stimulated with CpG-ODN ([51] paper II). In addition, our results from mice studies show that $CD25^+$ B cells secrete IL-10 following CpG-ODN, LPS or Pam_3Cys stimulation ([52] paper IV), suggesting that $CD25^+$ B cells may perhaps be a part of both regulatory and innate B cell responses in human and mice.

Memory B cells

During the last part of T cell dependent immune responses to an antigen the immunological memory is formed. Immunological memory in the humoral immune system is based on two different pathways of cellular differentiation. One results in the generation of antigen independent, long-lived memory cells that respond to the new antigenic challenge by rapid differentiation into plasma cells. The other pathway results in generation of a population of long-lived antibody producing plasma cells that are independent of sustained antigenic challenge.

Memory B cells have been characterised by expression of CD27 in humans [53, 54]. CD27, a type 1 glycoprotein, is a member of the tumour necrosis factor (TNF) receptor family with unique cysteine rich motifs and is expressed on B and T cells. In adults, 32% of circulating B cells express CD27 as compared to 4% of B cells in cord blood. Morphologically, CD27 positive B cells are larger with abundant cytoplasm when compared to CD27 negative B cells. Functional difference has also been identified between CD27 positive and negative B cells. When stimulated with *Staphylococcus aureus* Cowan strain (SAC) plus IL-2 *in vitro*, CD27 positive B cells are quickly activated and produce high levels of IgA, IgM and IgG [55]. We have shown that human CD27 expressing B cells coexpress CD25, figure 2. $CD25^{+}CD27^{+}$ memory B cells are mature and immunoglobulin class switched ([51] paper II). The expression of CD25 on memory B cells can be an indication that these cells respond to IL-2. Alternatively, CD25 may be a specific marker for a memory B cell subpopulation. It has been shown earlier that neutralization of CD25 prior to mixed lymphocyte reaction (MLR) will abolish the antigen presenting ability of $CD25^{+}$ B cells [56]. This is strong evidence that CD25 has other roles on the B cell surface than only to be a part of the high affinity IL-2 receptor.

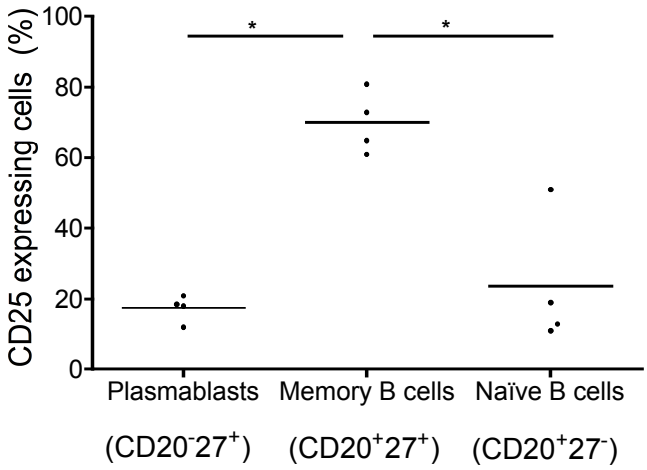

Figure 2: The expression of CD25 on different B cell subsets.

Memory B cells produce high affinity antibodies, this is primary achieved by somatic mutation of Ig genes during B cell development. It has been demonstrated that the majority of $IgM^+IgD^+CD27^+$ peripheral blood B cells carry mutated V-region genes compared to $IgM^+IgD^+CD27^-$ B cells [54]. B cells need to be activated by antigens via their B cell receptor, cytokines and direct cell-to-cell contact in germinal centre (GC) or by Toll-like receptors (TLRs) to develop into memory B cells or plasma cells. GC formation and CD40-CD40L interaction is necessary for somatic hypermutations and generation of long-lived memory B cells [57, 58].

In contrast to human memory B cells there are no good phenotypic markers for memory B cells in mice. So far analyses of somatic hypermutations have been the only way to identify memory B cells in mice. However, using a transgenic mice model Anderson et al have recently shown that $CD80^+CD35^{low}$ B cell population in mice is somatically mutated belonging to the memory B cell population [59]. We have shown that $CD25^+$ B cells in mice have properties of memory B cells. $CD25^+$ B cells secrete and express higher levels of IgA and IgG

immunoglobulins. Furthermore, in response to immunisation $CD25^+$ B cells produce higher levels of antigen specific antibodies of IgG and IgM subclasses ([52] paper IV). We suggest that CD25 can be used as a memory marker for mice B cells, however it is important to point out that genetic studies will be needed to confirm that.

Plasma cells

The final differentiation state for a B cell is to become an antibody secreting plasma cell. Marginal zone B cells responding to T cell independent antigens and circulating mature follicular B cells responding to T cell dependent antigens are the first B cells to differentiate into plasma cells. Plasma cells are formed either by follicular or marginal zone B cells in early extrafollicular response. These cells do not go through somatic mutation of their immunoglobulin genes and are considered to be short-lived, IgM producing plasma cells. In contrast, plasma cells originating from germinal centre reactions go through somatic mutations and may become long-lived plasma cells that home for example to bone marrow.

Antibodies secreted by plasma cells have high affinity for a specific antigen and are an important part of the humoral adaptive immunity. However, the half-life of the antibodies in blood is short and is estimated to only few weeks. Since antigen specific antibodies can be detected for decades after immunization this indicates a continuous *de novo* production suggesting regeneration or a steady state of plasma cells. Exactly how long a plasma cells can live is unknown, but it has been suggested that they can survive between a few days and up to years [60]. Many studies have been performed in order to explain the stable long time concentration of specific antibodies found within the circulation. One theory is that there is a constant generation of new short-lived plasma cells from activated memory B cells. An existing mechanism for generating short-lived plasma cells from

memory B cells may be the presence of low quantities of an antigen over a long period of time [61]. Also, memory B cells can proliferate and differentiate into plasma cells in response to polyclonal stimuli via their Toll-like receptors and cytokine receptors [62]. Long-lived plasma cells that have homed to the bone marrow and constantly secret antibodies are also part of this immunological memory.

During differentiation to plasma cells, B cells lose the expression of surface molecules such as surface immunoglobulins, major histocompatibility complex class II (MHC-II), CD19 and CD20. Another surface marker CD38 is kept on human plasma cells but is not detected on mouse plasma cells. The only protein that is increased on plasma cells in man and mouse is Syndecan-I (CD138) [63, 64].

Developing plasma cells have to exit the germinal centres and migrate to the bone marrow, to the mucosa or to the sites of inflammation [65]. As plasma cells are developing, the chemokine receptor 5 (CXCR5) and CC-chemokine receptor 7 (CCR7) expression is down regulated. These chemokines control germinal centre trafficking and down regulation of these molecules allows post-germinal centre cells to exit from the follicles. The expression of CXCR4 is important for plasma cells to enable homing to the splenic red pulp, lymph node medulla and to the bone marrow [65, 66]. Human and mouse plasma cells that home to bone marrow express CXCR3, a chemokine receptor that also is upregulated on plasmablasts upon stimulation with IFN-γ which is mainly produced by inflammatory cells, indicating that IFN-γ might be important for attraction of plasmablasts to the inflamed tissue [67].

B CELLS IN THE AUTOIMMUNITY

When the self-tolerance is broken and cells of the immune system recognise self proteins as foreign, the immune system may start an attack that will result in inflammation and destruction of the affected tissue giving rise to an autoimmune disease. B cells mediating autoimmune diseases can be specific for one organ like in diabetes mellitus [68] or may involve many organs as in SLE [69].

One of the systemic autoimmune diseases is RA. RA is characterised by chronic inflammation of the synovial membrane, resulting in synoviocyte proliferation, cartilage injury and bone erosion which can further result in deformations of joints [70]. The exact mechanisms of RA pathogenesis remain unclear, but involvement of cells like T cells, B cells, macrophages, neutrophils and synovial fibroblasts has been shown to be of importance [4, 71, 72].

SLE is also a systemic autoimmune disease affecting joints, skin, kidneys, lungs, blood cells, blood vessels and the central nervous system. B cells, T cells and monocytes are involved in the pathogenesis of the SLE but the exact mechanism behind the development of the disease is not clarified. Genetic factors, sex hormones, defective immune regulation such as clearance of apoptotic cells and immune complexes, loss of immune tolerance, defective B cells suppression and environmental factors has been considered as some of the factors that are required to trigger the disease [73].

B cells have been shown to be a part of RA and SLE pathogenesis [4, 69]. Exactly how they participate is presently unclear, however some suggestions have been made regarding B cells ability to produce autoantibodies, present antigen to T cells and secret proinflammatory cytokines [1, 74]. However, B cells targeted therapy can successfully ameliorate these conditions [75-77].

During the B cell development in the bone marrow randomly selected heavy and light chains are combined to form functional BCR. The specificity of combined chains is impossible to predict and it has been suggested that almost 50% of BCRs may carry high degree of autoreactivity [78]. To have autoreactive cells in the body is not desirable so self-tolerance control mechanism in bone marrow and later in the periphery exists. Autoreactive B cells will be deleted by receptor editing, clonal deletion, or inactivation (anergy). RA and SLE patients display a defective B cell tolerance in more than one of these mechanisms [79]. The levels of BAFF in sera and synovial tissue of RA patients are high and may cause the inappropriate survival of autoreactive B cell clones [80]. High levels of BAFF have also been shown to drive autoantibody production in RA and SLE [81]. The survival and proliferation of autoreactive B cells will result in an increased production of autoantibodies.

Autoantibodies

Rheumatoid factor (RF) is one of the autoantibodies that binds the Fc portion of IgG. RFs can be of any isotype but IgM is the most common one. They are found in about 70-80% of RA patients but may be detected in other autoimmune diseases, systemic infections as well as up to 4% of healthy individuals [82]. Other autoantibodies that may play a role in the pathogenesis of RA are antibodies to cyclic citrullinated peptides (anti-CCP) [83], anti-keratin, anti-filaggrin and many more, reviewed in [84]. In SLE, the most common autoantibody is antinuclear antibodies with a specificity to anti-double stranded DNA [85]. Autoantibodies form large immune complexes that activate B cells and follicular dendritic cells via Fc receptors and complement receptor 1, CR1 (CD21) and CR2 (CD35) [86, 87]. TLRs, together with a second signal delivered by BCR ligation have been shown to be important in the induction of RF producing B cells. It has been shown

that memory B cells express TLRs and can be activated by ligation of the TLRs alone [82]. B cells in most autoimmune diseases are mature and differentiated, it is thus possible that B cells producing autoantibodies will be activated through TLRs without the need for BCR ligation (T cell independent) and produce low affinity autoantibodies. The memory B cells ($CD19^+CD27^+CD25^+$) we have investigated from RA and SLE patients display a more mature phenotype ([51] paper II). Even if we lack the functional data regarding these cells it may be that CD25 expressing memory B cells are part of the memory B cell pool that may have the ability to become activated without T cell help and secrete autoantibodies and/or differentiate to autoantibody secreting plasma cells.

Cytokines

In the past B cells have not been considered to be a major source of cytokines. However, many studies have shown that B cell can produce a wide spectrum of cytokines. Cytokines are polypeptides produced by different cells in response to foreign antigens, mediating and regulating the immune system and inflammatory reactions. Peripheral stimulated B cells from healthy individuals secrete IL-6, TNF and lymphotoxin (LT) upon BCR and CD40 engagement [50]. These cytokines act not only as differentiation and autocrine factors but may also amplify the immune responses. IL-10 produced by B cells suppresses harmful immune responses and regulates the balance of Th1/Th2 [46]. IL-10 can also activate follicular dendritic cells (FDCs) and stimulate B cells. The effect of IL-10 on mice B cells is still discussed. It has been shown that IL-10 inhibits the antibody production by B cells [88] but also that IL-10 increases the expression of IgG3 in LPS activated B cells [89]. IL-10 is known to have an inhibitory effect on other cells of immune system like the T cells. Reports on regulatory B cells producing IL-10 has shown that these cells play a key role in controlling autoimmunity [45, 90]. We have

shown that both human and mouse CD25 expressing B cells have the ability to produce IL-10 after stimulation by TLR agonists ([51, 52] papers II and III). This can give them a role in the regulation of autoimmune diseases. Cytokines like IL-6, produced by activated human B cells, functions in an autocrine fashion by inducing differentiation into antibody producing B cells on those B cells that do express IL-6 receptor [91, 92]. The bacterial stimuli CpG-ODN induced production of IL-6 from mice B cells [93]. Also, INF-γ can act directly on the B cells to produce antibodies predominantly of IgM isotype, with IgG3 and IgG2a being the majority of non-IgM antibodies secreted [94].

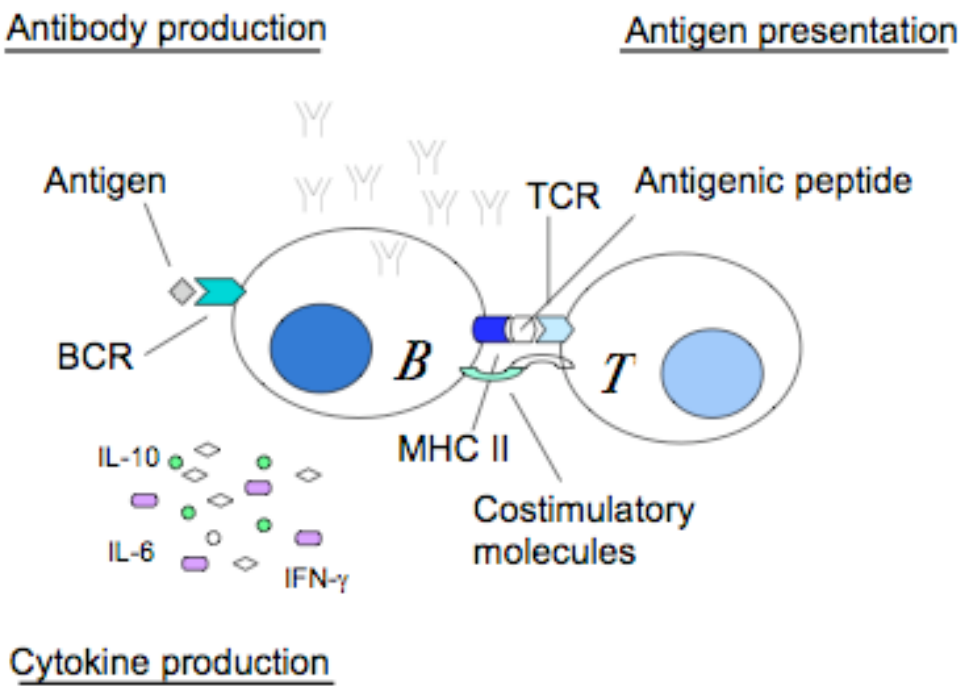

Figure 3: The function of a B cell in immune system and disease.

Chemokines

Binding of LT-β to its receptor induces expression of chemokines (CCL19, CCL21, CXCL12 and CXCL13) and adhesion molecules that

regulate lymphocyte homing and compartmentalization in lymphoid tissues. Chemokines are small proteins that stimulate the migration of phagocytes and lymphocytes. They have a central role in early inflammatory response directing cells to the inflamed tissue. It has been shown that B cells from RA and SLE patients have a distinct expression pattern of the chemokine receptors CXCR3, CXCR4 and CXCR5 when compared to healthy controls [95]. This is an indication that there are changes in lymphocyte trafficking in these patients. CXCL13, involved in the recruitment of B cells and LT-β have been shown to contribute to the formation of ectopic lymphoid tissue in RA patients [96, 97].

Antigen presentation

Another important function of B cells is the ability to present antigen. B cell takes up, internalize and process antigen into small peptides, which are then presented on MHC-II molecules. One of them, HLA-DR4, have shown a strong association with occurrence of RA [98]. RF positive B cells have also been shown to be antigen presenting cells [99]. Both human and mouse CD25 expressing B cells have the ability to present alloantigen in contrast to $CD25^-$ B cells [56] ([52] paper IV). We have not examined the ability of these cells to present autoantigen in RA or in SLE as it has been shown that neutralisation of CD25 on the surface of human B cells before performing MLR abolished the ability of these cells to present alloantigen. We hypothesise that $CD25^+$ B cells also may present autoantigen in RA and SLE.

IL-2 RECEPTOR

Studies in the eighties of human spleen, tonsils, and blood B cells reported that a monoclonal antibody reacting with IL-2 receptor molecule expressed by activated T cells (anti-Tac) also bound *S. aureus* activated B cells. These B cells proliferated in response to IL-2 and IL-2 induced proliferation was inhibited by the addition of anti-Tac in the cell culture [100, 101]. Lowenthal *et al,* showed that mitogen activated mouse B and T cells expressed both high and low affinity IL-2 receptors [102].

The interleukin-2 receptor (IL-2R) is expressed on B cells, T cells, NK cells and eosinophils [103-105] and also on certain tumour cells [106]. Interleukin-2 (IL-2), the ligand for IL-2R, is a cytokine with distinct activities on the immune system stimulating proliferation of B and T lymphocytes, and NK cells.

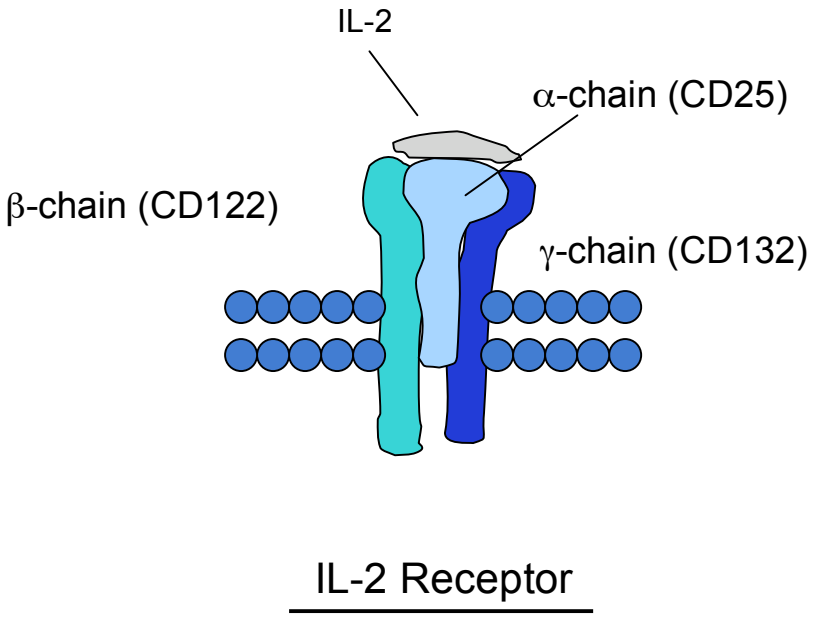

Figure 4: High affinity IL-2 receptor.

The receptor consists of three distinct sub-chains, alpha (IL-2Rα, CD25, Tac antigen), beta (IL-2Rβ, CD122) and gamma (IL-2Rγ, CD132). CD25

alone binds IL-2 with low affinity but is not capable of signal transmission. CD122 and CD132 together form an intermediate affinity IL-2 receptor complex. The high affinity IL-2R is formed when all three subunits CD25, CD122 and CD132 come together [107]. CD122 and CD132 are also part of other cytokine receptors. CD122 is a part of the receptor for IL-15 and CD132, (also known as common gamma chain), is a part of IL-4, IL-7, IL-9, IL-15 and IL-21 receptors [108]. When IL-2 is present in low concentration it will bind to high affinity IL-2R, whereas IL-2 at high concentrations binds intermediate and high affinity IL-2Rs on murine B cells [109].

CD25 FUNCTION

IL-2Rα (CD25) is a 55 kDa polypeptide with an extracellular domain (219 amino acids), transmembrane domain (19 amino acids) and cytoplasmic domain (13 amino acids). The gene encoding CD25 is located on chromosome 10 in humans and chromosome 2 in mice and is highly conserved between the species [110]. In the thymus, thymocytes that go through a stage that marks the first step in T cell receptor (TCR) rearrangement and irreversible commitment to the T cell lineage, express CD25 [111]. CD25 is also expressed on mouse bone marrow developing B cells (pre-B cells) [112]. These B cells do not proliferate in response to IL-2 [17]. So far the function of CD25 on these early B cells is unknown. Neither is it known if human B cells express CD25 during the development in bone marrow and if they do during which part of the differentiation. One study using different human cells lines mirroring B cell maturation stages have shown that CD25 was expressed in the final stage of B cell lineage maturation, suggesting that the IL-2 and IL-2R was not required early during human B cell development [113]. However, it is important to keep in

mind that B cell lines, even if very similar to the normal B cells have important differences.

Mice having disruption in the CD25 coding gene have similar phenotype as mice that lack the IL-2 gene [114, 115]. This suggest, that even if CD25 expression is not required for binding of IL-2 and further mediating of intracellular signalling, it will still be needed as a part of high affinity IL-2 receptor to mediate biological effect of IL-2 for the cell. In fact lymphocyte development in bone marrow is normal in IL-2/CD25 deficient animals. However, young mice develop polyclonal expansion of the peripheral lymphoid compartment with increased numbers of all major cells types suggesting a global defect in lymphoid homeostasis. B cell activity in these animals is increased, detected by higher levels of immunoglobulins in the serum [114]. Several mouse studies have shown that IL-2/CD25 deficient mice develop different kinds of autoreactivity, they produce autoantibodies to red blood cells, antibodies specific for colonic tissue, many develop inflammatory bowel disease, and antibodies to DNA [107, 114]. In a mouse model of collagen type II induce arthritis when $CD25^+$ cells were depleted, a more sever disease development was observed. However, there was no difference in the antibody titers against collagen in the absence of CD25 expression [116], suggesting a role for $CD25^+$ T cells but a normal function of $CD25^+$ B cells.

CD25 deficiency in humans is more severe than in mice. Patients suffer from recurrent infections and lymphocyte infiltration in multiple tissues. It has been shown that humans deficient in CD25 have a decreased apoptosis of developing T cells in thymus which affects the negative selection and results in release of autoreactive T cells causing inflammation in different tissues [117]. Although the effect of CD25 seems to be important for T cells, the somehow normal levels of immunoglobulins in serum indicates that CD25 expression in

B cells is not needed for class switch or secretion of immunoglobulins by B cells.

IL-2 has been identified as a T cell growth factor promoting T cell growth *in vitro*. Signals provided by the TCR upregulate CD25 expression, resulting in high affinity IL-2R expression, increased secretion of IL-2, and also the expression of Jak3, a critical signal inducing proliferation of T cells. In addition, CD25 can also be upregulated by TCR independent signalling through IL-1 and TNF [107]. Once the T cells express the high affinity IL-2R they will respond to IL-2 and start to proliferate. Importantly, IL-2 together with T cell help also promotes proliferation and differentiation of B cells, including secretion of IgM in primary B cell cultures [103]. Expression of CD25 on mature B cells have been considered as a marker for activated B cells [103]. However, most of the studies showing expression of CD25 on mature B cells are performed following B cell stimulation *in vitro*. The question if this is true *in vivo* is not resolved. In fact, about 30% of circulating B cells in healthy individuals express CD25 [56]. Whether, CD25 expression on B cells changes during an infection is still unknown in humans. It has been shown that neutralisation of CD25 on human B cells prior to performing MLR will abolish the $CD4^+$ T cell proliferation suggesting the role for CD25 in alloantigen presentation [56]. This can be an indication that CD25 may have other function than only being a part of the high affinity IL-2R. In healthy mice 2% of splenic B cells express CD25 ([18] paper III). Interestingly *in vitro* exposure of splenic B cells to medium alone increases the CD25 expression to around 25%. Certain stimuli, for example LPS, CpG-ODN, and Pam_3Cys, increase expression of B cell CD25 even more (our unpublished data). In contrast, *in vivo* experiments using *S. aureus* infected mice did not show any major upregulation of CD25 on B cells. B cells need CD25 expression for their differentiation but this may be strictly regulated during an infection

and the reason why B cell CD25 expression still is limited ([52] paper IV).

Soluble CD25

CD25 is present not only as surface molecule but also in a soluble form. Serum levels of soluble CD25 (sCD25) has been shown to be higher in individuals with RA and SLE compared to normal controls. In SLE, the presence of sCD25 in the serum increases with disease activation and drops when the patient recovers [118]. *In vitro*, the release of sCD25 is higher from peripheral blood mononuclear cells (PBMCs) in RA patients but less from cells of SLE patients when compared to controls. However, the serum levels were high in both RA and SLE patient when compared to controls. In RA patients it has been suggested that high serum levels of sCD25 will be a result of an increase in the spontaneous release of this protein from the activated cells, while in SLE patients this can depend on reduced clearance of the complex from the blood [119]. Even if these studies do not show any data regarding B cells alone, the involvement of these cells cannot be ruled out. However, the high concentration of sCD25 in biological fluids has so far been consider as an indication of T cell activation. There are no studies dissecting exactly which cells that are responsible for the release of sCD25. It can be speculated that in some autoimmune diseases, B cells can be the main source of sCD25 as they become activated in the presence of autoantigen and differentiate to plasma cells losing many of their surface molecules. Although, sCD25 binds IL-2 with low affinity it still may act as an IL-2 antagonist *in vivo* by binding and neutralising its biological activity. IL-2/sCD25 complex may also form a deposit of IL-2 as the complex clearance from the circulation is much lower in SLE patients [120]. However, the role of sCD25 in the pathogenesis of the diseases such as RA and SLE has not been addressed. In addition, the serum levels of sCD25 is not just

increased in RA and SLE patients. Indeed, studies have shown that sCD25 is also higher during respiratory syncytial virus (RSV) infection [121] and in different forms of cancer [122] .

HUMAN CD25$^+$ B CELLS

Phenotype of CD25$^+$ B cells

There are conflicting data regarding the expression of IL-2R subunits on human peripheral blood mononuclear cells in healthy individuals [56, 123]. In the peripheral blood from healthy individuals approximately 30% of the B cells express CD25 [56]. CD25 expressing B cells display a mature and class switched phenotype, having high expression of membrane bound IgA and IgG, and low expression of IgD and IgM when compared to CD25$^-$ B cells. The expression of MHC-II does not differ between CD25$^+$ and CD25$^-$ B cells. However, CD25$^+$ B cell have higher expression of costimulatory molecule CD80 and CD86 ([124] paper I). Approximately 60% of the memory B cell population identified by the expression of CD27 also coexpresses CD25, suggesting that CD25 expressing B cells are a part of memory B cell subpopulation ([51] paper II). The expression of costimulatory molecules are higher on the surface of these memory cells indicating that they have the ability to present antigens to T cells. Also, the expression of CD138, surface marker for plasma cells, was decreased strengthen the theory of these cells being in a mature/memory stage but not yet differentiated into plasmablasts. It seems like the plasmablasts downregulate the expression of CD25. In fact, B cells that lack CD25 expression have high surface CD138. The phenotype of CD25$^+$CD27$^+$ memory B cells suggests that these are highly activated and class switched memory B cells ([51] paper II).

Function of $CD25^+$ B cells

If human B cells are activated with anti-μ Ab and separated into $CD25^+$ and $CD25^-$ populations, the $CD25^+$ B cells responded to IL-2 but not to B cell growth factor (BCGF). In contrast, $CD25^-$ B cells responded to BCGF but not to IL-2 [125]. This suggest a fully functional high affinity IL-2 receptor on $CD25^+$ B cells. IL-4 and PHA can also induce expression of CD25 on human tonsil B cells, while after stimulation with IL-2, IFN-γ and PWM, B cells do not express CD25. The ability of IL-4 to induce CD25 on B cells was abolished by addition of IFN-γ to the culture [126]. The expression of CD25 on B cells was shown to be upregulated by stimulation of B cells with CpG-ODN, LPS and Pam_3Cys, while GpC-ODN, EBV and polyIC did not upregulate the expression [56]. This shows a high degree of selectivity for expression of CD25 on B cells and suggests that these cells will be able to differentiate without T cell help. It was also shown that the expression of CD25 is NF-κB dependent [56].

Functional differences between $CD25^+$ and $CD25^-$ B cells have been demonstrated in healthy individuals. We have recently shown that RA

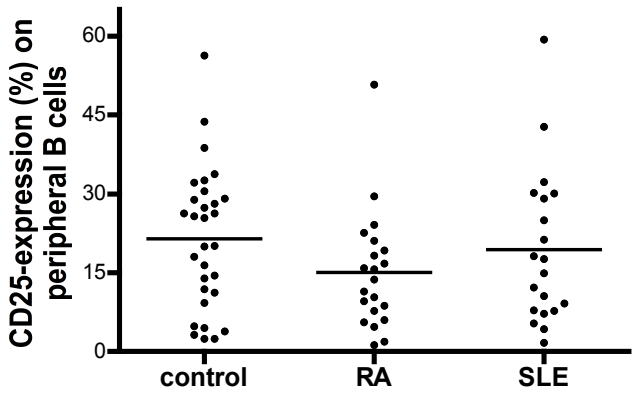

Figure 5: CD25 expression on peripheral B cells of healthy individuals and patients with RA and SLE.

and SLE patients have $CD25^+$ B cells in their circulation and consequently these B cells may be involved in the pathogenesis of these diseases, figure 5 ([51, 124] papers II and I).

Unstimulated human $CD25^+$ B cells secrete low levels of immunoglobulins of IgM, IgG and IgA subtypes when compared to $CD25^-$ B cells. However, they have high expression of membrane bound immunoglobulins of IgA and IgG classes ([56, 124] paper I) . As $CD25^+$ B cells are a part of the memory B cell compartment not only in healthy controls but also in patients with RA and SLE, figure 6, ([51] paper II), it is expected that they will secrete less immunoglobulins until they receive an appropriate signal and differentiate to plasmablasts or plasma cells. Memory B cells have a lower threshold to become activated and differentiate into antibody secreting cells.

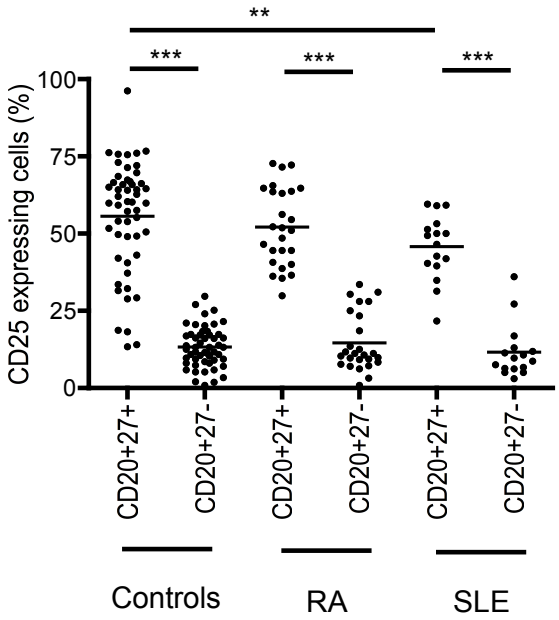

Figure 6: The CD25 expression on memory and naïve B cells of healthy controls and patients with RA and SLE.

Memory B cells can be an important player in autoimmune diseases such as RA and SLE. In these diseases the B cell tolerance is broken

resulting in the generation of autoreactive memory B cells. In addition, the autoantigen is constantly present activating mature and memory B cells. Comparing $CD25^+$ B cells from RA and SLE patients to healthy subjects, we detected that the surface immunoglobulin expression of IgA and IgM in RA, and IgD and IgM in SLE patients were lower compared to healthy controls ([124] paper I). It is possible that $CD25^+$ B cells in patients with autoimmune diseases display a faster turn-over of their surface molecules, leading to increased differentiation.

When analysing the expression of the other two IL-2R subunits, CD122 and CD132, we detected an increase expression of these two subchains on CD25 expressing B cells from RA and SLE as compared to healthy controls ([124] paper I). CD122 and CD132 are, in addition to the high affinity IL-2 receptor, also part of other cytokine receptors IL-4, IL-7, IL-9, IL-15 and IL-21. Cytokines affect B cells in different ways including differentiation to memory B cells or plasma cells and may thereby play a role in the disease. Memory B cells can be long lived, and together with the ability of a high turn-over rate and activation without T cell help they might be a major player in autoimmune disease if reacting to self antigens.

When stimulated with CpG-ODN, $CD25^+$ B cells secreted higher levels of IL-10 in contrast to $CD25^-$ B cells which secreted higher levels of IL-2 ([51] paper II). Why we detect lower secretion of IL-2 from $CD25^+$ B cells may be an effect of autocrine consumption, as CD25 expressing B cells do express high affinity IL-2 receptor. These results indicate that CD25 expressing B cells may have a significant effect on themselves in an autocrine manner initiating proliferation.

$CD25^+$ B cells display high expression of the costimulatory molecules CD80 and CD86, and MHC-II on their surface ([124] paper I). This gives an indication that $CD25^+$ B cell are activated and more efficient to present antigens. Indeed when MLR was performed using allogenic T cells, $CD25^+$ B cells originating form healthy individuals performed

better as alloantigen presenting cells compared to $CD25^-$ B cells. The use of anti-CD25 antibody, neutralising CD25 expression on B cells, prior to MLR almost totally abolished the T cell response indicated that CD25 is not just a part of IL-2 receptor but may also play an important role during antigen presentation [56]. If this is the case for $CD25^+$ B cells of RA and SLE patients is presently unknown. Interestingly, the expression of CD80 was higher on $CD25^+$ B cells from RA and SLE patients compared to $CD25^+$ B cells of healthy controls suggesting similar properties ([124] paper I). Keeping in mind the better ability of $CD25^+$ B cells as alloantigen presenting cells it is reasonable to think that these cells are involved in the pathogenesis of the autoimmune diseases.

The intracellular expression of Foxp3 together with CD25 on T cells have during a long time been consider as a marker for regulatory T cells [127, 128], so the question if CD25 expression on B cells will be a marker of regulatory B cells is obvious. First, $CD25^+$ B cells do not express Foxp3 [56]. Secondly, while T regulatory cells suppress other cells of the immune system, it has been shown that $CD25^+$ B cells activate the immune system. This contradictory findings makes it difficult to use the terminology regulatory regarding $CD25^+$ B cells [56].

It has been shown that $CD5^+$ B cells of RA patient produce autoreactive antibodies after stimulation *in vitro* [129]. However, we did not find any difference regarding CD5 expression between $CD25^+$ and $CD25^-$ B cells in RA and SLE patients ([124] paper I). In mice $CD5^+$ B cells belong to the B1 subpopulation and as mentioned before, this population of B cells form short lives plasma cells which after activation secrete low affinity antibodies [27]. However, the existence of B-1 cells in humans remains to be elucidated.

MOUSE CD25$^+$ B CELLS

Phenotype of murine CD25$^+$ B cells

During the murine B cell development CD25 is expressed on pre-B cells [17]. However, no one has so far investigated the phenotype or function of mature CD25 expressing B cells in healthy mice. Therefore, we phenotypically analysed CD25 expressing B cells from primary (bone marrow) and secondary (spleen, lymph nodes, Peyer´s patches and peritoneal cavity) lymphoid organs and blood from healthy mice. The frequency of CD25$^+$ B cells varied considerably between primary and different secondary lymphoid organs. In bone marrow, 49% of B cells expressed CD25, 15,8% of B cells in peritoneal cavity, 2% of spleen B cells and 0,9% of lymph nodes B cells. There were no CD25 expressing B cells present in blood or Peyer´s patches, figure 7, ([18] paper III). In agreement with published data, CD25 expressing B cells in bone marrow were of naive phenotype expressing high levels of AA4.1 when compared to CD25$^-$ B cells. B cells expressing CD25 in spleen, lymph nodes and peritoneal cavity cells on the other hand, display a more mature and activated phenotype.

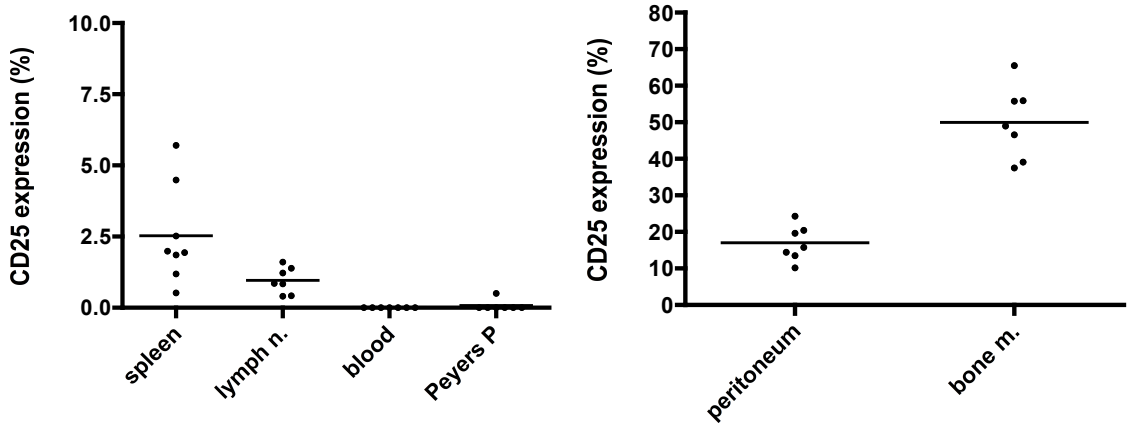

Figure 7: Frequency of CD25 expression on B cells from primary and secondary lymphoid organs from mice.

These $CD25^+$ B cells expressed high levels of the costimulatory molecules CD80 and CD86, membrane bound IgA and IgG and CD69 ([18] paper III). MHC-II was highly expressed on both $CD25^+$ and $CD25^-$ B cells. The quadruple staining for high affinity IL-2 receptor subunits (CD25, CD122 and CD132 in combination with CD19) showed that in spleen, lymph nodes and peritoneal cavity cells around 10% of the B cells stained positive for entire receptor ([18] paper III).

Function of murine $CD25^+$ B cells

B cells have the ability to produce cytokines of various types. Therefore we analysed the cytokine production pattern in response to different stimuli of murine $CD25^+$ B cells isolated from the spleen of healthy mice. CD25 expressing B cells produced higher levels of IL-6, IFN-γ and IL-10 in response to the TLR9 ligand CpG-ODN, TLR 4 ligand LPS and TLR2 ligand Pam_3Cys as compared to $CD25^-$ B cells. IL-4 production of $CD25^+$ B cells was higher after stimulation with CpG-ODN only. In contrast, we did not detect any production of IL-2 or TNF by these cells ([52] paper IV). This suggests that murine CD25 expressing B cells may be an important source of cytokines during inflammation. However, whether this finding is consistent in autoimmune models remains to be elucidated.

It is known that B cells are APC and contribute to the differentiation of T cells [2, 130]. We have shown that murine CD25 expressing B cells are significantly better at presenting alloantigen to $CD4^+$ T cells when compared to CD25 negative B cells in the MLR assay ([52] paper IV). $CD25^+$ B cells also express higher levels of the costimulatory molecules CD80 and CD86 together with MHC-II when compared to CD25 negative B cells and produce different cytokines ([18, 52] papers III and IV). Having all the signals needed to activate a T cell, the impact of CD25 expressing B cells is of interest in forming memory formation

to a specific antigen as well as its potential role in autoimmune diseases. In mice, $CD25^+$ B cells secrete increased levels of immunoglobulins of classes IgA, IgG and IgM when compared to $CD25^-$ B cells ([52] paper IV). In fact, we have shown that murine $CD25^+$ B cells coexpress CD5, which suggest that these cells may in fact belong to the B-1a subpopulation of B cells secreting natural antibodies. This is in contrast to the results form the human studies showing that $CD25^+$ B cells do not secrete high levels of immunoglobulins [56]. It can be due to differences between the species, or an indication that CD25 expression on human and mice B cells have different functions.

Since the phenotypical classification states $CD25^+$ B cells as more mature due to the surface immunoglobulin expression and the functional characterisation shows higher immunoglobulin secretion we suggest that murine $CD25^+$ B cells may in fact belong to the memory B cell population. Therefore, it is tempting to suggest that CD25 can be used as a memory marker for B cells from secondary lymphoid organs in mice.

To further investigate this question we immunised mice with the protein antigen ovalbumin (OVA) and analysed $CD25^+$ versus $CD25^-$ B cells with respect to the ability to secrete OVA specific antibodies. We found that CD25 expressing B cells produce higher levels of OVA specific antibodies of class IgG and IgM when compared to CD25 negative B cells ([52] paper IV). A B cell that produces antigen specific antibodies has gone through a phase of development including immunoglobulin class switch and somatic hypermutation, and in support to our theory, belongs to memory B cell population.

We have also shown that there is no difference in how the subsets $CD25^+$ and $CD25^-$ B cells respond to different stimuli targeting TLR2 (Pam_3Cys), TLR4 (LPS) and TLR9 (CpG-ODN) ([52] paper IV). Even if

CD25 expressing B cells are more mature the expansion of the CD25 negative B cells seem to be of equal important when a foreign antigen has entered the body and the immune system has to take care of it. This is just an indication that when it is needed, all B cell subsets will respond although in different ways.

CD69 has long been used as a marker for activated cells. Mitogen stimulation of mature B cells *in vitro* leads to CD25 expression and it has been accepted that CD25 is a marker of activated B cells. The question we asked was if this is also true *in vivo*. To answer this we infected mice with *S. aureus* collected spleen cells at different time points after inoculation and stained for CD25 and CD69 expression. Interestingly, we did not see any upregulation of CD25 on B cells *in vivo*. In contrast to the CD25 expression, the expression of CD69 on B cells raised strongly after infection *in vivo* ([52] paper IV). These findings together with our other data regarding $CD25^+$ B cells in mice make us believe that CD25 is not just a marker for activated B cells but represents an unique subpopulation with very distinct phenotypical and functional properties and belongs perhaps within the memory B cell population.

CONCLUDING REMARKS

B cells play an important role in the immune system and in the immunological memory by producing antibodies that makes up a crucial defence against foreign antigens attacking the body. However, if B cells recognize and produce antibodies to self-antigens the attack will be shifted towards the body itself and an autoimmune disease might evolve. There are many different subpopulations of B cells simultaneously present in the body. Each one of these subsets has its own function.
Depletion of B cells has so far been proven to be efficient treatments in B cell lymphomas and also in certain autoimmune diseases such as RA and SLE. The depletion of B cells is a relatively new treatment. Even if the risk of infection has thus far not been increased, the imbalance of the immune system introduced by lack of mature B cells in circulation can not be ignored. It may be the time to start looking for more precise treatment, for example depleting only certain B cell subpopulations.

In this thesis, a new subset of B cells identified by its surface expression of CD25 has been studied phenotypically in healthy individuals and in patients with the autoimmune diseases RA and SLE. This population of B cells show a mature and activated phenotype and is also a part of the memory B cells population. Memory B cells are antigen specific, long lived and may play a role in an autoimmune disease. Further work is needed to investigate if CD25 expressing B cells are specific for autoantigens in patients with RA or SLE.
Another finding that should not be ignored is the ability of $CD25^{+}$ B cells to produce IL-10. It has recently been shown that IL-10 producing B cells in mouse models of RA and SLE play an anti-inflammatory role. The presence of IL-10 producing human B cells in autoimmunity has

also been proved, but if they play a beneficial or pathogenic role is presently unknown. The mouse studies performed showed that also in mice $CD25^+$ B cells display an activated and mature phenotype and are able to become antigen specific antibody secreting cells upon active immunisation. In addition, $CD25^+$ B cells also produce IL-10 after stimulation *in vitro*. Characterisation of CD25 expressing B cells in mice can be a first step in development of a mouse model to further investigate the role of these B cells in the immune system.

Overall we suggest that CD25 expressing B cells both in human and mice are part of a unique population of memory B cells. However, further studies are needed to understand the function of these cells and their role in the normal immune system and in the disease.

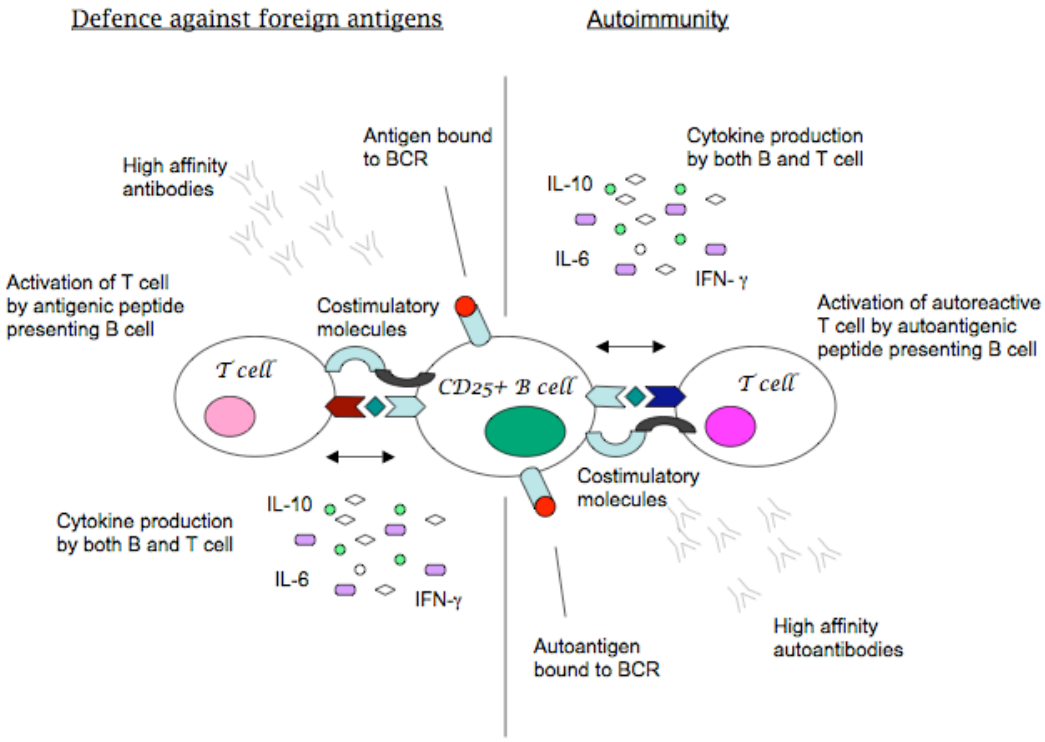

Figure 8: CD25 expressing B cells may be involved in the defence against foreign antigens but if autoreactive may play an important part in autoimmunity and its pathogenesis.

There are two important differences we have found so far during our studies of CD25 expressing B cells in human and mice. The $CD25^+$ B cells are present in the blood of humans but are not in mice. The second difference is regarding immunoglobulin secretion. Human $CD25^+$ B cells secrete low levels of immunoglobulins in contrast to their murine counterparts that secrete high levels. We can almost certainly say that human CD25 expressing B cells belong to the memory B cell population and their presence in circulation and low secretion of immunoglobulins support our hypothesis. Murine $CD25^+$ B cells also resemble memory B cells, even though they secrete immunoglobulins. However, further studies are needed to confirm these findings. The ability of murine $CD25^+$ B cells to produce high levels of immunoglobulins may suggest that these cells are activated or on their way to become plasma cells. One explanation can be the biological difference between the species. At the moment we do not have any explanation for this differences but we hope that further studies will give the answers.

MAIN CONCLUSIONS FROM THE THESIS

Paper I

During normal conditions the development, differentiation and selection process of B cells is relatively well controlled. However, autoreactive B cells occur in the process of autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) giving rise to pathology. Therefore it is important to understand the different role of B cell subpopulations. In this study we phenotypically characterised B cells from healthy controls and patients with RA and SLE regarding their expression of surface CD25. Our results indicate that $CD25^+$ B cells originating from RA and SLE patients and from healthy controls are in a highly activated state.

Paper II

Following our first study we asked to which B cells subset CD25 expressing B cells belong. Our results show that $CD25^+$ B cells from healthy controls, RA and SLE patients, constitute a unique subpopulation preferentially occurring within the $CD20^+CD27^+$ memory B cells. These cells may play role in the defence against microbial agents in the normal immune system but also may be involved in the pathogenesis of different autoimmune diseases by being a part of the immunological memory recognizing autoantigens.

Paper III

Mouse B cells are relatively well characterised. They are in analogy with T cells capable of expressing the functional IL-2 receptor. CD25 (IL-2R α-chain) positive T cells have been studied in detail but knowledge about murine CD25 expressing B cells is scarce. Our results indicate that B cells expressing CD25 are phenotypically distinctly different from those that are CD25 negative. Our findings suggest that

$CD25^+$ B cells are more prone to present antigen and display a more mature phenotype.

Paper IV

B cells are an important part of both the innate and adaptive immune system. Their ability to produce antibodies, cytokines, and to present antigens makes them a crucial part in defence against pathogens. In this study we have functionally characterised CD25 expressing murine B cells. We found that $CD25^+$ B cells secrete high levels of IL-4, IL-6, IL-10, INF-γ and are better at presenting alloantigen to $CD4^+$ T cells. They also spontaneously produce higher levels of IgA, IgG and IgM when compared to $CD25^-$ B cells. In addition, $CD25^+$ B cells are significantly better at secreting antigen-specific antibodies of the IgM and IgG class after immunisation. Our results demonstrate that $CD25^+$ B cells are highly activated and functionally mature. Therefore, we suggest that this population plays a major role in the immune system belonging to the memory B cell population and that CD25 may be used a memory marker.

POPULÄRVETENSKAPLIG SAMMANFATTNING

För att kunna försvara sig mot de stora mänger bakterier och virus som vi människor dagligen kommer i kontakt med har ett väl fungerande immunförsvar utvecklats. Immunförsvaret består av ett stort antal olika vita blodkroppar och deras produkter. Den viktigaste uppgiften för immunförsvaret är att försvara kroppen mot främmande farliga ämnen samtidig som den måste känna igen och ignorera ämnen som inte är farliga eller som är kroppsegna.
Vita blodkoppar utbildas tidigt att inte reagera på kroppsegna ämnen och de ofarliga främmande ämnen som finns runt omkring oss, till exempel ämnen i vår mat. Tyvärr fungerar inte alltid kontrollen av cellerna och ibland blir det fel. Reagerar immunförsvaret på ett fullständig ofarlig ämne som björkpollen kommer det leda till en allergisk reaktion medan om den angriper organen i den egna kroppen leder det till utveckling av en autoimmun sjukdom som reumatism.

Immunförsvaret är uppdelat i två delar, det medfödda, ospecifika immunförsvaret och det förvärvade immunförsvaret, vilken är specifikt och är en del av det immunologiska minnet. En typ av vita blodkroppar som är delaktig både i det medfödda och förvärvade immunförsvaret kallas B-celler. B-cellers främsta uppgift är att producera antikroppar (immunoglobuliner). I det medfödda immunförsvaret producerar B-celler naturliga antikroppar som reagerar på olika delar av bakterier eller virus. I det förvärvade immunförsvaret är antikropparna specifika och känner igen bara en enda struktur. B-celler kan också utsöndra signalämnen, så kallade cytokiner och kemokiner, som talar om för andra celler i immunförsvaret vad som pågår och hur de skall bete sig och vart de skall ta vägen. Den tredje uppgiften B-celler har är att ta in olika ämnen och vissa upp de för T-celler, som är en annan viktig cell i det

förvärvade immunförsvaret, och aktivera dessa så att immunsvaret mot ett ämne blir starkare och mer effektivt.

Det är naturligtvist mycket viktig att kroppen försvaras men ännu viktigare är att kroppens egna organ inte angrips och förstörs. För att förhindra detta har immunförsvaret ett antal skyddsmekanismer. Efter att B-celler bildas i benmärgen går de genom en kontroll där de celler som känner igen kroppsegna ämnen och bildar antikroppar mot dem tas bort. Tyvärr fungerar inte alltid de kontrollmekanismer kroppen har. Det leder till att B-celler, som reagerar på kroppsegna ämnen på samma sätt som främmande, släpps ut från benmärgen och börjar bilda antikroppar som i sin tur angriper kroppens olika organ vilket kan leda fram till utveckling av en så kallad autoimmun sjukdom. Att B-celler är en viktig del av autoimmuna sjukdomar som reumatism har visats med hjälp av en specifik behandling där B celler slås ut hos patienter med dessa sjukdomar. De patienter som svarar bra på behandlingen blir symptomfria och mår bättre i upp till nio månader eller mer efter behandlingen. Symptomen för sjukdomen kommer tillbaka när B-celler hittas i blodet igen.

Vi är intresserade av en särskild B-cell som kan identifieras med hjälp av en markör (protein) på dess yta. Den markör vi har använt oss av kallas för CD25. Vi undersökte B-cellers utseende och egenskaper med hjälp av CD25 och andra markörer inte bara hos friska vuxna utan också hos patienter som hade fått diagnosen reumatism (RA) eller systemic lupus erythematosus (SLE). Vi fann att B-celler som utryckte CD25 var mogna och aktiverade. Vidare kunde vi också vissa att dessa B-celler är så kallade minnes B-celler. Det immunologiska minnet är viktigt för att kunna känna igen, snabbt reagera och bekämpa ett främmande ämnen som redan har blivit bekämpat av immunförsvaret tidigare och som på nytt skulle kunna utgöra en fara. Minnesceller är viktig del av immunförsvaret hos friska, samtidigt kan dessa celler

vara riktigt farliga för de som lider av olika autoimmuna sjukdomar, då minnescellerna istället känner igen kroppsegna ämne och reagerar på dem. Detta innebär att de kommer att vara aktiva och bilda antikroppar och cytokiner och på så sätt ökar angreppen mot kroppen. Vi tror att de B-celler som vi har undersökt kan vara involverade i sjukdoms mekanismerna hos RA and SLE patienter. Fler studier behövs för att säkert kunna säga vad dessa B-celler har för funktion i immunförsvaret hos friska personer och hos dem som lider av autoimmuna sjukdomar.

Möss har länge används som modell för olika mänskliga sjukdomar. De har studerats för att bättre förstå en sjukdoms utveckling samt de olika vita blodkropparnas funktion under sjukdomen. Vi har använt oss av samma ytmarkör, CD25, som vi har använt i våra studier av mänskliga B-celler för att kunna identifiera dessa specifika B-celler hos möss. Målet har varit att undersöka B-celler som bär på CD25 i möss och förhoppningsvis kunna utveckla en modell för autoimmuna sjukdomar och vidare studera deras funktion. Vi fann att CD25 uttryckande B celler hos möss liksom hos människor är mer mogna och aktiverade än de som saknar CD25 på sin yta. De är också bättre på att bilda antikroppar och signalämnen, cytokiner. Vidare kunde vi se att dessa B-celler kunde aktivera T-celler och få dem att föröka sig. Dessa resultat indikerar att de B-cells vi har studerat har en klar funktion i immunförsvaret. Dessa B-celler verkar ha det som behövs för att kunna vara med i vanliga immunologiska reaktioner men också i olika autoimmuna sjukdomar.

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