THE ROLE OF FLT3L AND BAFF IN B CELL DEVELOPMENT AND HOMEOSTASIS

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Research is to see what everybody else has seen,

and to think what nobody else has thought.

Albert Szent-Györgi (1893-1986)

Summary

The maintenance of homeostasis in the immune system is a complex process. The control of B cell numbers occurs at many different stages in the developmental pathway, and numerous factors are involved. In this thesis, certain aspects of FLT3L, BAFF-R and BAFF on B cells were analyzed.

In the first part (chapter 3), the effect of increased availability of FLT3L on EPLMs, which have been found previously to be B cell progenitors, was investigated. Upon FLT3L administration in mice, the number of EPLMs increased in a dose-dependent manner, but the number of B cells decreased. This decrease was reversible as B cell numbers recovered after cessation of FLT3L treatment. *In vitro* studies revealed, that EPLMs from FLT3L treated mice had a reduction of about 20-fold in B-lineage potential, while the potential for the T cell lineage was only slightly reduced and myeloid potential was unchanged. Transplantation of EPLMs from FLT3L treated mice into lymphopenic hosts showed, that these cells are able to give rise to B and T cells, but not to myeloid cells. From these experiments we concluded, that FLT3L is able to increase the number of EPLMs, and that these progenitor cells can reconstitute B and T cell compartments *in vivo*.

In the second part (chapter 4), novel sets of monoclonal antibodies specific for mouse BAFF-R or human BAFF are described. Among the anti-mBAFF-R antibodies, several different clones that were able to block the binding of ligand to BAFF-R could be identified. With the anti-hBAFF antibodies, a sensitive ELISA for the detection of hBAFF in human sera was developed.

In the third part (chapter 5), the role of BAFF-R signaling in the *in vivo* maintenance of mature B cells was investigated with the use of the new monoclonal anti-mBAFF-R antibodies. We showed that 14 days following a single injection of a monoclonal anti-mBAFF-R antibody that prevents BAFF binding, both follicular and marginal zone B cell numbers were drastically reduced, whereas B-1 cells were not affected. Injection of control, isotype-matched but non-blocking anti-mBAFF-R monoclonal antibodies did not result in B cell depletion. We also showed that this depletion is neither due to antibody-dependent cellular cytotoxicity nor to complement-mediated lysis. Moreover, prevention of BAFF binding lead to a decrease in the size of the B cell follicles and to a strong impairment of T cell dependent humoral immune response. These results establish a central role for BAFF -

BAFF-R signaling in the *in vivo* survival and maintenance of both follicular and marginal zone B cell pools.

In the fourth part (chapter 6), the precise expression of BAFF-R on murine BM B cells was investigated with the use of the new monoclonal anti-mBAFF-R antibodies. We found that expression of BAFF-R is first detectable by flow cytometry on a fraction of $CD19^+$ $CD93^+$ $CD23^-$ bone marrow (BM) B cells. This BAFF-R^+ BM B cell population showed higher levels of surface IgM expression and decreased recombination-activating gene 2 (RAG-2) transcripts than BAFF-R^- immature B cells. When cultured *in vitro*, BAFF-R^+ immature B cells did not undergo further B cell receptor rearrangement, while BAFF-R^- immature B cells did. However, when cultured in the presence of an anti-kappa light chain antibody, BAFF-R^+ immature B cells could be induced to undergo receptor editing and this correlated with the downregulation of both surface IgM and BAFF-R expression. Addition of BAFF did not inhibit this induced receptor editing. We concluded, that expression of BAFF-R can be used as a marker to identify immature B cells, which under normal conditions no longer undergo BCR editing, but can still be induced to do so by BCR engagement.

In the last part (chapter 7), serum levels of hBAFF from patients with diseases connected with disturbed B cell homeostasis were analyzed. We found, that in a subset of patients with SLE, Sjögrens syndrome or autoimmune thyroid diseases, hBAFF levels were elevated. Also, hBAFF levels were elevated in most patients suffering from Hodgkin or non-Hodgkin lymphomas. In patients with chronic hepatitis C, hBAFF levels were elevated in a subgroup of patients. Furthermore, we found no correlation between hBAFF levels and anti-dsDNA autoantibody titers in SLE patients. In addition, hBAFF levels did not correlate with age or gender. Taken together, this analysis suggests, that hBAFF might be involved in the genesis and/or maintenance of autoimmune and chronic infectious diseases only in a subgroup of patients, while its role in B cell leukemias might be more pronounced.

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1 GENERAL INTRODUCTION

The necessity of developing a defense mechanism against life-threatening intruders is as old as life itself. Starting down in the phylogenetic tree with bacteria, more and more complex immune systems have evolved on the way up to vertebrates. Already the bacterial defense mechanism, the restriction modification system, fulfills one of the most important requirements of such a system, which is discrimination between self and non-self. The more complex immune systems of higher plants and invertebrates employ several mechanisms to recognize and eliminate pathogenic agents, including pattern recognition receptors, secreted antimicrobial peptides and phagocytosing cells. All these mechanisms are, among others, components of the so-called innate immune system, which is characterized by its fast but unspecific action. The innate immune system comprises highly conserved mechanisms and is also part of the immune system of vertebrates, where it provides the first line of defense. About 450 million years ago, a second, more specific immune system developed in vertebrates. This so-called adaptive immune system is able to react highly specificly to nearly all types of pathogenic molecules and microorganisms and possesses a memory function. The specificity of the adaptive immune system is achieved through the generation of numerous extremely diverse antigen receptors, the B and T cell receptors, which are expressed by the key players of adaptive immunity, the B and T lymphocytes, respectively.

1.1 B cell development

B cell development proceeds via several intermediate stages that can be distinguished by the differential expression of intracellular and cell surface markers. Several nomenclatures are used in different laboratories due to the usage of other sets of markers in order to delineate B cell developmental stages. In this work, the nomenclature established by Rolink and Melchers, which is based on the expression of cell surface markers and the rearrangement status of the immunoglobulin (Ig) genes, is used (1).

In mice and man, two major types of B lymphocytes are distinguished, namely B-1 and B-2 B cells. B-1 cells are less abundant than B-2 B cells and differ in many aspects from B-2 B cells. B-2 B cells, which is the major group of B cells both in mice and man, mature in the

bone marrow (BM), a primary lymphoid organ. The cells then migrate to the spleen, where further developmental steps take place. Mature B-2 B cells then either stay in the spleen or migrate to other secondary lymphoid organs like lymph nodes or mucosal-associated lymphoid tissues (MALT), which include among others the appendix, the tonsils and the Peyer's patches. Upon encountering of antigen in the secondary lymphoid organs, mature B cells further develop into highly specialized effector cells.

1.1.1 Early developmental steps in the bone marrow

B cells derive, as all other cells of the hematopoietic system, from multipotent long-term hematopoietic stem cells (LT-HSCs), which reside in the BM (see fig. 1). These cells have self-renewing capacity and are Sca1^{high} c-Kit^{high} Flt3^{-} CD34^{-} VCAM1^{+} Lin^{-} (2-4). LT-HSCs develop further into short-term HSCs (ST-HSCs) and then into multipotent progenitors (MPPs), a heterogeneous group of Sca1^{high} c-Kit^{high} Flt3$^{low/high}$ CD34^{+} VCAM1$^{-/+}$ Lin^{-} precursor cells (5). The gradual upregulation of Flt3 expression and downregulation of VCAM1 expression in ST-HSCs (Sca1^{high} c-Kit^{high} Flt3$^{-/low}$ CD34^{+} VCAM1^{+} Lin^{-}) and MPPs coincides with gradual reduction of self-renewal capacity and lineage potential (6, 7). A subgroup of MPPs, the lymphoid primed multipotent progenitors (LMPPs), are Sca1^{high} c-Kit^{high} Flt3^{high} CD34^{+} VCAM1^{-} Lin^{-}.

LMPPs can be further subdived into early lymphoid progenitors (ELPs), which are defined by the expression of recombination activating gene 1 (RAG-1), terminal deoxynucleotidyl transferase (TdT) or both (9, 10). Phenotypically, ELPs resemble LMPPs. LMPPs have no self-renewal capacity anymore and show a reduced lineage potential as they develop efficiently only into lymphoid and myeloid cells, but not into cells of the megakaryocyte-erythrocyte lineage (11). Further progenitors along the B cell lineage path encompass the common lymphoid progenitor (CLP), being Sca1^{low} c-Kit^{low} Flt3^{+} CD93^{+} B220^{-} IL-7Rα^{+} and the early progenitor with lymphoid and myeloid potential (EPLM), which is Sca1^{low} c-Kit^{low} Flt3^{+} CD93^{+} B220^{+} IL-7Rα^{+} CD19^{-} (12). EPLMs are sometimes also refered to as pre-pro-B cells, as both are B220^{+} and CD19^{-}.

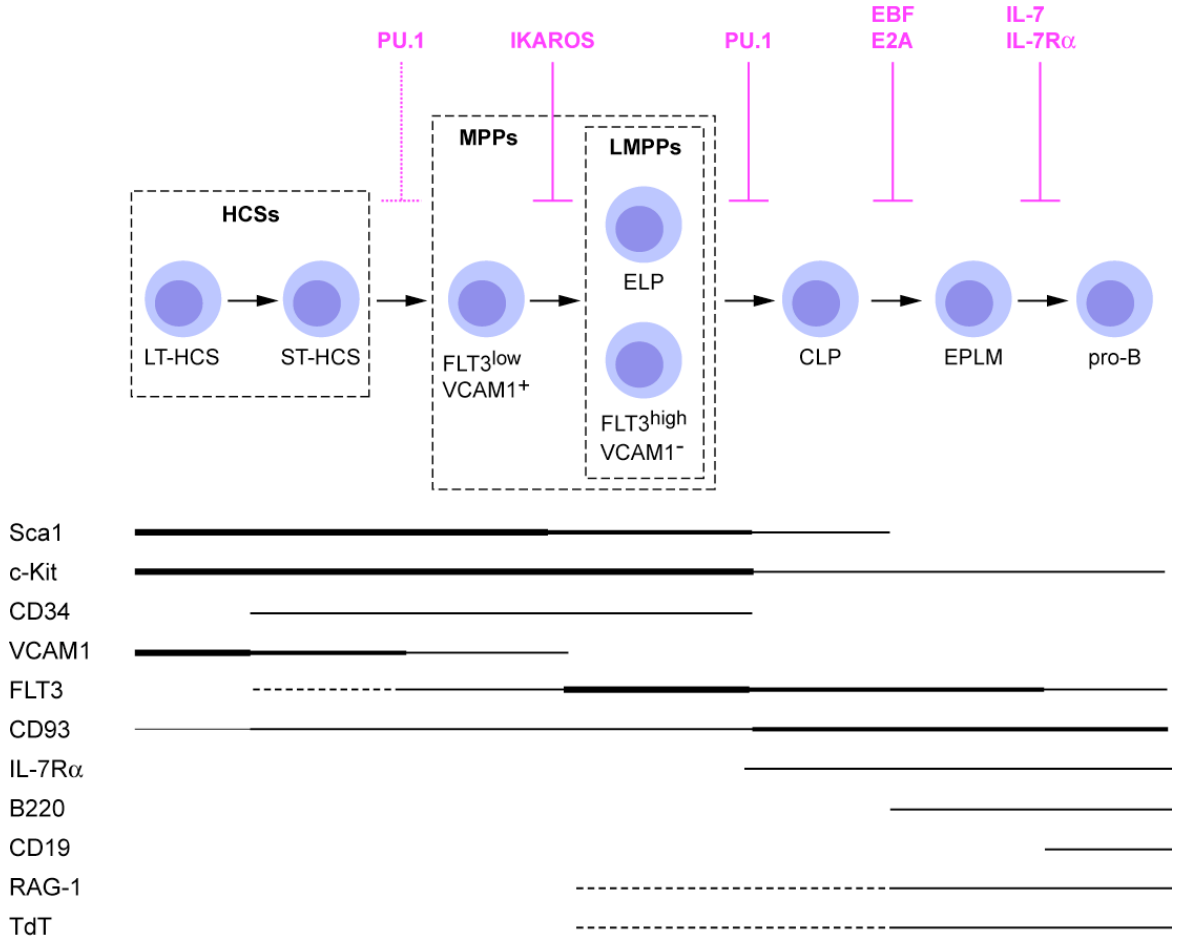

Figure 1: Early stages of B cell development. *Hematopoietic stem cells (HSCs) develop via several intermediate stages into B cell lineage committed pro-B cells. Multipotent progenitors (MPPs) comprise VCAM1^{+} FLT3^{low} and VCAM1^{-} FLT3^{high} cells, the latter are defined as lymphoid-primed multipotent progenitors (LMPPs). A subgroup of the LMPPs are early lymphoid progenitors (ELPs), which express RAG-1, TdT or both. Further precursor cells are the common lymphoid progenitor (CLP) and the early progenitor with lymphoid and myeloid potential (EPLM). Pro-B cells are the first cells that express CD19, a marker that defines B lineage committment. The expression of several surface markers and intracellular proteins is indicated with horizontal lines and the thickness of the lines indicates expression strenght. Factors that cause a complete or partial block at certain stages of B cell development are shown in pink (see chapter 1.1.5 for a description). Adapted from Welner et al., 2008 (8).*

The next characterized precursors are the pro-B cells, which gain expression of CD19 and are thus defined as Sca1^{low} c-Kit^{low} Flt3^{+} CD93^{+} B220^{+} IL-7Rα^{+} CD19^{+} progenitor B cells (see fig. 2). CD19 is part of a coreceptor for the B cell receptor (BCR), which is formed at a later stage. Expression of CD19 is directly regulated by the transcription factor PAX5 (paired box protein 5), which is thought to be responsible for B lineage comittment (13). In pro-B cells, D_H to J_H rearrangement of the IgH chain locus, which usually occurs on both alleles, is completed. Rearrangement of the immunoglobulin heavy (IgH) chain genes starts

already in some ELPs with joining D_H to J_H elements at the IgH chain locus. This DNA rearrangement is directed by the RAG-1 and RAG-2 proteins and the enzyme TdT catalyses the insertion of nucleotides at the coding joints. Pro-B cells express VpreB and λ5, which together form the so-called surrogate light (SL) chain. The next stage are pre-BI cells, which have both IgH chain genes D_HJ_H rearranged and are phenotypically defined as c-Kit^{low} Flt3$^-$ CD93$^+$ B220$^+$ IL-7Rα$^+$ CD19$^+$. Pre-BI cells continue IgH chain rearrangement with V_H to D_HJ_H-rearrangement on only one allele and start to express a μH chain. Association of the μH chain with the SL chain results in formation of the cell surface expressed pre-BCR. Pre-BI cells expressing a functional pre-BCR enter the cell cycle and thus become large pre-BII cells. Large preBII cells lose c-Kit expression and start to express CD25, which is the IL-2Rα chain, and thus are c-Kit$^-$ Flt3$^-$ CD93^{high} B220$^+$ IL-7Rα$^+$ CD19$^+$ CD25$^+$. *Rag-1* and *Rag-2* genes are downregulated rapidly in order to prevent further rearrangements on the H chain loci (14), thereby avoiding the production of cells bearing two different types of pre-BCRs, a mechanism refered to as allelic exclusion (15). Also, the genes encoding the SL chain proteins, VpreB and λ5, and TdT are downregulated. After about 5-7 divisions, due to dilution of the long-lived SL chain proteins and the resultant decrease in pre-BCR numbers formed, the large pre-BII cells become resting, small pre-BII cells (16). Phenotypically, small pre-BII cells resemble large pre-BII cells. In small pre-BII cells, *Rag-1* and *Rag-2* are upregulated again and the cells rearrange their immunoglobulin light (IgL) chain loci. In contrast to the H chain locus, two different L chain loci exist, named κ and λ. Also, both L chain genes do not contain D elements and thus, their V-regions consist only of V and J elements, in this way enabling repeated V-J rearrangements on the same locus, thereby increasing the chance for a productive L chain gene rearrangement. In mice, rearrangement is initiated more often at the κL chain locus, resulting in a ratio of 10:1 for κL chain to λL chain expressing B cells (17). Pairing of the L chain with the μH chain forms the cell surface expressed BCR and cells become immature B cells, which express intermediate levels of B220 but have lost CD25 expression. Immature B cells are thus CD93^{high} B220^{int} CD19$^+$ CD25$^-$ IgM$^+$. Expression levels of IgM are extremely variable and differ from low to high. Low expression of surface IgM (sIgM) is a result of either impaired BCR signaling or due to downregulation of an autoreactive BCR (18). Only immature B cells with high expression of sIgM leave the bone marrow to enter the spleen (19).

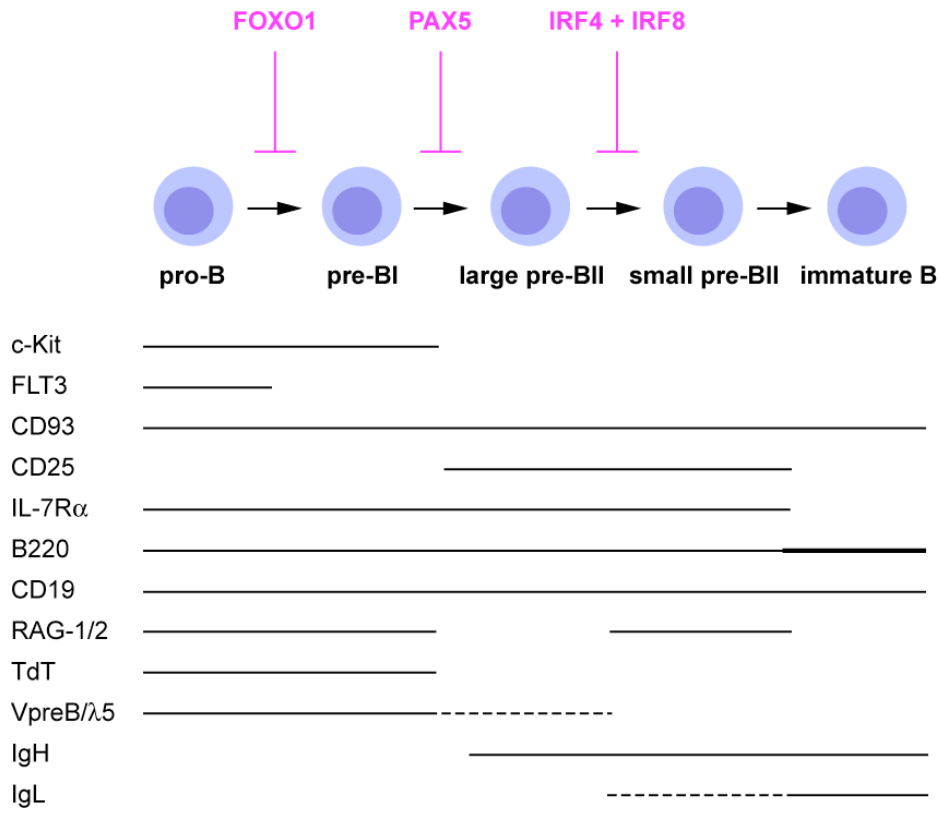

Figure 2: Later stages of B cell development in the bone marrow. *B cell lineage committed pro-B cells develop via several intermediate stages into immature B cells. The immature B cell stage is the first B cell developmental stage where the B cell receptor is expressed. The expression of several surface markers and intracellular proteins is indicated with horizontal lines and the thickness of the lines indicates expression strenght. Factors that cause a complete or partial block at certain developmental stages are shown in pink (see chapter 1.1.5 for a description).*

Several checkpoints exist along the B cell developmental pathway where the developing B cells are screened for bearing non-functional or autoreactive BCRs, being generated due to the random way of assembling the V, D and J Ig gene segments. The mechanisms employed to eliminate these cells involve both positive and negative selection methods. A first checkpoint exists at the pre-BI cell stage. The newly formed μH chain must be able to pair with the SL chain and a pre-BCR has to be expressed on the cell surface. Only cells with a signaling competent pre-BCR can induce cell cycling und efficiently transit to the next stage of development, the cycling large pre-BII cells. Cells that are not able to generate a functional pre-BCR are unable to enter the expansion phase. The next checkpoint is at the transition from the small pre-BII to the immature B cell stage, when the IgL chain genes are rearranged. This rearrangement has to be productive and the resulting IgL chain must be able to pair with the μH chain in order to form a surface expressed BCR. Cells being unable

to express a functional BCR can continue IgL chain gene rearrangement, a mechanism called receptor editing (20). Thereby, the cells have the chance to produce a new IgL chain protein that might be functional in combination with the μH chain already present. The number of possible rearrangements is limited by the survival time of the pre-BII cells. It could be shown that an increase in the survival time due to overexpression of the anti-apoptotic factor Bcl-2 leads to enhanced L chain editing (21).

The first tolerance checkpoint is at the immature B cell stage, when a new complete BCR is expressed for the first time. Tolerance checkpoints are intended to test BCRs for their auto-reactivity, what is achieved by providing them with autoantigens, which in the BM are expressed on stromal cells. Immature B cells expressing a BCR binding with too high avidity to a multivalent (and hence crosslinking) self-molecule undergo receptor editing and might thus be able to express an innocuous BCR, resulting in positive selection. In case a cell can not alter its autoreactive BCR it is eliminated by apoptosis, a process refered to as clonal deletion. It was found that most of the newly produced immature B cells express an autoreactive BCR and that 25-50% of all B cells in the periphery went through receptor editing (22). Nonetheless, of the approximately 2 x 10^7 immature B cells that develop per day in the BM (23), only around 10-20% enter the spleen (24), meaning that 80-90% of the newly formed immature B cells become negatively selected at the immature B cell stage.

1.1.2 Late developmental steps in the spleen

Immature B cells that survived negative selection leave the bone marrow and migrate via the blood stream to the spleen, where they penetrate the marginal zone blood sinus to enter the outer region of the periarteriolar lymphoid sheath (PALS). In the spleen, immature B cells go through two short-lived transitional stages and finally become long-lived mature B cells (see fig. 3). The first developmental stage consist of transitional type 1 (T1) B cells, which are characterized by expressing $CD19^+$ $B220^+$ $CD93^{high}$ $CD21^-$ $CD23^-$ IgM^{high} IgD^{low}. The next stage constitutes T2 B cells, which gained expression of CD21 and CD23. They also show a lower expression of CD93 and increased levels of IgD. Thus, T2 B cells are $CD19^+$ $B220^+$ $CD93^+$ $CD21^+$ $CD23^+$ IgM^{high} IgD^{high}. CD21, also called complement receptor 2 (CR2), forms together with CD19 and CD81 the B cell co-receptor. The ligand for CD21 is the complement cleavage product C3d. Crosslinking of the B cell co-receptor by binding of CD21 to C3d coated to an antigen that is captured by the BCR, leads to

phosphorylation of CD19, thus initiating a signaling cascade supporting BCR signaling (25). CD23, the low affinity IgE receptor or FcεRII, regulates IgE production and was found to be an important modulator of allergic responses. Initially, CD23 is expressed as a membrane bound molecule but then the majority is shed of by the metalloproteinase ADAM10 (26). Another type of B cells, which are $CD93^{+}$ and thus were considered to belong to the transitional types of B cells, were designated T3 B cells ($CD19^{+}$ $B220^{+}$ $CD93^{+}$ $CD21^{+}$ $CD23^{+}$ IgM^{low} IgD^{high}). Recent findings, however, showed that these T3 cells are not able to give rise to mature B cells and therefore are no precursors of mature B cells (27, 28).

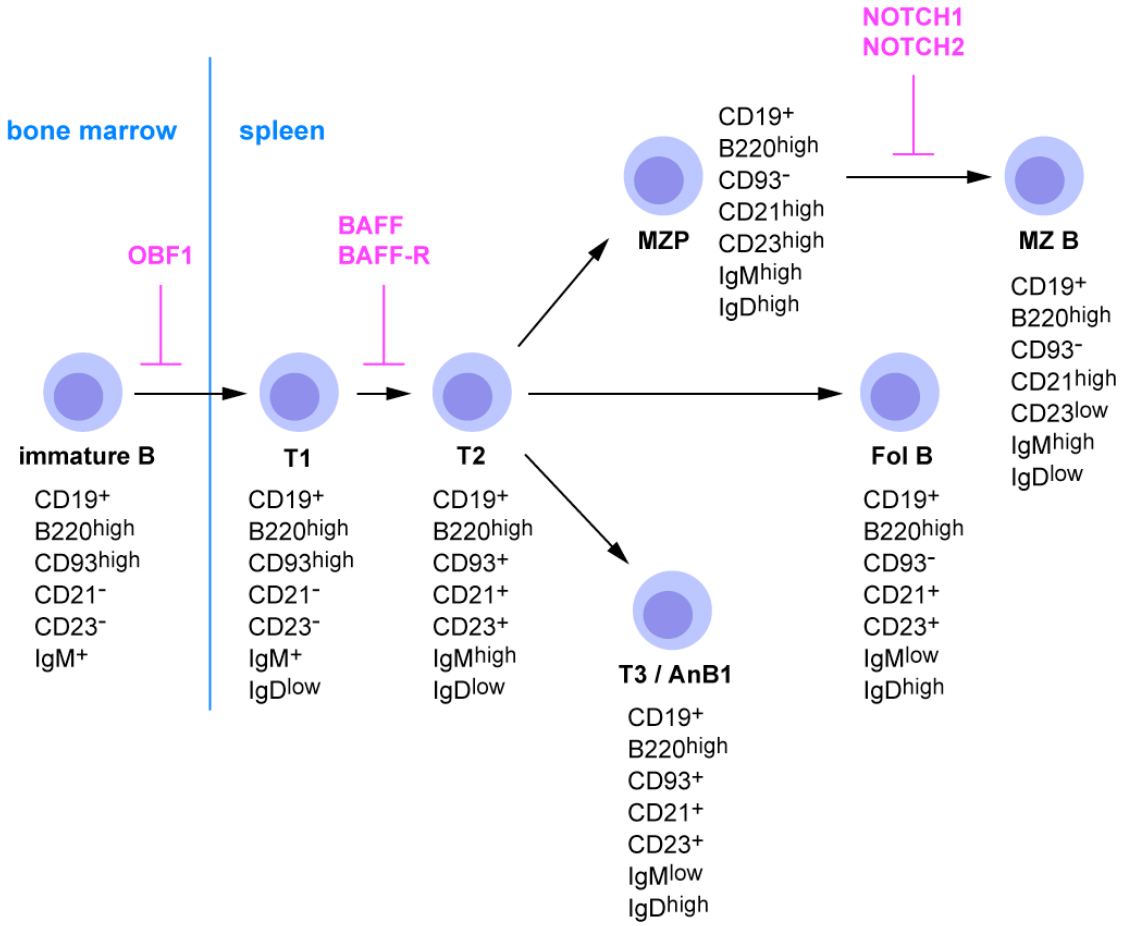

Figure 3: Stages of B cell development in the spleen. *Immature B cells leave the bone marrow and enter the spleen, where they develop first into short lived transitional type B cells, T1 and T2. From the T2 stage, the cells develop either into mature follicular (Fol) B cells or via the marginal zone precursor (MZP) B cells into mature marginal zone (MZ) B cells. T2 B cells that recognize autoantigen are rendered anergic and are now designated anergic population 1 B cells (AnB1 cells) instead of T3 B cells. The expression of several surface markers and intracellular proteins is indicated. Factors that cause a complete or partial block at certain developmental stages are shown in pink (see chapter 1.1.5 for a description).*

From the T2 stage B cells develop into one of two mature B cell stages, which are follicular (Fol) B cells and marginal zone (MZ) B cells. Both mature B cell types are negative for CD93. Fol B cells develop directly from T2 B cells and differ phenotypically from them by the loss of CD93 expression and by the expression levels of IgM and IgD and are $CD19^{+}$ $B220^{+}$ $CD93^{-}$ $CD21^{+}$ $CD23^{+}$ IgM^{low} IgD^{high}. In the spleen, Fol B cells are located inside the splenic follicles and represent the majority of B cells. Fol B cells circulate between the splenic follicles, the lymph nodes and the BM until they either die or encounter antigen and undergo further maturation (see chapter 1.1.4). On the way to MZ B cells, T2 B cells develop initially into a marginal zone precursor (MZP) B cell, defined by the expression of $CD19^{+}$ $B220^{+}$ $CD93^{-}$ $CD21^{high}$ $CD23^{high}$ IgM^{high} IgD^{high} (29), and then into mature MZ B cells. MZ B cells are characterized by the expression of $CD19^{+}$ $B220^{+}$ $CD93^{-}$ $CD21^{high}$ $CD23^{low}$ IgM^{high} IgD^{low} and are located in the outer area of the follicles, next to the marginal sinus and in close vicinity to the marginal zone macrophages and dendritic cells. In contrast to Fol B cells, MZ B cells do not migrate to other organs but have been shown to shuttle between the surrounding area of the marginal sinus and the Fol B cell area in the splenic follicles (30, 31).

The developmental pathway of both Fol and MZ B cells is not yet clearly defined. For many years, the spleen was considered to be the only site where the final steps in B cell development occur, but recent findings showed that Fol B cell maturation takes place both in the spleen and the BM (32, 33). In contrast, MZ B cells mature exclusively in the spleen (34). Also, some authors claim that MZ B cells can develop either directly from T2 progenitor B cells or from Fol B cells via MZP B cells (35).

The immature transitional B cell stages represent further tolerance checkpoints. On their way from the BM to the spleen, newly formed immature B cells encounter a number of peripheral antigens that might have not have been present in the BM. Cells bearing a BCR reacting with a peripheral antigen are eliminated by apoptosis or anergy. Which of these mechanisms is applied depends on several factors, including strength of the BCR signal and availability of the self-antigen, the latter is directly linked to the degree of receptor occupancy. A strong signal from the BCR usually leads to induction of apoptosis. Both T1 and T2 B cells have been shown to undergo apoptosis upon crosslinking of the BCR (24). A lower BCR signal in combination with a high enough occupancy of the BCR leads to induction of anergy, which is a state of unresponsiveness to antigen. It was shown that a constant occupancy of the BCR is required to maintain the anergic state (36). Anergic B cells have a low level of IgM expression and a short life span of about 4-5 days. The cells previously

identified as T3 B cells are now thought to represent anergic B cells and thus were renamed anergic population 1 (An1) B cells (27).
It was shown that about 50% of the immature B cells which enter the spleen are selected into the mature B cell pool (24), thus the more important tolerance checkpoint is at the immature B cell stage, where, as mentioned above, more than 80% of the newly formed B cells are negatively selected.

1.1.3 B-1 B cells

B-1 B cells represent a second type of B cells which are primarily found in the peritoneal and pleural cavities but also in the spleen and parts of the intestine. Two different subsets can be phenotypically distinguished, namely B-1a and B-1b B cells. B-1a B cells are $CD5^{+}$ $CD19^{+}$ $B220^{low}$ $CD23^{-}$ $CD11b^{+}$ IgM^{high} IgD^{low}, whereas B-1b B cells are negative for CD5. Splenic B-1 cells however do not express CD11b. The developmental pathway of B-1 B cells is still a matter of debate. Early studies concluded that B-1 cells arise only during fetal life and further persist in the adult as a self-replenishing population (37, 38). Later, B-1 B cells were supposed to develop in the adult from the same progenitor as B-2 B cells and that the decision for either lineage depends on the nature of signals delivered via the BCR (39-41). Recent findings report the existence of a B-1 restricted progenitor (B1P), being Lin^{-} $CD93^{+}$ $CD19^{+}$ $B220^{-/low}$, which is present in fetal liver and BM and adult BM (42). B1Ps are a heterogenous population and can be generated in vitro and in vivo from adult BM derived ELPs and CLPs, but not from pro-B cells or T1 B cells (43). These results argue for a developmental pathway similar to that observed for B-2 B cells, although other routes might still be possible.

1.1.4 Antigen dependent development

Upon encountering of antigen, mature B cells develop via several intermediate stages, into either antibody-secreting cells (ASCs), which are plasmablasts and plasma cells, or memory B cells. Plasmablasts and plasma cells differ by their cell-cycle status and the amount of antibody they can secrete. Plasmablast are dividing cells that secrete low amounts of antibody, while plasma cells are non-dividing cells that characteristically secrete high

amounts of soluble immunoglobulin and can be short- or long-lived. Memory B cells are quiescent, long-lived cells expressing high-affinity BCRs. On differentiation into plasma cells the characteristic B cell surface markers are gradually downregulated, thus plasma cells are negative for CD19, B220, surface Ig, CD21, CD23 and CD5. Plasma cells and their precursors, the plasmablasts, were found to express high levels of CD138 and CD93 (44). For murine memory B cells, so far no specific marker has been found. Memory B cells still express CD19, and a subset was found to have increased expression levels of CD80 and CD73 (45). Which of these cells are generated depends on the location of the encounter with antigen and the elicited immune response. Depending on the nature of the antigen, either a T-cell dependent (TD) or T-cell independent (TI) immune response is generated. TD antigens are mainly proteins that are processed and presented on MHC class II molecules to $CD4^+$ T-helper (T_H) cells. TI responses are subdivided into two categories referred to as type 1 (TI-1) and type 2 (TI-2). TI-1 antigens are polyclonal B cell activators (mitogens) such as LPS or CpG, that activate B cells via Toll-like receptors (TLRs), regardless of their BCR specificity, whereas TI-2 antigens are highly repetitive molecules like polymeric proteins (e. g. flagellin) or polysaccharides that activate B cells by engagement and cross-linking of their BCR. The three types of mature B cells, which are Fol, MZ and B-1 B cells, respond differently to TI and TD antigens.

In a TD immune response, mainly Fol B cells are involved, but MZ and B-1 B cells can also respond. Upon encountering of antigen and stimulation by a T_H cell via interaction of B cell expressed CD40 with T_H cell expressed CD40L, Fol B cells start to proliferate. The antigen activated B cells can then either develop directly into ASCs within so-called extrafollicular regions or enter a germinal center (GC). MZ B cells responding to a TD antigen presumably differentiate directly into extrafollicular ASCs and do not participate in a GC reaction. For Fol B cells, the decision of which pathway to take seems to depend on the affinity of the BCR (46). B cells with a high affinity preferentially undergo an extrafollicular plasma cell differentiation, while cells with a weaker affinity are directed towards a GC. Within an extrafollicular region, activated B cells undergo rapid proliferation leading to plasmablasts and finally to plasma cell development. Plasma cells formed in extrafollicular regions express a low affinity antibody mainly of the IgM class and have a short life span of about 3 days. GCs are specialized structures that can develop in the follicles of peripheral lymphoid tissues, including the spleen, lymph nodes, Peyer's patches and/or tonsils (47). A fully developed GC (see fig. 4) consists of a dark zone comprised almost exclusively of densely packed proliferating B cells, the so-called centroblasts, and a light zone, which is made up

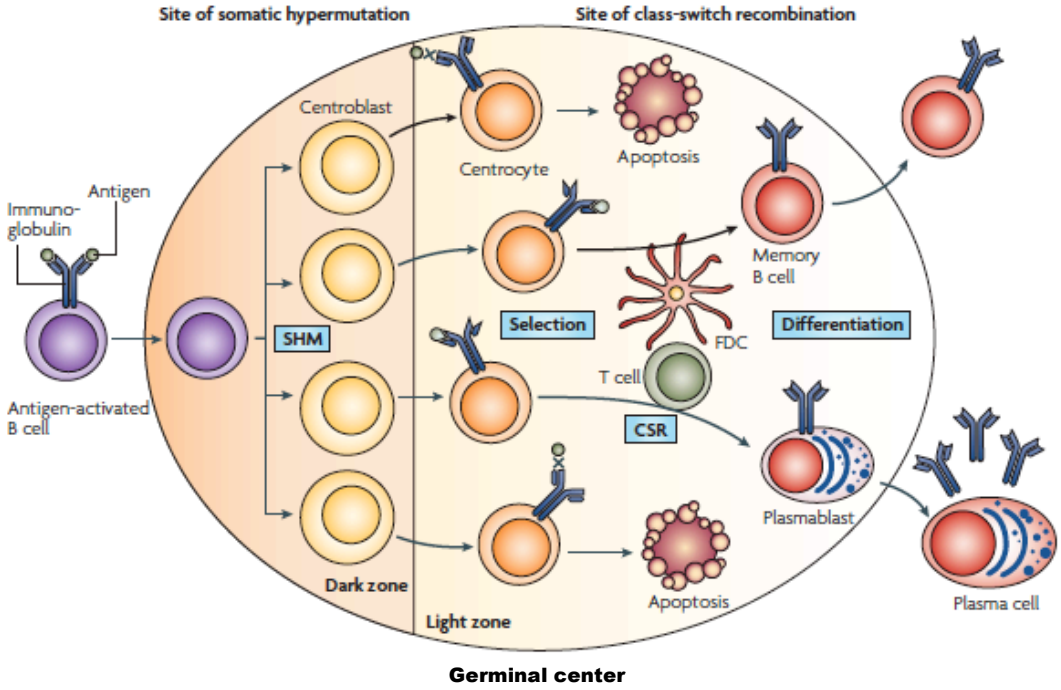

Figure 4: Schematic representation of B cell development in the germinal center. *Upon antigen activation, B cells differentiate into centroblasts, which undergo clonal expansion in the dark zone of the germinal center (GC). In the centroblasts, somatic hypermutation (SHM) takes place, a process that induces base-pair changes into the V(D)J region of the rearranged immunoglobulin heavy and light chain genes. Centroblasts differentiate further into centrocytes, which are found in the light zone of the GC. Here, the modified antigen receptor is tested for improved binding of the immunizing antigen, which is presented by T cells and follicular dendritic cells (FDCs). Centrocytes expressing a low affinity antibody are eliminated by apoptosis. Some of the centrocytes undergo immunoglobulin class-switch recombination (CSR). Eventually, the selected centrocytes differentiate into memory or plasma cells. Adapted from Klein et al., 2008 (47).*

of non-dividing centrocytes, follicular dendritic cells (FDCs), T cells and macrophages. Activated Fol B cells that enter a GC develop first into centroblasts, which are characterized by their fast proliferation and their apoptosis-prone phenotype. In centroblasts a process modifying the variable region of the rearranged IgH and IgL chain genes, termed somatic hypermutation (SHM), takes place. SHM is initiated by the enzyme activation-induced cytidine deaminase (AID), which catalyzes the targeted deamination of deoxycytidine residues. The resulting U:G mismatch is then repaired by different error-prone DNA repair mechanisms, leading to a nucleotide exchange and occasionally to an amino-acid substitution. Centroblasts then differentiate into centrocytes and migrate to the light zone of the GC, where cells with a mutated BCR that have aquired a high affinity for the antigen are selected. Centrocytes bearing a BCR with a low affinity for the antigen die by apoptosis, while centrocytes with an intermediate affinity might develop back into centroblasts and

undergo another round of SHM. In this way, high-affinity variants of the original antigen-specific BCR are rapidly formed. The combination of introduction of mutations by SHM and subsequent selection of cells with a mutated BCR accounts for the so-called affinity maturation of antibodies. Within the positively selected centrocytes another important process, the so-called class-switch recombination (CSR) takes place. CSR is an irreversible mechanism also dependent on AID expression, by which B cells switch the class of the expressed immunoglobulin from IgM or IgD to one of the other classes, which have different effector functions. The determination of the resulting Ig class depends mainly on the combination of specific cytokines that are secreted by follicular T_H cells (48).

The further development of centrocytes seems to depend on the affinity of the mutated BCR. Cells with a high-affinity BCR preferentially develop via plasmablasts into plasma cells (49), while memory B cells are formed from centrocytes within a more broad affinity spectrum (50). The plasma cells formed during a GC reaction secrete high-affinity antibodies and have an increased life span compared to the plasma cells formed in extracellular foci. While some of the GC formed plasma cells stay in the organ where they developed, others migrate via the blood stream to the inflamed tissue or the BM. Within the secondary lymphoid organs and the BM, GC-derived plasma cells develop into long-lived plasma cells that continue with secretion of antibody, thus contributing to the establishment and maintenance of humoral immunity. Also, memory B cells reside in spleen, lymph nodes and BM. Upon re-encountering of the specific antigen, at least a subset of the previously formed memory B cells can rapidly develop into plasma cells which then secrete huge amounts of high-affinity antibodies.

In a TI immune response, mainly MZ and B-1 B cells are involved. Generally, no GCs are formed and only short-lived plasma cells originating from extrafollicular regions develop. In contrast to Fol B cells, MZ and B-1 B cells can develop into short-lived plasma cells when activated via Toll-like receptors by a TI-1 antigen. Some authors claim the formation of memory B cells also in a TI response (51), but this subject remains controversial. Both MZ and B-1 B cells are thought to be a more innate-like B cell type that have a phenotype similar to activated Fol B cells, which is obvious from the high level of CD80 and CD86 expression. Thus, both cell types can easily be activated and respond rapidly to antigenic stimuli (52). The location of MZ B cells and their functional properties facilitates early encounter with blood-borne antigens and a rapid response, thereby bridging the time interval between the immediate innate immune response and the delayed TD GC response.

B-1 cells can develop into ASCs without the stimulation of exogenous antigen, resulting in the production of natural IgM in the serum, thus providing a first line of defense.

1.1.5 Important transcription factors and other proteins influencing lineage and cell fate decisions

As outlined above, a HSC has to go through numerous intermediate stages until finally becoming a terminally differentiated B cell. To date, numerous transcription factors and other proteins, including cell surface receptors, integrins and cytokines, have been identified as important components, but the mechanisms selecting a HSC for the B cell lineage and later for a specific compartment are not yet completely resolved.

For many years it was thought that the decision for a specific hematopoietic lineage is made at a very early step during differentiation, a process that was termed lineage commitment. Based on several factors including the expression of surface molecules and the grade of lineage potential, hierarchical tree models for hematopoiesis were developed. The discovery of new intermediate progenitors made it necessary to adjust these models during the years, but newer findings do not fit into these models anymore. It seems that the potential to differentiate into other hematopoietic lineages is irretrievably lost, if at all, then only in terminally differentiated cells such as plasma cells, since even mature B cells are not restricted to the B cell lineage. It has been shown that mature B cells are able to undergo dedifferentiation into uncommited precursors, which then can develop into T cells (53).

Recently, a completely different model of hematopoiesis was presented (54). This so-called pairwise relationships model (fig. 5) suggests that no strict branching points exist and instead there is a continuum of lineage relationships. HSCs developing towards a specific cell fate successively loose lineage potentials, keeping the cell fate potentials that are directly adjacent in the illustration until the final step of differentiation. During differentiation each cell fate potential varies between being dominant, available or having been lost, meaning latent. The pairwise relationships model also shows that contiguous cell fates are regulated by the same set of transcription factors. In general, a specific transcription factor promotes the development towards a specific cell fate while at the same time repressing the potential to differentiate into another cell fate.

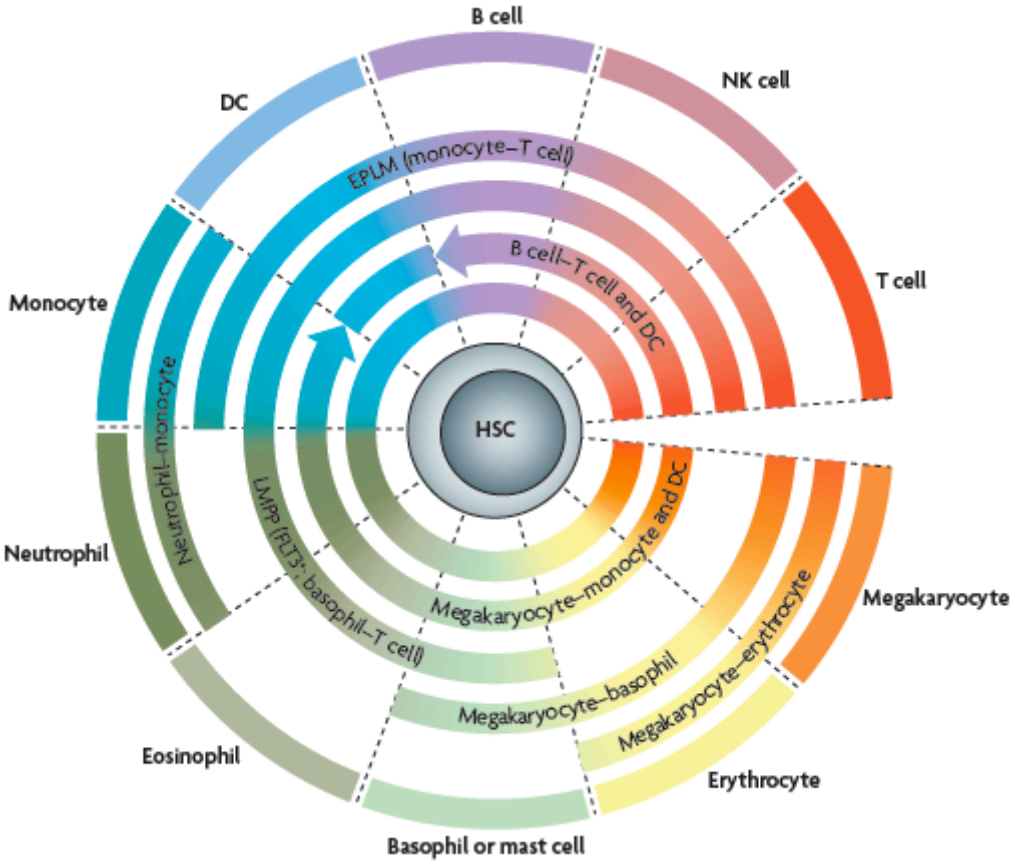

Figure 5: The pairwise relationships model of hematopoiesis*. Instead of a hierarchical tree structure, this model depicts hematopoiesis as a continuum of lineage relationships, where no strict branching points exist. The known oligopotent progenitor cells are indicated by arcs. DC, dendritic cell; NK cell, natural killer cell. Adapted from Ceredig et al., 2009 (54).*

One of the earliest transcription factors shown to be important for differentiation into the B cell lineage is PU.1, which belongs to the ETS family. Expression of *Pu.1* in a subset of MPPs directs them to the myelo-lymphoid differentiation pathway (55). Mice deficient in *Pu.1* die during embryonic development at day 18.5 and have a reduced number of MPPs in the fetal liver, resulting in the absence of B cells, T cells, monocytes and granulocytes. The impairment of proliferation and differentiation capacities can be explained at least in part by a reduced expression of the cytokine receptor FLT3 and an inability to express IL-7Rα, as the IL-7Rα gene is thought to be regulated directly by PU.1 (56). Interestingly, the level of PU.1 expression seems to be crucial for the decision between the B cell and the myeloid cell pathway. Precursors expressing low levels of PU.1 tend to B lymphopoiesis, while high levels of PU.1 expression inhibit B cell development and favor the myeloid cell development (57).

The zinc-finger transcription factor IKAROS is widely expressed in all cells of the hematopoietic system. IKAROS exists in many isoforms which are generated by alternative splicing of the *Ikfz1* gene. Mice expressing a mutant form of IKAROS, which is unable to bind to DNA, die before birth. In the embryos, B and T cells are completely absent and development of myeloid and erythroid cells is severely impaired. The differentiation block is downstream of the block observed in *Pu.1*$^{-/-}$ mice at the LMPP stage (58) and *Pu.1*$^{-/-}$ cells show normal levels of IKAROS (56). *Ikfz1*$^{-/-}$ progenitor cells upregulated factors important in myeloid cell differentiation. Enforced expression of EBF (early B-cell factor) in *Ikfz1*$^{-/-}$ cells can restore the B cell lineage potential, which is probably due to the ability of EBF to redirect the myeloid potential towards the B cell lineage (59, 60).

Another important transcription factor essential for B cell lineage differentiation is E2A, a member of the basic helix-loop-helix (bHLH) protein family. Actually, two transcription factors, namely E12 and E47, are collectively termed E2A and are generated from a single gene by alternative splicing. Deficiency in the *E2a* gene results in a differentiation block at an early B cell precursor stage (61). These cells have not yet started to rearrange their IgH chain genes and several B cell lineage specific proteins are absent. E2A is required for the initiation and maintenance of the expression of another transcription factor, EBF (early B cell factor) and expression of EBF in E2A deficient mice rescues the observed developmental block, but the obtained B cells show an impaired response to IL-7 (62). E2A is furthermore directly involved in the recombination of IgH and Igκ chain, accomplished most likely by the binding of E2A to the Eμ enhancer (63) and the intronic κ enhancer (iEκ) (64), respectively. Also, E2A seems to be important in secondary Igκ rearrangements during receptor editing (65) and in GC reactions, as it is supossed to regulate the expression of AID (66).

EBF regulates the expression of several essential transcription factors, which are PAX5 (paired box protein 5), FOXO1 (Forkhead O1) and OBF1 (Oct-binding factor 1) (67). Mice deficient in the gene encoding EBF show a phenotyp that is similar to the one observed in E2A deficient mice, with a block at the CD19^{-} pre-pro B cell stage and the absence of IgH chain recombination (68). This phenotype can only partially be rescued by expression of the downstream transcription factor PAX5 and recently EBF has been shown to be important for commitment to the B cell fate independent of PAX5 (59).

PAX5 is a paired homeodomain protein that mediates commitment to the B cell lineage (13) and is indispensable for maintaining the B cell fate (53, 69). This transcription factor

directly activates several B cell specific genes such as those encoding CD19 and Igα and represses the expression of genes encoding for proteins important for other lineages like M-CSF receptor and NOTCH-1, which are specific for the myeloid and T cell lineage, respectively (70). Moreover, PAX5 establishes a positive feedback loop by activating the genes for E2A and EBF, thus promoting B cell lineage commitment. *Pax5* deficient mice have a complete block in B cell differentiation downstream of the one seen in E2A and EBF1 deficient mice. The most mature B cells found in these mice are $CD19^-$ and have rearranged D_HJ_H gene segment, while V_H to D_HJ_H rearrangement is severely impaired. These $Pax5^{-/-}$ B cells can differentiate *in vitro* and *in vivo* into almost all hematopoietic cell types, including macrophages, osteoclasts, dendritic cells (DCs), granulocytes, natural killer (NK) cells, T cells and erythrocytes (13, 71, 72). The importance of maintaining *Pax5* expression in order to keep B cell lineage commitment has been shown by several experiments. The enforced retroviral expression of the transcription factors C/EBP-α and C/EBP-β in differentiated B cells resulted in inhibition of PAX5 and down-regulation of its target genes, leading to the reprogramming of the B cells and their differentiation into macrophages (69). In another experiment, conditional deletion of *Pax5* in mature B cells led to the loss of B cell specification, resulting in de-differentiation to uncommitted progenitors and differentiation into the T cell lineage (53).

The transcription factor FOXO1 is like PAX5 directly regulated by EBF. FOXO1 is considered to be important at several steps during B cell development (73). Mice deficient in FOXO1 have a reduced level of IL-7Rα expression resulting in a block at the pro-B cell stage. Furthermore, these cells show decreased levels of RAG-1 and RAG-2 proteins resulting in impaired V(D)J recombination. FOXO1 was found to directly regulate the expression of the RAG proteins (74). Deletion of *FoxO1* at later stages in B cell development showed a complete failure of CSR, both in TD in TI-2 responses, which was assigned to a direct involvement of FOXO1 in the upregulation of the gene encoding AID (73).

Interferon-regulatory factor 4 (IRF4) and IRF8 are highly related transcription factors that are expressed in lymphoid and myeloid lineage cells. Binding to recognition sites requires the interaction with other transcription factors such as PU.1 or the related SPI-B, as IRF4 and IRF8 bind only weakly on their own. Due to redundancy, only mice deficient for both *Irf4* and *Irf8* show a block in B cell differentiation at the cycling large pre-BII cell stage. These cells showed upregulated expression of *VpreB* and *λ5* and failed to downregulate the pre-BCR and to rearrange the IgL chain loci (75). Recently, it was shown that IRF4 and

IRF8 induce the expression of the transcription factors IKAROS and AIOLOS, which mediate the downregulation of surrogate LC expression and induce cell-cycle arrest (76).

OBF1 is a coactivator of the transcription factors OCT1 and OCT2 and its expression is induced by EBF. Despite this early activation, mice deficient in OBF1 have a nearly normal early B cell development until the stage of BM immature B cells (77). However, in later developmental stages several defects have been observed. *Obf1*$^{-/-}$ mice have severely reduced numbers of peripheral B cells and an impaired TD immune response with a lack of GC formation (78). Absence of OBF1 in combination with genetic factors, meaning only on a C57/BL6 background, results in an almost complete loss of MZ B cells, while B-1 B cells are not affected (79). In contrast, development of B-1 cells is dependent on OCT-2, as cells from *Oct2*$^{-/-}$ mice are unable to reconstitute peritoneal B-1 cells (80). Furthermore, OCT2 has more recently been shown to be involved in the formation of ASCs through the direct regulation of the expression of the IL-5R α-chain (81). Activation of this receptor by its ligand IL-5 resulted in increased expression of BLIMP1, a transcription factor known to be essential for plasma cell differentiation (see below).

The Notch family represent a special kind of transcription factors. Upon activation of the receptor by ligand binding, the Notch receptor is cleaved by the proteases TACE and γ-secretase and the so-called Notch intracellular domain (NICD) is released. NICD then translocates to the nucleus where it acts as a transcription factor. Engagement of either NOTCH1 or NOTCH2 by the ligand DELTA-LIKE1 (DLL1) has been shown to be necessary for MZ B cell generation (82, 83). Furthermore, engagement of NOTCH1 by DLL1 promotes the differentiation of activated B cells into ASCs (84).

The transcriptional control of ASC and plasma cell generation is quite complex, as it is apparent from the transcription factors described so far (47, 85). Upon entry into the GC reaction, B cells express high levels of PAX5 and BCL6 (B cell lymphoma 6). As already mentioned above, PAX5 is necessary for maintaining B cell lineage commitment but also for the expression of AID (86) and the repression of X-box-binding protein 1 (XBP1), a transcription factor required for plasma cell differentiation. Despite several other functions, BCL6 is expressed in centroblasts and centrocytes. It was shown that BCL6 directly suppresses apoptotic proteins such as p53, and also the protein ATR, which is one of the main sensors of DNA damage, thus BCL6 expression makes the centroblasts and centrocytes less sensitive to genotoxic stress, caused by high proliferation rates and genomic remodelling within SHM and CSR. Both PAX5 and BCL6 inhibit the differentiation into

plasma cells, making it necessary to downregulate these proteins to achieve further differentiation. BCL6 represses the expression of B-lymphocyte-induced maturation protein 1 (BLIMP1), which was shown to be an essential regulator of plasma cell differentiation. Ectopic expression of BLIMP1 drives differentiation into plasma cells (87) and suppresses the expression of both *Pax5* and *Bcl6*, revealling the existence of a regulatory circuit. BLIMP1 deficient B cells were shown to develop into cells secreting Ig, but full differentiation into plasma cells could not be achieved. IRF4 has been found to be essential for plasma cell differentiation and CSR and both BLIMP1 and IRF4, which both are suppossed to be upstream of XBP1, seem to be required for the development of fully differentiated plasma cells (88). Although much is known about the genetic regulation of plasma cell differentiation, a clear picture did not yet emerge and several factors remain to be identified.

Despite the transcription factors, numerous cytokines have been identified that regulate B cell development, among which are FLT3 ligand (FLT3L), which is the ligand for Fms-like tyrosine kinase 3 (FLT3), interleukin 7 (IL-7) and B cell activating factor belonging to the TNF family (BAFF). FL acts on very early progenitors and BAFF is important in the splenic transitional and later developmental stages (see below). Mice deficient in IL-7 or IL-7Rα have a block in B cell development at the stage of pre-proB cells (89). IL-7 has been shown to be necessary for the maintenance of B cell potential in CLPs (90), and signaling via the IL-7R was found to be necessary to keep EBF levels above a certain threshold, as IL-7Rα$^{-/-}$ pre-pro B cells had undetectable levels of EBF and PAX5 expression, which resulted in absence or severe reduction of Igα, λ5, VpreB and RAG1/2 protein levels (89). Analysis of IL-7 and IL-7Rα deficient mice also revealed, that fetal and adult hematopoiesis differs with respect to IL-7 dependence. During fetal hematopoiesis mature B cells develop in the absence of IL-7. These cells have been shown to be either B-1 or MZ B cells (91), indicating that the development of Fol B cells depends on IL-7R signaling.

1.2 FLT3 and FLT3L

1.2.1 Structure and expression pattern

FLT3 (FMS-like tyrosine kinase), also known as FLK-2 (fetal liver kinase 2) is a receptor tyrosine kinase and belongs to the family of class III receptor tyrosine kinases. A subset of this family, characterized by an extracellular domain composed of 5 Ig-like domains and by a cytoplasmic domain consisting of a split tyrosine kinase motif, comprises in addition to FLT3 also c-KIT, M-CSFR (macrophage colony stimulating factor receptor; also known as FMS) and PDGFR (platelet-derived growth factor receptor). FLT3 was found to be expressed in a variety of tissues including BM, fetal liver, thymus, spleen, gonads, placenta and brain. Within the hematopoietic compartment, FLT3 expression is found on early B and T cell progenitors in BM and thymus and on peritoneal macrophages and monocytes (92). LT-HSCs from the BM have been found to be negative for FLT3 and upregulation of FLT3 correlates with the loss of self-renewal capacity and lineage potential (see chapter 1.1.1).
FLT3 ligand (FLT3L) is a type I transmembrane protein that exists either in a transmembrane, a membrane-bound or a soluble form. In mice, the most abundant isoform is the membrane-bound protein, which arises from alternative splicing resulting in a protein that terminates in a stretch of hydrophobic amino acids that serve as membrane anchor. Soluble FLT3L is produced by proteolycical cleavage of the transmembrane form or more rarely by alternative splicing. All isoforms are biologically active and can activate FLT3 signaling. Structurally, FLT3L is similar to stem cell factor (SCF), the ligand of c-KIT, and to M-CSF. In contrast to its receptor, FLT3L is ubiquitously expressed at the mRNA level, but FLT3L protein has only been found in stromal fibroblasts in the BM and in T cells (93).

1.2.2 Biological function

Mice deficient for FLT3 are viable and healthy and have an almost normal B cell compartment with mature B cells in normal numbers being present in peripheral blood, spleen and BM. $B220^+$ B cell progenitors in the BM were diminished and more primitive progenitors showed *in vitro* a decreased potential for development into the B cell lineage. Moreover, competitive repopulation experiments revealed a defect at the stem cell level, as *Flt3*$^{-/-}$ BM cells were not able to effectively reconstitute the hematopoietic system (94).

Mice deficient for FLT3L are also viable and healthy but have a slightly different phenotype than FLT3 deficient mice, although results might not be comparable as mice were bred on different genetic backgrounds. In C57BL/6 *Flt3L*$^{-/-}$ mice, leukocyte cellularity was reduced in peripheral blood, spleen, BM and lymph nodes and the number of dendritic cells (DCs) and natural killer (NK) cells was severely reduced. As in mice deficient in FLT3, the number of B cell progenitors in the BM was significantly decreased, albeit mature B cells that were able to mount a TD immune response were present. Furthermore, BM-derived myeloid progenitors were also reduced (95).

In vitro experiments showed that FLT3L synergistically stimulates the growth of lymphoid and myeloid progenitors in combination with the appropriate cytokines or interleukins, but that it is not efficient when used alone (96, 97). Interestingly, FLT3L has no effect on erythroid, mast cell or eosinophil development. Stimulation of B lymphopoiesis was observed when FLT3L was used in combination with IL-7, IL-3 and IL-11. Likewise, the expansion of CD34^{+} cord blood progenitors and the progenitors of granulocytes, macrophages, T cells, NK cells and DCs was supported when FLT3L was used in combination with SCF or TPO (thrombopoietin), G-CSF, M-CSF, IL-12, IL-15 and GM-CSF or IL-4, respectively (93, 98). *In vivo* studies with mice showed that administration of FLT3L results in the expansion of hematopoietic progenitor cells (HPCs) and stimulation of hematopoiesis, leading to splenomegaly, BM hyperplasia and enlargement of LNs and liver. An increase in immature B cell numbers is observed in BM and spleen, but the greatest effect is observed within the DC compartment, with extremely large numbers of functional mature DCs (99). Administration of FLT3L and G-CSF has a strong synergistic effect on the mobilization of HPCs to the blood stream and these cells are able to reconstitute lethally irradiated donors upon transfer.

1.3 BAFF and its receptors, BAFF-R, TACI and BCMA

1.3.1 Structure and expression of BAFF

B cell activating factor belonging to the tumor necrosis factor (TNF) family (BAFF) was discovered a decade ago by several different groups and thus it is also known as BLyS (B lymphocyte stimulator), TALL-1 (TNF- and ApoL-related leucocyte-expressed ligand 1), THANK (TNF homologue that activates apoptosis, NF-B, and JNK) and zTNF4 (100-104). BAFF is a type II transmembrane protein of the TNF ligand superfamily (TNFSF) and it is highly homologous to APRIL (a proliferation inducing ligand), another member of the TNF ligand family (105). BAFF and APRIL share about 50% similarity within the TNF homology domain (THD), which is a C-terminal sequence characteristic for the TNFSF. BAFF exists in a transmembrane and a soluble form, generated by cleavage of the transmembrane form by a furin-like protease. Furthermore, a shorter isoform termed deltaBAFF exists, which arises from alternative splicing (106). DeltaBAFF is also a transmembrane protein, but it lacks the furin protease recognition motif and thus can not be released as a soluble molecule. All forms of BAFF adopt a trimeric structure, which is mediated by the THD. Soluble BAFF exist as a homotrimer (107, 108), but it is reported that these homotrimers are able to form virus like clusters consisting of up to 60 monomers (109). The question of which form is prevalent is still unanswered. Furthermore, BAFF can also form heterotrimers with deltaBAFF or APRIL. BAFF - deltaBAFF heterotrimers exist only as transmembrane proteins, and the heteromultimerization is thought to inhibit the release of soluble BAFF trimers, in that way regulating the function of BAFF (110). Soluble BAFF - APRIL heterotrimers are biologically active and have been found in patients with an autoimmune disease (111). How these soluble heterotrimers are formed is unclear, as APRIL is only initially transcribed as a transmembrane protein, but is then processed already in the Golgi apparatus by a furin convertase and secreted as homotrimer (112). A fusion protein named TWE-PRIL, consisting of the cytoplasmic and transmembrane domain of TWEAK, which is another TNFSF member, and the extracellular domain of APRIL, exists as a membrane bound form (113). It is assumed that full-lenght BAFF and TWE-PRIL form transmembrane heterotrimers that upon cleavage by a protease give rise to soluble BAFF - APRIL heterotrimers.

BAFF is produced mainly by myeloid cells such as monocytes, macrophages and DCs, but it was also found in activated T cells. Moreover, activation of B cells with LPS or CpG

resulted in a MyD88 dependent upregulation of BAFF expression and secretion (114). In humans, the expression pattern is largely identical. hBAFF is produced by macrophages and DCs and upon activation with IFNγ or G-CSF also by neutrophils (115). Human DCs and monocytes were shown to upregulate hBAFF production upon stimulation with IFNα, IFNγ, CD40L or LPS (116). Beside the myeloid cells, several other human cell types have also been found to produce hBAFF upon activation, including airway (117) and salivary gland epithelial cells (118), fibroblast-like synoviocytes (119) and astrocytes (120). Furthermore, hBAFF is also produced by VCAM1 positive stromal cells from human BM (121) and by osteoclasts (122). Several malignant human B cell lines, such as B cell chronic lymphatic leukemia, multiple myeloma and Hodgkin's lymphoma cells, also produce hBAFF (123-125).

1.3.2 The BAFF receptors BAFF-R, TACI and BCMA

BAFF can bind to three receptors called BAFF receptor (BAFF-R, also known as BR3), transmembrane activator and CAML (calcium modulator and cyclophilin ligand) interactor (TACI) and B cell maturation antigen (BCMA). While BAFF-R specifically binds BAFF, TACI and BCMA can bind both BAFF and APRIL (fig. 6). TWE-PRIL is also thought to be bound by TACI and BCMA, while BAFF - APRIL heterotrimers bind to TACI (111). Both the transmembrane and soluble form of BAFF can bind to BAFF-R and probably also BCMA, while TACI has been shown to be activated only by membrane bound or oligomeric BAFF and APRIL, but not by the soluble trimeric form of these ligands (126). DeltaBAFF and its heterotrimers are unable to bind to BAFF-R or TACI (106).

BAFF-R, TACI and BCMA belong to the TNF receptor superfamily (TNFRSF), characterized by a cysteine-rich extracellular part. This extracellular part usually contains several cysteine-rich domains (CRDs), which is made up of two structural modules. The extracellular domain of TACI consists of two CRDs, while BCMA has only one CRD in its extracellular domain. The CRDs of TACI and BCMA contain a specific module that is not commonly found in other receptors of the TNFSF. BAFF-R contains only a partial, atypical module and thus not a complete CRD, making it an unusual member of the TNFSF. All three receptors are type III transmembrane proteins that are expressed on B cells (127). In mice, BAFF-R is expressed on splenic B cells and a subset of $CD4^+$ T cells, while TACI is highly expressed on splenic T2 and MZ B cells and at lower levels on Fol B cells, but not on

T1 B cells nor on resting or activated $CD4^+$ T cells (128). BCMA is found on plasmablasts and plasma cells, but not on earlier developmental stages, including mature splenic B cells (129).

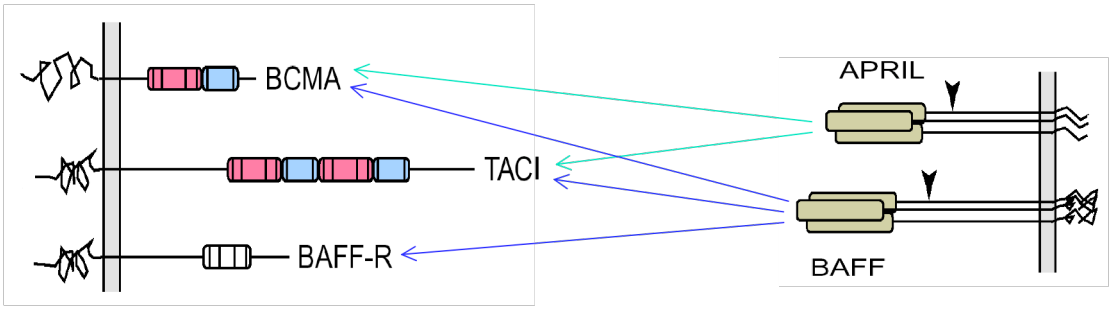

Figure 6: BAFF and APRIL and their receptors. *BAFF can bind to all three receptors, while APRIL only binds to TACI and BCMA. BCMA, B cell maturation antigen; TACI, transmembrane activator and CAML interactor; BAFF-R, BAFF receptor; APRIL, a proliferation inducing ligand; BAFF, B cell activating factor belonging to the TNF family.*

All BAFF receptors belong to a subgoup of the TNFRSF that trigger intracellular signaling events via interaction with TNF receptor associated factors (TRAFs). The six known TRAF molecules have a conserved C terminal domain termed TRAF domain, which enables the interaction with various receptors and multiple intracellular signaling molecules (130). BAFF-R was found to interact only with TRAF3, but none of the other TRAFs (131) and activates the alternative NF-κB pathway (132). In the absence of BAFF-R signaling, TRAF3 is distributed in the cytoplasm and interacts with NF-κB inducing kinase (NIK), thus inducing proteasome-mediated degradation of NIK, resulting in the inhibition of the NF-κB2 pathway. Upon engagement of BAFF-R, TRAF3 is recruited to the receptor and then degraded by TRAF2 dependent proteolysis (133), thus allowing NIK to activate the IκB kinase α (IKKα), which results in the processing of the NF-κB2 p100 precursor protein to p52 and the translocation of the p52/RelB dimer to the nucleus (134). Furthermore, BAFF-R signaling inhibits the nuclear translocation of the pro-apoptotic protein kinase Cδ (PKCδ) (135), which also seems to be mediated by TRAF3, as B cell specific deletion of TRAF3 results in decreased levels of nuclear PKCδ (136). It is suppossed, that interaction of PKCδ with a yet unknown factor inhibits its nuclear translocation, while interaction of TRAF3 with the unknown factor releases PKCδ from the complex, allowing PKCδ to enter the nucleus (130), but this hypothesis has still to be proven. BAFF-R also activates the

kinase AKT and the MAPK pathway with c-RAF and ERK, resulting in downregulation of the pro-apoptotic protein BIM (137, 138). Recently, it was shown that BAFF-R can also be found in the nucleus where it interacts with several proteins including histone H3, IKKβ and NF-κB/c-Rel, thus functioning as transcriptional regulator (139). *In vitro* experiments with a transfected cell line showed that TACI interacts with TRAF2, TRAF5 and TRAF6 (140) while BCMA binds TRAF1, TRAF2 and TRAF3 and activates the MAPK pathway with JNK and p38 and the transcription factors NF-κB and ELK-1 (141), but the signaling pathways activated by TACI or BCMA in a more natural situation are not yet well defined.

1.3.3 Biological activity of BAFF and its receptors

BAFF is an important survival and maturation factor for B cells beyond the stage of immature BM B cells. Mice transgenic for BAFF have enlarged spleen, lymph nodes and Payer's patches, resulting from massively expanded B cell numbers. All stages beyond the T1 stage are affected, while elevated BAFF levels do not have any effect on progenitor B cell stages in the BM. The largest increase is seen in T2, MZ and splenic $B220^+$ $CD5^+$ B cell numbers, whereas Fol B cell numbers are increased to a lesser extend. BAFF transgenic mice exhibit also increased levels of serum immunoglobulin of all isotypes and IgG sub-classes. Furthermore, BAFF transgenic mice develop an autoimmune disease which closely resembles human systemic lupus erythematosus (SLE), characterized by anti-nuclear and anti-dsDNA autoantibodies, circulating immune complexes and Ig deposition in the kidney, resulting in glomerulonephritis, kidney destruction and proteinurea (103, 142, 143). Mice deficient for BAFF have a severely reduced mature B cell compartment. Splenic Fol and MZ B cells and T2 B cells are almost completely absent, while T1 B cell numbers are only slightly reduced. Both B-1a and B-1b B cells from the peritoneal cavity are present in normal numbers. BAFF deficient mice also have extremely low serum Ig levels and the few mature B cells that are still present in these mice are not able to mount a normal TD or TI immune response (144). *In vitro* studies showed that BAFF prolongs the survival of BM immature and splenic transitional and mature B cells by prevention of apoptosis. Furthermore, incubation of BM immature or splenic transitional B cells resulted in maturation of the cells, obvious from phenotypical and functional changes (145). BAFF was also found to act synergistically with IL-6 to maintain BM plasma cell survival *in vitro* (129).

In contrast, APRIL deficient mice have normal numbers of mature B cells and show only a mild phenotype with impaired class switching to IgA and enlarged GCs (146), thus APRIL is not important for the development of mature Fol and MZ B cells, but for the antigen dependent developmental process.

From the receptor knock-out mice, only mice deficient for BAFF-R show a phenotype that is similar to the one observed for BAFF deficient mice, thus BAFF-R was considered to be the main receptor responsible for the observed B cell survival and maturation effects of BAFF. BAFF-$R^{-/-}$ mice have significantly reduced numbers of splenic T2 and mature Fol and MZ B cells, and the numbers of peritoneal B-1, BM progenitor and splenic T1 B cells are unchanged. Serum Ig levels are reduced, but in contrast to BAFF deficient mice, immune responses to TI-II antigens and IgM responses against some TD antigens are nearly identical to WT mice (147). Mice deficient for BCMA have a normal mature B cell compartment with a normal splenic architecture. Furthermore, BM progenitor B cells and peritoneal B-1 cells are normal in numbers. Serum Ig levels are not changed and primary and secondary immune responses against TD and TI antigens proceed normally (144, 148). TACI deficient mice have a phenotype that closely resembles the one observed for BAFF transgenic mice, with enlarged lymphoid organs and increased numbers of mature B cells. Serum Ig levels are almost normal, only IgA levels are decreased. TD immune resonses in general are nearly normal, but IgM and IgG responses to a TI-II antigen are significantly reduced. Also, $TACI^{-/-}$ mice develop an SLE-like autoimmune disease (149, 150). Thus, TACI signaling has an inhibitory function for peripheral B cell survival.

Both BAFF and APRIL were shown to mediate isotype switching to IgA, IgE and IgG1, in part in synergism with IL-4, by engagement of BAFF-R and TACI, respectively (151) and signaling via the APRIL - TACI axis was shown to be important for the regulation of IgA production (152). The expression of BAFR-R and TACI is up-regulated in response to TLR engagement in Fol, MZ and B-1a cells, leading to increased cell proliferation and Ig secretion (153, 154), thus connecting the innate with the adaptive immune system.

BAFF signaling via BAFF-R was also shown to co-stimulate activated T cells (128) and thus to influence T_H1-type immune responses (155).

In the BAFF-APRIL system, several species differences have been observed comparing the mouse with the human system. While in the murine system TACI was found to be a negative regulator, in humans a defective TACI signaling leads to immunodeficiencies (151). Furthermore, murine BCMA binds APRIL with a low affinity, while human APRIL is bound with high affinity to human BCMA (156).

1.4 Aim of thesis

B cells are an indispensable component of the adaptive immune system and the development of mature and functional B cells is important for humoral immunity. Numerous facts are known about the process of differentiation of HSCs into mature B cells, including intermediate stages and their regulation by transcription factors, cytokines and interleukins. Nonetheless, certain aspects of this differentiation process are still unresolved. A better understanding of the underlying mechanisms might also help to develop new substances for the treatment of human disorders known to be associated with a dysregulated B cell development, including autoimmune diseases and malignant disorders such as lymphomas and leukemias.

The discovery of EPLMs, a new type of progenitor cells with developmental potential for B cells, T cells and myeloid cells and expression of FLT3 (12), raised the question of how signaling via the FLT3 - FLT3L axis would influence the EPLM compartment and developmental potential. FLT3L has long been known to affect B lymphopoiesis. Administration of FLT3L results in an increased number of immature B cell in BM and spleen and *in vitro* experiments showed a synergistic stimulation of B lymphopoiesis when FLT3L was used in combination with IL-7, IL-3 and IL-11. Mice deficient for FLT3L or FLT3 have diminished numbers of BM derived B cell progenitors. Due to the fact that FLT3L administration massively increases DC development, most studies on FLT3L and FLT3 deal with DCs. The analysis of EPLMs from FLT3L treated mice should give the answer to what extend signaling via the FLT3 - FLT3L axis influences non-myeloid cell development and especially B cell development.

Although much is known about the factors that regulate B cell development in the BM, the factors regulating splenic B cell development remain largely unknown. BAFF has been found in *in vitro* experiments to be a survival and maturation factor for BM immature and splenic transitional and mature B cells and signaling via BAFF-R has been shown to mediate this function. BAFF-R deficient mice show a severely impaired B cell development starting with the T2 stage, which results in an almost complete loss of mature B cells, thus the analysis of an involvement of BAFF-R signaling in later stages of B cell development is not possible in BAFF-$R^{-/-}$ mice. The use of monoclonal α-mBAFF-R antibodies that are able to block the binding of BAFF to BAFF-R *in vivo* should help to analyze the role of

signaling via the BAFF - BAFF-R axis in development of B cells into mature Fol and MZ B cells and their maintenance and also in formation and maintaining memory and plasma cells during TD and TI immune responses.

Furthermore, BAFF seemed to have no effect on immature B cells, although a subset of BM immature B cells expresses BAFF-R (157). The first stage at which BAFF seemed to be important was at the transition from the T1 to T2 stage in the spleen, as both BAFF and BAFF-R deficient mice show a block at the T1 cell stage. With the help of monoclonal antibodies directed against murine BAFF-R, the expression pattern of mBAFF-R should be analyzed in detail and the developmental stage at which BAFF-R is first expressed should be identified. Also, the question of why BAFF-R is expressed already on BM immature B cells while its function is essential not until the splenic T1 stage should be answered.

The finding that increased expression of BAFF in mice results in the development of autoimmune disorders resembling SLE (142) and, in aged mice, Sjögren's syndrome, led to the discovery of increased BAFF levels in humans affected with these autoimmune diseases (158, 159). To further elucidate the role of BAFF in human autoimmune diseases and other disorders associated with perturbation of B cell homeostasis, monoclonal antibodies against hBAFF should be made in order to develop an ELISA for hBAFF detection. With this ELISA, the reported observations should be confirmed and serum hBAFF levels in further disorders should be analyzed in order to determine an involvement of BAFF in selected human autoimmune, infectious and cancerous diseases.

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2 MATERIALS AND METHODS

2.1 General buffers and solutions

ABI loading buffer	25 mM EDTA, pH 8.0 50 mg/ml blue dextran in deionized formamide
ACK (red blood cell lysis buffer)	0.15 M NH_4Cl 1.0 mM $KHCO_3$ 0.1 mM EDTA sterile filter through a 0.22 µm filter
Coomassie blue staining solution	0.25 g Coomassie brilliant blue R-250 45 ml H_2O 45 ml methanol 10 ml glacial acetic acid
Coomassie destaining solution	45 ml H_2O 45 ml methanol 10 ml acetic acid
DNA loading buffer (6x)	0.25% bromphenol blue 0.25% xylene cyanol 1 mM EDTA 30% glycerol
ECL stock solution A	250 mM Luminol in DMSO stored at RT in the dark
ECL stock solution B	90 mM p-Coumaric acid in DMSO stored at RT in the dark

ECL stock solution C	1 M Tris/HCl, pH 8.5
ECL stock solution D	35% H_2O_2
ECL solution 1	200 µl ECL stock solution A 89 µl ECL stock solution B 2 ml ECL stock solution C 8 ml H_2O stored at 4°C
ELISA blocking buffer	4% BSA 0.2% Tween 20 10 mM NaN_3 in PBS
ELISA substrate buffer for alkaline phosphatase	0.1 g $MgCl_2$ x 6 H_2O 10 mM NaN_3 10% diethanolamine adjust to pH 9.8 with HCl fill up to 1l with ddH_2O
ELISA substrate stock solution (100x)	100 mg/ml Dinitrophenylphosphate in H_2O stored at -20°C
FACS buffer	2% FCS 10 mM NaN_3 in PBS
FACS sorting buffer	2% FCS in PBS sterile filter through 0.22 µm filter

IPTG	1 M IPTG in ddH_2O sterile filter through 0.22 µm filter stored in aliquots at -20°C use 4 µl per agar plate
Lysis buffer for mouse tails	50 mM Tris/HCl, pH 8.0 5 mM EDTA 100 mM NaCl 0.5% SDS add 20 µl Proteinase K (10 mg/ml) to 750 µl lysis buffer directly before use
PBS (10x)	1.37 M NaCl 27 mM KCl 15 mM KH_2PO_4 80 mM Na_2HPO_4 pH 7.2
PBST	0.1% Tween 20 in PBS
SDS-PAGE lower buffer	1 l Tris/HCl (1.5 M), pH 8.7 40 ml SDS (10%)
SDS-PAGE reducing sample buffer (6x)	300 mM Tris/HCl, pH 6.8 30% glycerol 12% 2-Mercaptoethanol 12% SDS 0.02% bromphenolblue
SDS-PAGE running buffer	0.2 M Glycine 25 mM TRIS 0.1% SDS

SDS-PAGE upper buffer	1 l Tris/HCl (0.5 M), pH 6.8 40 ml SDS (10%)
Silver staining-developer solution	6 g Na_2CO_3 in 98 ml ddH_2O add 50 µl formaldehyde (37%) add 2 ml Silver staining-thiosulfate solution prepare fresh before use
Silver staining-fixative solution	50 ml methanol 12 ml glacial acetic acid fill up to 100 ml with ddH_2O add 50 µl formaldehyde (37%) prepare fresh before use
Silver staining-silver nitrate solution	200 mg $AgNO_3$ in 100 ml ddH_2O add 75 µl formaldehyde (37%) prepare fresh before use
Silver staining-stop solution	50% methanol 12% acetic acid in ddH_2O prepare fresh before use
Silver staining-thiosulfate solution	20 mg $Na_2S_2O_3$ * 5 H_2O in 100 ml ddH_2O prepare fresh before use
TAE	1 mM EDTA 40 mM Tris-acetate, pH 8.0

TBE (10x)	21.6 g Tris 11 g Boric acid 8 ml EDTA (0.5 M, pH 8.0) fill up to 200 ml with dd H_2O
TBS	10 mM $MgCl_2$ 10 mM $MgSO_4$ 10% PEG 4000 5% DMSO 15% glycerol in LB medium sterile filter through 0.22 µm filter
TE	1 mM EDTA 10 mM Tris/HCl, pH 8.0
Western blot transfer buffer	0.2 M Glycine 25 mM TRIS 20% Methanol
Western blot blocking buffer	5% milk powder 0.1% Tween 20 dissolve in PBS
X-Gal	20 mg X-Gal in 1 ml DMF store at -20°C in the dark use 40 µl per agar plate

2.2 Bacterial media and supplements

LB medium	10 g tryptone (BD Difco) 5 g yeast extract (BD Difco) 5 g NaCl fill up to 1 l H_2O and autoclave
LB-Agar	15 g agar (Sigma) added to 1 l LB medium and autoclave
Ampicilin	stock 100 mg/ml in H_2O, used at 100 µg/ml
Kanamycin	stock 50 mg/ml in H_2O, used at 50 µg/ml

2.3 Cell culture media and supplements

IMDM medium, SF	176.6 g IMDM powder (Gibco BRL) 30.24 g $NaHCO_3$ (Fluka) 100 ml Penicillin/Streptomycin solution (100x) (Gibco BRL) 100 ml MEM Non Essential Amino Acids (100x) (Gibco BRL) 10 ml Insulin (5 mg/ml) (Sigma) 10 ml β-Mercapthoethanol (50 mM) (Fluka) 30 ml Primatone RL (10%) (Quest International) adjust to pH 7.0 with NaOH (Fluka) fill up to 10 l with fresh H_2O, tridistilled sterile filter through 0.22 µm filter
RPMI medium	Gibco BRL
DMEM with Glutamax	Gibco BRL

HAT medium	1 l SF-IMDM medium 20 ml FCS 5 ml Ciproxin 2 x HAT Media Supplement (50x) (Sigma) 10 ml IL6-supernatant sterile filter through 0.22 µm filter
SF-900 medium	1 x SF-900 II SFM powder for 10 l (Gibco BRL) 3.5 g $NaHCO_3$ adjust to pH 6.20 - 6.30 with NaOH fill up to 10 l with fresh H_2O, tridistilled sterile filter through 0.22 µm filter
Freezing medium	10% DMSO in FCS
FCS	Amimed
Cellfectin	Gibco BRL
$CuSO_4$ solution	500 mM $CuSO_4$ sterile filtered through 0.22 µm syringe filter used at 1.25 mM
Cyproxin	2 mg/ml solution (Bayer AG)
IL6-supernatant	IL6 producing X63 cells (X63-IL6) were grown in 2% FCS/SF-IMDM medium for 5-7 days, then the suspension was sterile filtered through diatomaceous earth (Highflow Super Cell Medium, Fluka). The resulting cleared supernatant was kept at 4°C.
Lipofectamine Reagent	Invitrogen

Plus Reagent	Invitrogen
Trypsin solution	Gibco BRL
PEG 1500, 50%, sterile solution	Boehringer Mannheim
Penicillin/ Streptomycin (100x)	Gibco BRL
Puromycin	stock 10 mg/ml in H_2O sterile filtered through 0.22 µm syringe filter used at 5 µg/ml

2.4 Primers

Name	Sequence (5′- 3′)	Application
hBAFF-1	GAT AAC AGG AAA TGA TCC ATT C	amplification of hBAFF cDNA, 5′-UTR
hBAFF-2r	CTT AGA GGT ACA GAG AAA GG	amplification of hBAFF cDNA, 3′-UTR
hBAFF-5	*GGG CCC* **AGG GTC CAG AAG AAA C**	soluble hBAFF cDNA, 5′-end, introduction of ApaI site
hBAFF-6r	*GCG GCC GC*T **CAC AGC AGT TTC AAT GC**	soluble hBAFF cDNA, 3′-end, introduction of NotI site
hBAFF-S1	CAA GTG AAT CAT CTC AGT GC	sequencing downstream of pRmHa-3 promoter
hBAFF-S2r	TAT TAT CGT ATT AGG AGA AG	sequencing upstream of pRmHa-3 ADH-polyA site
mBAFF-R4	ATT *AGA TCT* GAA **ATG GGC GCC AGG AGA CTC C**	mBAFF-R cDNA, coding start, introduction of BglII site
mBAFF-R5r	GAT *GAA TTC* **CTA TTG CTC TGG GCC AGC TG**	mBAFF-R cDNA, 3′-end, introduction of EcoRI site

Remarks: coding sequence in bold, sequence of restriction sites in italics

Primers were obtained from Microsynth GmbH, Switzerland or from QIAGEN, Germany.

2.5 Plasmids

Name	Desciption	Insert	Reference	Application
pCR4-TOPO (4 kb)	amp^R, kan^R, $lacZ^+$	---	Invitrogen	Subcloning of PCR products with TA-overhang
pCR-Blunt II-TOPO (3.5 kb)	kan^R, $lacZ^+$	---	Invitrogen	Subcloning of PCR products with blunt ends
phshs-puro	$puro^R$	---	Steller et al., 1985 (1)	Co-transfection of Drosophila Schneider cells
pGEM-hBAFF (4 kb)	based on pGEM-T Easy amp^R, $lacZ^+$	hBAFF (984 bp)	M. Rauch this thesis	Storage of hBAFF cDNA
pRmHa3-HA/myc/TM (4.2 kb)	based on pRmHa3	---	Puig et al., 2001 (2)	Expression vector for Drosophila Schneider cells
pRmHa3-HA-hBAFF (4.6 kb)	based on pRmHa3-HA/myc/TM	soluble hBAFF, aa 136-285 (453 bp)	M. Rauch this thesis	Production of HA-hBAFF protein in Drosophila SL-3 cells
pMIG-R1 (6.2 kb)	MSCV based retroviral vector containing the complete GFP coding sequence preceded by the encephalomyocarditis internal ribosome entry site	---	Addgene #9044 Pear et al., 1998 (3)	Expression vector for eukaryotic cell lines
pMIG-mBAFF-R (6.7 kb)	based on pMIG-R1	mBAFF-R (528 bp)	M. Rauch this thesis	Expression of mBAFF-R in different cell lines

2.6 Antibodies and recombinant proteins

2.6.1 Antibodies for FACS

Commercial antibodies were diluted in FACS buffer and used at a final dilution of 1:200.

anti-mouse CD3ε	142-2C11	BD Pharmingen
anti-mouse CD4	RM4-5	BD Pharmingen
anti-mouse CD5	53-7.3	BD Pharmingen
anti-mouse CD8α	53-6.7	BD Pharmingen
anti-mouse CD11b	M1/70	BD Pharmingen
anti-mouse CD11c	HL3	BD Biosciences
anti-mouse CD21	7G6	BD Pharmingen
anti-mouse CD23	B3B4	BD Pharmingen
anti-mouse CD25 α-chain	7D4	BD Pharmingen
anti-mouse CD44	IM7	BD Biosciences
anti-mouse CD45R (B220)	RA3-6B2	BD Pharmingen
anti-mouse CD117 (c-kit)	2B8	eBiosciences
anti-mouse CD127 (IL-7Rα)	A7R34	eBiosciences
anti-mouse CD135 (FLT3)	A2F10	eBiosciences
anti-mouse λ_{1+2}-LC		BD Biosciences
anti mouse TCRβ	H57	BD Biosciences
anti-mouse TCRγ/δ	GL3	BD Biosciences
anti-mouse NK1.1	PK 136	BD Biosciences
mouse-anti-rat IgG		Jackson ImmunoResearch Laboratories
Streptavidin-PE	used at 1:1000	BD Biosciences
Streptavidin-PE-Cy7	used at 1:400	BD Biosciences
Streptavidin-APC	used at 1:200	Becton Dickinson

Antibodies produced in the laboratory were individually titrated before use.

anti-HA	12CA5
anti-mouse CD19	1D3

anti-mouse CD90	T24
anti-mouse CD93	PB493
anti-mouse CD117 (c-Kit)	ACK4
anti-mouse IgM	M41
anti-mouse IgD	1.19
anti-mouseκ-LC	187.1

2.6.2 Antibodies and proteins used for ELISA or Western Blot

anti-ratIgG-2A	BD biosciences
anti-ratIgG-2B	BD biosciences
Biotin-SP mouse anti-rat IgG	Jackson ImmunoResearch
hBAFF, soluble	PeproTech
Streptavidin-AP	Amersham biosciences
anti-ratIgG-AP	Jackson ImmunoResearch
Avidin-HRP	BD biosciences

2.7 Kits

Big Dye 1.1 Terminator v 1.1 Cycle Sequencing Kit	Applied Biosystems
Geneclean Kit	Q-Biogene
NucleoSpin Plasmid kit	Macherey-Nagel
Pure Yield Plasmid Midiprep System	Promega
QIAEX II Gel Extraction Kit	QIAGEN
QIAfilter Plasmid Midi Kit	QIAGEN
QIAprep Spin Miniprep Kit	QIAGEN
QIAquick Gel Extraction Kit	QIAGEN
QIAquick PCR Purification Kit	QIAGEN
SuperScript First-Strand Synthesis System for RT-PCR	Invitrogen

2.8 Cell lines

Name	Description	Culture medium
Sp2/0 (Sp2/0-Ag14)	mouse myeloma ATCC CRL-1581	SF-IMDM, 2% FCS, 0.5% Cyproxin
Y3 (Y3-Ag 1.2.3)	rat myeloma ATCC CRL-1631	SF-IMDM, 2% FCS, 0.5% Cyproxin
Phoenix	amphotropic retroviral packaging cell line ATCC SD 3443	RPMI, 10% FCS, 50 µM β-Mercapthoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.5% Cyproxin
SL-3	Drosophila melanogaster ATCC CRL-1963	SF-900, 2% FCS
40E1	pre-B cell Alt et al., 1981 (4)	SF-IMDM, 2% FCS, 0.5% Cyproxin
OP9	stroma cell line does not produce M-CSF Kodama et al., 1994 (5)	SF-IMDM, 2% FCS, 0.5% Cyproxin
OP9-DL1	OP9 expressing the Notch ligand Delta-like 1 (DL1) Schmitt et al., 2002 (6)	SF-IMDM, 2% FCS, 0.5% Cyproxin
ST-2	stroma cell line Hardy et al., 1987 (7)	SF-IMDM, 2% FCS, 0.5% Cyproxin

2.9 Animal strains

Name	Description	Source
C57BL/6	wild type mice	RCC Ltd., Füllinsdorf
$FcR\gamma^{-/-}$	mice deficient for FcRγ-chain	Taconic, Ejby, Denmark
Bcl-2 tg	mice transgenic for Bcl-2 gene under $H2K^b$ promoter	Dr. A. Trump, ISREC, Lausanne
Lewis	wild type rat	RCC Ltd., Füllinsdorf

2.10 Molecular biology methods

2.10.1 RNA isolation

RNA from tissue or cultured cells was isolated with Tri Reagent (MRC) according to the manufacturer's description, dissolved in 15 µl DEPC-treated ddH_2O per 1 ml initially used Tri Reagent and stored at -80°C.

2.10.2 Polymerase chain reaction (PCR)

2.10.2.1 Analytical PCR

For analytical purposes, PCR reactions were mixed on ice in 0.2 µl thermo PCR tubes and were carried out with Taq DNA polymerase. The reaction mixture was set up as follows:

1-5 µl	DNA template
3 µl	forward primer (5 µM)
3 µl	reverse primer (5 µM)
1.5 µl	dNTP mix (5 mM)
2 µl	PCR buffer (10x)
0.5 µl	Taq DNA polymerase
fill up to 20µl with ddH_2O	

The PCR reaction was performed according to the following standard program:

1 cycle	94°C	45 sec (3 min for genomic DNA template)
35 cycles	92°C	45 sec
	T_m-3°C	30 sec
	72°C	1 min/kb of product lenght
1 cycle	72°C	10 min
unlimited	10°C	

The melting temperature T_m of each primer was calculated based on the primer sequence. Therefore, each nucleotide in the primer sequence that was complementary to the target

DNA was counted, with A and T counting 2°C and G and C counting 4°C. The annealing temperature was then set 2-5°C lower than the lowest melting temperature calculated for the used primer pair.
For screening of bacteria colonies after transformation, the PCR reaction mixture was set up as described above without DNA template. With a small tip, one colony was picked from the plate and the tip was dipped into the appropriate PCR tube containing the reaction mixture, before dipping the tip into one well of a 96-well plate containing 100 µl of LB/Amp medium. This plate was then kept at RT until the positive clones could be selected based on the result of the PCR. The content of the appropriate wells was added to 3 ml of LB/Amp medium in a 12 ml tube each and cultured O/N.

2.10.2.2 Preparative PCR

For preparative purposes, PCR reactions were mixed on ice in 0.2 µl thermo PCR tubes and carried out with Pfu DNA polymerase (Stratagene). The reaction mixture was set up as follows:

50-200 ng	DNA template
1 µl	forward primer (10µM)
1 µl	reverse primer (10 µM)
4 µl	dNTP mix (5 mM each dNTP)
5 µl	PCR buffer (10x)
1 µl	Pfu DNA polymerase (2.5 U/µl)
fill up to 50µl with ddH_2O	

The PCR reaction was performed according to the following program, which was suggested by the manufacturer:

1 cycle	94°C	45 sec
25-30 cycles	94°C	45 sec
	T_m-5°C	45 sec
	72°C	1.5 min/kb of product lenght
1 cycle	72°C	10 min
unlimited	4°C	

For preparative amplification of demanding templates, such as GC-rich templates, Platinum Pfx DNA polymerase (Invitrogen) and if necessary, the corresponding PCRx Enhancer Solution (Invitrogen) were used, according to the manufacturer's protocol. As the optimal composition of the PCR reaction mixture as well as the optimal PCR cycling conditions are highly dependent on the DNA template that has to be amplified, these settings had to be determined empirically.
Amplification products were subsequently either cloned directly into pCR-Blunt II-TOPO according to the manufacturer's protocol or purified with QIAquick PCR Purification Kit as recommended by the manufacturer and then subjected to restriction enzyme digestion.

2.10.2.3 Sequencing PCR

For sequencing, the PCR was carried out using a ready-to-use solution (Big Dye Terminator Cycle Sequencing Kit, Applied Biosystems), containing fluorescent terminator dideoxy-nucleotides. The set-up of the reaction mixture was the following:

200-500 ng	plasmid DNA (3 µl mini-prep DNA)
3.5 µl	primer (1µM)
2 µl	1/2 BD
2 µl	BD sequencing mix
fill up to 20 µl with ddH_2O	

The PCR reaction was performed according to the following standard program:

1 cycle	96°C	1 min
35 cycles	96°C	30 sec
	50°C	20 sec
	60°C	4 min
1 cycle	60°C	5 min
unlimited	10°C	

To remove free fluorescent terminator dideoxynucleotides, the PCR reaction was purified by DNA precipitation. Therefore, 2 µl sodium acetate (3 M, pH 4.6) and then 50 µl ethanol (100%, RT) were added to each tube, mixed and incubated for 20 min at RT. After

centrifugation at full speed in a table top centrifuge (21 000 x g) for 30 min at 20°C, the supernatant was removed and the pellet was washed by adding 50 µl ethanol (70%, RT) to each tube. Immediately after that, a further centrifugation step for 10 min at full speed and 20°C was performed. Next, the supernatant was removed and the pellet was dried in a speed vac for 5 min. Pellets were then resuspended either in 40 µl ddH_2O for sequencing on a capillary sequencer, or in 3.5 µl ABI loading buffer for sequencing on a gel sequencer.

2.10.3 Preparation and transformation of competent bacteria

2.10.3.1 Preparation of chemical-competent E. coli

100 ml of pre-warmed LB medium were inoculated with 0.5 ml of a fresh overnight culture of *E. coli* DH5α or *E. coli* XL-2. The bacteria cells were grown at 37°C with vigorous shaking to early log phase, which corresponds to an OD_{600} of 0.3-0.6. Cells were then pelleted by centrifugation at 3500 x g (20 min, 4°C). The supernatant was discarded and the cell pellet was resuspended in 10 ml of cold and sterile TBS buffer. 100 µl aliquots of the bacteria suspension were shock frozen in liquid nitrogen and stored at -80°C.

2.10.3.2 Preparation of electro-competent E. coli

600 ml of pre-warmed LB medium were inoculated with 5 ml of a fresh overnight culture of *E. coli* DH10B. The bacteria culture was grown at 37°C with vigorous shaking to an OD_{600} of 0.6-0.8 and the cells were then cooled down on ice for 30 min. Subsequently, the cell pellet was washed successively in 600 ml of ice cold and sterile ddH_2O, in 300 ml of ice cold and sterile ddH_2O and in 12 ml of ice cold and sterile 10% glycerol. Bacteria cells were pelleted in between the different washing steps by centrifugation at 3500 x g (20 min, 4°C). Finally, the pellet was resuspended in 3 ml of ice cold and sterile 10% glycerol and 50 µl aliquots of the bacteria suspension were shock frozen in liquid nitrogen and stored at -80°C.

2.10.3.3 *Transformation of chemical-competent* E. coli

An aliquot of chemical competent *E. coli* DH5α or *E. coli* XL-2 in TBS buffer was thawed on ice and then 1-10 µl of a ligation mix were given to 50 µl bacteria suspension. After incubation on ice for 15-30 min, a heat shock was performed by putting the bacteria to 42°C for 90 sec. Subsequently, the bacteria were cooled down on ice for 2 min. After that, 900 µl

LB medium without antibiotics were added and the bacteria were incubated for 40-60 min at 37°C with shaking. Then, 50-100 µl of the transformation mixture were plated on a selecting agar plate, containing the appropriate antibiotic. The remaining transformation mixture was centrifuged at 10 000 g for 20 sec and the supernatant was discarded except for 100 µl in which the pellet was resuspended. These 100 µl were then also plated on a selecting agar plate and all plates were incubated O/N at 37°C.

2.10.3.4 *Transformation of electro-competent* E. coli

A 50 µl aliquot of electro-competent *E. coli* DH10B was thawed on ice for not more than 5 min. Then, 1-2 µl of ligation reaction was added and the mixture was transfered to a pre-cooled 0.1 cm gap Gene Pulser cuvette (Bio-Rad), making sure that no air bubbles were present in the bacteria suspension. Subsequently, the cuvette was placed into the electroporator and pulsed with 1.8 kV, 25 µF and 200 Ω. Immediately after pulsing, 900 µl of cold LB medium without antibiotics were given into the cuvette and the bacteria were transfered to a 1.5 ml tube. After incubation for 60 min at 37°C with shaking, 50-100 µl of the transformation mixture were plated on an agar plate containing the appropriate antibiotic. The remaining transformation mixture was centrifuged at 10 000 g for 20 sec and the supernatant was discarded except for 100 µl in which the pellet was resuspended. These 100 µl were then also plated on a selecting agar plate and all plates were incubated O/N at 37°C.

2.10.4 Agarose gel electrophoresis

Electrophoretic separation of DNA for analytical or preparative purposes was done by using agarose gels of different concentrations in 1 x TAE. Before pouring, ethidium bromide (10 µg/ml) was added to the gel resulting in a final concentration of 0.1 µg/ml. Prior to loading, 6 x DNA loading buffer was added to each sample. Electrophoresis was performed at 3-6 V/cm. As molecular weight marker, 5 µl of prediluted 1 kb Plus DNA ladder was used, or for quantification of DNA concentration 5 µl SmartLadder.

2.10.5 Purification of DNA from agarose gels

To avoid DNA damage by UV light, the agarose gels were examined under less energetic UV light (366 nm) and the bands of interest were excised quickly. Purification was then performed using either QIAquick Gel Extraction Kit (Qiagen) or for larger DNA fragments QIAEX II Gel Extraction kit (Qiagen), in each case according to the manufacturer's instruction.

2.10.6 Restriction enzyme digestion of plasmid DNA and PCR fragments

For analytical purposes, DNA digestion with restriction enzymes was carried out in 10-20 µl volume according to the enzyme manufacturer's (NEB) recommendations. Usually, the digestion reactions were performed for 30-60 min, unless the enzyme used required other conditions.
For preparative purposes, the DNA amount needed was diluted as recommended by the enzyme manufacturer. Reaction conditions were set as advised, and digestions were performed for at least 2 h to O/N. DNA fragments where then purified by agarose gel electrophoresis.

2.10.7 Ligation of DNA fragments into plasmids

Ligations were usually performed using 50 ng plasmid DNA and 1 µl (400 U) T4 DNA ligase (NEB) in a volume of 20 µl. PCR products that have been directly digested with restriction enzymes were ligated into the plasmid using a molecular ratio between insert and vector of 5-10:1. DNA fragments that were cut out of plasmids were ligated into the desired plasmid with a molecular ratio between insert and vector of 2-4:1. The ligation was incubated either for 2 h at RT or O/N at 16°C.

2.10.8 Preparation of plasmid DNA from *E. coli* cultures

Plasmid DNA was prepared from fresh O/N cultures or from cultures kept at 4°C for up to 48 h after overnight culturing. For Mini-preps, the QIAprep Spin Miniprep Kit (QIAGEN)

or the NucleoSpin Plasmid kit (Macherey-Nagel) were used, for preparation of larger DNA amounts the QIAfilter Plasmid Midi Kit (QIAGEN) or the Pure Yield Plasmid Midiprep System (Promega) were used. All preparations were performed according to the instructions of the manufacturer.

2.10.9 Ethanol precipitation of DNA

DNA precipitation was performed by adding 1/10 volume of 3 M Na-acetate (pH 4.8) and 2 volumes of 100% ethanol to the DNA solution and subsequent incubation for 15-30 min at -20°C. Next the DNA precipitate was pelleted by centrifugation (21 000 x g, 4°C, 15 min) and washed with ice-cold 70% ethanol. After air-drying for about 10 min, the DNA was dissolved in TE. To obtain sterile DNA solutions, as necessary for transfections, the precipitated DNA was dissolved in sterile buffer or dH_2O.

2.10.10 Sequencing

Sequencing of plasmid DNA was performed either by capillary or gel electrophoresis. For using the capillary sequencer (ABI Prism Genetic Analyzer 310), the purified PCR reactions had to be dissolved in H_2O and sequencing was performed with an injection time of 3-4 seconds usually. Using the gel sequencer (ABI 377), the sequencing gel was prepared by dissolving 18 g urea and 7.5 ml Acrylamid/Bis (30%, 37.5:1) in 23 ml H_2O. The gel was then deionized by adding a spoon of amberlite and stirring it for 20 min at RT. 200 ml of fresh 10x TBE was prepared and filtered through a 0.22 µm sterile filter. The gel was filtered through the same filter, and 6 ml of the filtered 10x TBS was added to the filtered gel. This mixture was then degased for 5 min and subsequently cooled on ice. The glas plates were freshly washed with Biorad cleaning solution and rinsed with a 9:1 isopropanol-water mixture. Next, the glas plates were assembled with 0.2 mm spacers inbetween in the pouring chamber. To the ice cold gel-TBS mix, 200 µl APS (10%) and 20 µl TEMED were added and mixed well. Immediately afterwards, the gel was poured and then left for 2 h at RT to allow the gel to polymerize. Subsequently, the gel was mounted in the sequencer and the rest of the freshly prepared 10x TBE was diluted to obtain 1x TBE, which was used as running buffer. The purified PCR reactions, which were resuspended in 3.5 µl ABI loading

buffer, were denaturated by heating to 95°C for 3 min. The samples were then cooled down on ice and 1 µl of each sample was loaded on the gel.

2.10.11 Preparation of genomic DNA from mouse tails

700 µl lysis buffer for mouse tails were added to the mouse tail and incubated O/N at 56°C without shaking. The next day, 250 µl saturated NaCl were added and mixed by inverting the tube. After centrifugation for 10 min at full speed and RT in a table top centrifuge (Eppendorf), the DNA was precipitated by transfering 750 µl supernatant into a new tube already containing 500 µl isopropanol. The solutions were then mixed by inverting the tube and centrifuged for 5 min at 4°C and full speed. The supernatant was removed and the precipitated DNA was washed by adding 500 µl of cold 75% ethanol. After another centrifugation step for 5 min at 4°C and full speed, the supernatant was removed and the pellet was dried. To resolve the DNA, 100 µl of 1x TE buffer was added and the tube was incubated for 10 min at 56°C.
When toes were used instead of tails, 350 µl of DNA lysis buffer were used and incubation at 56°C was reduced to 3 h. Then 125 µl saturated NaCl were added. After centrifugation, 450 µl supernatant and 300 µl isopropanol were mixed. For washing the pellet, 350 µl of cold 75% ethanol were used. finally, the pellet was dissolved in 50-60 µl 1x TE buffer.

2.11 Biochemical methods

2.11.1 Affinity chromatography

Affinity chromatography was used to either purify proteins provided with an HA-tag or for antibodies of mouse or rat origin.
To purify proteins tagged with the hemagglutinin (HA) epitope, the monoclonal anti-HA antibody 12CA5 (Roche) was coupled to CNBr-activated Sepharose™ 4B, fast flow (GE Healthcare) according to the manufacturer's protocol and the material was then packed into a column.
For purification of HA-hBAFF protein from Drosophila SL-3 cell medium, the supernatant was first cleared by filtering through diatomaceous earth (Highflow Super Cell Medium,

Fluka) and then added to the column. Next, the column was washed with about 300 ml PBS. For protein elution, 0.1 M Glycin/HCl, pH 3.0 was used, and 500 µl samples were collected in 1.5 ml Eppendorf tubes containing 50 µl 1M Tris/HCl, pH 8.6. For each sample collected, OD_{280} was meassured and samples showing the highest OD values (usually above 0.7) were pooled. Likewise, all other samples having an OD value above 0.2 were pooled. The pooled fractions were then dialysed against 1x PBS for 48 h at 4°C with changing the PBS 3-4 times.

Antibodies of the IgG subtype of either mouse or rat origin were purified using ProteinG sepharose 4 fast flow (GE healthcare) according to the manufacturer's protocol.

2.11.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Glass plates were washed with a 9:1 isopropanol-H_2O mixture and assebled in the pouring chamber of the Protean II minigel system (Bio-Rad). For two resolving gels, one the following mixture was prepared, depending on the size of the proteins to be separated:

	10% resolving gel	**15% resolving gel**
H_2O	7.35 ml	5.5 ml
lower buffer	3.9 ml	3.9 ml
Acrylamid/Bis (40%, 29:1)	3.75 ml	5.6 ml
APS	90 µl	90 µl
TEMED	15 µl	15 µl

Immediately after preparation, the mixture was poured into the glas plate assembly, leaving a space of 2.5 cm to the top of the smaller glas plate. The resolving gel was then overlaid with 200 µl of 0.1% SDS. After polymerization, the SDS solution was removed, the stacking gel, prepared according to the protocol below, was poured on top of the resolving gel and a comb was inserted immediately.

	4% stacking gel
H_2O	4.5 ml
upper buffer	1.8 ml
Acrylamid/Bis (40%, 29:1)	0.7 ml
APS	50 µl
TEMED	14 µl

After polymerization, the comb was removed carefully and the gel slots were washed with running buffer. Samples were mixed with 6 x reducing sample buffer, boiled for 5 min at 95°C, spun down and loaded. Also, 10 µl of BenchMark Pre-Stained Protein ladder (Invitrogen) were loaded. The gel was run with a constant voltage of 80 V for about 2.5 h.

2.11.3 Coomassie staining

After electrophoresis, the resolving gel was separated from the stacking gel and stained in Coomassie blue staining solution for 30 min with gentle agitation. Subsequently, the gel was transfered to the destaining solution and incubated with gentle agitation until bands were visible on a clear background. If necessary, the destaining solution was replaced with fresh liquid. The gel was then rehydrated in 2% glycerol and dried on Whatmann 3MM paper in a vacuum system.

2.11.4 Silver staining

Silver staining is much more sensitive than Coomassie staining, thus it was used if low amounts of protein had to be detected. To avoid high background staining, every solution was made with fresh ddH_2O and p. a. grade solvents. All steps were performed with a large excess (10 x gel volume) of incubation liquid in a freshly cleaned plastic container and with gentle agitation. Both unfixed or already fixed and Coomassie blue stained gels were stained. Unfixed gels were fixed by incubation in fixative solution for 30 min. Fixed gels were washed three times in 50% ethanol for 20 min and once in deionized H_2O for 5-10 min. The gels were then incubated for exactly 1 min in thiosulfate solution and then washed four times in deionized H_2O for 20 sec. Staining of the gels was then performed by

incubation in silver nitrate solution for 20 min in the dark. Afterwards, gels were washed twice in deionized H_2O for 20 sec. Then the staining of the protein bands was developed by incubating the gels in developer solution with continuous visual inspection until the bands became visible, which could take up to 10 min. To stop further staining, the gels were washed twice in deionized H_2O for 30 sec and then incubated in stop solution for 10 min. Gels were then either kept in 50% methanol at 4°C or rehydrated in deionized H_2O for 10 min, soaked in 2% glycerol for 15-30 min and dried on Whatman 3MM filter paper in a vaccum system.

2.11.5 Western blot

2.11.5.1 Transfer

To transfer proteins from a SDS-polyacrylamide gel to a Nitrocellulose membrane, the Trans-Blot SD Semi-Dry Electrophoretic Transfer System (BioRad) was used. Therefore, the resolving gel was separated from the stacking gel and soaked in transfer buffer, together with the membrane (Hybond C super nitrocellulose membrane, Amersham) and 2 pieces of filter paper (Whatmann, 3MM). These items were then assembled in a sandwich formation in this order: black side/anode - foamed material - filter paper - gel (inverted) - NC membrane - filter paper - foamed material - transparent side/cathode. To avoid air bubbles, the assembling was done under transfer buffer and potential air bubbles were removed before the cathode was fitted. The transfer was performed with a constant current of 200 mA for 1-2 h.

2.11.5.2 Immunodetection

After the transfer, the sandwich formation was disassembled and the membrane was incubated in 5% milk/PBST for 1 h at RT. Then the primary antibody diluted in 5% milk/PBST was added and the membrane was incubated O/N at 4°C. Next, the membrane was washed 3 x 10 min with milk/PBST and then incubated with the secondary reagent Avidin-HRP diluted 1:1000 in 5% milk/PBST for 1 h at RT. Finally, the membrane was washed 3 x 10 min with milk/PBST and 1 x 10 min with PBST. For development, ECL stock solution D was diluted 1:1000 in H_2O and mixed 1:1 with ECL solution 1. Of this mix, 2 ml were given onto the membrane and incubated for 1 min. Subsequently, excessive

liquid was removed with a paper towel. The membrane was then wrapped into saran wrap and exposed to films (Hyperfilm ECL, Amersham) for different time periods.

2.11.6 Qualitative and quantitative ELISA

Initially, a 96-well flat-bottom Maxisorp immuno-plate (Nunc) was coated with the capturing anti–body. Therefore, 50 µl of the antibody dilution (1-10 µg/ml in PBS) were added to each well and the plate was incubated for 1 h at RT or O/N at 4°C. Subsequently, the plates were washed three times with tap water containing 0.05% Tween 20 and then 50 µl ELISA buffer were added to each well. Next, 50 µl of cell culture supernatant or protein dilution in ELISA buffer were added and the plate was incubated for 2-4 h at RT or O/N at 4°C. In case a quantitative ELISA was performed also a dilution series of a standardized solution was applied. After the incubation, the plate was washed three times and then 50 µl of of the secondary antibody, diluted in ELISA buffer, was added to each well. The plate was incubated for 2 h at RT and washed again three times. In case the secondary antibody was not coupled to the enzyme alkaline phosphatase, a further incubation step with Strep-AP or an AP-coupled antibody was performed, using 50 µl/well of AP-coupled reagent diluted in ELISA buffer for 30 min (when using Strep-AP) or 2 h (when using another antibody) at RT. Then, the plate was washed five times with tap water containing 0.05% Tween 20, this time with an incubation of 1 min between the single washing steps. Subsequently, 50 µl substrate solution were added per well and incubated at RT under visual inspection until the dye development was found to be optimal. The reaction was then stopped by adding 50 µl of 1 M NaOH per well and the plate was read at 405 nm in a $THERMO_{max}$ microplate reader (Molecular Devices).

2.11.7 Development of a hBAFF ELISA

For the development of a two-step ELISA to detect hBAFF, the anti-hBAFF antibodies described in this thesis have been used. The best combination of these antibodies had to be found empirically. To determine the antibodies best suited as capture antibody, ELISA plates (Nunc MaxiSorb) were coated with 0.5 µg/ml or 5 µg/ml of anti-hBAFF antibody clones 1-35, 1-62, 2-18, 2-43, 2-44, 2-56, 2-67, 2-81, 4-62, 5-73, 6-23 and 6-46 in PBS

using 100 µl/well and then incubated o/n at 4°C. Subsequently, plates were washed 5 times in PBS with 0.5% Tween 20 and then 100 µl/well of a 1:4 dilution series of HA-hBAFF in ELISA buffer, ranging from about 100 ng/ml to 25 pg/ml, and ELISA buffer alone as negative control were applied. Plates were incubated for 2 h at RT and then washed as described above. To detect bound HA-hBAFF, 100 µl/well of a 1:200 dilution of anti-HAbio antibody in ELISA buffer was used. After an incubation time of 1.5 h at RT, the plates were washed again as described. Then 100 µl/well of a 1:200 dilution of Strep-AP in ELISA buffer was applied. Plates were again incubated at RT for 1 h and washed, this time with incubation of the plates for 1 min between each washing step. Finally, 100 µl/well of substrate solution were applied and plates were incubated in the dark at RT. After 30 min and 60 min the plates were read in an ELISA reader at 405 nm. Antibodies selected as coating antibodies for further testing had to fulfill the following criteria: a) background value (meassured in the well incubated with ELISA buffer alone) should not exceed 0.1 OD, b) the concentration of HA-hBAFF that could be detected should be as low as possible (whereat an OD of at least twice the value for the background was considered to be significant detection of an antigen), c) the OD range between the highest and the lowest concentration detected should be wide. Based on these criteria, antibodies 1-35, 2-18, 2-43, 2-56, 2-81, 4-62 and 5-73 were chosen as capture antibodies for further testing. Furthermore, only a concentration of 5 µg/ml antibody for coating turned out to be suitable.
To determine which of the anti-hBAFF antibodies is best suited as detection antibody, plates were coated o/n at 4°C with the antibodies 1-35, 2-18, 2-43, 2-56, 2-81, 4-62 and 5-73 at 5 µg/µl in PBS using 100 µl/well. Plates were washed in PBS with 0.05% Tween 20 as described above, then a 1:10 dilution series, ranging from 50 ng/ml to 5 pg/ml, of untagged hBAFF (recombinant protein, Peprotech) in ELISA buffer was applied. Plates were incubated for 2.5 h at RT and afterwards washed in PBS with 0.05% Tween 20. As detection antibodies, biotinylated anti-hBAFF antibody clones 1-35, 1-62, 2-18, 2-43, 2-44, 2-56, 2-67, 2-81, 4-62, 5-73, 6-23 and 6-46 were applied at a concentration of about 5 µg/ml in ELISA buffer using 100 µl/well. Further washing in PBS with 0.05% Tween 20 and development was done as described before. Based on the criteria listed above, coating with clone 2-81 and detection with clones 4-62 or 5-73, or coating with clone 4-62 and detection with clone 2-81 gave the best results. These antibody combinations were used for further optimization of the ELISA using a different ELISA buffer (containing different amounts of rat serum instead of BSA). At the end, the following protocol turned out to be suitable to detect hBAFF down to the range of pg/ml: ELISA plates are coated with anti-hBAFF

antibody clone 2-81 at 5 µg/ml in cold and sterile PBS and 100 µl/well at 4°C o/n. Washing steps are done in 0.5x PBS, 0.05% Tween 20. Plates are washed 2x and then 3x with incubation at RT for 1 min inbetween. Antigen is diluted in buffer containing 1x PBS, 0.05% Tween 20, 5% rat serum (RS-ELISA buffer) and 100 µl is added per well. As standard, untagged recombinant hBAFF (Peprotech) is used in a serial 1:2 dilution ranging from 1000 pg/ml to 62.5 pg /ml. Plates are incubated at 4°C o/n. After a washing step as described before, 100µl/well biotinylated anti-hBAFF antibody clone 4-62 diluted 1:200 (about 4-5 µg/ml final concentration) in RS-ELISA buffer is applied and plateas are incubated for 3-4 h at RT. After another washing step, 100 µl/well Strep-AP, diluted 1:2000 in buffer containing 1x PBS, 0.05% Tween 20, 2% rat serum, are added and plates are incubated for 1 h at RT. Plates are washed 5x with incubation times of 2 min inbetween each washing step and then developed by adding 100 µl/well substrate solution (DNPP in substrate buffer). Plates are incubated in the dark for 30 to 90 min and then the reaction is stopped by adding 100 µl/well 1M NaOH. Plates are read in an ELISA microplate reader at 405 nm.

For the analysis of serum samples from patients, samples were usually diluted 1:10, 1:100 and 1:1000 in RS-ELISA buffer and applied in duplicates using 100 µl/well. The ELISA was then developed as described above. For calculation of hBAFF concentration, the OD values within the linear range of the standard curve, which were obtained from the lowest dilutions were used.

2.12 Animal work

2.12.1 BrdU labeling of mice

Initially, mice recived an intraperitoneal injection of 1 mg BrdU in PBS and where then fed with drinking water containing 1 mg/ml BrdU for different periods of time. The BrdU-containing water was protected from light by wrapping the bottle in aluminum foil and exchanged every 4 days. Cell suspensions were then prepared from various lymphoid organs and stained for intracellular BrdU as described below.

2.12.2 Thymus dependent and independent immunization of mice

Mice were immunized i.p. with 100 μg NIP-OVA (Biosearch Technologies Inc., Novato, CA) in alum (T dependent immune response) or i.v. with 50 μg NIP-FICOLL (Biosearch Technologies Inc., Novato, CA) in PBS (T independent immune response). At day 14 after immunization, mice were bled and the serum IgG anti-NIP titer was determined by ELISA as previously described (8). For a recall response in the case of a T dependent immune response, 6 weeks after immunization mice were boosted with 10 μg NIP-OVA in PBS and 10 days thereafter the serum IgG anti-NIP titer was determined by ELISA.

2.12.3 Immunization of rats for hybridoma production

Lewis rats were immunized either with 0.4-1 mg/ml purified protein in PBS, mixed 1:1 with CFA, or with 1-2 x 10^7 transfected Y3-cells mixed with CFA. 50-100 μl were injected in the upper side of one foot. To boost the reaction, one and two weeks after the first injection, 100 μl of a solution containing either 10 μg purified protein in 300 μl PBS or 5-10 x 10^7 transfected Y3-cells in 1 ml PBS were injected into the foot. The day after the second boost, the popliteal and inguinal lymph nodes of the leg in which the antigen had been injected were taken out and treated as described below.

2.13 Cell culture

2.13.1 Freezing and thawing of cells

For freezing, the pelleted cells were resuspended in cold freezing medium at a cell density that was dependent on the cell line, and 1 ml aliquots were transfered to 2 ml cryotubes (Nunc), cooled down on ice and incubated at -80°C for at least 12 h. Subsequently, the tubes were transfered to their final storage place in a liquid nitrogen tank.
For thawing, cells were unfrozen either in a 37°C water bath or at RT and transfered immediately to 10 ml of the appropriate medium. After centrifugation at 1200 rpm and 4°C for 10 min, the cell pellet was gently resuspended in the appropriate medium and transfered to a culture dish.

2.13.2 Determination of cell numbers

Cell numbers were determined using a Neubauer counting chamber with 0.1 mm depth. An aliquot of the cell suspension was diluted 1:5-10 with trypan blue solution and loaded into the chamber. Cells in two of the outer squares, consisting of 16 small squares each, were counted. The cell number per ml was then calculated by multiplying the average cell number in an outer square with 10^4.

2.13.3 Transfection and Transduction

2.13.3.1 Transfection of Drosophila Schneider cells SL-3

SL-3 cells were split 1:5-10 the day before transfection and plated out in a 24-well plate with 1 ml SF-900 medium containing 2% FCS per well. Usually, three wells per plasmid were prepared. The next day, 12 µg of supercoiled plasmid DNA (ethanol precipitated and dissolved in sterile H_2O), 100 µl SF-900 medium, 1.2 µg phshs-puro and 20 µl Plus Reagent were mixed together in a sterile tube and incubated for 15 min at RT. After the incubation, 8 µl Lipofectamine and 200 µl SF-900 medium were mixed, given to the plasmid-mix and incubated for another 15 min at RT. In the meanwhile, the SL-3 cells were carefully washed three times with 1 ml SF-900 medium per well and finally 300 µl SF-900 medium was added. Then, 100 µl/well of the DNA-Lipofectamin-mix was added, and the cells were incubated O/N at 25°C. The next day, the supernatant was removed and to each well 1 ml SF-900 medium with 2% FCS was given. After 2-3 days, the medium was changed again and cells were now grown in SF-900/2% FCS medium containing 5 µg/ml puromycin for selection. Cells were kept for 2-3 weeks in selection medium and were split during this period when necessary.

2.13.3.2 Transfection of Phoenix cells

The day before transfection, Phoenix cells were plated out in a 6-well plate with 5-7.5 x 10^5 cells/well in 1-2 ml RPMI medium containing 10% FCS and 50 µM β-Mercapthoethanol. Usually, 2-3 wells per plasmid were prepared. The next day, 2 µg supercoiled plasmid DNA (ethanol precipitated and dissolved in sterile H_2O), 100 µl SF-RPMI medium and 10 µl Plus Reagent per well were mixed together in a sterile tube and incubated for 15 min at RT. After

the incubation, 2.5 µl Lipofectamine and 100 µl SF-RPMI medium were mixed, given to the plasmid-mix and incubated for another 15-45 min at RT. In the meanwhile, the Phoenix cells were carefully washed twice times with 1.5 ml/well SF-RPMI medium and finally 1 ml SF-RPMI medium was added per well. Then, 200 µl DNA-Lipofectamin-mix were added per well, and the cells were incubated for 5-6 h at 37°C and 5% CO_2. Next, the supernatant was removed and to each well 3 ml RPMI medium containing 10% FCS and 50 µM β-Mercapthoethanol were given. After 20-24 h, the medium was changed again and 2 ml fresh RPMI medium with 10% FCS and 50 µM β-Mercapthoethanol were added. Cells were incubated for another 24 h and then the virus containing supernatant was taken, centrifuged at 10 000 rpm and 4°C for 1 min and either used directly for transduction or transfered to freezing vials and stored at -80°C.

2.13.3.3 Transduction of eucaryotic cell lines

For transduction of cells by spininfection, 1-5 x 10^5 cells in 10-20 µl were transfered to a sterile 1.5 ml tube and mixed with 1 ml of virus-containing Phoenix cell supernatant. Cells were then centrifuged for 4 h at 3300 rpm (1160 x g) and 30°C. Afterwards the supernatant was removed carefully. The cell pellet was eventually resuspended in 1 ml of the appropriate medium and transfered to a culture dish.

2.13.4 Hybridoma production

From the popliteal and inguinal lymph nodes of a rat immunized as described above, a cell suspension was prepared in 50 ml IMDM/2% FCS medium. the suspension was transfered to 50 ml tubes and cells were centrifuged at 1000 rpm and 4°C for 10 min. To get rid of the fat contained in the lymph nodes, cell pellets were resuspended in 10 ml SF-DMEM (with glutamax, Gibco BRL) and transfered to a fresh 50 ml tube, combining all cell suspensions. The tube was then filled up to 50 ml with SF-DMEM and centrifuged again. Subsequently, another washing step was performed. In parallel, Sp2/0 cells were harvested by centrifugation at 1000 rpm and 4°C for 10 min and then washed twice with SF-DMEM. Then Sp2/0 cells were combined with the lymph node cells, using an amount of Sp2/0 cells that was slightly greater than that of the lymph node cells, and centrifuged again. Afterwards, the supernatant was completely removed and the tube was shaken in a 37°C

water bath, while at the same time 1 ml of pre-warmed 50% PEG solution was added dropwise during 1 min to the pellet. The dissolved pellet was shaken for an additional 1 min in the water bath, and then 10 ml of pre-warmed alkaline SF-DMEM were added dropwise to the cell suspension while shaking the tube carefully. Subsequently, the cell suspension was centrifuged at 800 rpm and RT for 5 min. The pellet was resuspended carefully in 5000 ml pre-warmed HAT medium and plated out into 96-well U-bottom plates adding 200 µl per well. Plates were then incubated at 37°C for 10-12 days until cell clusters became visible by the naked eye. Finally, supernatants were analyzed by FACS or ELISA, positive cell clusters were subcloned, re-tested and frozen.

2.13.5 Staining of sections for fluorescence microscopy

Organs were taken out of sacrificed mice, snap frozen in OCT compound and kept at -80°C until further usage. Cryostat sections of 4 µm were prepared, fixed for 10 min in aceton and stored at -20°C. Slides were allowed to thaw for 10 min at RT before staining and then a hydrophobic ring was drawn around the tissue section with a Dako Pen (DAKO) to minimize the amount of antibody solution needed. Sections were stained with anti-IgM^{FITC} (M41) and anti-IgD^{Biotin} (1.19) or anti-$Thy1^{FITC}$ (T24) and anti-IgM^{Biotin} (M41). Therefore, sections were covered with antibody dilution (1:50 in FACS buffer) and incubated for 30 min at RT in the dark. Then slides were rinsed in PBS and washed with shaking in PBS for 15 min at RT. Biotin-labeled antibodies were visualized using streptavidin Texas red (BD Biosciences) at a dilution of 1:2000 in FACS-buffer for 15 min at RT. Slides were washed again as described and then covered with 1-2 drops of a 1:1 mixture of PBS and glycerol and a 24 x 45 mm cover glass (Medite). Stained sections were analyzed using an Axioskop Immunofluorescence (Zeis, Feldbach, CH) equipped with a Nikon digital camera.

2.13.6 Flow cytometry

2.13.6.1 Lysis of red blood cells

The pelleted cells were resuspended in 1-2 ml of ACK buffer and incubated at RT for 1 min. To stop the lysis reaction, 10 ml of medium or FACS buffer were added. After centrifugation at 1000 rpm and 4°C for 10 min, the cells were resuspended in the appropriate medium.

2.13.6.2 Surface staining of cells

For staining of cell surface antigens, usually 50-100 µl cell suspension (1-2 x 10^7 cells/ml) were mixed together with the appropriate volume of antibody dilution in a 96-well U-bottom plate and incubated on ice for at least 30 min, covered with aluminum foil to protect the fluorescent dyes. To wash the cells, 100 µl of FACS buffer were added to each well and the plate was centrifuged at 250 x g and 4°C for 5 min. The supernatant was then poured out quickly and the plate was dried carefully on a paper towel. Cells were resuspended in the remaining supernatant by vortexing the plate. If necessary, a further staining step with a Streptavidin-coupled fluoreszent dye was performed, with an incubation for 15 min on ice, followed by another washing step. Finally, cells were resuspended in an appropriate volume of FACS buffer. Unless PE-Cy7 was used as fluorescent dye, 10 µg/ml propidium iodide (PI) were added to the FACS buffer, in order to be able to exclude dead cells. Negative and single stained compensation controls were always included. FACS analysis was carried out on a FACSCalibur (Becton Dickinson) and analyzed using the CellQuest Pro v4.0.1 software (Becton Dickinson). Cells were gated on the lymphoid FSC/SSC gate and on PI-negative living cells, if PI was included.
The appropriate dilutions of the antibodies that were produced in the lab were determined by titration before. Commercial antibodies were mostly used at a final concentration of 1:200. Strep-PE was used at a final concentration of 1:1000, Strep-APC at 1:200 and Strep-PE-Cy7 at 1:400.

2.13.6.3 Intracellular anti-BrdU staining

For detecting incorporated BrdU, intracellular staining using the BrdU Flow Kit (BD Biosciences) was performed. Therefore, 2-3 x 10^6 cells were stained for surface antigens as described above but without addition of PI, including all control stainings. Stained cells were then transfered into tubes and centrifuged. The cell pellets were resuspended in 100 µl Cytofix/Cytoperm and incubated for 20 min on ice. For washing the cells, 1 ml of Permwash buffer was added and cells were pelleted by centrifugation, resuspended in 100 µl Cytoperm Plus and incubated for 10 min on ice. Subsequently, cells were washed again with 1 ml of Permwash buffer and resuspended in 100 µl DNAse solution (provided in the kit or alternatively DNAse I, 2000 U/ml in PBS) and incubated at 37°C for 1 h. After another washing step with 1 ml Permwash buffer, cells were resuspended in 50 µl anti-BrdU antibody dilution (1:100) and incubated for 20 min at RT. Cells were then washed again in 1

ml Permwash buffer and analyzed on a FACSCalibur (Becton Dickinson) using the CellQuest Pro v4.0.1 software (Becton Dickinson).

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3 FLT3 LIGAND AND B CELL DEVELOPMENT

3.1 Increasing Flt3L availability alters composition of a novel bone marrow lymphoid progenitor compartment

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HEMATOPOIESIS

Increasing Flt3L availability alters composition of a novel bone marrow lymphoid progenitor compartment

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We have recently described a CD19$^-$ B220$^+$CD117^{low} bone marrow subpopulation with B, T, and myeloid developmental potential, which we have called "early progenitors with lymphoid and myeloid potential" or EPLM. These cells also expressed Fms-like tyrosine kinase 3, Flt3, or CD135. Treatment of mice with the corresponding ligand, Flt3L, showed a 50-fold increase in EPLM. In addition to the expected increase in dendritic cell numbers, Flt3L treatment had a reversible inhibitory effect on B lymphopoiesis. Limiting dilution analysis of sorted EPLM from Flt3L-treated mice showed that B-lymphocyte progenitor activity was reduced 20-fold, but that myeloid and T-cell progenitor activity was largely preserved. EPLM from treated mice transiently reconstituted the thymus and bone marrow of recipient mice, generating cohorts of functional T and B cells in peripheral lymphoid organs. Thus, Flt3L treatment results in a dramatic increase in a novel bone marrow cell with lymphoid and myeloid progenitor activity. (Blood. 2006; 108:1216-1222)

Introduction

In the bone marrow (BM) of normal adult mice, we have recently characterized a subpopulation of B220$^+$CD117^{low} cells with T, B, and myeloid developmental potential.[1] As a population, B220$^+$CD117^{low} BM cells are heterogeneous, containing about 95% CD93$^+$CD19$^+$ committed B-cell progenitors, about 3% CD93$^-$NK1.1$^+$ natural killer (NK) progenitors, and about 2% CD19$^-$NK1.1$^-$ cells. Further analysis indicated that this latter CD19$^-$NK1.1$^-$ subpopulation expressed CD127$^+$ (IL-7Rα^+) and CD135, the so-called fetal liver kinase 2 (flk-2), also known as Fms-like tyrosine kinase 3 (Flt3). When cultured on different stromal cells and in the presence of appropriate cytokines, including Flt3 ligand (Flt3L), sorted CD19$^-$NK1.1$^-$B220$^+$CD117^{low} cells showed, in addition to potent B-cell potential, T and myeloid potential. When injected intravenously into sublethally irradiated immunodeficient *Rag2*.γc recipients, freshly isolated cells transiently reconstituted both the T- and B-cell progenitor compartments, giving rise to cohorts of both mature T and B cells. Myeloid reconstitution in vivo was difficult to demonstrate. Based on these phenotypic and functional results, we have called these cells early progenitors with lymphoid and myeloid potential, or EPLM.

In the adult BM, the Flt3 kinase (CD135) is expressed by cells at many different stages of lymphoid and myeloid development,[2-4] starting with CD117^{high}, Lin$^-$ multipotent high proliferative potential progenitors and including both CD117^{low}, Lin$^-$ so-called common lymphoid progenitors (CLPs)[5] and EPLM.[1] Expression levels of CD135 tend to decrease with increasing differentiation. In the B-cell lineage CD135 expression is lost on CD19 acquisition but in the myeloid lineage CD135 expression persists particularly during dendritic cell (DC) development.[4] Thus, treatment of mice with the ligand for Flt3 (Flt3 ligand [Flt3L]) is a frequently used protocol for increasing the number of BM DC progenitors.[6]

Flt3L is a type 1 membrane protein expressed on multiple cell types including BM stroma and lymphoid cells.[7,8] Flt3L can also exist in soluble form and both membrane-bound and soluble forms activate the corresponding CD135 receptor.[9] Generally, Flt3L acts in combination with other cytokines,[10] for example, stem cell factor,[4] GM-CSF, IL-4, IL-7,[11] and IL-15.[12] It has been reported that B lymphopoiesis increases following Flt3L treatment[11] and decreases in *Flt3* knock-out (KO) mice.[13] In addition, Flt3L treatment is known to result in the accumulation of B220$^+$CD4$^+$ and B220$^+$CD8$^+$, so-called "plasmacytoid" DCs, in both BM and spleen[6,14]; there are also reports of an increase in NK cells.[15] However, to our knowledge, a detailed phenotypic and functional analysis of the effect of Flt3L treatment on nondendritic BM progenitors has not been carried out.

Because the in vitro culture systems for the analysis of the commitment status of EPLM contained Flt3L[1] and because freshly isolated cells expressed the corresponding receptor, CD135, we wanted to see what effect in vivo treatment with Flt3L would have on the B220$^+$CD117^{low} BM compartment. Results obtained indicated that after 10 days of Flt3L injection, the proportion and absolute number of EPLM among B220$^+$CD117^{low} cells increased dramatically. However, the frequency of B-cell progenitors among these EPLM was reduced 20-fold. Associated with the reduced in vitro B-cell potential of EPLM and reduced proportion of CD19$^+$ cells among B220$^+$CD117^{low} cells, BM B lymphopoiesis was drastically reduced. Freshly isolated EPLM from Flt3L-treated mice were fully capable of functionally reconstituting the T- and

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B-cell compartments of immunodeficient recipient mice. Thus, treatment with Flt3L provides a valuable way of expanding a novel BM cell that has both B- and T-cell progenitor activity.

Materials and methods

Mice

Female C57BL/6, C57BL/6-*Rag2*–deficient (*Rag2*$^{-/-}$),[16] and C57BL/6.Ly5.1 congenic mice of 5 to 8 weeks of age were used. All mice were bred and maintained in our animal facility under specific pathogen–free conditions. All animal experiments were carried out within institutional guidelines.

Flt3L treatment

Recombinant human Flt3L (rFlt3L) was a kind gift of Amgen (Thousand Oaks, CA). A stock solution containing 50 μg rFlt3L/mL was prepared in PBS and aliquots stored at −20°C until use. For Flt3L treatment, mice generally received 10 μg rFlt3L (0.2 mL) by intraperitoneal injection daily for 10 days, a treatment schedule previously used to increase DC number.[6] In titration experiments ("Results"), mice were treated with graded doses of 0.4, 2, or 10 μg/0.2-mL injection. Control mice received 0.2 mL PBS. Mice were humanely killed by CO_2 inhalation and organs removed by standard procedures.

Stromal cell lines

OP9 or OP9 stromal cells expressing the Notch ligand δ-like-1 (OP9-DL1) were kindly provided by Professor Juan-Carlos Zúñiga-Pflücker (University of Toronto, ON, Canada) and maintained in IMDM (Life Technologies, Basel, Switzerland) supplemented with 5×10^{-5} M β-mercaptoethanol, 1 mM glutamine, 0.03% (wt/vol) Primatone (Quest, Naarden, The Netherlands), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2% heat-inactivated fetal bovine serum (FBS). ST-2 stroma was also maintained in the medium.

Flow cytometry and cell sorting

FITC-, PE-, APC-, or biotin-conjugated monoclonal antibody (mAb) specific for B220, NK1.1 (PK 136), CD11b (M1/70), CD11c (HL3), CD3ε (2C-11), CD4 (GK1.5), CD8α (53-6.7), CD19 (1D3), CD21 (CR2/CR1), CD23 (B3B4), CD25 (7D4), CD44 (IM7), CD45.1 (A20), TCRβ (H57), and TCRγ/δ (GL3) were purchased from BD Biosciences (Milan, Italy); APC-conjugated CD117 (2B8), PE-conjugated CD127 (A7R34), and PE-conjugated CD135 (A2F10) were purchased from eBioscience (San Diego, CA); anti-C1qRp (PB493, CD93) antibody was purified from the hybridoma supernatant and labeled with biotin by standard methods. Staining of cells was performed as previously described. Flow cytometry was performed using a fluorescence-activated cell sorting FACSCalibur (BD Biosciences) and data were analyzed using the CellQuest Pro Software (BD Biosciences). For cell sorting the FACS Aria (BD Biosciences) was used. Erythrocyte-depleted BM cells were stained in IMDM 2% FBS with saturating concentrations of anti-B220^{FITC}, anti-CD19^{PE} plus anti-NK1.1^{PE}, anti-CD117^{APC}, and biotinylated anti-CD93 (PB493/AA4.1). Following a 30-minute incubation at 4°C, cells were washed in PBS 2% FBS and resuspended in PBS containing SA$^{PE/Cy7}$. After a further 30 minutes at 4°C, cells were washed in IMDM, filtered through a 20-μm diameter nylon mesh, and resuspended at about 20×10^6/mL in filtered PBS 2% FBS prior to sorting. Reanalysis of sorted cells indicated that in all instances they were more than 98% pure.

Stromal cell cocultures

Two days prior to coculture, 2×10^3 stromal cells were seeded per well of a 96-well flat bottom plate or 10^4 per well of a 24-well plate. At day 0, stromal cells had grown to semiconfluency and were then γ-irradiated with 3000 rad. Then, culture medium was removed and replaced by IMDM supplemented with 5×10^{-5} M β-mercaptoethanol, 1 mM glutamine, 0.03% (wt/vol) primatone, 100 U/mL penicillin, 100 μg/mL streptomycin, 2% FBS. To these cultures were added various numbers of sorted BM cells. For limiting dilution analysis (LDA), 48 replicate cultures containing graded numbers of sorted EPLM were plated in 96-well microtiter plates containing irradiated ST2 stroma plus Flt3L for myeloid, OP9 plus IL-7 and Flt3L for B, or OP9-DL1 stroma plus IL-7 and Flt-3L for T cells. The presence of Flt3L in stromal cell cultures did not alter results obtained. At day 14 of culture, all wells were inspected using an inverted microscope. Wells containing colonies of more than 50 cells were scored positive.

Transplantation

Groups of C57Bl/6.*Rag2*$^{-/-}$ mice were irradiated with 450 rad and 1 to 10×10^5 cells injected intravenously. After the indicated time, mice were humanely killed and organ cell suspensions prepared by mechanical disruption, stained, and subsequently analyzed by flow cytometry. In some experiments ("Results"), recipients also received daily injections of 4 μg Flt3L.

Immunizations

To induce a T-dependent antibody response, reconstituted mice were injected intraperitoneally with 50 μg alum-precipitated NIP-ovalbumin. Sera were obtained from tail bleeding prior to and 14 days after immunization and stored at −20°C. Total and hapten-specific IgG levels were determined by enzyme-linked immunosorbent assay (ELISA).

Results

Cells obtained from the BM of PBS control or Flt3L-treated mice were stained with combinations of mAb used to define early lymphoid progenitors. As shown in Figure 1A, upper left panels, staining with B220^{FITC} versus CD117^{APC} revealed the expected 1.5% B220$^+$CD117^{low} subpopulation (boxed insert). Further analysis (right panel) of gated B220$^+$CD117^{low} cells shows that they contained about 93% CD93$^+$CD19$^+$, 3% CD93$^-$NK1.1$^+$, and about 4% cells with a broad CD93 staining pattern but negative for both CD19 and NK1.1. As previously shown,[1] the latter cells comprise EPLM.

The lower panels of Figure 1A show the strikingly different staining pattern obtained with BM cells from mice treated with 10 μg/injection of Flt3L, a dose taken from previously published reports.[6] First, the proportion of B220$^+$CD117^{low} cells increased to 4.9% of gated cells and, second, there is a drastic alteration in CD93 versus CD19/NK1.1 distribution. Whereas the proportion of NK1.1$^+$ cells is about the same (2.2%), the proportion of CD19$^+$ cells is reduced to only 6% and cells negative for both CD19 and NK1.1 increased from about 4% to over 90%. Again, the CD93 distribution on CD19$^-$ cells is unchanged compared with controls (upper panel). Tables 1 and 2 summarize the numbers of cells in each subpopulation from a series of 5 independent groups of treated mice.

Reduction in in vivo B lymphopoiesis

Staining of BM cell suspensions with a panel of markers defining the different stages of B lymphopoiesis unexpectedly revealed a drastic reduction in B lymphopoiesis in treated mice (Figure 1B). Shown are cytogram displays of cells stained with B220 versus CD19 (left panels) or B220 versus IgM (right panels). Classically, in PBS-treated controls (upper panels), 45% BM cells were B220$^+$CD19$^+$ B cells and 3.5% mostly B220^{bright}, CD19$^-$ cells. As previously shown,[17] B220^{bright}, CD19$^-$ cells comprise a heterogeneous mixture of both mature CD4$^-$ NK and plasmacytoid DCs. Staining with B220 and IgM shows the expected pattern of B220$^+$IgM$^-$ (mostly pre-BII), B220$^+$IgM^{low} (immature B), B220$^+$IgM^{bright} (mature B), and B220^{bright}IgM^{low} (recirculating)

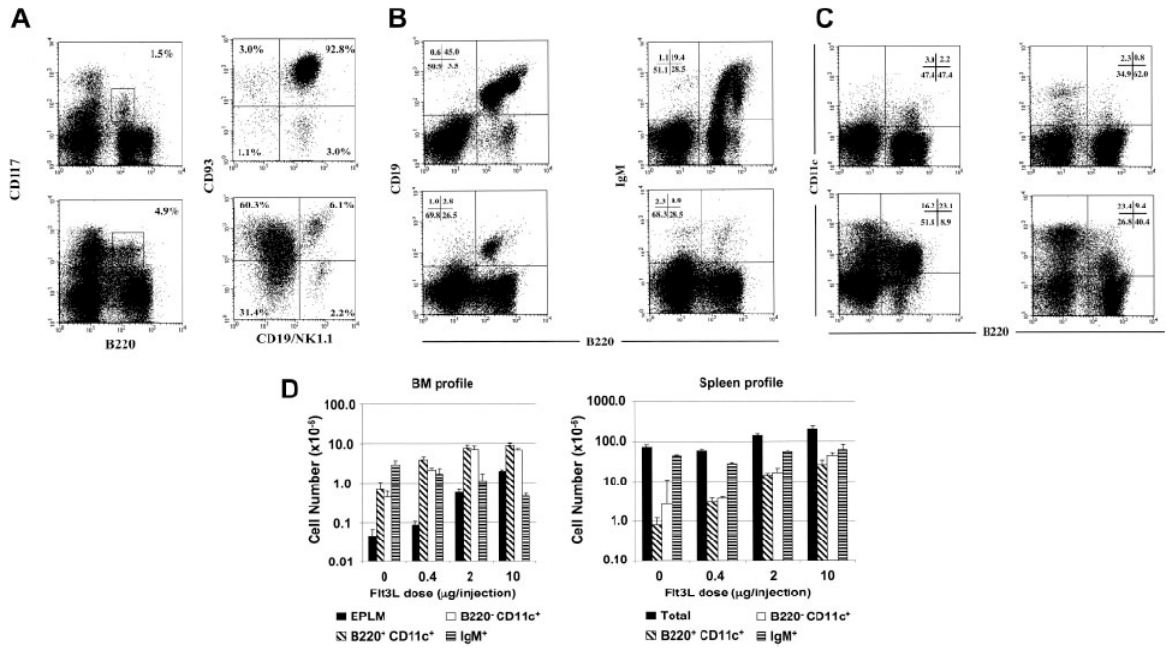

Figure 1. Effects of Flt3L treatment on EPLM, B lymphopoiesis, and DCs. (A) Effect of Flt3L treatment on EPLM. The left cytograms show the B220 versus CD117 distribution on forward/side scatter-gated bone marrow cells from PBS-injected (top panels) or Flt3L-injected mice (bottom panels). The boxed areas indicate $B220^{+}CD117^{low}$ cells and the figures the percent cells in these areas. The right cytograms show the CD93 versus CD19/NK1.1 distribution on gated $B220^{+}CD117^{low}$ cells and the percent cells in each quadrant. (B) Effect of Flt3L treatment on B lymphopoiesis. Shown are cytogram displays of BM cells from PBS-treated (top panels) or Flt3L-treated (bottom panels) stained with the indicated markers. In each panel, the figures represent the percent cells in each quadrant. (C) Effect of Flt3L treatment on DCs. Shown are cytogram displays of B220 versus CD11c on BM (left) or spleen cells (right) from PBS- (top) or Flt3L-treated mice. The figures in each panel indicate the percent cells in each quadrant. (D) Flt3L dose response. Shown is a summary of the BM (left) or spleen (right) profile of mice treated with 10 daily injections of the indicated dose of Flt3L. Each histogram shows, on a logarithmic scale, the mean ± SD number of cells in the indicated subpopulations from groups of 3 mice. Note the different vertical scale for BM and spleen.

B cells. In the BM of treated mice (lower panels), the proportion of $CD19^{+}$ B cells was reduced from 45% to only 2.8% gated cells. Additional staining with IgM showed that only a small population of immature B cells remained, with an almost total absence (0.9%) of IgM^{bright} mature and $B220^{bright}$ IgM^{low} recirculating B cells.

Increase in in vivo DC development

As expected, treatment with Flt3L resulted in a dramatic increase in both BM and spleen DC subsets.[14,18] Confirming previous reports,[6,19] in the BM of control mice (Figure 1C upper left) 2.2% cells were $B220^{bright}CD11c^{low}$ DCs, whereas the spleen (right panel) contained 2.3% $B220^{-}CD11c^{bright}$ and 0.8% $B220^{+}CD11c^{dull}$ DC subpopulations. After Flt3L treatment, the BM (lower left) contained not only 23.1% $B220^{bright}CD11c^{low}$, but also 16.2% $B220^{-}CD11c^{+}$ cells; the CD11c expression of the latter extended from $CD11c^{dull}$ to $CD11c^{bright}$. Likewise the spleen contained 23% $B220^{+}$ and 9.4% $B220^{-}$ subpopulations of $CD11c^{+}$ cells.

Tables 1 and 2 summarize the mean cell numbers in the different BM and spleen subpopulations. For B cells, results indicate the severe (approximately 7-fold) reduction in BM B-cell numbers, which is mostly manifest as a reduction in B-cell development beyond the pre–B cell stage. In contrast, the dramatic increase in DC development is seen by the 17-fold increase in $CD11c^{+}$ cells. For the spleen, B-cell development, as measured by total numbers of $CD19^{+}$, $CD23^{+}CD21^{+}$ follicular B (FB), or $CD23^{low}CD21^{+}$ marginal zone B (MZB) cells is not drastically altered. The number of mature spleen T cells was unchanged and there was a slight increase in $NK1.1^{+}$ cell numbers. However, the most dramatic changes were seen in DC numbers, with an approximately 20-fold increase in both plasmacytoid $B220^{+}$ and conventional $B220^{-}CD11c^{+}$ cells. There was no discernible effect on the T-cell progenitor compartment of the thymus (data not shown).

To monitor how the dose of injected Flt3L affected BM and spleen cell recoveries, groups of mice were treated with graded doses of Flt3L and the cellularity and phenotype of BM and spleen directly compared. As shown in Figure 1D, left panel, there was a dose-dependent increase of BM EPLM, $B220^{+}$, and $B220^{-}$ DC numbers and concomitant decrease in IgM^{+} B cells. For DCs, there was a plateau in cell number between the 2- and 10-μg dose. In the spleen (right panel), there was a slight (< 3-fold) increase in total

Table 1. BM cell numbers in Flt3L-treated mice

Group	Total, 2 femora	$CD19^{+}$	$B220^{+}$ $CD19^{-}$	$B220^{+}$ $CD117^{+}$	$B220^{+}CD19^{-}$ $CD117^{+}NK1.1^{-}$	IgM^{+}	RecB*	$CD11c^{+}$
PBS	25.6	9.8	1	0.38	0.02	3.2	1.6	0.5
Flt3L	25.7	1.4	7.2	1.26	1.15	0.35	0.05	8.5
Ratio†	1.0	0.14	7.2	3.3	57	0.1	0.03	17

BM cell numbers are shown as $\times 10^{-6}$.
*RecB refers to $B220^{bright}$ IgM^{low} recirculating B cells.
†Ratio is of cell number in Flt3L-treated versus PBS control.

Table 2. Spleen cell numbers in Flt3L-treated mice

Group	Total	CD19+	B220+ CD19−	FoIB	MZB	CD4+	CD8+	CD11c	NK
PBS	76	41.2	0.6	32.5	2	12.6	7.5	1.5	2.6
Flt3L	138	56.9	11.9	43.6	3	12.1	10.1	32.3	7.3
Ratio*	1.8	1.4	19.8	1.3	1.5	0.96	1.35	21.5	2.8

Spleen cell numbers are shown as $\times 10^{-6}$.
*Ratio is of cell number in Flt3L-treated versus PBS control.

cellularity with a relatively constant number of IgM+ B cells. As expected, most of the additional cellularity was accounted for by plasmacytoid and conventional DCs, whose number progressively increased with increasing Flt3L dose.

To show that Flt3L treatment had only transient effects on BM B lymphopoiesis and DC generation, mice initially treated for 10 days with Flt3L were allowed to recover for 2 weeks and BM and spleen cellularity and phenotype were monitored. It is known that the turnover of B cells in the BM is relatively rapid, whereas most peripheral B cells are long-lived.[20,21] Short-term treatment of mice with Flt3L had no discernible effect on mature B cells and 2 weeks after stopping treatment, B lymphopoiesis in the BM had returned to normal (data not shown). For DCs, it is known that their turnover in the periphery is relatively rapid.[22,23] Therefore, not surprisingly, following the cessation of Flt3L treatment, the BM and spleen DC compartments were indistinguishable from controls (data not shown).

In vitro functional analysis of CD19−NK1.1−B220+CD117low cells from Flt3L-treated mice

We have used appropriate high plating efficiency stromal cell culture systems and LDA to assess the developmental potential of various sorted BM and thymus-derived lymphoid progenitors.[1,24] In this way, we could show that CD19−NK1.1−B220+CD117low BM cells, or EPLM, possessed potent B, T, and myeloid developmental potential. Therefore, we wished to compare the developmental potential of EPLM from PBS-treated, control, with those from Flt3L-treated mice. To this end, we sorted CD19−NK1.1−B220+CD117low BM cells from Flt3L-treated mice and placed them at limiting dilution in cultures of OP9 stroma plus IL-7 and Flt3L to measure B-cell potential, ST-2 stroma and Flt3L for myeloid and OP9-DL1 plus IL-7 and Flt3L to measure T-cell potential. In every experiment, the same cells were sorted from PBS-treated mice and cultured in parallel. In addition, CD19+NK1.1−B220+CD117low BM cells, which have a high plating efficiency for B-cell progenitors, were included.

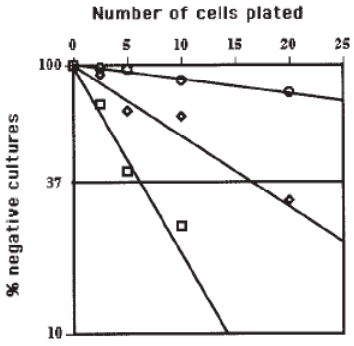

Figure 2. LDA of EPLM from Flt3L-treated mice. The figure summarizes the LDA for myeloid (□), T cell (◇), and B cell (○) among EPLM from Flt3L-treated mice. Shown are the semilogarithmic plots of cell dose (horizontal linear scale) versus the percent negative cultures (vertical logarithmic scale) of 48 replicate cultures containing on average 2.5, 5, 10, or 20 sorted EPLM plated in 96-well microtiter plates containing irradiated ST2 stroma plus Flt3L for myeloid, OP9 plus IL-7 and Flt3L for B, or OP9-DL1 stroma plus IL-7 and Flt-3L for T cells. After 10 to 14 days, wells were scored for growth by microscopic examination. When 37% of replicate cultures are negative (horizontal line), according to Poisson statistics, each well would on average contain one precursor.

Whereas EPLM from normal mice generated myeloid, T, and B cells with a frequency of approximately 1 in 5 to 10, CD19−NK1.1−B220+CD117low BM cells from FLT3L-treated mice showed high myeloid- (1 in 5), intermediate T- (1 in about 18), and low B-lineage (1 in 180) potential (Figure 2). Thus, the most significant difference in developmental potential of CD19−NK1.1−B220+CD117low BM cells from Flt3L-treated mice was the reduction in B-cell progenitor activity. It is important to note that the presence of Flt3L in limiting dilution cultures influences neither the plating efficiency nor clone size of committed (CD19+B220+CD117low) BM cells used as controls (not shown).

Does Flt3L induce myeloid differentiation of EPLM in vivo?

The in vitro finding that EPLM derived from Flt3L-treated mice had a bias in myeloid differentiation potential might suggest that Flt3L is myeloid instructive for EPLM. To test this possibility, Ly5.2.C57Bl/6.RAG-2−/− mice were treated daily for 3 days with either PBS or 4 μg Flt3L, irradiated and then injected intravenously with 10^4 EPLM derived from the BM of wild-type C57Bl/6.Ly5.1 mice. Injections of PBS or Flt3L were continued for a further 5 days prior to analysis. Results obtained (Figure 3) showed that in the 3 control recipients, the BM contained 0.7% ±0.5%, or $3 \pm 2 \times 10^4$ donor-derived CD19+ cells, whereas in the Flt3L-treated mice, values were 0.4% ± 0.2%, or $7 \pm 3 \times 10^4$ cells. Note that following irradiation, Flt3L treatment increased BM cellularity 4-fold. In addition, as seen by the increase in B220 expression among host (Ly5.1−) cells (lower left cytogram), the myeloid compartment was particularly affected. However, despite these changes, we failed to detect donor-derived myeloid cells in either BM (Figure 3) or spleen (data not shown). Therefore, increasing Flt3L availability in vivo does not seem to influence EPLM differentiation.

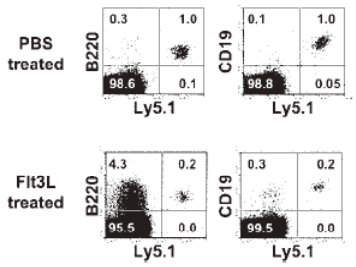

Figure 3. No effect of Flt3L treatment on EPLM injected in vivo. Shown are cytogram displays of BM from one of 3 PBS (top panels) or Flt3L-treated (bottom panels) Ly5.2.C57Bl/6.*Rag2*−/− mice injected 5 days previously with 10^4 EPLM from C57Bl/6.Ly5.1 mice stained for Ly5.2 (horizontal scale) and either B220 (left panels) or CD19 (right panels). Figures in each panel represent the percent positive cells.

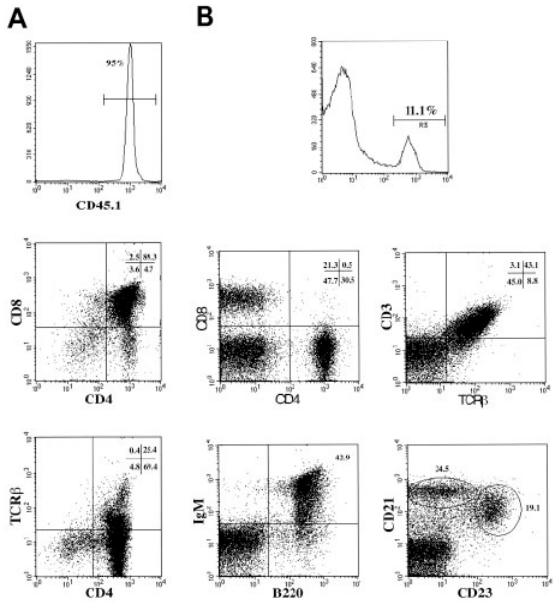

Figure 4. Thymus and T- and B-cell reconstitution. (A) Thymus reconstitution by EPLM from Flt3L-treated mice. A total of 2×10^4 EPLM from C57Bl/6CD45.1 mice were injected intravenously into C57Bl/6.*Rag2*$^{-/-}$.Ly5.2 recipients. Three weeks after injection, the thymus contained 95% CD45.1$^+$ cells (left histogram). The cytogram displays show the CD4 versus CD8 (middle panel) and CD4 versus TCR-β distribution on gated CD45.1$^+$ cells. The figures in each panel indicate the percent cells in each quadrant. (B) EPLM reconstitute both T and B spleen cells. Shown are results of staining spleen cells from C57Bl/6.*Rag2*KO.Ly5.2 mice 7 weeks after reconstitution with 2×10^4 sorted EPLM from C57Bl/6.Ly5.1 Flt3L-treated mice. The spleen contained 11.1% donor derived CD45.1$^+$ cells (top histogram) and cytogram displays show the CD4 versus CD8 (top left), CD3 versus TCR-β (top right), B220 versus IgM (lower left), and CD23 versus CD21 (bottom right) distribution on gated CD45.1$^+$ cells. Figures in each panel represent the percent positive cells in each quadrant. In the lower right cytogram, the regions show the 19.1% CD23$^+$CD21$^+$ FB and 24.5% CD23$^-$CD21$^+$ MZB cells.

In vivo functional analysis of CD19$^-$NK1.1$^-$B220$^+$CD117^{low} cells from FLT3L-treated mice

Previously we showed that intravenous injection of 2×10^4 ex vivo sorted B220$^+$CD117$^+$CD19$^-$NK1.1$^-$ BM cells from wild-type mice transiently reconstituted the T- and B-cell progenitor compartments and resulted in the formation of cohorts of long-lived peripheral B and T cells.[1] To test the ability of EPLM from Flt3L-treated mice to reconstitute mice, we injected 2×10^4 ex vivo sorted B220$^+$CD117$^+$CD19$^-$NK1.1$^-$ BM cells from FLT3L-treated C57Bl/6.Ly5.1 mice into C57Bl/6.*Rag2*KO.Ly5.2 recipients. We detected T- and B-cell reconstitution in all recipient mice. As previously reported,[1] we did not detect myeloid reconstitution in vivo. The thymuses of recipients were analyzed between 3 and 4 weeks after reconstitution (Figure 4A left histogram) showing that the thymus was predominantly (95%) composed of donor (CD45.1$^+$) cells. Gated CD45.1$^+$ cells (right panels) contained all 4 CD4- and CD8-defined thymocyte subsets (middle panel), each expressing the appropriate levels of surface TCR-β (right panel). Likewise, the BM at this time contained CD45.1$^+$ B cell progenitors (not shown).

At 7 weeks following reconstitution, whereas the thymus only contained either CD4$^+$ or CD8$^+$ single-positive cells (not shown), the spleen contained 11.1% CD45.1$^+$ cells (Figure 4B upper histogram) and of these, 30% were CD4 and 21% CD8 (left middle cytogram) and TCR-β$^+$ (right middle cytogram). The spleen also contained donor-derived mature B cells that were for the most part IgM^{high} (left lower cytogram). As expected in situations where the B-cell compartment was not fully reconstituted,[25] there was a relative enrichment in MZB, comprising 24.5% of gated B220$^+$ cells (lower right cytogram). Thus, after 7 weeks, the spleens of reconstituted RAG-2$^{-/-}$ mice contained populations of mature T and B cells.

Immune responsiveness of reconstituted mice

To assess the combined function of T- and B-cell compartments, groups of 4 reconstituted or control C57Bl/6 mice were immunized with the T cell–dependent antigen NIP-ovalbumin. The magnitude of the antigen-specific serum IgG antibody responses of individual mice measured by ELISA showed that reconstituted mice were as capable as controls in mounting a T cell–dependent B-cell response (Figure 5). This shows that for responsiveness to this prototype antigen, both T- and B-cell compartments of reconstituted mice were fully functional.

Discussion

Recently, we have described in the BM of normal adult mice a CD19$^-$NK1.1$^-$B220$^+$CD117^{low} cell, designated EPLM, which has B, T, and myeloid developmental potential.[1] Furthermore, phenotypic analysis of EPLM indicated that they expressed CD135, the so-called Flt3 receptor. Therefore, we wished to see if increasing the in vivo availability of Flt3L, the corresponding ligand for Flt3, would alter the number or functional activity of EPLM.

Increasing Flt3L availability in vivo was achieved by administration of graded quantities of recombinant human Flt3L and resulted in a dose-dependent increase of BM lymphopoiesis, characterized by an approximately 50-fold increase in EPLM number (Tables 1 and 2) and an expected dramatic effect on DC generation. Unexpectedly, however, Flt3L treatment resulted in a concomitant dramatic reduction in B lymphopoiesis. The effects on both B and DC compartments were transient as shown by the rapid recovery to normal of BM and spleen cellularity and phenotype following cessation of Flt3L treatment. Functionally, when tested in LDA and compared with cells from controls, EPLM from mice treated with 10 μg Flt3L showed a dramatic (~20-fold) reduction in frequency of B progenitors, a slight reduction (~1.5-fold) in T progenitors, and slightly increased myeloid developmental potential. Combining the changes in EPLM cell recovery and precursor frequency, this amounts to an approximately 2.5-fold increase in total B progenitors, a 30-fold increase in T and a 50-fold increase in myeloid progenitors. The apparently high number of B-cell progenitors still present among EPLM from Flt3L-treated mice despite a massive decrease in in vivo B lymphopoiesis might

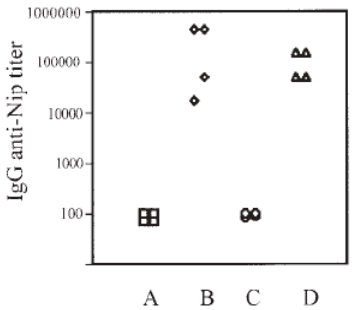

Figure 5. T cell–dependent antibody responses in EPLM-reconstituted mice. Shown are the IgG anti-Nip–specific antibody titers from 4 individual control C57Bl/6 (A-B) or EPLM-reconstituted C57Bl/6.RAG-2KO mice (C-D) before (A,C) or 14 days after (B,D) immunization with NIP-ovalbumin.

seem paradoxical. However, it must be remembered that in vivo and in vitro microenvironments are completely different and, in addition, that the concentration (20 ng/mL) and bioavailability of Flt3L used for in vitro cultures probably differ significantly from those in vivo. The presence of Flt3L in the in vitro cultures did not alter the cloning efficiency or burst size of colonies. Taken together, it is clear that in vitro cultures can reveal B- and T-cell developmental potentials unavailable or not used in vivo.

The failure to reveal myeloid potential of EPLM following intravenous injection is rather surprising. By treating irradiated recipients with Flt3L injections and by analyzing mice soon after reconstitution, we tried to provide optimal conditions for revealing the myeloid potential of injected cells. However, despite showing an effect of Flt3L injections on the recovering hemopoietic system of the host (Figure 3), we were unable to detect EPLM-derived myeloid cells. One simple explanation for this is that despite being able to find donor-derived B cells in the BM, injected EPLM were unable to enter the correct microenvironment supporting myelopoiesis. Thus the apparent lineage potential of a cell is very much influenced by the assay used; this is particularly acute for myeloid cells.

These results raise the issue of how cytokine availability in vivo might alter the developmental potential of hemopoietic progenitors. The "instructive" role played by cytokines in guiding hemopoiesis was invoked many years ago.[26,27] Progenitor cells frequently express extremely low numbers of cytokine receptor molecules,[28] often below the reliable detection limits of flow cytometry. In this regard, bioresposiveness of cells is a far more sensitive assay than staining by FACS. One such example is the evident IL-7 responsiveness of the earliest population of $CD117^+$, $CD44^+$, $CD25^-$, so-called DN1, progenitor thymocytes, which by flow cytometry appear IL-7Rα (CD127) negative.[24] Interestingly, in the BM of IL-7 overexpressing transgenic mice,[29] where B lymphopoiesis is massively increased, the proportion of $CD19^-NK1.1^-$ EPLM among $B220^+CD117^{low}$ cells is reduced and treatment of IL-7 transgenics with Flt3L did not alter this phenotype (not shown). When added to in vitro cultures or injected into recipient mice, increasing Flt3L availability did not discernibly alter the differentiation of EPLM. Taken together, these results suggest that Flt3L has little direct effect on EPLM, but rather acts on an upstream precursor. This would appear logical because Flt3L receptor (CD135) expression progressively decreases along the B-cell developmental pathway.[2-4] By promoting development along a particular lineage, for example, IL-7 toward B or Flt3L toward myeloid, the cytokine milieu surrounding particular BM progenitors might significantly alter their cellular composition and developmental potential, thereby favoring the notion that cytokines can play an instructive role in lymphoid lineage commitment.[27]

Using in vitro experiments, we have shown that potent T-cell potential can be revealed among EPLM when they were cultured on OP9 stroma expressing the Notch ligand δ-like 1 (DL1) and that following prolonged Notch signaling, there was a dramatic up-regulation of c-kit expression and loss of B-lineage potential.[1] In addition, using EPLM and *Pax5*$^{-/-}$ pre–B cell lines, we have recently shown that up-regulation of c-kit gene transcription and protein expression is a direct consequence of DL1 signalling.[30] Thus, in addition to cytokines, Notch signaling is known to influence the T versus B, or lineage commitment status, of lymphoid progenitors from both mice[31] and humans.[32] However, in Flt3L-treated mice, we did not observe changes to the thymus T-cell progenitor compartment. In addition, we have not been able to find a thymocyte with the EPLM phenotype. Therefore, under physiologic conditions, we do not think that EPLM contribute to thymopoiesis. Rather, EPLM probably represent the last stage along a lymphoid developmental pathway at which a Notch signal can rescue T-cell commitment.

The most significant observation from these studies, however, is the increased number of BM EPLM induced by Flt3L. Freshly isolated EPLM from both control and Flt3L-treated mice could reconstitute both the T- and B-cell compartments of recipients, generating cohorts of functional B and T cells. Immune responses in EPLM-reconstituted mice showed the characteristic features of T/B cell collaboration. Taken together, these results may have considerable practical, perhaps clinical, applications in the context of BM transplantation.

Acknowledgments

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3.2 Addendum

The most important cytokines regulating early B cell development are FLT3L and IL-7. Mice deficient for FLT3L have reduced numbers of CLPs, but normal numbers of common myeloid progenitors (CMPs). Serial transplantation experiments showed that HSCs from *Flt3L* deficient mice are present in normal numbers and have no intrinsic defect, indicating that FLT3L does not act on HSCs (1). Therefore, the reduced number of CLPs in *Flt3L* deficient mice results from the missing survival effect of FLT3L on CLPs directly or on one of the immediate progenitors of CLPs. More recently it was shown that LMPPs, which are progenitors of CLPs, are reduced in *Flt3L*$^{-/-}$ mice and show a reduced expression of the genes encoding IL-7Rα and RAG-1 at the mRNA level (2). The work presented here shows that FLT3L also has a potent stimulatory effect on EPLMs, a B cell progenitor stage downstream of CLPs (3). A synergistic effect between FLT3L and IL-7 was reported a decade ago (4), and this effect is most obvious in mice double deficient for FLT3L and IL-7 or IL-7Rα. Both the *Flt3L* x *Il-7* and *Flt3L* x *Il-7rα* double deficient mice completely lack B220^{+} CD19^{+}, B220^{+} IgM^{+} and B220^{+} CD5^{+} B cells. Furthermore, the pre-pro B cell population is extremely reduced, while later developmental stages are completely absent and no *Pax5* expression is detectable in BM cells (5, 6). The analysis of these double deficient mice in combination with mice deficient for the gene encoding the TSLP receptor showed that FLT3L is responsible for IL-7 independent B lymphopoiesis and not TSLP (6). This data strongly suggest that FLT3L has a synergistic and nonredundant role in B lymphopoiesis and that signaling via FLT3 is important for survival, proliferation and lineage commitment of several early B cell progenitor stages.

Interestingly, increased availability of FLT3L results in reduced numbers of mature B cells. The reason for this observation is presumably related to the fact that repression of FLT3 signaling by PAX5 is important for B cell lineage commitment. PAX5 directly regulates the expression of *Flt3*, leading to downregulation of *Flt3* (7). Furthermore, enforced retroviral expression of FLT3 in HSCs reduced their ability to develop into the B cell lineage both in vivo and in vitro (7). Thus, it might be that intensified signaling via FLT3 through increased availability of FLT3L interferes with either *Pax5* expression or the PAX5 mediated downmodulation of *Flt3*, leading to the observed reduction in the number of mature B cells and the potential to develop into the B cell lineage. Whether this assumption is correct and how the intensified FLT3 signaling disturbs the normal B cell developmental pathway remains to be elucidated.

3.2.1 References

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4 PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST HUMAN BAFF AND MURINE BAFF-R AND DEVELOPMENT OF A HUMAN BAFF ELISA

4.1 Monoclonal antibodies against human BAFF

4.1.1 Hybridoma production

BAFF mainly exists in a soluble form generated by cleaving the membrane bound form, thus it was decided to use the extracellular part of the hBAFF protein (aa 136-285) as antigen for immunization. Cloning, production and purification of soluble hBAFF, and production and selection of hybridomas is described in chapter 2. Finally, 49 hybridoma clones producing a monoclonal anti-hBAFF antibody remained.

4.1.2 Specification of anti-hBAFF antibody characteristics

Most likely, within the 49 anti-hBAFF producing hybridomas obtained, several are of the same origin, meaning that some of the antibodies produced are identical. Instead of sequencing the V-regions of the Ig genes from individual anti-hBAFF hybridomas, it was attempted to identify independent clones by characterizing the binding properties of the various anti-hBAFF antibodies obtained.

4.1.2.1 Cross-reactivity with HA-mBAFF

The protein sequence of the secreted extracellular part of human and mouse BAFF is to 85% identical, thus the possibility that a certain antibody possesses affinity for both human and mouse BAFF is very likely. To determine whether any of the anti-hBAFF antibodies produced could also bind to mBAFF, an ELISA with Fc-tagged mBAFF was performed.
Of the 35 monoclonal antibodies tested, seven showed affinity to mBAFF (fig. 1). However, compared to the signal for hBAFF, only one antibody (clone 2-44-14) possesses pronounced

specificity for mBAFF, obvious from the comparable high signals observed. Clone 5-85 gave an intermediate signal for mBAFF, and clone 6-23 seemed to react better with mBAFF than with hBAFF, but the signal strength for binding to mBAFF observed for these clones was in both cases rather low. The four other positive clones all showed a low signal, indicating only a marginal specificity for mBAFF (fig. 1 and tab. 1).

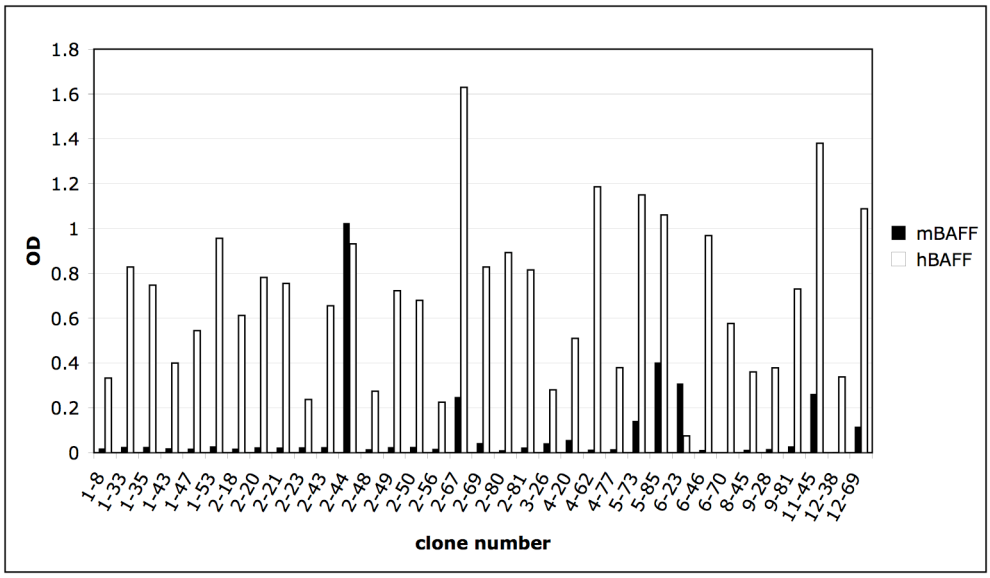

Figure 1. Crossreactivity of anti-hBAFF antibodies to mBAFF. *The supernatant of the indicated anti-hBAFF hybridoma clones was tested in an ELISA for its capacity to bind to Fc-tagged mBAFF (black bars) or Fc-tagged hBAFF (white bars).*

4.1.2.2 Ability to block hBAFF from binding to receptor

Binding of an anti-hBAFF antibody to a hBAFF molecule might interfere with binding of this hBAFF molecule to its cellular receptor, BAFF-R. Blocking of hBAFF binding to its receptor by an antibody indicates that this antibody recognizes an epitope that is either necessary for receptor binding or in close vicinity thereto. Thus, the observation of interference allows to draw conclusions on the epitope recognized by a certain anti-hBAFF antibody.

To determine the blocking ability of the anti-hBAFF antibodies, recombinant HA-hBAFF was pre-incubated with hybridoma supernatant and then used for staining splenic B cells, which were identified by using B220 as a marker. Among the 49 antibodies tested, the vast majority (about 70%) were non-blocking antibodies, giving FACS stainings which were almost indistinguishable from the positive control staining (fig. 2A, middle panel and upper

right). In contrast, the 15 antibodies classified as blocking antibodies showed an almost complete absence of HA-hBAFF binding, resembling the FACS staining obtained for the negative control (fig. 2A, lower panel and upper left). In figure 2B, an overlay of the results obtained for several non-blocking (left) and blocking (right) anti-hBAFF antibodies is shown. As expected, the curves for the non-blocking antibodies superimpose the curve given by the positive control staining, whereas the curves from the blocking antibodies superimpose the negative control staining curve.

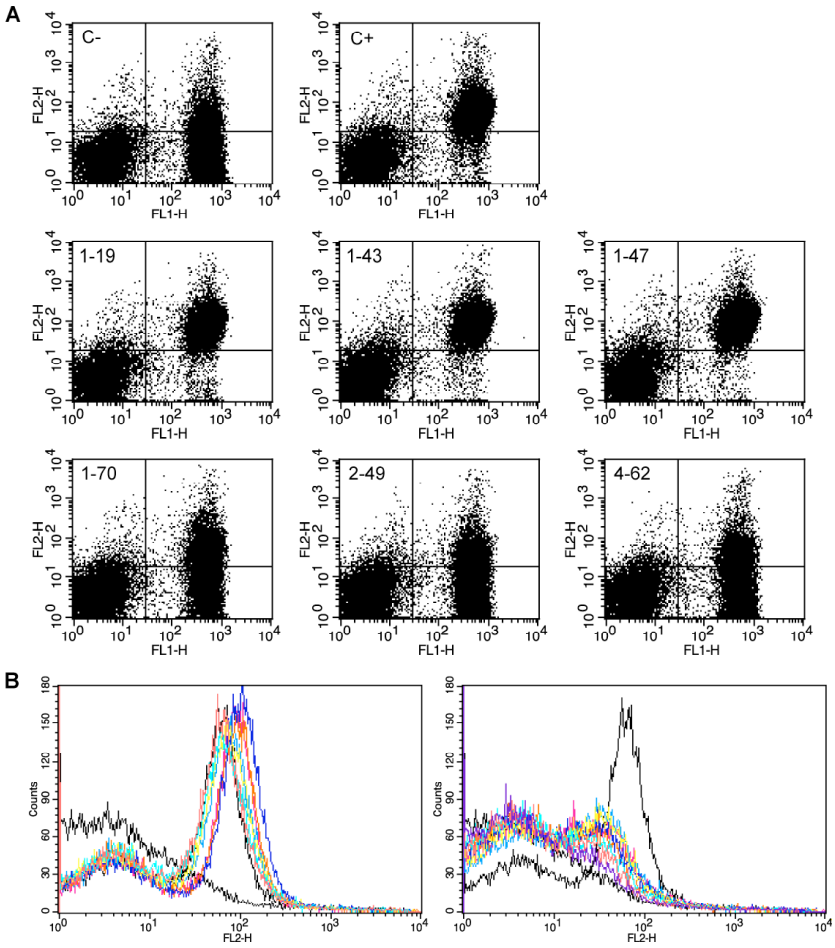

Figure 2. Blocking of HA-hBAFF binding to splenic B cells. *Total spleen cells were stained with anti-B220^{FITC} and HA-hBAFF which was pre-incubated with supernatant of different anti-hBAFF hybridoma cell lines. As controls, cells were stained with anti-B220^{FITC} alone (C-) or with anti-B220^{FITC} and HA-hBAFF pre-incubated in medium (C+). A) FACS plots of a selection of anti-hBAFF antibodies classified as non-blocking (middle panel) or blocking (lower panel) are shown. B) Overlay of several non-blocking (left) or blocking (right) hybridomas, gated on B220^{+} cells.*

On the basis of this result several blocking and non-blocking antibody producing hybridomas were chosen and the antibodies were purified in order to be able to quantify the

amount of antibody used. Then, HA-hBAFF was pre-incubated with different amounts of antibody and afterwards used to stain the human Burkitt lymphoma cell line, Raji, known to express large amounts of hBAFF-R on its surface (1).

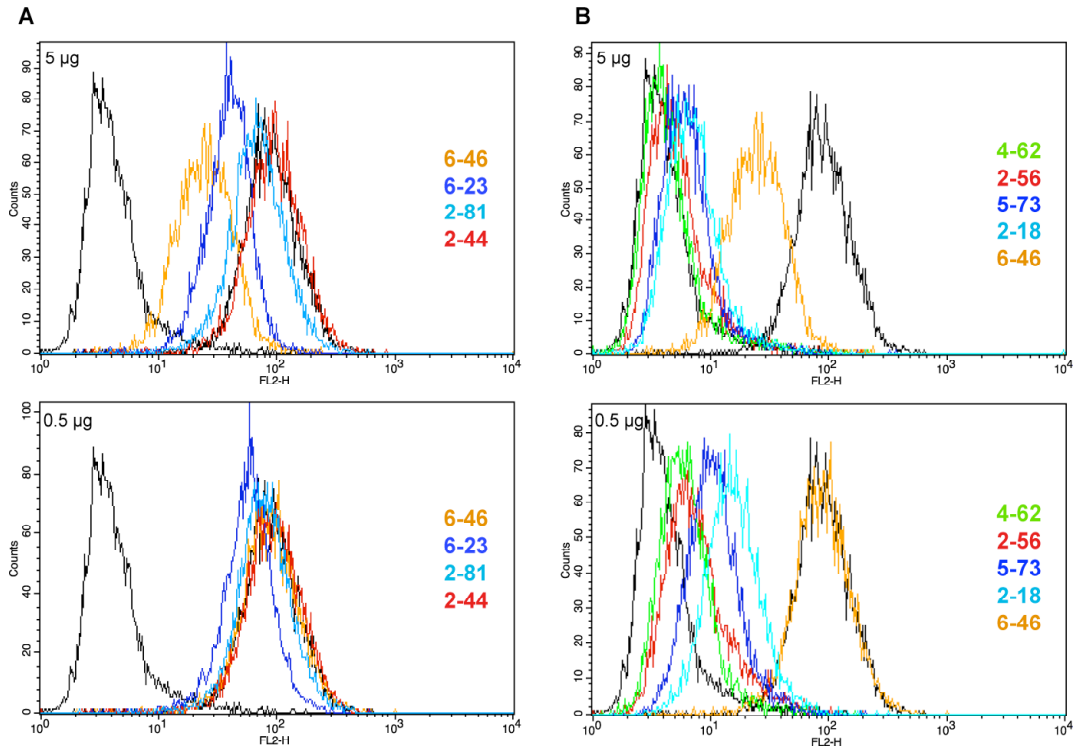

Figure 3. Concentration-dependent blocking of HA-hBAFF binding to Raji cells. *A) and B) 5 µg or 0.5 µg of the indicated anti-hBAFF antibodies were incubated with HA-hBAFF and the resulting antibody-antigen complex was used to stain human Raji cells. Unstained cells and cells stained with HA-hBAFF were used as controls (black lines). HA-hBAFF binding was revealed using a biotinylated anti-HA antibody and Strep-PE. Anti-hBAFF antibodies were divided into A) non-blocking antibodies and B) blocking antibodies.*

The extent of blocking observed was dependent on the nature of the antibody, as shown before, and also on the antibody amount used (fig. 3). The antibody clones categorized as non-blocking (fig. 3A) showed only little if any blocking at 5 µg (upper picture) and no blocking at 0.5 µg (lower picture). In particular, even when used at 5 µg, clone 2-44-14 did not at all block the binding of HA-hBAFF to Raji cells at all. In contrast, antibody clones 4-62 and 2-56 showed an almost complete block of hBAFF binding when used at 5 µg and still prevented to a large extend the binding of hBAFF when used at 0.5 µg (fig. 3B, green and red curves). Clones 5-73 and 2-18 also blocked the binding of HA-hBAFF when used at 5 µg and 0.5 µg, but in both cases less efficient than the aforementioned antibody clones

(fig. 3B). Clone 6-46, which showed an intermediate level of blocking at 5 µg, did not at all block the binding of hBAFF to Raji cells at all, when used at 0.5 µg (fig. 3A and B).

To further confirm the above observation that of the four blocking antibodies clones 4-62 and 2-56 perform better than clones 5-73 and 2-18, for each of these antibodies six different amounts were used for a blocking experiment as described above. As shown in figure 4, all of these antibody clones block the binding of hBAFF completely or at least considerably when used at 5 to 0.3 µg and no blocking can be observed when used at 0.03 µg or below. The results of this experiment confirm the above findings, that clones 4-62 and 2-56 are the most efficient ones for blocking hBAFF binding, followed by clones 5-73 and 2-18.

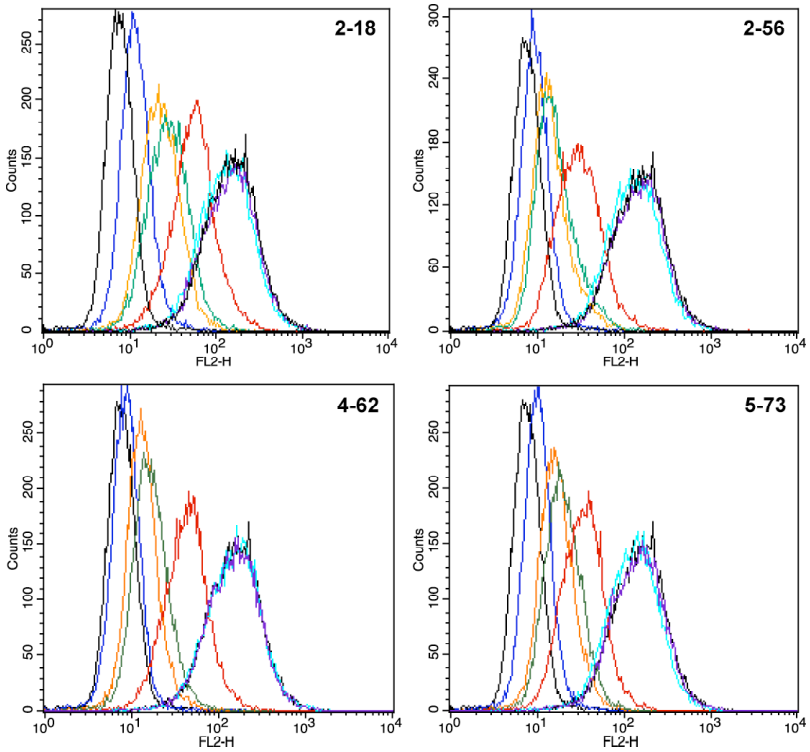

Figure 4. Correlation between concentration and blocking ability of several blocking anti-hBAFF antibodies. *Different amounts of the indicated antibodies, namely 5 µg (blue), 0.5 µg (orange), 0.3 µg (green), 0.1 µg (red), 0.03 µg (light blue) and 0.01 µg (purple), were incubated with HA-hBAFF and the resulting complex was then used to stain human Raji cells. Binding of HA-hBAFF was revealed using anti-HA^{bio} and Strep-PE. As controls, unstained cells and cells stained with HA-hBAFF where used, which both are depicted in black.*

The combination of all results indicated that diverse anti-hBAFF antibody clones were obtained. For further usage eight independent clones were chosen. The characteristics of the selected anti-hBAFF antibodies are summarized in table 1.

Clone	Subclones	Blocking of hBAFF binding	Cross-reactivity with mBAFF
2-18	**2-18-1**	+	-
2-44	2-44-6 **2-44-14**	- - -	+++
2-56	**2-56-9** 2-56-10	+++	-
2-81	**2-81-5** 2-81-8	- -	-
4-62	none	+++	-
5-73	none	++	+
6-23	6-23-2 **6-23-7**	-	+
6-46	none	+/-	-

Table 1. Monoclonal anti-hBAFF antibodies. *Overview of the selected anti-hBAFF monoclonal antibodies and their features.*

4.2 Development and validation of a human BAFF ELISA

For the detection of hBAFF in blood serum, a two-step or Sandwich ELISA had to be developed. As described in detail in chapter 2, from the anti-hBAFF antibodies obtained the antibodies best suited as capture and detection antibody were identified using an empirical method. Finally, antibodies 2-81 and 4-62 in combination with an ELISA buffer containing rat serum instead of BSA were found to work best. In order to test the functionality, reliability, specificity and sensitivity of the newly developed ELISA assay, several tests were made.

Initially serum and plasma of hBAFF transgenic mice were analysed. Therefore, two-fold serial dilutions of each sample, ranging from 1:10 to 1:80, were analyzed in duplicates. For the standard curve, commercially available untagged hBAFF in concentrations from 2 ng/ml to 125 pg/ml was used. For the first analysis, only the sample values obtained for the 1:20 dilution were taken. As shown in figure 5, hBAFF could be detected in serum and in plasma

samples of all transgenic mice. No signal was detected when serum of WT mice was used (data not shown).

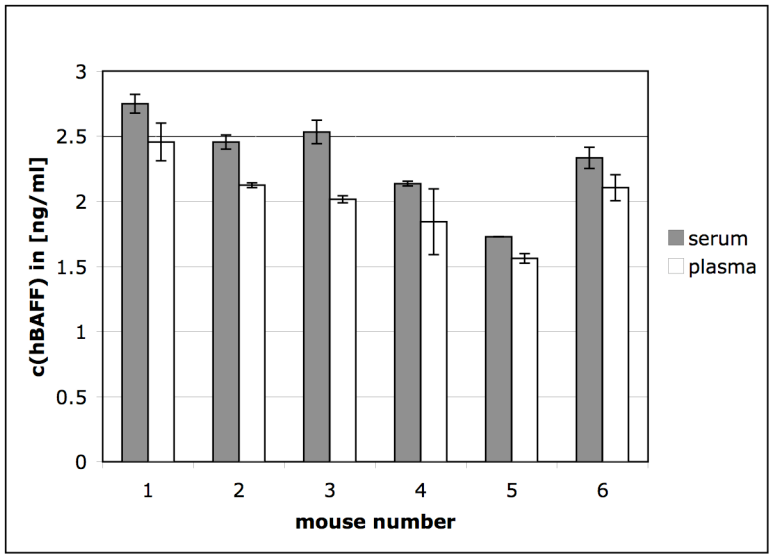

Figure 5. hBAFF concentration in serum and plasma of hBAFF transgenic mice. *Serum and plasma were taken at the same time. From each sample two independently made dilutions of 1:20 were analyzed in parallel. Mean values and the corresponding standard deviations are given.*

These results indicate that the new ELISA is sensitive enough to detect hBAFF in the range of pg/ml in serum and plasma of mice. Furthermore, the new ELISA is highly specific for hBAFF, as it does not detect murine BAFF nor does it show any crossreactivity with other proteins present in serum of WT mice. Moreover, the low SD values obtained indicate that intra-assay variations are only marginal. The analysis of the serum and plasma samples showed, that in all cases the signal strength found for plasma was lower than the values for the corresponding serum sample, but no statistically significant difference could be observed (fig. 5). Thus, plasma and serum are comparably well suited for hBAFF measurements.

Next the values obtained for the different dilutions were compared. As the concentration of hBAFF in samples from patients may be several folds higher than in healthy individuals, it might be necessary to compare values obtained from different dilutions, depending on which sample values are within the linear range of the standard curve. Thus, from the experiment mentioned above, the mean hBAFF concentration was calculated from the duplicate OD values determined for each dilution, omitting OD values which were not within the linear range of the standard curve. Like this, for each sample three or four dilution specific mean concentrations were obtained. From these so-called dilution specific mean concentrations, the mean value and SD value was calculated. This SD value indicates how

wide the dilution specific mean values obtained for a single sample differ from each other. As can be seen in figure 6, the SD values are in most cases relatively small, meaning less than 10% and the maximum value for the deviation never exceeds 20%. Thus, the observed deviation values are within the range of 5-20%, which is given in the literature as the typical range for routine variations (2). This value has to be kept in mind when data from different dilutions have to be compared.

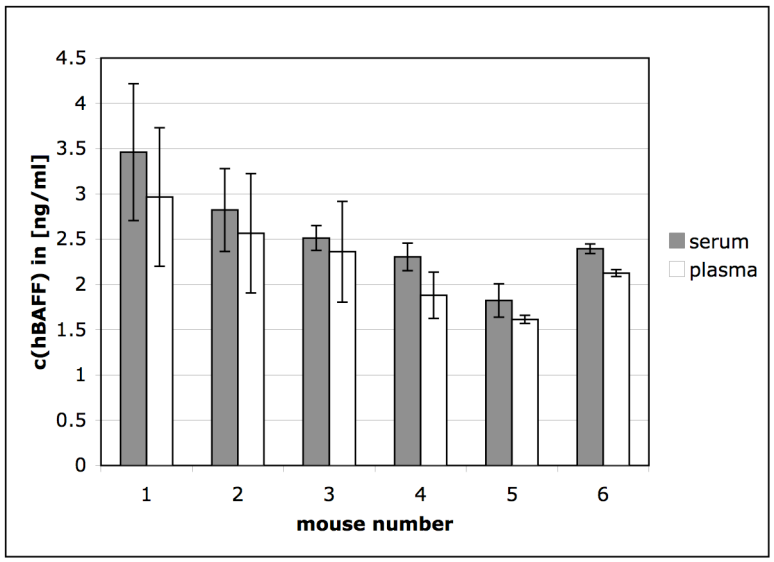

Figure 6. Influence of the dilution factor on hBAFF concentration in serum and plasma of hBAFF transgenic mice. *Serum and plasma were taken at the same day. From all samples two independently made dilution series ranging from 1:10 to 1:80 were analyzed. For each dilution a mean concentration value was calculated, and from these values a mean value and the SD value was determined. SD values are an indication for the variation of the dilution specific concentrations.*

The analysis of blood samples sometimes requires the prevention of blood clotting. Several different anticoagulants are commonly used, including lithium-heparin, sodium-citrate and EDTA. It is known that these additives can influence test results. In order to find out whether or not any of these anti-coagulants influence the measurement of hBAFF concentration with the newly developed ELISA, blood from a patient suffering from Hashimoto's thyroiditis was taken at the same time into several blood collection tubes provided with different anti-coagulants. Blood from this patient has been analyzed before and a high hBAFF concentration was determined. For the determination of the hBAFF concentration, all blood samples were diluted 1:10, 1:100 and 1:1000 and analyzed in duplicates. Only the OD values obtained for the 1:1000 dilution were within the linear range of the standard curve and have thus been taken for analysis. As shown in figure 7, the values determined for serum, lithium-heparin plasma and citrate plasma gave almost the same value. EDTA plasma gave a roughly 25% higher value compared to serum and the other plasma preparations. Thus, for the determination of hBAFF concentration in blood from patients, preparations

done as serum or as plasma using heparin or citrate are to prefer. Data obtained from these preparations can be compared between each other, which is not possible if EDTA plasma was used for the analysis.

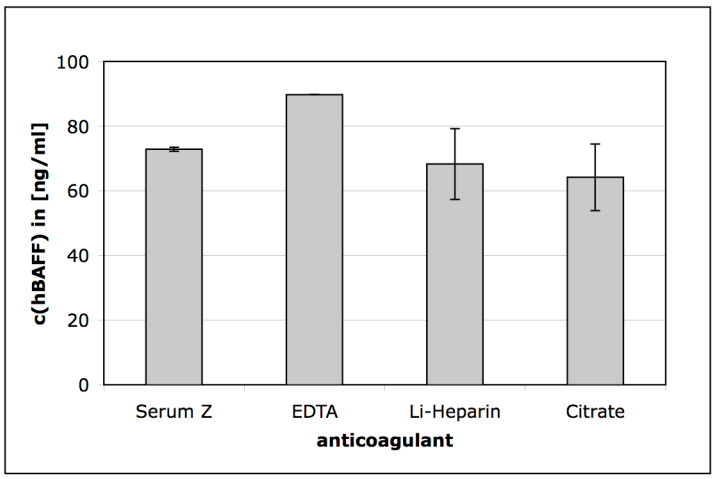

Figure 7. Influence of different anticoagulants on hBAFF concentration meassured in human serum or plasma. *Serum and plasma were taken at the same time from a patient suffering from Hashimoto's thyroiditis. Blood was collected in different tubes to obtain serum, EDTA plasma, lithium-heparin plasma and citrate plasma. Samples were analyzed in duplicates at a dilution of 1:1000. Shown are mean values and the corresponding SD values.*

Taken together, the results obtained in the aforementioned experiments showed that the newly developed hBAFF ELISA is suitable for detection of hBAFF in serum and plasma of human patients.

4.3 Monoclonal antibodies against murine BAFF-R

4.3.1 Hybridoma production and selection by FACS

BAFF-R exists only as a membrane bound protein, thus it was decided to use a rat myeloma cell line expressing membrane bound mouse BAFF-R as antigen. Cloning of mBAFF-R, transduction of cell lines and hybridoma production is described in chapter 2. For selection, hybridoma supernatants were screened with FACS using a mixture of untransduced (GFP^-) 40E1 and mBAFF-R transduced (GFP^+) 40E1 cells, in order to find antibodies against mBAFF-R in one step with excluding antibodies giving unspecific staining. As shown in figure 8, several antibodies specific for mBAFF-R could be found. Specific antibodies, e. g. clones 1G10 and 5A12, gave a positive signal only on GFP^+ but not on GFP^- 40E1 cells. Unspecific binding, obvious from a positive signal on both GFP^+ and GFP^- cells (fig. 8,

clone 1A2), was also observed in many cases. Besides the non-specific binding antibodies, also the ones giving low signals (fig. 8, clone 1B7) or no signal at all (fig. 8, clone 1A1) were excluded. Hybridomas producing specific anti-mBAFF-R antibodies were subcloned and the corresponding antibody was purified by affinity chromatography.

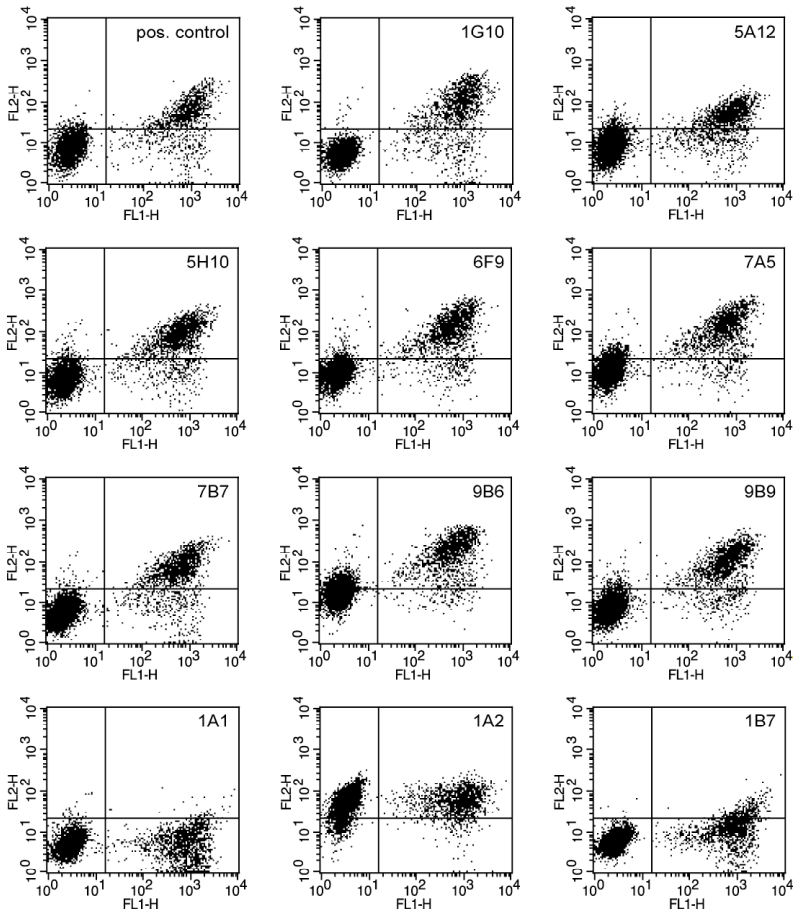

Figure 8. Selection of FACS plots from screening supernatants of hybridoma cultures possibly producing an anti-mBAFF-R antibody. *Hybridoma supernatants were used to stain a mixture of normal (GFP^-) and mBAFF-R expressing (GFP^+) 40E1 cells. Binding of an antibody was revealed using anti-ratIgG^{bio} and Strep^{PE}. As positive control, a staining with HA-hBAFF, anti-HA^{bio} and Strep^{PE} was performed.*

4.3.2 Specification of anti-mBAFF-R antibody characteristics

In order to find independent hybridoma clones producing antibodies with distinct features, several characteristics of the new anti-mBAFF-R antibodies were determined.

4.3.2.1 Determination of IgG subclass

Rat antibodies of the IgG class can be further subdivided into the subclasses IgG1, IgG2a, IgG2b and IgG2c. For the determination of the IgG subclasses, an ELISA using rat IgG subclass specific antibodies and supernatant from previously subcloned hybridomas was

performed. All of the antibodies tested were either of rat IgG2a or rat IgG2b subclass (data not shown and tab. 2).

4.3.2.2 Determination of ability to block ligand binding

BAFF-R is a tiny molecule, thus the possibility that binding of an antibody to this receptor inhibits the simultaneous binding of the corresponding ligand is relatively high. To analyze the blocking capacity of the anti-mBAFF-R antibodies, a mixture of un-transduced and mBAFF-R expressing Sp2/0 cells were incubated with different amounts of purified antibodies and then stained with HA-hBAFF. Bound HA-hBAFF was detected by staining with an monoclonal anti-HA^{bio} antibody, followed by Strep-PE. In figure 2-6, the FACS plots for a selection of antibodies is shown. The variation of antibody amount in the range between10 µg and 0.5 µg had no influence on the outcome of the stainings (fig. 9B and data not shown). Compared with the control stainings, some of the antibodies, e.g. clones 5H10 and 9B9, completely blocked the binding of ligand. In these cases no signal coming from bound HA-hBAFF could be detected (fig. 9, A and B, left panels). One antibody, namely clone 5A12, did not at all interfere with ligand binding at all, which was obvious from a high positive signal comparable to that of the positive control staining (fig. 9A). Other antibodies like clones 1G10 and 9B6 showed a low level of blocking ability, apparent from the reduced levels of HA-hBAFF staining (fig. 9A). All results are summarized in table 2.

4.3.2.3 Investigations on anti-mBAFF-R antibody interference

The fact that not all antibodies could block the binding of ligand to the receptor indicates that among the anti-mBAFF-R antibodies obtained there are independent clones recognizing different epitopes on the mBAFF-R receptor. This makes it possible that one antibody can bind to mBAFF-R receptor even if another antibody is already bound. To test this, mBAFF-R expressing (GFP^+) 40E1 cells were pre-incubated with unlabeled anti-mBAFF-R antibody 5A12 or 7A5 and then with different biotin labeled anti-mBAFF-R antibodies. As depicted in figure 10, cells pre-incubated with anti-mBAFF-R antibody clone 5A12 could still bind all other clones of anti-mBAFF-R antibodies tested, except biotin-labeled 5A12 antibody. In contrast, cells pre-incubated with clone 7A5 could bind only clone $5A12^{bio}$, all other clones tested showed either a severely reduced signal or could not be bound at all. Therefore we

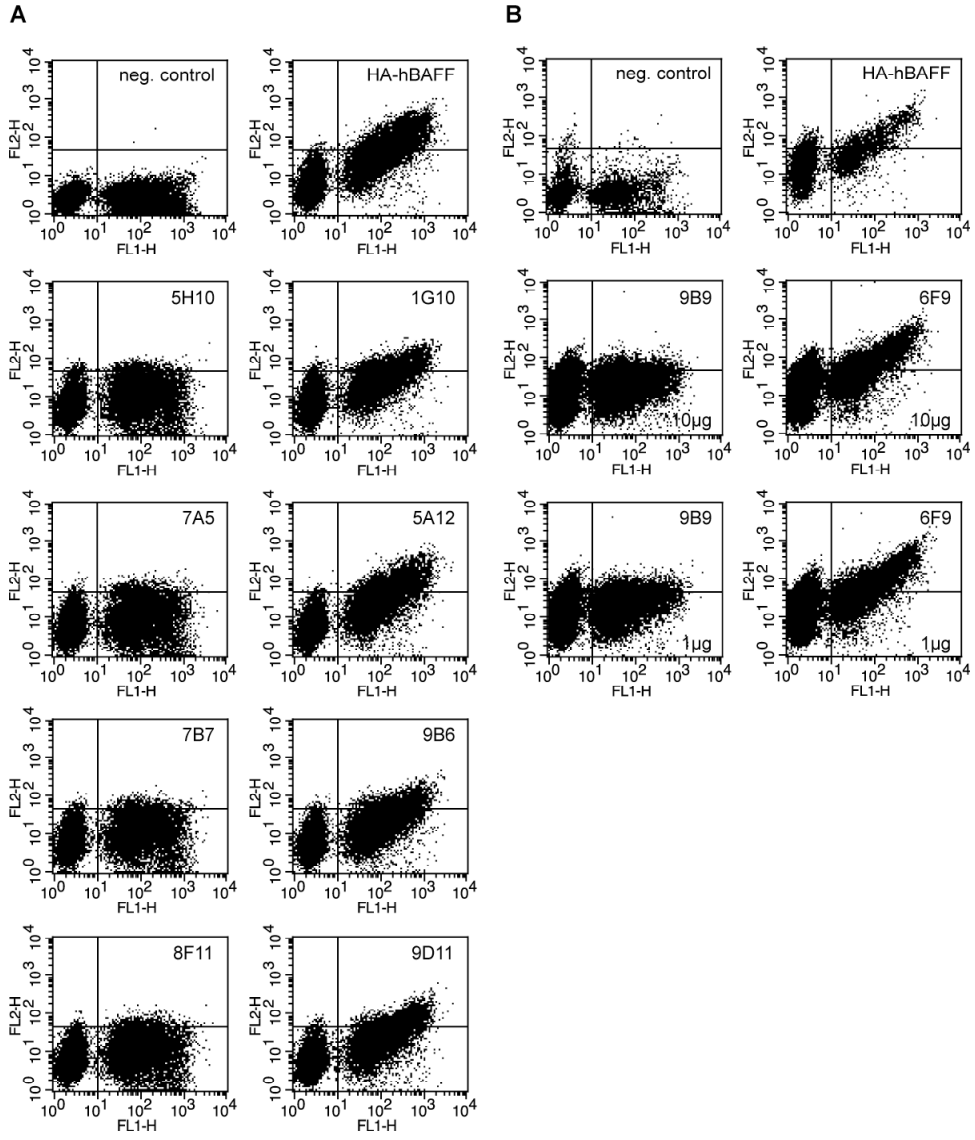

Figure 9. Blocking of hBAFF binding to mBAFF-R by anti-mBAFF-R antibodies. *A) and B) A mixture of un-transduced (GFP$^-$) and transduced (GFP$^+$) Sp2/0 cells was pre-incubated with different amounts of purified anti-mBAFF-R antibodies and then stained with HA-hBAFF, anti-HA^{bio} and Strep-PE. As controls, cells were stained with anti-HA^{bio} and Strep-PE (negative control) or with HA-hBAFF, anti-HA^{bio} and Strep-PE (positive control). A) Cells were pre-incubated with 10 µg, 5 µg or 0.5 µg of anti-mBAFF-R antibody. Results for 0.5 µg are shown. B) Cells were pre-incubated with 10 µg or 1 µg of anti-mBAFF-R antibody.*

concluded that clone 5A12 recognizes an epitope on mBAFF-R which is completely different from the epitopes recognized by the other antibody clones tested. The epitope recognized by clone 7A5 on the other hand is either very similar to the epitopes recognized by the other clones tested, or in close vicinity thereto. The complete absence of any positive signal in the case of antibodies 5H10 and 7B7 makes it very possible that these two antibodies recognize the same epitope as clone 7A5, or in other words, antibodies 5H10,

7A5 and 7B7 may not represent independent clones. A reduction of signal strength in comparison to the positive control, as was observed for the other antibodies tested, is very likely attributed to the close vicinity of the different epitopes recognized. This would mean that clones 1G10, 7A5, 9B6 and 14H7 recognize different but closely located epitopes.

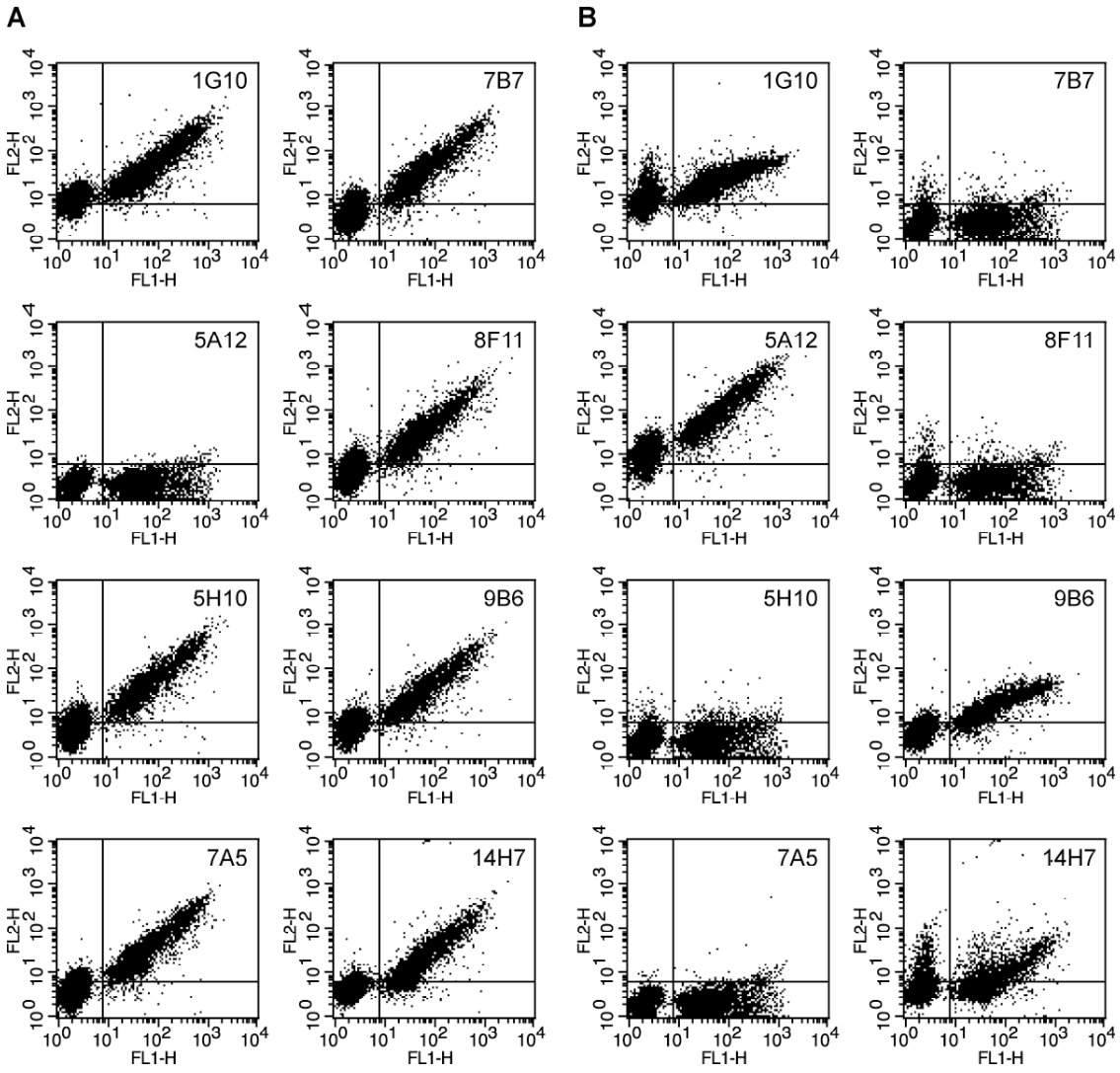

Figure 10. Crossreactivity of several anti-mBAFF-R antibody clones. *A mixture of un-transduced (GFP$^-$) and transduced (GFP$^+$) 40E1 cells was pre-incubated with A) antibody 5A12 or B) antibody 7A5. Cells were then stained with the indicated biotinylated anti-mBAFF-R antibody clones and Strep-PE.*

Taken together, the combination of the obtained results (tab. 2) indicate that several independent anti-mBAFF-R antibodies have been obtained. Antibody clones 1G10 and 7A5 are both of IgG subclass 2a and represent a ligand non-blocking and blocking clone, respec-

tively. Clones 5A12, 5H10 and 9B9 are all of IgG2b subclass. While clone 5A12 does not block ligand binding, clones 5H10 and 9B9 efficiently block the binding of ligand.

clone	IgG subclass	blocking of hBAFF binding	binding blocked by pre-bound 5A12	binding blocked by pre-bound 7A5	subclones
1G10	2a	+/-	-	+/-	**D4**, D11, E4, E6, E8
5A12	2b	-	+	-	A11, B2, C1, **D6**, H8
5H10	2b	+	-	+	**G8**
6F9	2a	-	n.d.	n.d.	none
7A5	2a	+	-	+	A1, B3, **E7**
7B7	2b	+	-	+	none
8F11	2b	+	-	+	none
9B6	2a	+/-	-	+/-	B12, **G1**, G2, H12
9B9	2b	+	n.d.	n.d.	A11, **D7**, E7, H8, H9
14H7	2a	-	-	+/-	none

Table 2: Overview of anti-mBAFF-R antibodies and their features. *Subclones chosen for production are indicated in bold.*

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5 CRUCIAL ROLE FOR BAFF - BAFF-R SIGNALING IN THE SURVIVAL AND MAINTENANCE OF MATURE B CELLS

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5.1 Introduction

The pool of peripheral B cells is continuously replenished by newly-formed immature B cells generated in the bone marrow. In the adult mouse, about $2x10^7$ B cells are produced per day (1, 2). Following several steps of antigen-independent differentiation and depending upon successful rearrangement of the corresponding genes and expression of the B cell receptor (BCR) protein on their surface, only about 20% of the newly-generated bone marrow B cells migrate to the spleen as immature B cells (3-7). These cells are characterized by a short half-life of about 2-4 days and upon further differentiation steps develop into mature, naïve B cells. It has been shown that upon engagement of their BCR, immature B cells undergo apoptosis whereas mature B cells, under the same conditions, are induced to proliferate (3-7). As for the early stages in the bone marrow, in the periphery the BCR signal is not the only requirement for the progression of B cells along their developmental pathway. The surrounding stromal microenvironment, the presence of appropriate growth factors, as well as their ability to respond to them, are all crucial players in the final maturation steps of developing B cells.

Surface expression of CD93 is a hallmark for immature B cells and on splenic B cells is a phenotypic characteristic for so called transitional B cells (3, 5). The latter can be further subdivided according to the expression of CD21, CD23, IgM and IgD. Thus, transitional type 1 (T1) cells are $CD21^-$ $CD23^-$ IgM^{high} and IgD^{low}, T2 are $CD21^+$ $CD23^+$ IgM^{high} and IgD^{high}, and T3 are $CD21^+$ $CD23^+$ IgM^{low} and IgD^{high} cells (3, 5, 6). Recently, it has been suggested that T3 cells, rather than representing an intermediate in the formation of mature B cells, might identify an independent pool of anergic B cells (8). Therefore, only T1 and T2 cells would represent the immediate precursors of Follicular and marginal zone B cells, the two major mature splenic B cell subsets.

BAFF (B cell activating factor), a member of the TNF family (also termed TALL-1, THANK, BlyS and zTNF4) and BAFF receptor (BAFF-R) play a fundamental role during the transition from immature T1 to T2 B cells and therefore for the generation of mature B cells in the spleen. This was clearly demonstrated by an almost complete lack of follicular and marginal zone B cells and by a block at the T1 cell stage in BAFF as well as in BAFF-R deficient mice (9-13). In these mice, the B-1 compartment was not affected, indicating that the development of this subset was independent of BAFF-BAFF-R signaling. On the other hand, transgenic mice over-expressing BAFF display an overall increase in all B cell

subsets, suggesting that all mature B cells express BAFF-R on their surface or are able to respond to BAFF (10, 14-16).

The binding of BAFF to the BAFF-R leads to the activation of the NF-κB pathway and ultimately to the transcription of the anti-apoptotic factor Bcl-2 (17-19). The finding that Bcl-2 overexpression can, to a large extent, rescue the mature B cell compartment in BAFF signaling deficient mice, indicates that Bcl-2 expression induced by BAFF is crucial for the survival of B cells during the transition from immature to mature stages (18).

Since BAFF-R is expressed on all mature peripheral B cells and its signaling promotes *in vitro* survival of immature as well as mature B-2 cells, we hypothesised that BAFF-BAFF-R signaling was also playing a central role in the *in vivo* maintenance of the peripheral mature B cell pool. However, the potential survival role of BAFF in the mature B cell pool is masked in both BAFF- and BAFF-R deficient animals due to the associated developmental block at the T1 stage. Therefore, to address this question, we generated a collection of anti-BAFF-R mAbs, some of which blocked and others failed to block BAFF binding.

Administration of these blocking antibodies to wild-type mice resulted in an almost complete depletion of follicular B cells and a reduction of about 50% in the MZB cell compartment. Non-blocking antibodies had no, or only minor effects on the mature B cell pool. Moreover, by using FcRγ-deficient or Bcl-2-transgenic mice, we could show that this depletion was Fc-Receptor (FcR) and complement independent. Taken together, beyond its essential role in allowing the developmental progression from immature T1 cells into T2-T3 and mature B cells, we formally demonstrate the essential role of the BAFF-BAFF-R signaling in the long-term survival and homeostasis of mature B-2 and marginal zone B cells.

5.2 Results

5.2.1 Characterization of anti-BAFF-R monoclonal antibodies

A mixture of un-transfected and mouse BAFF-R-expressing Y3 rat myeloma cells was used to screen supernatants of individual hybridomas generated as described in Materials and Methods. As shown in figure 1A, BAFF-R expressing Y3 (GFP+) cells but not control un-transfected cells (GFP-) stained with two of the generated anti-BAFF-R antibodies, 9B9 and 5A12. After subcloning and re-testing, the supernatants of positively-identified clones were

used to stain the BAFF-R-expressing Abelson transformed pre-B cell line 40-E1. In total, eleven hybridomas producing anti-BAFF-R mAbs were obtained and of these, five were of rat IgG2a and 6 of IgG2b isotype. To evaluate their blocking capacity, again a mixture of Y3 (GFP-) and BAFF-R expressing Y3 cells (GFP+) was pre-incubated with these mAbs and subsequently with a saturating concentration of HA-tagged BAFF. These experiments were performed using human BAFF, but similar results were obtained using mouse BAFF. FACS analysis with an anti-HA mAb then allowed us to determine which of the generated anti-BAFF-R mAbs blocked BAFF binding. As depicted in figure 1B, pre-incubation of BAFF-R-expressing Y3 cells with mAbs 5A12 and 9B6 did not affect BAFF binding, whereas mAbs 9B9 and 5H10 could block BAFF binding. Of the eleven anti-BAFF-R mAbs generated, five were able to block BAFF binding.

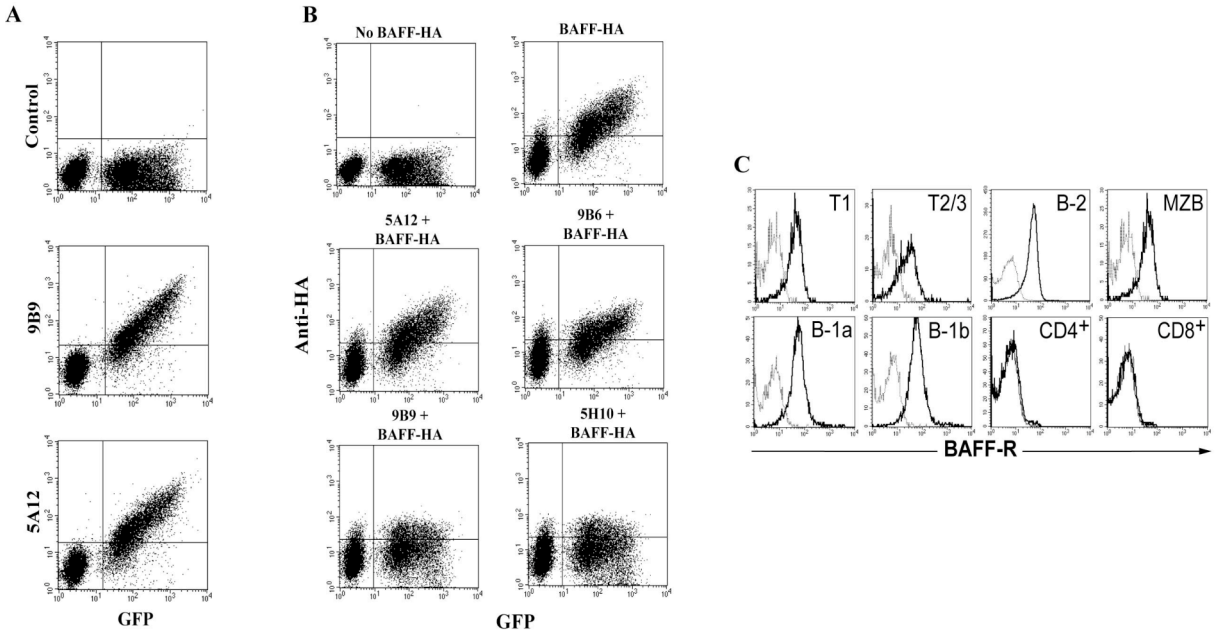

Figure 1. Binding of anti-BAFF-R antibodies to BAFF-R expressing Y3 rat myeloma cells. Panel A*. FACS analysis of a 1:1 mixture of BAFF-R-IRES-GFP transfected and untransfected Y3 myeloma cells. Upper plot: irrelevant isotype control antibody. Lower plots: the anti-BAFF-R antibodies 9B9 and 5A12 stained the BAFF-R expressing BAFF-R-IRES-GFP transfected Y3 myeloma cells but not the untransfected GFP negative cells.* ***Panel B****. The same mixture of BAFF-R-IRES-GFP transfected and untransfected Y3 myeloma cells was pre-incubated with or without the four different anti BAFF-R antibodies as indicated: 5A12, 9B6, 9B9 and 5H10 followed by HA-tagged BAFF, which was revealed by a PE labeled anti-HA antibody (upper plot right). 9B9 and 5H10 (lower plots), but not 5A12 and 9B6 (middle plots) were preventing the binding of HA-tagged BAFF to BAFF-R, revealing blocking capacity.* ***Panel C****. FACS analysis on the different B and T cell subsets for BAFF-R surface expression, as indicated for each plot. Dotted histograms represent isotype control stainings.*

All BAFF-blocking and non-blocking mAbs were used to reveal the expression of BAFF-R on ex vivo isolated spleen, lymph node, bone marrow, peripheral blood and peritoneal cells. All mature B cells, irrespective of their localization within the lymphatic compartments, namely B-2, MZB, B-1a and B-1b B cells as well as the three immature transitional splenic B cell subsets (T1, T2 and T3) expressed similar levels of BAFF-R (figure 1C). Bone marrow precursor B cells and haematopoietic cells of other lineages did not express detectable surface BAFF-R (figure 1C and data not shown).

5.2.2 *In vivo* depletion of circulating mature B cells with anti-BAFF-R mAbs that block BAFF binding.

Since BAFF was shown to be a potent survival factor for mature and immature B cells *in vitro*, we reasoned that the *in vivo* use of blocking anti-BAFF-R mAbs would affect the B cell pool. Therefore wild-type C57BL/6 mice were treated with two BAFF-blocking and two BAFF non-blocking anti-BAFF-R mAbs; all four of the same isotype. At day 14 after treatment, the percentage of mature circulating peripheral blood B cells, characterized as $CD19^+$ $CD93^-$ cells, was determined by flow cytometry. Mature B cells in the control group of PBS treated mice represent about 40% of the circulating leukocytes. Similar percentages were obtained with the non-blocking 5A12 mAb (figure 2, upper graph). For 9B6, also a non-blocking mAb, mature B cells ranged from 25-35%, whereas treatment with either 9B9 or 5H10 mAb, both of which blocked BAFF binding, resulted in a dramatic decrease of up to 80-90% of circulating B cells (figure 2, upper graph). Therefore, whereas non-blocking mAbs had only a minimal or no effect, the use of mAbs that prevented BAFF binding drastically reduced circulating peripheral B cell numbers. Taken together, these results suggest that mature, circulating B cells require BAFF for their survival.

In order to determine the kinetics of mature B cells depletion, C57BL/6 mice were injected with the BAFF-blocking mAb 9B9 and at days 4, 7 and 10 blood lymphocyte subpopulations were analyzed. Mice injected with either PBS or the *in vivo*-depleting anti-CD4 mAb (GK1.5, rat IgG2b) were used as negative and positive controls, respectively. Results are summarized in figure 2, lower graph. Already by day 4 after treatment with GK1.5, almost all $CD4^+$ T cells had disappeared and as a consequence, the other lymphocyte sub-populations had proportionately increased. Treatment with 9B9 resulted in a 40% depletion of mature B cells at day 4, which increased to 70% by day 7 and reached its

maximal level of 80% by day 10. In accordance with the *in vivo* expected half-life of IgG antibodies, B cell numbers started to recover by day 25-30 after antibody treatment (data not shown).

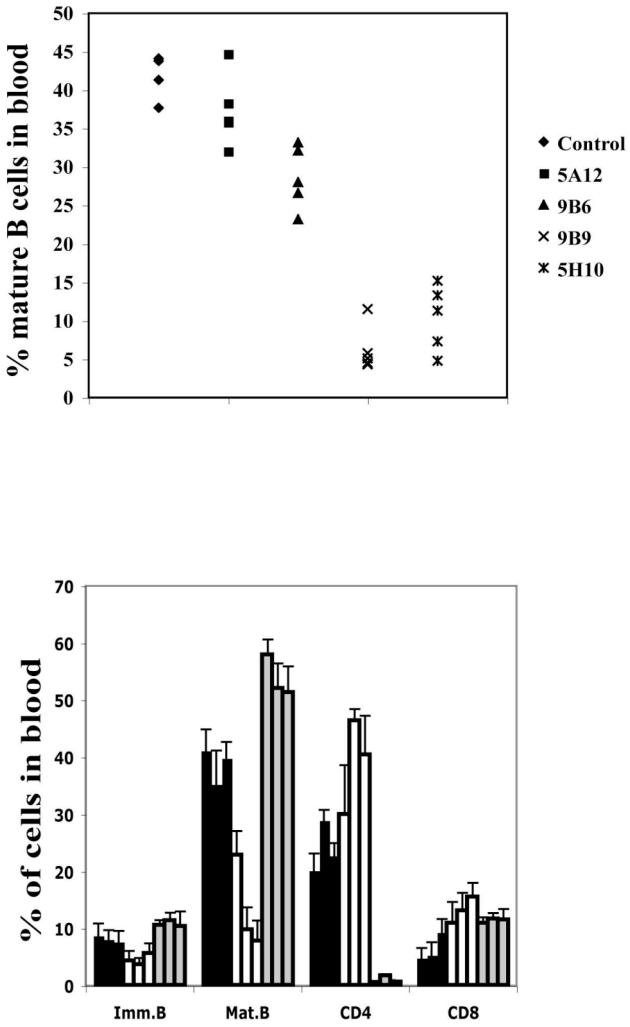

Figure 2. Circulating mature B cells and kinetic analysis of peripheral blood B and T cell depletion. Upper graph: *C57BL/6 mice were injected i.v. at day 0 with 0.5 mg of a given anti-BAFF-R antibody, as indicated. At day 14 the percentage of $CD93^{-}CD19^{+}$ mature B cells was determined by FACS analysis on the peripheral blood mononucleated cells. Each symbol represents an individual mouse. Statistical analysis revealed a significant difference for control versus 9B6, 9B9 and 5H10 ($p<0.05$).* ***Lower graph:*** *C57BL/6 mice were injected with either 0.5 mg of the anti-BAFF-R mAb 9B9 (white bars), or 0.5 mg of the anti-CD4 mAb GK1.5 (grey bars). Black bars represent PBS injected controls. The mAbs were injected at day 0 and the percentages of immature B cells ($CD93^{+}CD19^{+}$), mature B cells ($CD93^{-}CD19^{+}$), CD4 and CD8 T cells were determined by FACS at days 4, 7 and 10. Each column represents a time point: day 4 left column, day 7 middle and day 10 right column. Statistical analysis revealed a significant difference between PBS treated mice as compared to anti-BAFF-R mAb at each time point analyzed for mature B cells but not for immature B cells.*

5.2.3 The BAFF - BAFF-R interaction is essential for the maintenance of circulating mature B cells

The finding that mAbs that prevented BAFF binding caused a pronounced depletion of circulating B cells whereas isotype-matched non-blocking ones had only a minor effect strongly suggested, that this ablation was neither due to antibody-dependent cellular cytotoxicity (ADCC) nor to complement-mediated depletion. In order to test this hypothesis, FcR common γ chain-deficient mice were injected with the BAFF-blocking mAb 9B9 or the

non-blocking mAb 5A12. At day 14 after injection, the percentage of circulating mature B cells ($CD19^+CD93^-$) was determined in the blood and compared to untreated mice. In untreated or 5A12-treated mice a similar percentage of circulating mature B cells was detected, namely 35% and 25-38%, respectively. In contrast, there were only 7-12% circulating B cells in FcRγ-deficient mice treated with the BAFF-blocking mAb 9B9 (figure 3A). This result shows that the depletion of circulating mature B cells with BAFF-blocking mAb is FcR independent.

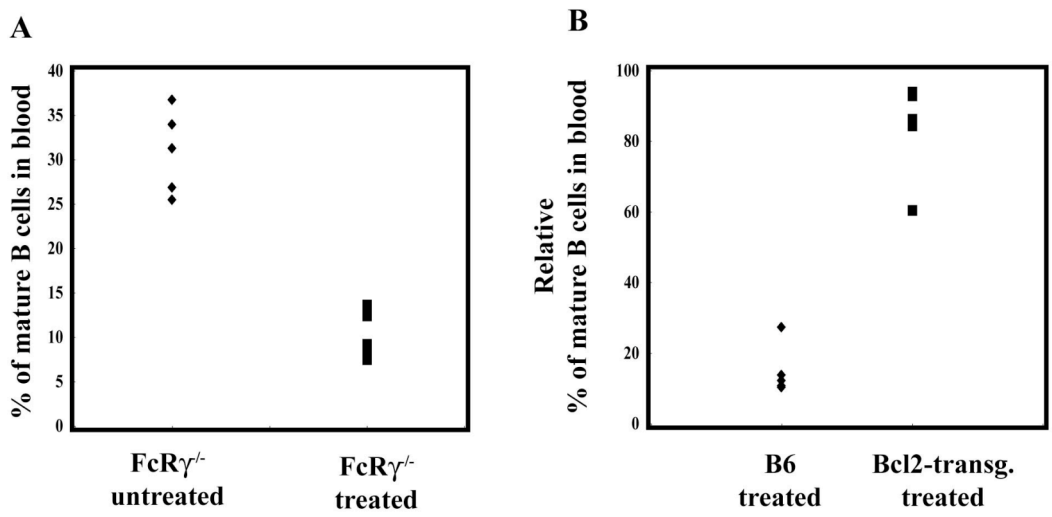

Figure 3. Circulating mature B cells in FcRγ deficient and Bcl2-transgenic mice following anti-BAFF-R 9B9 injection. Panel A. *$FcR\gamma^{-/-}$ mice were injected at day 0 and the percentage of $CD93^-$ $CD19^+$ mature B cells in the blood was determined at day 14 by FACS analysis. Non-injected $FcR\gamma^{-/-}$ mice were used as controls. The difference between $FcR\gamma^{-/-}$ treated versus untreated mice was statistically significant ($p<0.05$).* ***Panel B.*** *The percentage of mature B cells in the blood of C57BL/6 and Bcl2 transgenic mice at day 14 following the injection of 9B9 mAb. The difference between C57BL/6 and Bcl2 transgenic mice following 9B9 treatment was statistically significant ($p<0.01$).*

It has been shown that BAFF-induced B cell survival is achieved through an NF-κB mediated increase in anti-apoptotic molecules, including members of the Bcl-2 family. Based on this finding, we wondered whether B cell depletion with BAFF-R blocking mAbs would still be seen in transgenic mice over-expressing Bcl-2. Therefore, C57BL/6 and Bcl-2 transgenic mice were injected with the 9B9 mAb and analyzed after 14 days. As shown in figure 3B, the mature B cell pool in the blood of C57BL/6 mice treated with 9B9 was reduced by 80-90%. In marked contrast, only a 5-10% reduction was observed in 4 out of 5 Bcl-2 transgenic animals (figure 3B). Transgenic over-expression of the anti-apoptotic Bcl-

2 gene was therefore able to overcome to a large extent the B cell depleting effect of blocking anti-BAFF-R mAbs. Collectively, these results rule out a role for either ADCC or complement-mediated lysis in the observed depletion of recirculating B cells and strongly suggest that interactions between BAFF and BAFF-R are crucial for the survival and maintenance of the mature B cell pool.

5.2.4 BAFF is a survival factor for B-2 and marginal zone B cells *in vivo*

Mature, peripheral B cells in the mouse can be subdivided into B-2, also called follicular B (Fol B), MZB and B-1 B cells. In order to determine the effect of a blocking anti-BAFF-R mAb on the different B cell subsets, we injected C57BL/6 mice with the mAb 9B9 and analyzed the bone marrow, spleen, lymph nodes and peritoneal cavity lymphocytes at days 14 to 21. In the spleen, the immature transitional B cell subpopulations were only slightly affected with no reduction of T1 and a two fold reduction in T2/T3 subsets (figure 4A and B). Mirroring the depletion of circulating B cells, injections with the mAb 9B9 resulted in a 4 -5 fold reduction of $CD93^{-}CD19^{+}$ mature splenic B cells. The highest reduction, 80-90%, was observed among follicular ($CD21^{+}CD23^{+}$) B cells, whereas MZB ($CD21^{high}CD23^{low}$) cells were only decreased by 50%. The number of $CD21^{-}CD23^{-}$ mature splenic B cells, which to a large extent comprises B-1 B cells, was not affected at all. CD4 and CD8 T cells also remained unchanged (figure 4B).

Mature B cells in the bone marrow or lymph nodes, both of which consist almost entirely of B-2 B cells, were also reduced by 80-90% upon 9B9 treatment (data not shown). Bone marrow B cell progenitors were not affected by the treatment (data not shown).

Mature B cell subsets in the peritoneal cavity can be subdivided into B-2 ($CD19^{+}CD23^{+}$), B-1a ($CD19^{+}CD23^{-}CD11b^{+}CD5^{+}$) and B-1b ($CD19^{+}CD23^{-}CD11b^{+}CD5^{-}$) B cells (figure 4C upper plots). All these subsets express similar levels of BAFF-R (figure 1C). However, upon 9B9 treatment, whereas 70% of the B-2 B cells were depleted, both B-1a and B-1b cell subpopulations remained unaffected (figure 4C).

Taken together, these findings show that maintenance of the vast majority of B-2 and about half of the marginal zone B cells is highly dependent upon the interaction between BAFF and BAFF-R, whereas that of B-1 B cells is largely BAFF-R independent. The fact that

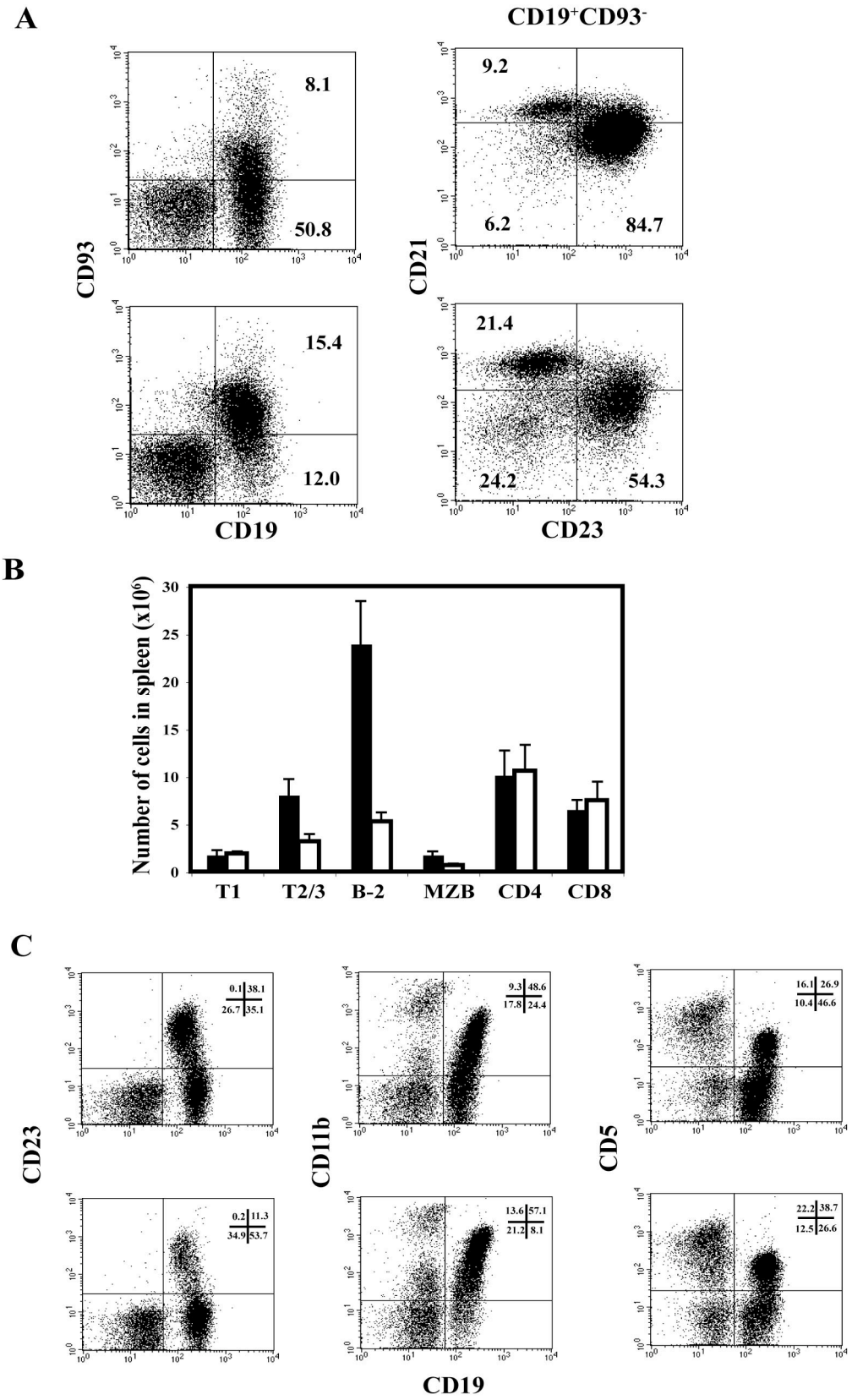

Figure 4. Depletion of the B and T cell subsets following anti BAFF-R 9B9 antibody injection. *For description see next page.*

some B-2 and about half MZB cells remained following antibody treatment might suggest either that some of these B cells do not require BAFF-BAFF-R interaction for their survival or that B-2 and MZB cells are constantly re-generated having a high turnover rate but are still dependent upon BAFF for their survival. In order to discriminate between these alternatives, we determined the turnover rate of splenic B cell subpopulations in control and 9B9 treated mice by BrdU labeling. In accordance to their high turn-over rate, after 10 days of continuous BrdU labeling, the vast majority of T1 and T2/T3 in control and 9B9 treated mice were found to be BrdU positive (figure 5A), whereas only about 10% of control and 18% of B-2 cells from 9B9 treated mice were BrdU positive (figure 5A). Taken together, the turnover rate of the remaining B-2 cells in 9B9 treated mice was similar to that of B-2 cells in control mice and is indicative of a BAFF-BAFF-R-independent mechanism for the maintenance of this small number of B-2 cells in treated mice. After 10 days of BrdU labeling, about 25% of the MZB cells from the control and 20% from the 9B9 treated mice, were positive (figure 5A).

In order to test whether prolonged treatment would improve the B cell depletion, mice were injected over a 5 months time. FACS analysis revealed that such a prolonged treatment did not alter the outcome of the B cell depletion. Meaning that, B-1 B cell compartment was not affected (data not shown), MZB cells were reduced by half and 10-20% of the B-2 B cell compartment was still present (figure 5B). Thus, the vast majority of B-2 cells are highly dependent for their survival on BAFF-BAFF-R signaling, and only about half of the MZB cells seem to be BAFF-BAFF-R dependent.

Figure 4. Depletion of the B and T cell subsets following anti BAFF-R 9B9 antibody injection. ***Panel A.*** *Representative FACS plot of the immature ($CD93^+CD19^+$) and mature ($CD93^-CD19^+$) B cell compartments in the spleen of C57BL/6 mice; upper plots untreated control, lower plots day 14 after 0.5mg of anti-BAFF-R 9B9 injection. On the left side total splenocytes are depicted. On the right side, gated on mature B cells ($CD93^-CD19^+$) follicular ($CD21^+CD23^+$) and MZB ($CD21^{high}CD23^{low}$) B cells are shown.* ***Panel B.*** *Absolute numbers of splenic T1 and T2/3 immature B cells, B-2 and MZ B cells, CD4 and CD8 T cells in controls (black bars) and 9B9 injected C57BL/6 mice at day 14 after injection (white bars). 5 mice were analyzed for each group. A significant difference could be observed for T2/T3, B-2 and MZB cell numbers in control as compared to 9B9 injected mice.* ***Panel C.*** *Representative FACS plot analysis indicating the percentages of $CD19^+CD23^+$ B-2, $CD19^+CD11b^+$ B-1b and $CD19^+CD5^+$ B-1a B cells in the peritoneal cavity of control (upper dot plots) and C57BL/6 mice injected with 9B9 mAb at day 14 after injection (lower dot plots).*

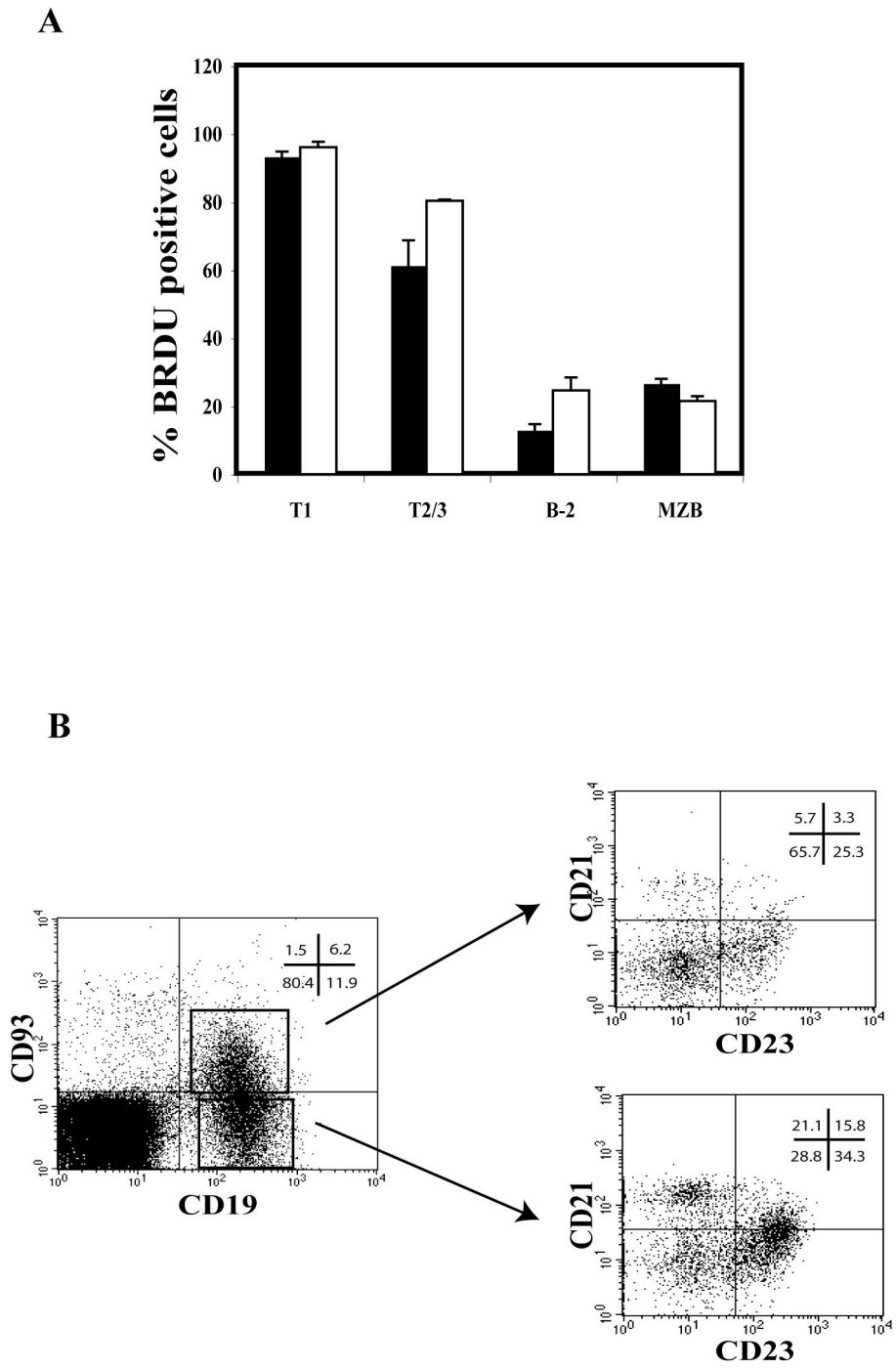

Figure 5. Turnover of splenic B cells following injection with anti-BAFF-R 9B9. Panel A. *Turnover of splenic B cell populations in control (black bars) and 9B9 injected C57BL/6 mice (white bars). C57BL/6 mice were injected with 1mg of BrdU and BrdU was added to the drinking water. 10 days after, splenic T1, T2/3, B-2 and MZ B cells were stained, sorted and the percentage of BrdU positive cells was determined by FACS analysis. Mean values with standard deviation are shown. 4 mice were analyzed for each group. Differences were statistically not significant.* ***Panel B.*** *Representative FACS plot analysis of the immature ($CD93^+CD19^+$) and mature ($CD93^-CD19^+$) B cell compartments in the spleen of C57BL/6 mice treated over a 5 months period with anti-BAFF-R 9B9 mAb. Depicted on the right side, CD21 and CD23 staining gated on immature B cells (upper plot) and on mature B cells (lower plot). Indicated are the percentages of the cells represented in each quadrant.*

5.2.5 Immune response in 9B9 treated mice

In the spleen of normal mice, IgM^{low} IgD^{high} follicular B (B-2) cells are clustered in B cell follicles and are surrounded by a rim of IgM^{high} IgD^{low} marginal zone B cells (figure 6). Plasma cells, characterized by strong cytoplasmic IgM expression, are localized outside the follicles within the red pulp. In mice treated with the BAFF-R blocking mAb 9B9 splenic B cell follicles were considerably smaller (figure 6) as a consequence of the drastic reduction of the follicular B cells. T cell areas did not show a significant reduction in size, confirming the results obtained from the FACS analysis (figure 6). As in controls, plasma cells in anti-BAFF-R treated mice were localized in the red pulp and were identical in numbers, as determined by ELISpot assay (data not shown). Collectively, these results show that treatment with a blocking anti-BAFF-R mAb perturbs the splenic follicular organization by severely depleting mature B cell numbers and thereby reducing follicular size.

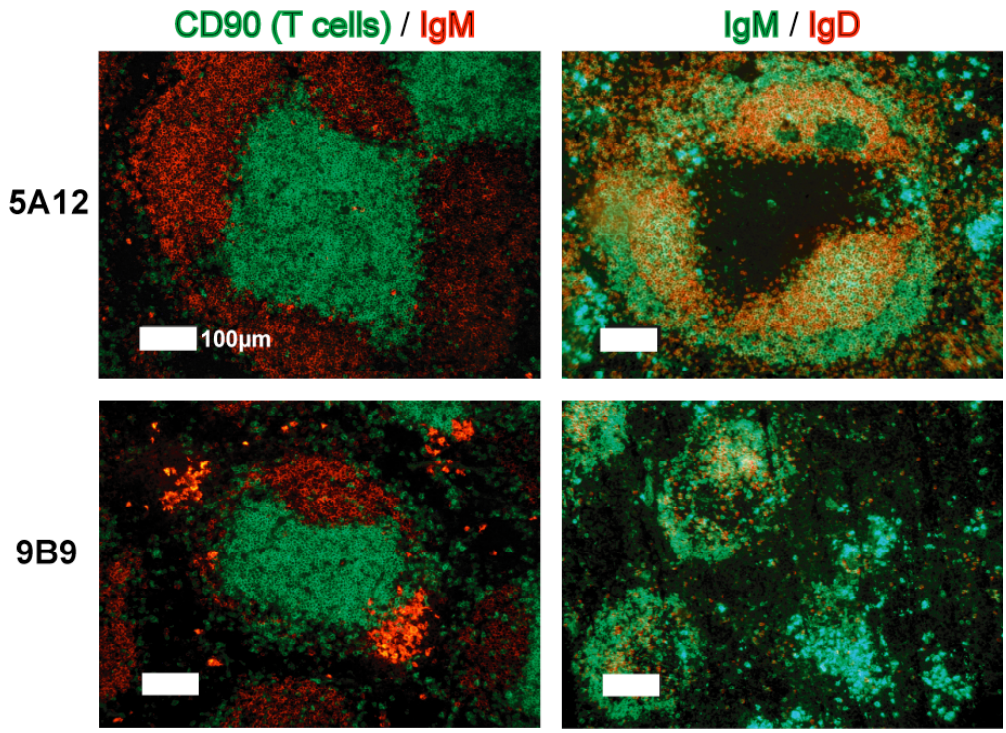

Figure 6. Immunohistochemistry of spleen sections. *Spleen histology of C57BL/6 mice 14 days after injection of either the non-blocking 5A12 or blocking 9B9 anti-BAFF-R mAbs, as indicated. Cryosections were stained with anti-IgM (red) and CD90 (T cells) (green), left panels, and with anti-IgM (green) and anti-IgD (red), right panels, as indicated above. Magnification 240x*

In order to test whether the B cell depletion and alteration of the splenic architecture, induced by the treatment, would influence humoral immune responses, mice were immunized with NIP-ficoll or NIP-ovalbumin 10 days after treatment with 9B9 mAb. Serum IgM and IgG anti-NIP titers were determined at day 12 after antigen administration. Depletion following anti-BAFF-R treatment was not affecting IgM titers upon challenge with T cell independent NIP-ficoll antigen (data not shown), whereas a 5 to 10 fold reduction in the titers was observed in 9B9 treated compared to control animals, following a T dependent NIP-ovalbumin (NIP-OVA) immunization (figure 7A). However, 9B9 injected mice were still able to mount a significantly higher immune response (3-5 fold), compared to mice depleted of CD4 T cells by GK1.5 mAb injections (figure 7A). Consistent with this finding, the presence of small germinal centers could be detected in 9B9 treated mice after immunization by histological analysis (data not shown). Thus, mice treated with an anti-BAFF-R mAb which prevents BAFF binding showed an impaired but not a completely abrogated ability to mount a primary antigen specific IgG response.

To evaluate the impact of B cell depletion on the formation of memory B cells mice were injected with the 9B9 blocking mAb or the 5A12 control non-blocking mAb 14 days before being immunized with NIP-OVA. The Ab treatment was continued over a two months period and at day 60 after priming mice were boosted, and the recall IgG anti-NIP response was determined at day 74. As shown in figure 7B (3 versus 6) the IgG anti-NIP titer of the memory response was 5-10 fold lower in the 9B9 treated group (figure 7B group 6) as compared to the 5A12 treated group (figure 7B group 3). Thus, treatment with an anti-BAFF-R mAb that prevents BAFF binding impaired the formation of memory B cells. Nevertheless the induction of memory formation was not completely abrogated by 9B9 treatment, since the antigen specific IgG response was still higher as compared to not immunized (figure 7B group 1), not immunized 9B9 treated (figure 7B group 4) mice as well as mice which received only the booster immunization, irrespective of the 9B9 treatment (figure 7B group 2 and 7B group 5, respectively).

To address the role of BAFF-BAFF-R signaling on the maintenance of memory B cells, NIP-OVA primed mice were injected with 9B9 mAb at day 60 and 74 following immunization. At day 80 after priming mice were boosted and the IgG anti-NIP titer was determined at day 94. As shown in figure 7B group 7, the mice mounted an IgG anti-NIP response that was not significantly different from control treated mice (figure 7B group 3). Collectively, these results suggest that formation of memory B cells requires BAFF - BAFF-

R signaling, while the maintenance and survival of memory B cells is not affected by 9B9 treatment and therefore BAFF independent.

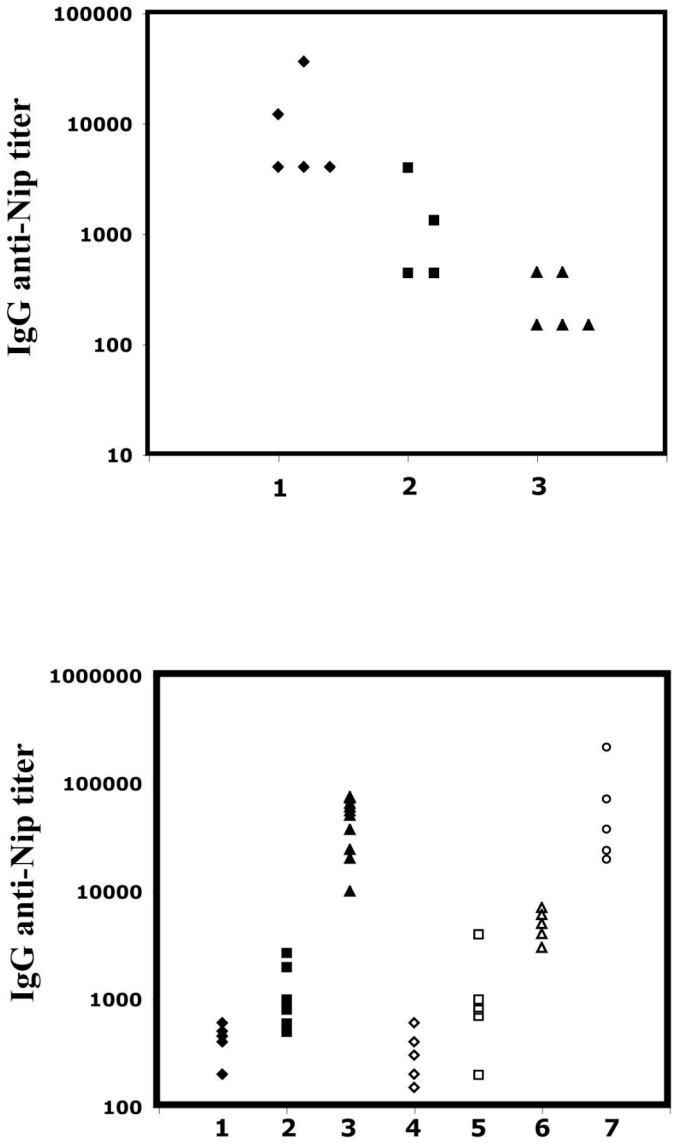

Figure 7. Humoral immune response. Panel A. *12 days after T dependent immunization serum levels of anti-NIP IgG were measured by ELISA in groups of 5 controls (1) and 5 C57BL/6 mice injected either with anti-BAFF-R mAb 9B9 (2) or anti-CD4 mAb GK1.5 (3), as indicated. Immunization with NIP-ovalbumin was performed 10 days after injection of the 9B9 or the GK1.5 mAbs. The titer is defined as the serum dilution that gives OD values twice the background and is depicted on a logarithmic scale. Each symbol represents a mouse. A significant difference could be observed in the response of 9B9 as well as GK1.5 mAbs treated mice as compared to untreated.* ***Panel B.*** *Sera of mice subjected to different treatments was collected at different time points as indicated for each group. Mice received repeated injections of the mAb every third week, over the indicated period of time. Levels of anti-NIP IgG were measured. Each symbol represents a mouse. The titer is defined as the serum dilution that gives OD values twice the background and is depicted on a logarithmic scale. Groups 1 and 4: serum collected at day 1. Mice were treated with 5A12 mAb (non-blocking) and 9B9 mAb (blocking), respectively, from day 1 to day 14. Mice were not immunized. Groups 2 and 5: serum was collected at day 74. Mice were treated with 5A12 mAb (non-blocking) and 9B9 mAb (blocking), respectively, from day 1 to day 74. Mice were only boosted at day 60. Groups 3 and 6: Serum was collected at day 74. Mice were treated with 5A12 mAb (non-blocking) and 9B9 mAb (blocking), respectively, from day 1 to day 74. Mice were primed with NIP-ovalbumin at day 14 and boosted at day 60. Group 7: Serum was collected at day 94. Mice were treated with 9B9 mAb (blocking) from day 60 to day 74. Mice were primed with NIP-ovalbumin at day 14 and boosted at day 80. Statistical analysis revealed a significant difference for group 3 versus group 6, but not for group 3 versus 7.*

5.3 Discussion

The role of BAFF in the development of mouse B cells was most clearly demonstrated by the generation of two different BAFF deficient strains and by the characterization of BAFF-R deficient mice (9-13). These mutant mice displayed a severe block in B cell development at the differentiation from, so called, T1 to T2 B cells in the spleen, whereas the development of B-1 B cells appeared to be unaffected. As a consequence of the profound decrease in T2 B cell numbers, their downstream mature B cell progeny, namely follicular and marginal zone B cells, were drastically depleted. Whether BAFF was necessary either for the survival of T2 cells or for promoting their differentiation and maturation, however, remained an open question. Furthermore, the role of BAFF in the survival of mature B cell *in vivo* could not be addressed using these mice, since their precursors depended on BAFF for their generation and development.

By the generation and administration of anti-BAFF-R monoclonal antibodies capable of preventing BAFF binding, we show in this report, that the *in vivo* survival of almost all follicular and half of the marginal zone B cells is dependent upon BAFF-BAFF-R signaling. Injection of mice with a blocking anti-BAFF-R antibody induced a profound depletion of the mature B cell compartment, whereas a non-blocking antibody had only a minimal or no effect. The possible scenarios which could explain this phenomenon include: an antibody-dependent cellular cytotoxicity (ADCC), a complement-mediated lysis, an impairment of the survival through prevention of BAFF binding or a limited generation of newly formed mature B cells as a consequence of the depletion of transitional B cells. The fact, that an isotype-matched non-blocking anti-BAFF-R monoclonal antibody is not affecting the peripheral mature B cell pool and that in addition the detectable presence of the non-blocking anti-BAFF-R antibody on the surface of non-depleted B cells (data not shown) taken together would indicate an ADCC mechanism is unlikely. Moreover, that B cell depletion is still occurring in FcRγ-chain (20) deficient mice confirms that ADCC is most likely not involved. In comparison to the rapid ADCC dependent anti-CD4-mediated depletion of T cells by GK1.5, the relatively slow kinetic of B cell deletion observed after the administration of blocking anti-BAFF-R antibody suggested a major role for BAFF-R signaling on mature B cell survival. Moreover, treatment of Bcl-2 transgenic mice (21), where B cells were only slightly reduced, further corroborates the hypothesis of a BAFF mediated survival on the one hand and makes complement mediated cell lysis improbable on the other hand.

Yet an alternative explanation for the observed depletion of mature B cells upon treatment would be that the numbers of differentiating T2/T3 immature B cells into mature cells decrease with time. However, based on BrdU *in vivo* labeling studies performed by us and others, this seems to be an unlikely scenario (3, 7). These studies showed that in an adult mouse about 1% of the mature B-2 cells are replaced per day. Upon treatment we see already an 80-90% reduction of mature B cells at day 10-14, whereas only a 10-14% reduction could be explained by the differentiation block of T2/T3 into mature B-2 cells. Therefore collectively our findings demonstrate, that the vast majority of B-2 cells and about half of MZB cells require BAFF for their survival.

Different experimental approaches, performed by other groups, were also suggestive of a survival role of BAFF-BAFF-R signaling in mature B cells. Treatment of mice with a TACI-Fc fusion protein was shown to lead to a reduction of B cells (22-24). However, in these studies the extent of B-2 and MZB cells depletion was not analyzed in detail (22, 23). Moreover, given that TACI can interact with both BAFF and APRIL (24), the potential role for APRIL in this depletion process could not be excluded. Several groups showed that treatment of mice with a BAFF-R-Fc fusion protein also resulted in a depletion of B cells (22, 25). Since BAFF is the only known ligand for BAFF-R, these studies strongly suggest that peripheral B cells require BAFF for their survival. In one of these studies a more detailed analysis of the extent of depletion of the various mature B cell subpopulations is reported (25). This analysis revealed that after such a treatment the B-2 and MZB compartments were largely reduced, whereas the B-1 cell numbers were practically not affected. In other words, treatment of mice with a BAFF-R-Fc fusion protein results in a very similar B cell depletion as we observed here upon treatment with the blocking anti-BAFF-R mAbs. Thus, our results confirm and extent previously reported findings on the role of BAFF - BAFF-R signaling in the survival and maintenance of the mature B cell compartments.

B-1 B cells express relatively high amounts of BAFF-R on their surface. However, as shown in BAFF as well as BAFF-R deficient mice their generation and maintenance is not affected (9-13). Also the here described short-term and long-term (5 months) treatment with anti-BAFF-R mAbs that block BAFF binding did not affect the B-1 B cell compartment. Moreover, we could rule out an inability of the injected mAbs to enter the peritoneal cavity since FACS analysis with an anti-rat IgG revealed the presence of the anti-BAFF-R mAb on the surface of B-1 B cells (data not shown). Thus the role of the BAFF-R in B-1 B cell biology still needs to be elucidated. However, the finding that the B-1 B cell compartment is

largely expanded in BAFF transgenic mice (10, 14-16) might suggest that BAFF can act as a B-1 B cell growth factor.

Following anti-BAFF-R treatment, we observed that the B-2 B cell compartment was the most affected B cell subset, indicating that the majority of B-2 cells rely on BAFF signaling for their survival. Marginal zone B cells were reduced only by half upon treatment, which compared to their almost complete absence observed in BAFF and BAFF-R deficient mice, is indicative for a crucial role of BAFF signaling during marginal zone B cell development or survival of their progeny, but dispensable for their survival subsequent to maturation. In BAFF-R deficient mice, over-expression of Bcl-2 could not overcome the marginal zone B cell defect (18), arguing for an instructive role of BAFF for their development, which still needs to be elucidated.

By BrdU labeling experiments and FACS analysis we show that the survival of a small subset of B-2 B cells seemed to be BAFF-independent and not reflecting newly formed mature B cells. A similar result was shown in BAFF as well as BAFF-R deficient mice, where the mature follicular B cell compartment was drastically reduced but still present in small numbers (26). The follicular B cells that survived this BAFF-R blockage could not be distinguished according to phenotypic criteria (data not shown). Because B-1 cells were not affected by anti-BAFF-R treatment and considering their predominant origin during fetal/neonatal development, we wondered whether the surviving B-2 cells were also of fetal origin. A hallmark of B cell development during fetal life is the lack of expression of deoxynucleotidyl transferase which prevents non-templated nucleotide additions in the V-D and D-J junction of the BCR heavy chain. No difference could be observed comparing junctional regions of untreated to 9B9 treated B-2 B cells, ruling out this hypothesis (data not shown).

Prolonged (5 months) treatment with the 9B9 mAb did not improve the depletion of these mature B cell subsets. FACS analysis with an anti-rat IgG mAb revealed the presence of 9B9 on the surface of B-2 and MZB cells surviving the treatment (data not shown). Moreover, the surface available BAFF-R seemed to be saturated by the injected 9B9, as other anti-BAFF-R mAbs were undetectable by FACS analysis (data not shown). Therefore, the survival of these mature B cell subsets seems to be BAFF - BAFF-R signaling independent.

It has been shown that surface BCR expression is mandatory for mature B cell survival, since conditional ablation of BCR expression subsequent to the establishment of steady-

state B cell numbers resulted in rapid death of most peripheral B cells (27). The mechanism by which BCR expression influences B cell longevity remains to be clarified.

The survival of mature B cells is dependent on signaling processes that use the NF-κB signal transduction pathway (28, 29). Two pathways leading to NF-κB activation in B cells have been described, namely the classical and the alternative pathways (30). Several mutations affecting one or both NF-κB signaling cascades were shown to affect the B cell compartment (12, 31-37). Since the two NF-κB pathways employ both shared and distinct components, the role of each activation pathway in B cells remains to be elucidated (18).

Several *in vitro* studies have demonstrated a survival role of BAFF on transitional as well as mature B cells (16, 38). The mechanism by which this increased survival is achieved seems to be dependent on the NF-κB mediated upregulation of anti-apoptotic Bcl-2 family proteins and inhibition of the nuclear translocation of the pro-apoptotic protein kinase Cδ (39, 40). Collectively, BCR as well as BAFF-R signaling seem to be essential for the maintenance of mature B cells and this is probably achieved by up-regulating anti-apoptotic pathways, where Bcl-2 might be a key player. In fact, transgenic expression of Bcl-2 was able to rescue the survival of B cells upon BCR deletion (27), and we show that it is able to overcome, to a large extent, the ablating effect of anti-BAFF-R blocking antibodies. Mature B cell survival seems to be regulated by the achievement of a certain activation state induced by a combination of basal BCR and BAFF - BAFF-R signaling, with a different contribution of the two pathways, which might also be dependent on the B cell subset. Nevertheless, it still remains to be clarified how BCR and BAFF-R signals act on one another and are integrated within the cell to maintain cell survival. The different impact of BCR and BAFF - BAFF-R signaling on the activation state and as a consequence on the survival of mature B cells could explain why some B cells survive anti-BAFF-R treatment as a BAFF-independent subset. In agreement with this hypothesis is the finding that many marginal zone B and B-1 B cells survived treatment with anti-BAFF-R mAb.

It is believed that formation of the different mature B cell compartments is influenced by specific BCR-ligand interactions (41-43). Thus, B cells in transgenic mice expressing recombinant BCRs for self-antigens tend to differentiate into B-1 and marginal zone B cells (44). Both, B-1 and MZB cells are enriched for self reactive clones, whereas follicular B cells generally require higher levels of BCR signaling for their formation. Therefore, if for the survival of mature B cells a certain threshold of activation is required and if this activation is a combination of basal BCR signaling and competition for as well as the availability of BAFF, prevention of BAFF binding would rather favor the survival of those

B cells with relatively higher affinity for self-antigens, such as B-1 and MZB cells. Nevertheless, we cannot rule out an alternative explanation for the BAFF independent survival of MZB cells, namely that their location next to metallophilic macrophages could provide them with a specific environmental niche favoring their survival. Moreover, considering that their development is dependent on Notch signaling (45, 46), different Notch ligands might also be involved in their maintenance.

As a consequence for the observed B cell depletion in anti-BAFF-R treated mice, we show that splenic follicles are greatly reduced in size. Moreover, the primary immune response to T dependent antigen was impaired, with IgG titers reduced by a factor of 5 to 10 following treatment with an anti-BAFF-R blocking antibody, whereas total immunoglobulin levels were not affected. This result is in line with what was shown for BAFF-R deficient mice, where the IgG_1 response to T-dependent antigen was significantly reduced (12). Moreover, we showed that in absence of BAFF-R signaling the induction memory B cells is strongly impaired. However, the maintenance of memory B cells seems to be BAFF - BAFF-R signaling independent, since 9B9 mAb treatment of mice primed before with a T cell dependent antigen did not impair the recall response. This finding confirms the very recent data published by Benson et al. (22), showing that treatment of antigen primed mice with TACI-Ig or BAFF-R-Ig does not impair their ability to mount an efficient recall response.

These findings enlighten a new role of BAFF - BAFF-R signaling as a crucial factor for the formation of memory or the survival of developing memory B cells, while confirming its dispensable role in the maintenance of memory B cells. Therefore, the question whether therapies based on BAFF as well as BAFF-R neutralization in B cell mediated autoimmune diseases could be successful remains uncertain. On the other hand, since elevated BAFF serum levels and deregulated BAFF-R signaling were shown to contribute to the pathogenic B survival in oncological as immunological disorders (47-52), a potential use of anti-BAFF-R mAb might represent an optimal targeted therapy, which would not compromise the ability of these patients to respond to already encountered antigens.

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6 DIFFERENTIAL BAFF-R EXPRESSION ENABLES THE DISCRIMINATION BETWEEN RECEPTOR EDITING AND NON-RECEPTOR EDITING IMMATURE BONE MARROW B CELLS

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Parts of the results presented here have been submitted for publication.

6.1 Introduction

The random assembly of V, D and J immunoglobulin (Ig) gene segments in developing lymphocytes results in the formation of an immense number of different B cell receptors (BCR) able to recognize a diverse repertoire of antigens. However, this random assembly of BCR can lead to the formation of Ig receptors that are either autoreactive or functionally impaired. In general, such cells are eliminated from the system by negative selection. Receptor editing is an important salvage mechanism to eliminate cells bearing potentially autoreactive or signaling-incompetent receptors, while at the same time preventing unnecessary deletion of cells. B cells expressing an inappropriate BCR can undergo secondary Ig gene rearrangements to form a BCR with a new specificity (1, 2). Thus, receptor editing plays a major role in both positive and negative selection (3). The main selection checkpoint seems to be at the immature B cell stage, even though a first checkpoint occurs already at the pre-BI cell stage. Appropriate signaling by the pre-BCR, which consist of μH and surrogate light (SL) chains, is important for the survival of pre-BI cells and their developmental progression to cycling large pre-BII cells, whereas insufficient pre-BCR signaling results in their developmental arrest.

Ig light chain (LC) locus rearrangement takes place at the pre-BII cell stage and the first cells expressing a complete BCR are newly formed immature B cells. Analyses of production and turnover rates revealed severe cell losses among immature B cells, as from the about 20 million immature B cells produced in the BM per day, only 20% exited the marrow (4, 5). These findings indicate that strong selection takes place at the immature B cell stage. Negative selection may not account for all of the losses observed at this point of development, since positive selection in form of continuous BCR signaling was shown to be important for survival of both immature and mature B cells (6, 7). The mechanism(s) underlying positive selection of B cells are less well characterized compared to those for negative selection. One of the main factors for positive selection seems to be ligand-independent (tonic) signaling via the BCR. Although several co-receptors and internal signaling molecules involved in positive selection have been identified (8), to date it is not clear whether B cell survival is directly accomplished by tonic signals, or whether these tonic signals lead to the expression and maintenance of survival-promoting intracellular proteins and/or cell surface receptors. One candidate for such a receptor is BAFF-R (B cell activating factor belonging to the TNF family receptor). For transitional and mature B cell subtypes, it has been shown that BAFF-R expression levels are regulated by BCR signaling

(9). BAFF signaling via BAFF-R is known to be important for the survival of immature B cells as well as their further development into mature B cells in the spleen. Both BAFF and BAFF-R deficient mice show a block in B cell differentiation at the transitional type 1 (T1) stage in the spleen, resulting in decreased numbers of down-stream transitional type 2 (T2), mature follicular (Fol) and marginal zone (MZ) B cells (10-12).

Here we report that during B cell development BAFF-R expression first occurs on a subpopulation of $CD19^+$ $CD93^+$ IgM^+ $CD23^-$ BM B cells and that BAFF-R expression in this subpopulation is regulated by signals of a BCR that must not be self-reactive, thus making BAFF-R a useful marker for immature B cells that might be positively selected.

6.2 Results and Discussion

6.2.1 BAFF-R is expressed only on IgM^{high} immature B cells in the BM

To characterize our newly generated monoclonal antibodies against mouse BAFF-R (13) we used five-color flow cytometric analyses with antibodies against CD19, CD21, CD23, CD93 and mBAFF-R (clones 7A5 and 9B9, respectively). As shown in figure 1A, anti-mBAFF-R clone 9B9 detects BAFF-R on splenic T1 ($CD19^+$ $CD93^+$ $CD21^-$ $CD23^-$), T2/3 ($CD19^+$ $CD93^+$ $CD21^+$ $CD23^+$), follicular ($CD19^+$ $CD93^-$ $CD21^+$ $CD23^+$) and marginal zone B cells ($CD19^+$ $CD93^-$ $CD21^+$ $CD23^-$). On all splenic B cell subsets, BAFF-R was expressed homogenously and to the same extent. BAFF-R expression could also be detected on both B-1a ($CD19^+$ $CD5^+$) and B-1b ($CD19^+$ $CD11b^+$) B cells from the peritoneal cavity. In contrast, no expression of BAFF-R could be detected on $CD4^+$ or $CD8^+$ splenic T cells. Similar results were obtained using another anti-mBAFF-R antibody (clone 7A5, data not shown).

Contrary to the splenic and peritoneal B cells, BAFF-R expression on BM B cells was heterogeneous. We found that there was no FACS-detectable expression of BAFF-R on $B220^+$ IgM^- B cells (Fig. 1B, gate A), but that BAFF-R was highly expressed on $B220^{high}$ IgM^+ recirculating B cells (Fig. 1B, gate C). Interestingly, by gating on $B220^{int}$ IgM^+ newly formed B cells we observed that this was a mixed population with regard to BAFF-R expression (Fig. 1B, gate B). A BAFF-R positive fraction could be clearly distinguished from a BAFF-R negative fraction, with about 40% of the newly formed B cells being

positive for BAFF-R in a 6-8 week old C57BL/6 mouse. BM B cells defined as $B220^{int}$ IgM^+ are the progeny of pre-BII cells and express for the first time a complete BCR. Thus, B cells in this compartment are in a developmental stage where BCR editing may occur. This prompted us to look for a correlation between BAFF-R expression and putative BCR editing.

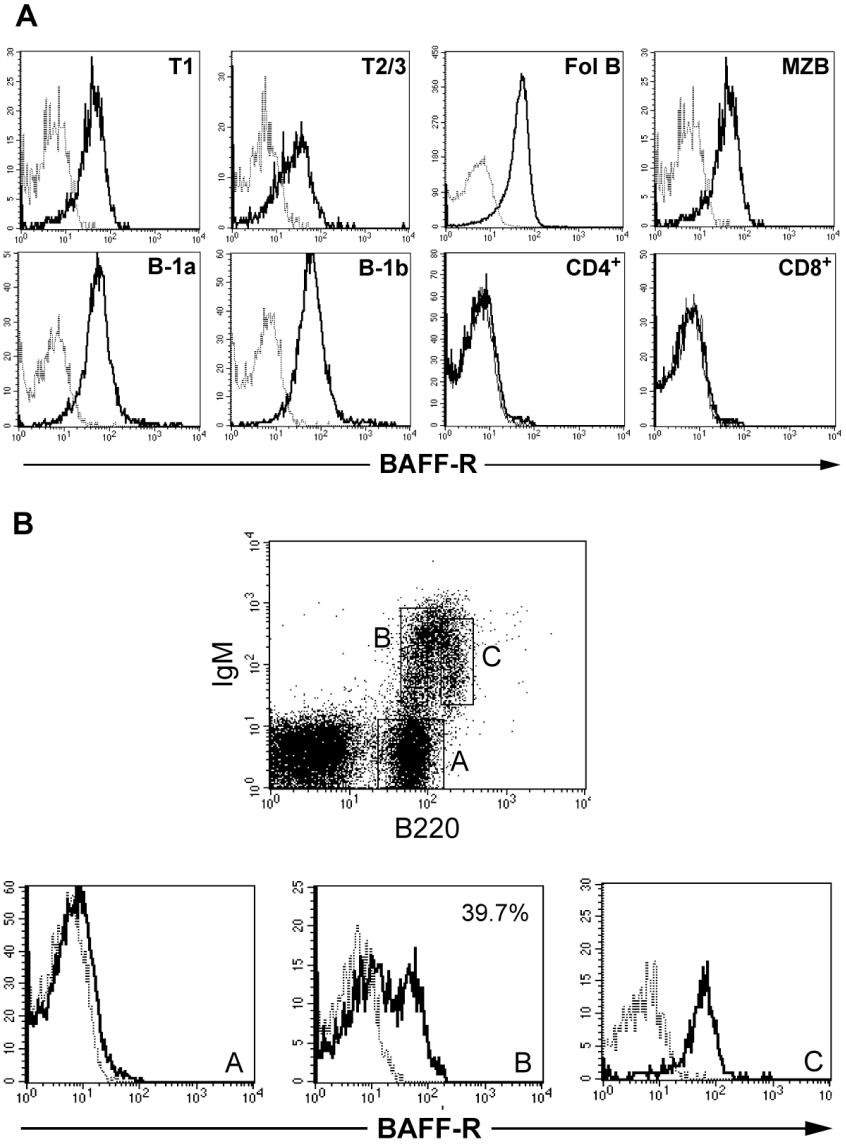

Figure 1. BAFF-R expression on B and T cell subsets. *A) Splenic B cell subsets (upper panel) were delineated using markers CD19, CD93, CD21 and CD23 and BAFF-R expression on transitional T1 and T2/3 B cells as well as mature MZ and Fol B cells was analyzed. Furthermore, BAFF-R expression was measured on B-1a ($CD19^+$ $CD5^+$) and B-1b ($CD19^+$ $CD11b^+$) B cells from the peritoneal cavity as well as on splenic $CD4^+$ and $CD8^+$ T cells. Dotted line histograms represent the negative controls. B) Bone marrow B cell subsets were delineated using markers B220 and IgM, and BAFF-R expression on IgM^- $B220^+$ cells (A), IgM^+ $B220^{low}$ newly formed B cells (B) and IgM^+ $B220^{high}$ recirculating B cells (C) was analyzed. Dotted line histograms represent the negative controls.*

6.2.2 BAFF-R expression levels correlate with surface IgM levels

BCR editing is known to be associated with low levels of surface IgM expression on B cells in the BM (14). If our assumption was correct, that there was a correlation between BAFF-R expression and BCR editing, there should also be a correlation between BAFF-R and IgM expression. More recently published data revealed that B cell maturation to mature, long-lived B cells occurs not only in the spleen but might be also occurring in the BM (15, 16). To rule out that our $B220^{int}$ IgM^+ newly formed B cell subpopulation contained more mature BM specific B cell developmental intermediates, we used the surface markers CD19, CD93, IgM and CD23 to delineate differentiation stages (Fig. 2A). We found that $CD19^+$ $CD93^+$ IgM^+ $CD23^+$ BM B cells were all positive for BAFF-R, with an expression level similar to splenic B cells (Fig. 2B, right), whereas $CD19^+$ $CD93^+$ IgM^+ $CD23^-$ BM B cells are heterogeneous concerning BAFF-R expression (Fig. 2B, left). Since it could only be assumed that within the $CD19^+$ $CD93^+$ IgM^+ $CD23^-$ B cells there is a BAFF-R^- and a BAFF-R^+ fraction, we sorted and reanalyzed the putative two populations. We found, that two clearly definable fractions exist, a BAFF-R^- and a BAFF-R^+ one (Fig. 2C, left). Upon analysis of the surface IgM expression we found that the level was lower on the BAFF-R^- fraction of $CD19^+$ $CD93^+$ IgM^+ $CD23^-$ B cells compared to the BAFF-R^+ fraction (Fig. 2C, right). Since cells showing low levels of IgM expression in BM have been indicated to undergo receptor editing (14), our findings might suggest that BAFF-R expression discriminates between receptor editing and non-editing immature B cells.

6.2.3 Analysis of BCR knock-in mice

Further evidence supporting our hypothesis comes from the analysis of BCR knock-in B1-8Hi; 3-83κi mice which carry pre-rearranged HC and LC genes in their appropriate genomic context and express only an innocuous BCR consisting of the B1-8 HC and the 3-83 κ-LC. As B cell receptor editing is the main mechanism through which B cells expressing autoreactive BCRs are eliminated, these mice do not have B cells that undergo receptor editing. Staining of BM B cells of B1-8Hi; 3-83κi mice for B220 and IgM revealed that there are almost no $B220^+$ IgM^- progenitor B cells (17). This observation has been explained by the direct and rapid maturation of progenitors into immature B cells due to the absence of negative selection and receptor editing. Our finding, that BAFF-R is expressed only on

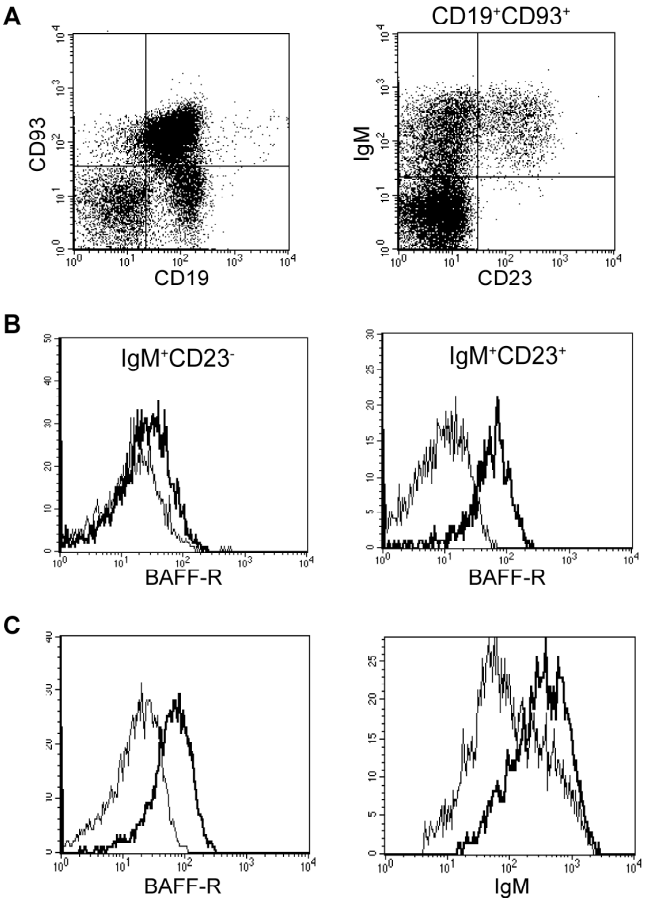

Figure 2. Newly formed B cells from the bone marrow can be subdivided into a BAFF-R$^-$ and a BAFF-R$^+$ fraction. *A) BM B cell subsets were delineated using markers CD19, CD93, CD23 and IgM. B) BAFF-R expression on newly formed CD19$^+$ CD93$^+$ IgM$^+$ CD23$^-$ B cells (left) and on mature CD19$^+$ CD93$^+$ IgM$^+$ CD23$^+$ B cells (right). Thin line histograms represent the negative controls. C) Newly formed CD19$^+$ CD93$^+$ IgM$^+$ CD23$^-$ B cells (B, left picture) where sorted according to BAFF-R expression and reanalyzed. The left overlay represents the reanalysis of the sorted BAFF-R$^-$ fraction (thin line histogram) and BAFF-R$^+$ fraction (thick line histogram). The right hand overlay shows the IgM expression on IgM$^+$ CD23$^-$ BAFF-R$^-$ (thin line histogram) and BAFF-R$^+$ (thick line histogram) cells.*

newly formed B cells that need not to be eliminated by negative selection, leads to the assumption that all of the newly formed immature B cells in the BM of these knock-in mice should homogenously express BAFF-R. FACS staining of the BM of the B1-8Hi; 3-83κi mice for IgM, B220 and mBAFF-R revealed that, in fact, the newly formed IgM^{high} B220^{int} B cells express BAFF-R homogenously (Fig. 3, fraction B), which is in contrast to the finding in BM of WT mice (Fig. 1B, fraction B). As expected and in accordance with the results obtained for WT mice (Fig. 1B, fractions A and C), IgM$^-$ B220$^+$ B cells in these knock-in mice are negative for BAFF-R expression (Fig. 3, fraction A), and all IgM$^+$ B220^{high} recirculating B cells express BAFF-R (Fig. 3, fraction C). This result supports our assumption, that BAFF-R is expressed first on immature BM B cells that have passed the negative selection process.

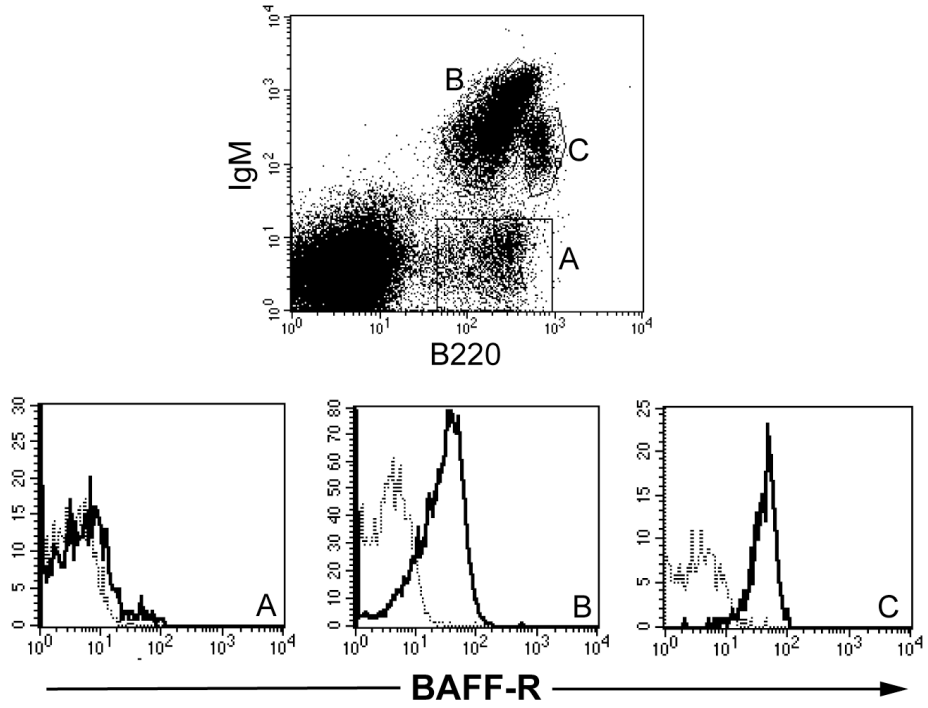

Figure 3. BAFF-R is expressed homogenously on newly formed BM B which do not undergo receptor editing. *Bone marrow B cell subsets from A) WT and B) B1-8 transgenic knock-in mice were delineated using markers B220 and IgM, and BAFF-R expression on IgM⁻ B220⁺ cells (A), IgM⁺ B220^{low} newly formed B cells (B) and IgM⁺ B220^{high} recirculating B cells (C) was analyzed.*

6.2.4 BAFF-R expression correlates negatively with RAG-2 expression and BCR editing

B cells that undergo receptor editing need to express RAG-1 and RAG-2, as these proteins are absolutely necessary for V(D)J recombination. Considering that BAFF-R expression might be related to BCR editing, there might also be a correlation with RAG expression. Therefore, we analyzed BCR LC editing and RAG-2 expression in B cell populations subjected to different in vitro conditions. Thus, we sorted κ-LC$^+$ CD19$^+$ CD93$^+$ CD23$^-$ BAFF-R$^-$ (referred to as CD23$^-$ BAFF-R$^-$), κ-LC$^+$ CD19$^+$ CD93$^+$ CD23$^-$ BAFF-R$^+$ (referred to as CD23$^-$ BAFF-R$^+$) and κ-LC$^+$ CD19$^+$ CD93$^+$ CD23$^+$ BAFF-R$^+$ (referred to as CD23$^+$ BAFF-R$^+$) B cells from the BM and incubated them either in the presence or absence of an anti-κ-LC antibody. After 36 h we analyzed the cells by FACS, using an anti-λ-LC antibody to follow LC editing from κ to λ. RAG-2 expression was determined by semi-quantitative

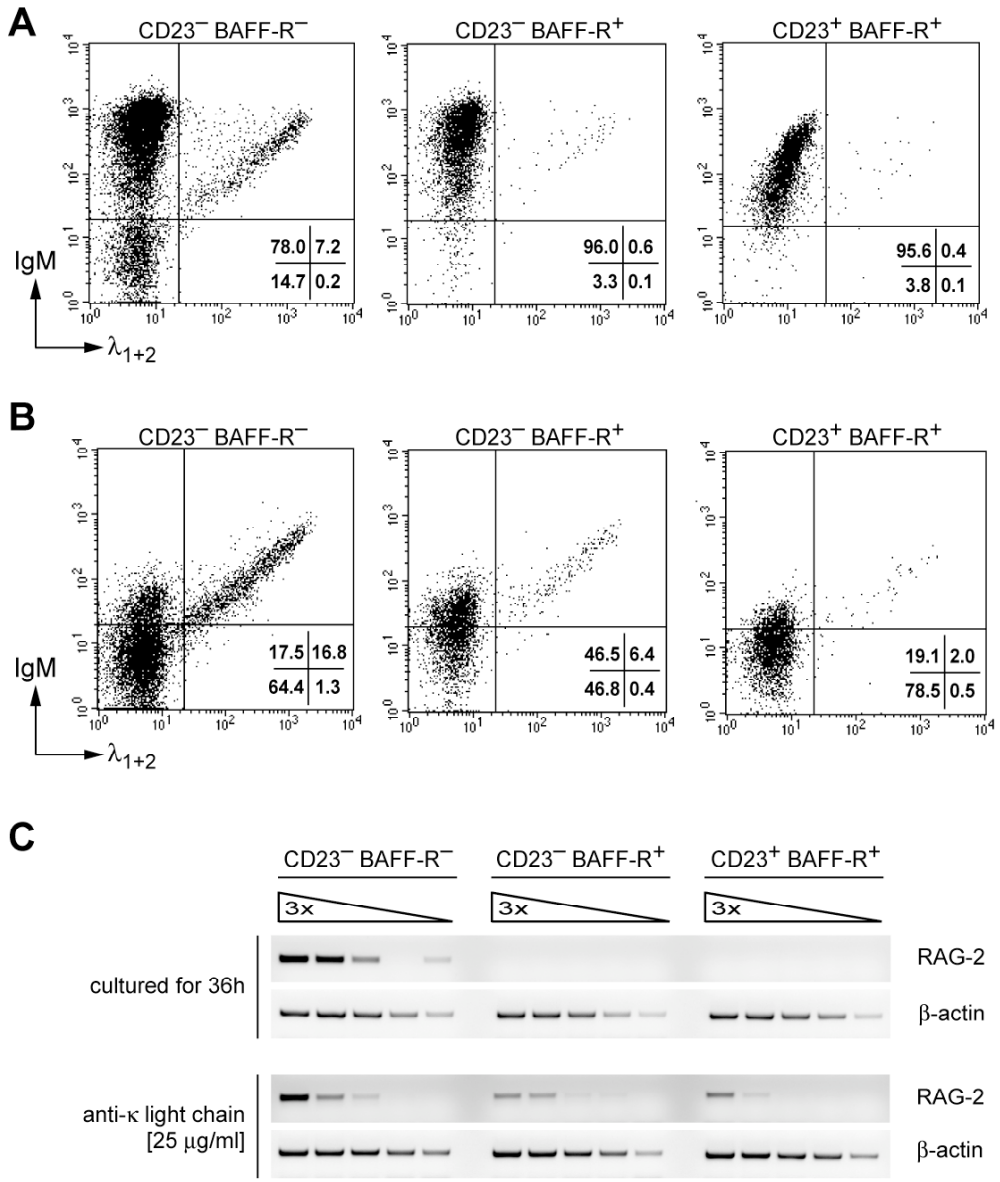

Figure 4. RAG-2 is expressed only in BAFF-R⁻ newly formed B cells from the BM, but can be induced in BAFF-R⁺ newly formed B cells from the BM by culturing with anti-κ-LC antibody. *A) and B) B cells from the BM were sorted as CD19⁺ CD93⁺ κ-LC⁺ CD23⁻ BAFF-R⁻ (left panel), CD19⁺ CD93⁺ κ-LC⁺ CD23⁻ BAFF-R⁺ (middle) and CD19⁺ CD93⁺ κ-LC⁺ CD23⁺ BAFF-R⁺ (right panel) and incubated for 36 h in medium alone (A) or in the presence of 25 µg/ml anti-κ-LC antibody (B). LC editing from κ- to λ-LC was analyzed using IgM and λ_{1+2}-LC specific antibodies. C) RAG-2 expression was measured by semi-quantitative PCR for BM B cell subpopulations sorted and cultured as mentioned in A and B.*

RT-PCR. When cultured in the absence of anti-κ-LC antibody, CD23⁻ BAFF-R⁻ B cells (Fig. 4A, left) underwent extensive LC editing, as was apparent by 7.2% of previously κ-LC⁺ cells that had become λ-LC⁺. About 15 % of the cells had down-regulated their BCR and were now IgM negative. These cells were probably not able to further edit their LCs

and presumably were in the process of apoptosis. Interestingly, both of the other B cell subtypes analyzed, which were both BAFF-R^+, did not show any sign of receptor editing and kept expressing κ-LC BCRs (Fig. 4A, middle and right). RT-PCR analysis revealed, that only the BAFF-R^- subpopulation expressed RAG-2, whereas both of the BAFF-R^+ subpopulations were negative for RAG-2 expression (Fig. 4C, upper panel).

This result supports the previous finding and confirms, that only $CD23^-$ BAFF-R^- BM B cells can undergo V(D)J recombination and LC editing, whereas $CD23^-$ BAFF-R^+ as well as $CD23^+$ BAFF-R^+ BM B cells did not undergo LC editing, probably because they expressed already a harmless (non-autoreactive) and functional BCR.

When we performed the same experiment in the presence of an anti-κ-LC antibody in order to induce receptor editing (Fig. 4B), then $CD23^-$ BAFF-R^- BM B cells showed increased LC editing, which was apparent from the about 17% λ-LC^+ B cells (Fig. 4B, left). As before, the two BAFF-R^+ subpopulations analyzed behaved almost the same, showing around 6% λ-LC^+ cells in the case of $CD23^-$ BAFF-R^+ cells and 2% λ-LC^+ cells for $CD23^+$ BAFF-R^+ cells (Fig. 4B, middle and right). Cells that were not able to edit the BCR from κ- to λ-LC showed reduced surface IgM expression levels (Fig. 4B). In the presence of the anti-κ-LC antibody, RAG-2 expression could be detected for all three subpopulations, but BAFF-R^+ cells showed lower levels than BAFF-R^- cells (Fig. 4C, lower panel).

These results clearly indicate that $CD23^-$ BAFF-R^- immature B cells do not yet express an appropriate BCR, as evidenced by the still existing RAG-2 expression and the high percentage of cells undergoing LC editing. Moreover, our findings indicate that BAFF-R expression does not inhibit LC editing, since BCR ligation of both $CD23^-$ BAFF-R^+ and $CD23^+$ BAFF-R^+ B cells was able to re-induce RAG expression and LC editing. In this context it is worthwhile noting that LC editing in $CD23^-$ BAFF-R^+ and $CD23^+$ BAFF-R^+ B cells by the anti-κ-LC antibody could not be prevented by the addition of BAFF (data not shown). These findings suggest, that the B cell autoimmunity observed in transgenic mice which over-express BAFF (18, 19) is not due to BAFF interfering with negative selection and/or receptor editing of autoreactive immature bone marrow B cells, but rather might be a result of BAFF rescuing anergic/self-reactive B cells in the periphery (20).

6.2.5 Changes of surface BAFF-R expression levels upon BCR ligation depends on the B cell maturation stage

To test whether BAFF-R expression levels change upon BCR ligation, IgM^+ $CD93^+$ BAFF-R^- $CD23^-$ and IgM^+ $CD93^+$ BAFF-R^+ $CD23^-$ as well as IgM^+ $CD93^+$ BAFF-R^+ $CD23^+$ B cells from the BM, and transitional T1 and T2/3 and mature follicular B cells from the spleen were purified by cell sorting. Cells were then incubated either in the presence or absence of anti-IgM antibody. FACS analysis after 36 h revealed, that all BM B cell subsets analyzed expressed BAFF-R at the same level when cultured in the absence of anti-Ig antibody (Fig. 5A, thick solid histogram). IgM^+ $CD93^+$ $CD23^-$ BAFF-R^- B cells become

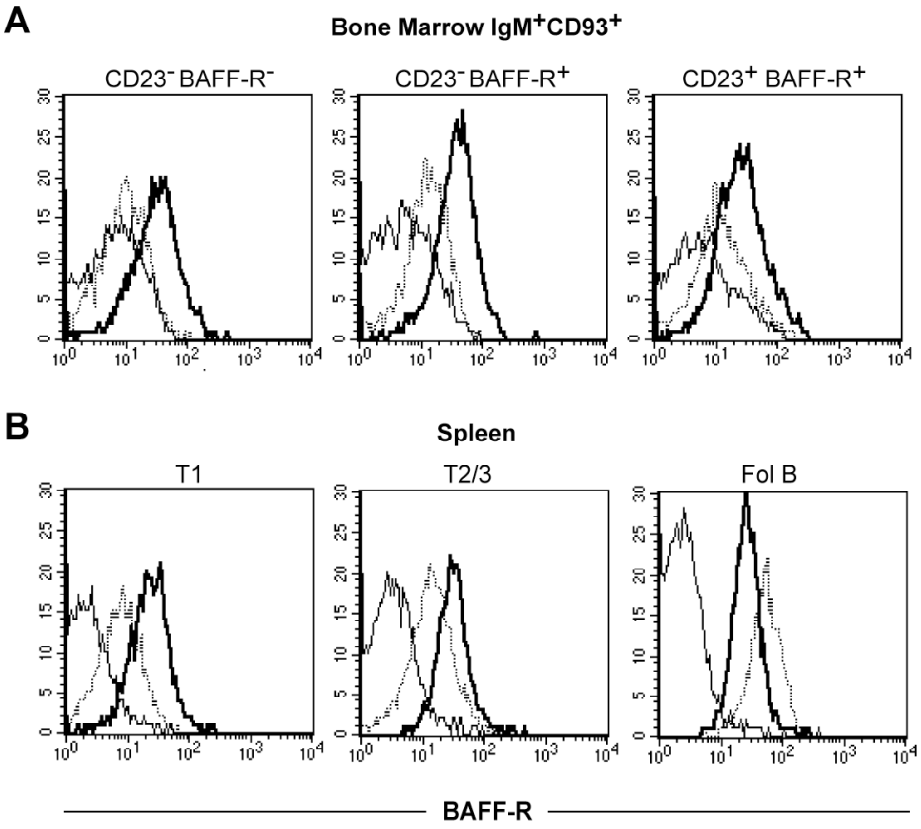

Figure 5. Surface BAFF-R levels on B cell subsets change during culturing with anti-IgM. *A) BM B cells were sorted as $CD19^+$ $CD93^+$ κ-LC^+ $CD23^-$ BAFF-R^- (left panel), $CD19^+$ $CD93^+$ κ-LC^+ $CD23^-$ BAFF-R^+ (middle) and $CD19^+$ $CD93^+$ κ-LC^+ $CD23^+$ BAFF-R^+ (right panel) and expression levels of BAFF-R were analyzed after culturing the cells in the absence (thick line histograms) or presence (dotted line histograms) of anti-IgM antibody for 36 h. Thin line histograms represent the negative controls. B) Splenic B cells were sorted as $CD19^+$ $CD93^+$ $CD23^-$ κ-LC^+ (T1), $CD19^+$ $CD93^+$ $CD23^+$ κ-LC^+ (T2/3) or $CD19^+$ $CD93^-$ $CD23^+$ κ-LC^+ (Fol B) and BAFF-R expression was analyzed after culturing the cells in the absence (thick line histograms) or presence (dotted line histograms) of anti-IgM antibody for 36 h. Thin line histograms represent the negative controls.*

BAFF-R+, since they continued their developmental program. When incubated in the presence of the anti-IgM antibody, we observed that BAFF-R was down modulated in all BM B cell subtypes analyzed (Fig. 5A, dotted histogram). Incubation of $CD23^-$ $BAFF\text{-}R^-$ cells in the presence of antibody (Fig. 4B, left) leads to down-modulation of IgM expression in more than 80% of the cells. This population also shows low to negative expression of BAFF-R. The splenic transitional T1 and T2/3 subpopulations behaved the same as the BM subpopulations, as they kept BAFF-R expression in the absence of anti-IgM antibody and down-regulated BAFF-R in the presence of the antibody (Fig. 5B, left and middle).

Interestingly, Fol B cells also kept BAFF-R expression when cultured in the absence of the anti-IgM antibody, but in contrast to BM and splenic transitional type B cells, they up-regulated BAFF-R expression in the presence of BCR ligation (Fig. 5B, right). Thus, our results suggest that BAFF-R expression is regulated by BCR signaling and that the outcome of BCR signaling on BAFF-R expression is B cell developmental stage dependent i.e. down-modulation on immature B cells and up-regulation on mature B cells.

Findings in support of this assumption come from the observations made in mice lacking both Rac-1 and Rac-2. Such mice show defective BCR signaling, resulting in diminished numbers of splenic B cells, but normal numbers of BM B cells. Furthermore, this impaired BCR signaling also leads to reduced levels of BAFF-R, pointing to a direct regulation of BAFF-R expression by BCR signaling via the Rac-1 and Rac-2 pathway (21).

Our finding that in B cells susceptible to negative selection, engagement of the BCR leads to down-regulation of BAFF-R expression might suggest that their survival time upon BCR ligation is reduced and therefore these cells might be more easily eliminated. This could be part of the mechanisms by which autoreactive B cells are deleted. On the other hand, however, absence of down-modulation of the BAFF-R upon BCR engagement by immature B cells might lead to autoimmunity.

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7 ANALYSIS OF SERUM BAFF LEVELS IN HUMANS WITH AUTOIMMUNE AND OTHER DISEASES WITH B CELL IMPLICATION

7.1 Introduction

Despite the numerous tolerance checkpoints that exist in the immune system in order to prevent the formation and activation of self-reactive B and T lymphocytes, inappropriate immune responses against normal components of the body occur in about 5% of the human population (1). These autoimmune diseases can either be directed against a single organ, as in autoimmune thyroid diseases (AITD), type 1 diabetes mellitus and myasthenia gravis, or be systemic illnesses, such as rheumatoid arthritis (RA), multiple sclerosis (MS), systemic lupus erythematosus (SLE) or Sjögren's syndrome (SS).

The etiopathogenic mechanisms initiating autoimmune diseases still remain undefined, due to their complex nature which is reflected in the various factors that have been found to be associated with these diseases. The influence of genetic factors was obvious from the observation of familial tendencies and the study of monozygotic twins. The results obtained from the studies in monozygotic twins also indicated that inherited factors alone are not sufficient for the development of an autoimmune disease. Several environmental factors like infections, vaccination, drugs, toxins and nutrition are discussed in this respect (2). Another possibility might be somatic mutations that occur during normal B- or T-cell development. The combination of this mutation together with an inherited defect or the accumulation of several somatic mutations could make it possible for the affected cell to evade tolerance checkpoints. Thus, a stepwise mechanism may underlay the pathogenesis of autoimmune diseases, resembling cancer development (1).

The genes which were associated with a higher susceptibility for autoimmune diseases encode for proteins that are involved in all steps of normal B- and T-cell maturation and activation. Of particular importance are HLA, AIRE, CTLA-4 and TLR. Polymorphisms and mutations in these genes lead to dysregulation of the normal developmental and activation process of B or T lymphocytes. In the case of HLA, specific alleles code for HLA molecules that allow the presentation of specific autoantigens. This has been investigated in detail for the HLA-DQ gene and the presence or absence of an Asp residue at position 57 in

type 1 diabetes mellitus (T1DM) (3, 4). Individuals with defects in the transcription factor AIRE show an impaired negative selection of T-cells in the thymus due to the failure to produce peripheral antigens in the medullary thymic epithelial cells. This breakdown of central tolerance results in an increased frequency of auto-reactive naive T-cells in the periphery. The autoimmune disorder APECED seems to be strongly associated with AIRE mutations (5). CTLA-4 is a negative regulator of T-cell activation, which competes against CD28 for binding to CD80 or CD86, respectively, and thus inhibits further activation of the T-cell. Polymorphisms in the gene encoding for CTLA-4 have been associated with a number of autoimmune diseases such as T1DM, myasthenia gravis, SS, SLE and AITD (6-12). Toll-like receptors belong to the pattern recognition receptors and usually are activated by pathogen-associated molecular patterns such as LPS, flaggelin or dsRNA. Recent evidence suggests that TLR7, TLR8 and TLR9 are associated with the induction of auto-immunity (13). TLRs have also been found to bind to endogenous antigens, such as fibrinogen, components of the extracellular matrix, heat shock proteins and host derived DNA and mRNA (14). The involvement of TLRs in the development of autoimmunity thus connects the innate immune system with autoimmune diseases. Infections seem to be of importance in the pathogenesis of at least some autoimmune diseases. Several mechanism by which an infection of the host can lead to autoimmunity have been proposed, including molecular mimicry, epitope spreading, bystander activation and cryptic antigen presentation. A strong evidence for a link between infection and induction of autoimmunity has to date only been found for *Campylobacter jejuni* infection and subsequent development of Guillain Barré Syndrome (15).

The role of B cells in pathogenesis of autoimmune diseases has long been neglected, but in recent years their considerable contribution became more and more evident. The production of autoantibodies and the resulting formation and deposition of immune complexes have long been considered to be the only part of central importance by which B cells participate in the pathogenesis of autoimmune diseases. Besides their antibody-producing capacity, B cells also have the capability to efficiently present antigens and secrete cytokines. Several studies in mice have shown that the presence of B-cells is required for the development of certain autoimmune diseases such as lupus, fibrosis or type 1 diabetes mellitus, but not their antibody-secreting capability (16-18). Nevertheless, both the autoantibody production and antigen-presenting capabilities of B-cells seem to be required for the induction of arthritis in mice (19). A contribution of B cells in human autoimmune disease development or main-

tenance became apparent when a B-cell depletion therapy showed significant efficacy in patients with rheumatoid arthritis (20).

A correlation between BAFF and autoimmune diseases was supposed when the analysis of BAFF transgenic mice revealed a phenotype which closely resembles human SLE. Like in humans afflicted with SLE, these mice show high levels of serum immunoglobulins including anti-DNA antibodies and rheumatoid factor, circulating immune complexes, Ig depositions in the kidney and proteinuria (21, 22). Moreover, with age BAFF transgenic mice also develop symptoms similar to human SS, with B cell infiltrates in salivary glands and reduced saliva production (23).

Besides the autoimmune diseases, BAFF might be involved in the genesis or maintenance of other diseases with B cell contribution. In this respect, the most obvious disorders are B cell malignancies. B cells from patients with B cell chronic lymphocytic leukemia (B-CLL) were found to express BAFF-R and TACI, and in contrast to normal B cells these cells also express BAFF and APRIL, thus establishing an autocrine survival pathway (24). Chronic infections are another type of disorders where BAFF might be involved, as patients with chronic infections often develop additional diseases, including autoimmune disorders.

Serum BAFF levels in patients afflicted with different autoimmune and cancerous diseases were already analyzed by several groups, but the reported data are inconsistent. To further elucidate whether elevated BAFF levels are associated with certain diseases in humans, a sensitive ELISA for hBAFF was developed and sera from patients suffering from an autoimmune disorder, lymphoma or chronic infection were analyzed.

7.2 Results

7.2.1 Analysis of hBAFF concentration in healthy blood donors

In order to determine the normal hBAFF concentration present in the blood of healthy persons, serum from 48 voluntary healthy blood donors was analyzed, using the newly developed hBAFF ELISA (see chapter 4). Therefore, two-fold serial dilutions ranging from 1:10 to 1:80 were analyzed in duplicates. As shown in figure 1, most of the donors showed a serum hBAFF concentration of 20 ng/ml or below, and only two persons presented with high levels of more than 50 ng/ml.

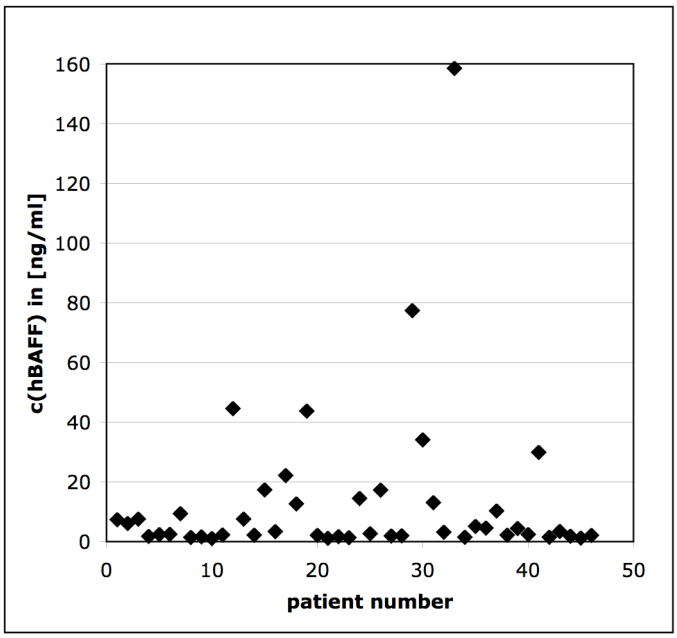

Figure 1. hBAFF concentration in serum of healthy blood donors. *48 samples from healthy individuals were analyzed in duplicates. Standard deviation values were to small to be depicted.*

A more detailed analysis of the distribution of hBAFF concentration levels is shown in figure 2. In 59% of all voluntary blood donors tested, the serum hBAFF concentration was 5 ng/ml or lower, 13% showed a value between 5 and 10 ng/ml, 9% had a value between 10 and 15 ng/ml, and 4% had a value between 15 and 20 ng/ml. Higher hBAFF values were found in seven persons. Two persons or 4% had values in the range of 20-30 ng/ml, one person or 2% had a value between 30 and 40 ng/ml, and two persons or 4% were within the range of 40-50 ng/ml or above 50 ng/ml, respectively. From this result it was concluded, that healthy individuals have in general a serum hBAFF concentration of 5 ng/ml or below.

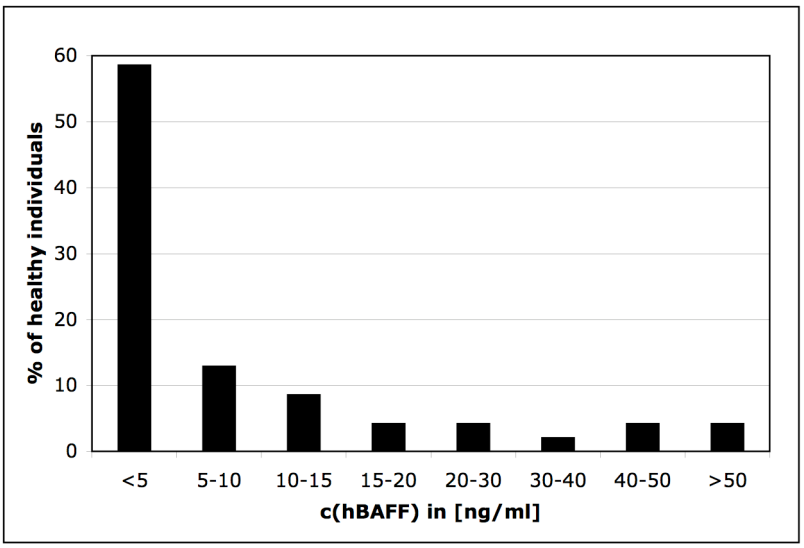

Figure 2. Percentage distribution of hBAFF concentration in serum of healthy blood donors. *Values of serum hBAFF concentration were grouped as indicated and the percentage of persons within one group was calculated based on the values shown in figure 1.*

7.2.2 Serum hBAFF concentration in patients suffering from SLE

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with extremely heterogeneous clinical manifestations and disease courses (revied in (25)). SLE affects most frequently the skin, joints, lungs, heart, kidneys and central nervous system but can involve almost any organ. The disease is commonly associated with periods of illness (flares) and remission. To assess disease phase, several different disease activity indices have been developed. These include the SLE disease activity index (SLEDAI), the British Isles lupus assessment group (BILAG), the systemic lupus activity measure (SLAM) and the European consensus lupus activity measurement (ECLAM). Clinical criteria for diagnosis include skin rashes, photosensitivity, non-erosive arthritis, oral ulcers, serositis, glomerulonephritis, hematological and neuronal disorders and the presence of antibodies to certain autoantigens, such as dsDNA, cardiolipin and ribonucleoproteins termed Smith (Sm). Production of numerous autoantibodies is the characteristic feature of SLE. In the sera of patients with SLE over 100 different autoantibody specificities have been detected, including antibodies against nuclear components, serum proteins and cellular antigens of mainly hematopoietic cells (26). The deposition of immune complexes or self-associating complexes formed by some of these autoantibodies is considered to cause chronic inflammation in the affected tissues, leading to the observed tissue damages. A relation between the production of a particular autoantibody and the occurrence of a certain tissue damage has to date only been shown for anti-dsDNA antibodies in glomerulonephritis and anti-cardiolipin antibodies in thrombosis (27).

The etiology of SLE is still poorly understood. Genetic factors have been shown to be involved, as was suggested by the observation of increased frequencies of SLE among first-degree relatives and in monozygotic twins (25). It turned out that not a single gene defect accounts for SLE pathogenesis but rather a number of genes and alleles on multiple loci. Genes controlling SLE can be of either susceptible or modifying type. Each SLE patient has therefore a specific set of susceptibility and disease modifying genes, which most likely explains the observed heterogeneity of SLE. Besides the genetic factors, also the involvement of environmental factors in SLE pathogenesis is discussed. Intense exposure to UV light (sun), emotional stress or infections can cause a flare of SLE symptoms.

Analysis of humans affected with SLE showed several signs for B cell hyperactivity, including abnormalities in B cell activation, signaling and migration, constitutively enhanced expression of costimulatory molecules like CD154 (CD40 ligand), enhanced

production of cytokines that stimulate B cells such as IL-6 and IL-10, and abnormalities in positive and negative selection of B cells. All the findings listed above are in accordance with a B-cell mediated disease and thus an involvement of BAFF in formation or maintenance of SLE is conceivable (27).

Sera from 46 patients suffering from SLE were analyzed. From eleven of these patients, two or three serum samples that were taken at diverse time points were available, so that in total 66 different serum samples were available for measurement. As can be seen in figure 3, around 65% of all samples analyzed showed a hBAFF concentration of 5 ng/ml or below, a value that was found in the majority of healthy persons. 85% of all samples are within the range of 0-10 ng/ml. Thus, about 15% of all samples showed an increased hBAFF concentration. These 10 samples belong to 8 different patients, because the three highest values found for male patients belong to different samples from the same patient (see patient I in fig. 4). Among the 46 patients, 41 were of female and five of male gender, reflecting the fact that SLE more frequently affects women than men. No difference can be seen when hBAFF serum concentrations of female and male samples are compared, taking into consideration that from one male patient with an increased hBAFF serum level three samples were measured (fig. 3).

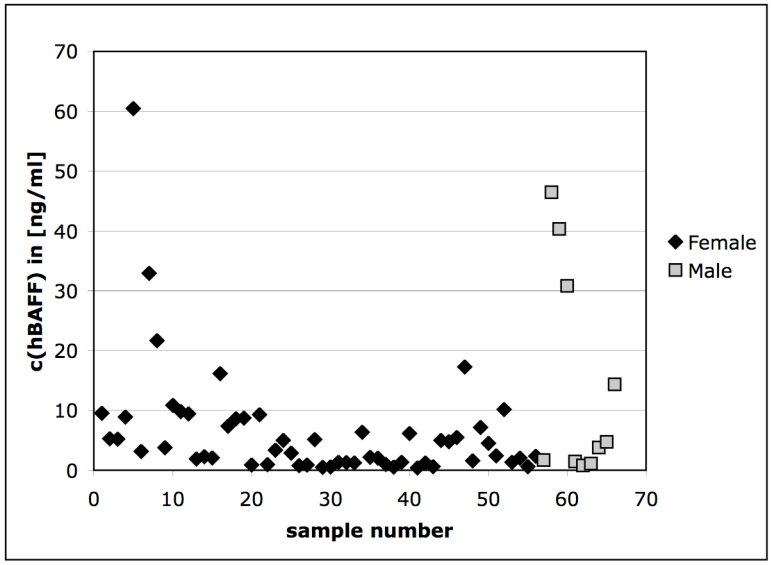

Figure 3. hBAFF concentration in serum of SLE patients. *Serum samples from 46 different female and male patients were analyzed in duplicates. As from some patients several samples taken at different time points were at hand, the total sample number was 66. Standard deviation values were to small to be depicted.*

As mentioned above, from eleven patients two or three serum samples were available, which were taken at different time points. One to two years lie between the single blood takes, so that hBAFF serum concentration could be followed over a period of two to three

years in the course of disease. hBAFF concentration values remained almost constant over the years or decreased (fig. 4). Only for one patient who presented with a considerably elevated hBAFF level from the beginning, an increase in hBAFF concentration of 1.5 fold within two years could be observed (fig. 4, patient I). Interestingly, ECLAM values, which are a measure for disease activity, changed in these patients over the years, without being reflected in hBAFF concentration values (see below and fig. 5).

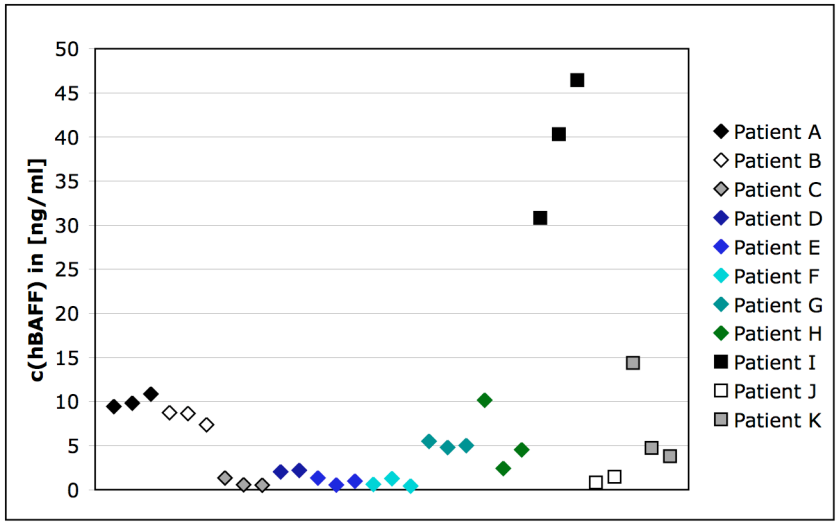

Figure 4. hBAFF concentration in serum of SLE patients followed over some years. *From eleven patients, samples taken in the course of the disease over a period of two to three years were analyzed. Within the data set for each patient, the value obtained for the earliest time point is shown at the left side and the value obtained for the latest time point is at the right side. Female (◆) and male (■) patients are depicted separately. Samples were analyzed in duplicates. Standard deviation values were to small to be depicted.*

ECLAM is a standardized measure to assess SLE disease activity, as already mentioned earlier. For the ECLAM value, 20 clinical manifestations and eleven laboratory tests are scored. Possible ECLAM values are within the range of 1-10, with a higher score reflecting more active disease. Looking at the correlation between ECLAM values and hBAFF concentration, including all patient samples, showed that no relation exists (fig. 5). The two patients with the highest ECLAM values of 9 and 10, respectively, had hBAFF concentration values below 10 ng/ml. The patient with the highest hBAFF concentration had an ECLAM value of 7, but three other patients with the same ECLAM value showed hBAFF concentrations below 10 ng/ml. Moreover, male patient I (see fig. 4) showed an increase in hBAFF concentration levels from 30 ng/ml to 46 ng/ml over a period of two years, while at the same time the corresponding ECLAM values decreased from 4 to 2. Besides, no difference could be observed regarding female or male samples.

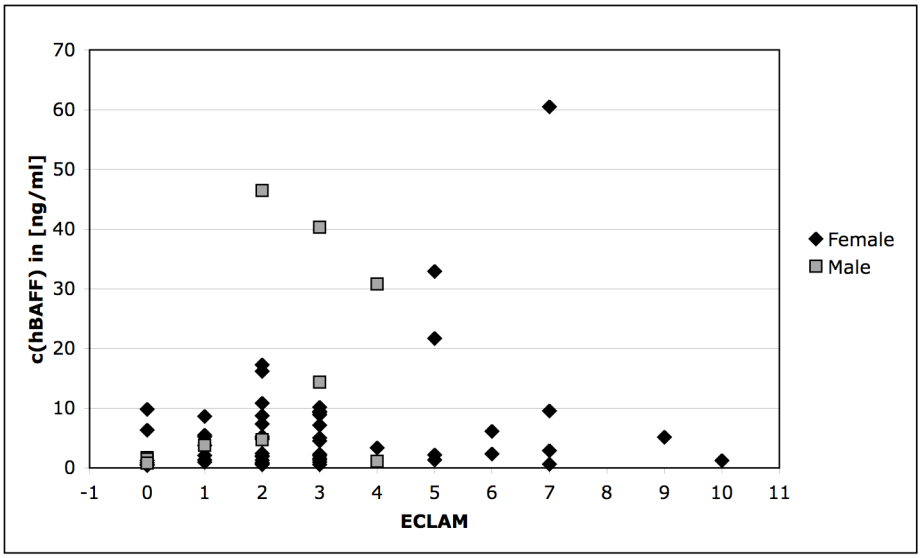

Figure 5. Correlation between hBAFF concentration and ECLAM values. *Female (◆) and male patients (■) are depicted separately. Samples were analyzed in duplicates. Standard deviation values were to small to be depicted.*

A hallmark of SLE is the formation of numerous different antibodies, particularly against nuclear components. The patient samples examined here have been analyzed for anti-dsDNA antibodies using the Farr assay. In this assay, anti-dsDNA antibodies are quantified through precipitation of antibody-antigen complexes with ammonium sulfate, using radio-actively labeled dsDNA as antigen. A standard anti-dsDNA antibody is used in order to indicate results as international units per ml. As depicted in figure 6, no direct correlation between anti-dsDNA antibody titer and serum hBAFF concentration can bee seen. The majority of patients showed hBAFF concentration values of 10 ng/ml or below in combination with an anti-dsDNA antibody concentration of 100 U/ml or below. Surprisingly, six of the seven patients with values above 300 U/ml for anti-dsDNA antibody concentration showed hBAFF concentration values of less than 10 ng/ml, which is within the normal range, and the remaining patient showed a value of less than 20 ng/ml. Furthermore, all patients with increased hBAFF concentration values showed relatively low values for anti-dsDNA antibody concentration.

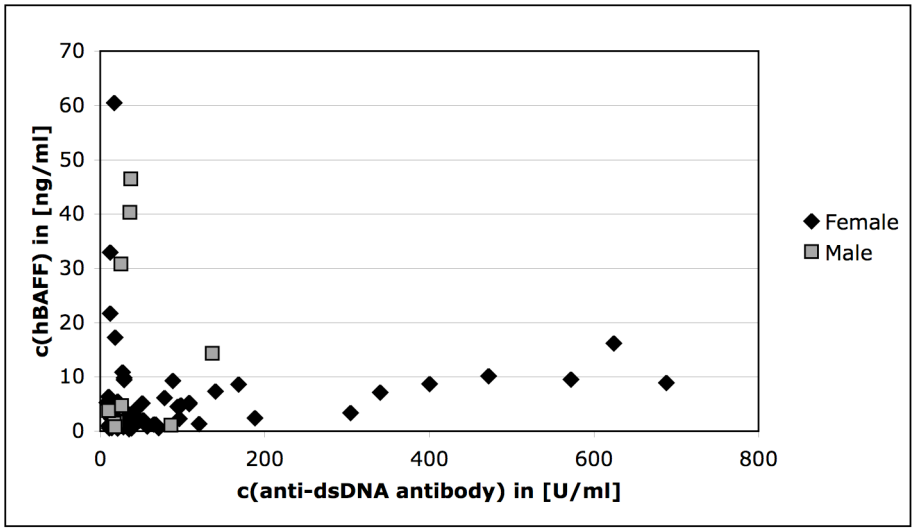

***Figure 6. Correlation between hBAFF concentration and anti-dsDNA antibody titer**. Samples were analyzed for their hBAFF concentration using an ELISA and for their anti-dsDNA antibody titer using the Farr assay. Female (◆) and male patients (■) are depicted separately. Standard deviation values were to small to be depicted.*

As the anti-dsDNA antibody concentration values obtained with the Farr assay are a measurement for antibody binding and thus are influenced by both antibody concentration and avidity, it is difficult to draw conclusions from the observations made. For medical evaluation, not a single anti-dsDNA concentration value is considered, but the chronological progression of these values. Thus, the correlation of hBAFF and anti-dsDNA antibody concentration during disease progression was analyzed, using only the data from patients of which several serum samples were available. Interestingly, even large changes in anti-dsDNA antibody concentration during the progression of disease did not correlate with hBAFF concentration values (fig. 7, patients B and H). Also, changes in hBAFF concentration are not connected with anti-dsDNA antibody concentration, meaning that neither more antibody nor antibodies with a higher avidity for dsDNA were produced. Looking for gender-specific differences revealed that all patients showing high values for anti-dsDNA antibody concentration were of female gender, whereas all male patients showed relatively low anti-dsDNA antibody concentration values. As the overall number of male patients analyzed was quite limited, further investigations are necessary in order to confirm this finding and to analyze its relevance.

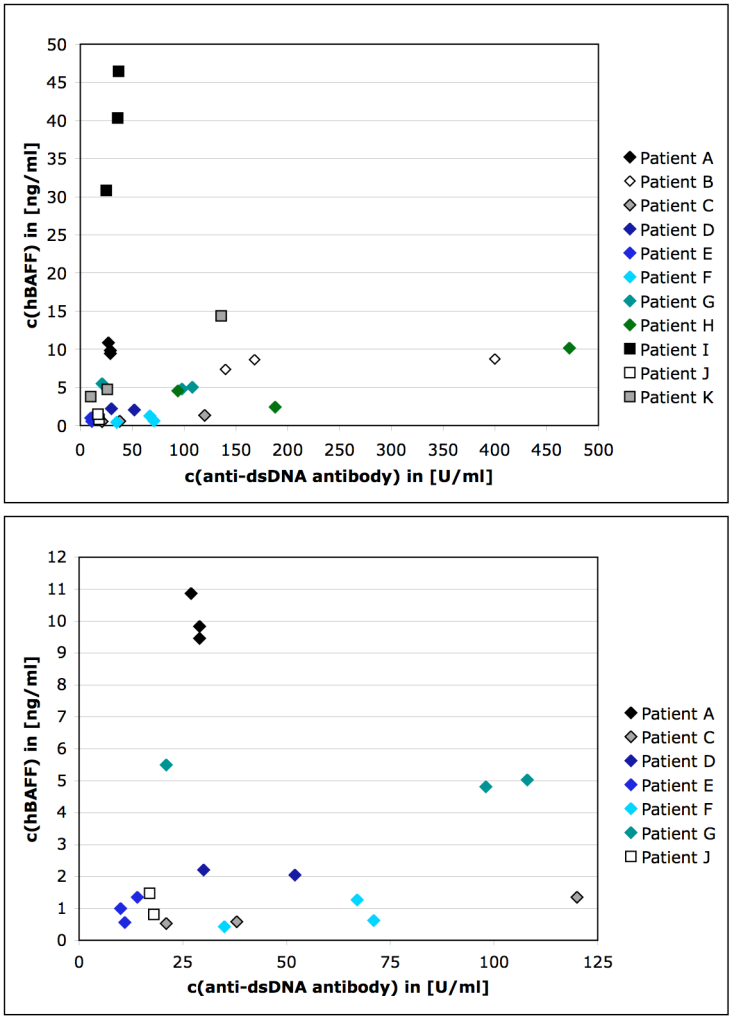

Figure 7. Correlation between hBAFF and anti-dsDNA antibody concentration during progression of disease. *Upper graph: From eleven different patients each two or three serum samples, taken at different time points, were at hand. Different patients are indicated by different colors, using different characters for female (◆) and male (■) gender. Within the data set of each patient, samples are ordered chronologically from left to right. Lower graph: Seperate depiction of selected patients.*

Finally, we looked for a correlation between hBAFF concentration levels and patient age. The analyzed patients were between 19 and 81 years old (fig. 8). Highest hBAFF concentration values were observed in patients around the age of 30, but most of the patients within this age showed hBAFF concentration levels of 10 ng/ml or below. No difference between female and male samples was observed. Thus, a relation between age of the patient and serum hBAFF concentration does not exist.

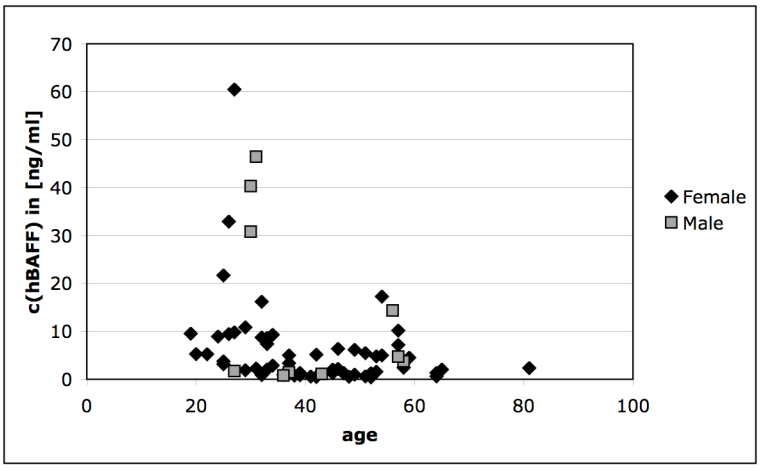

Figure 8. Correlation between hBAFF concentration and patient age. *Serum samples of 58 different patients suffering from SLE were analyzed. Female (◆) and male patients (■) are depicted separately. Standard deviation values were to small to be depicted.*

Taken together, the data obtained in this analysis do not support the idea of a general involvement of hBAFF in human SLE, as only a subpopulation of patients have increased hBAFF levels. Furthermore, no correlation between hBAFF levels and disease activity, reflected by the ECLAM values, or anti-dsDNA antibody concentration could be observed.

7.2.3 Serum hBAFF concentration in patients suffering from AITD

Up to 5% of the overall population are affected with autoimmune thyroid diseases (AITD), making it one of the most common autoimmune disorders (for a review see (28)). The thyroid is an exocrine gland, producing the thyroid hormones, thyroxine (T4) and triiodothyronine (T3), which control diverse metabolic pathways. For production of the thyroid hormones, iodine is oxidized by thyroid peroxidase (TPO) and then coupled to tyrosine residues found at the end of the glycoprotein thyroglobulin. T3 and T4 are formed in an oxidative reaction catalyzed by TPO and thyroglobulin is also the storage form of T3 and T4. The release of these hormones is regulated by thyroid stimulating hormone (TSH). Proteolysis of thyroglobulin releases T3 and T4, whereby some of the T4 is deiodinized to T3. Triiodothyronine is the active form of the thyroid hormones and is either released directly from the thyroid gland or formed by extrathyroidal deiodination of T4, mainly in liver and kidney.

Within the AITDs, Hashimoto's thyroiditis (HT) and Graves' disease (GD) are the most common disorders. As found for other autoimmune diseases, both genetic and environmental factors play a role in the etiology of HT and GD. Environmental factors include iodine excess and deficiency, selenium deficiency, use of certain drugs such as lithium or oral contraceptives, infections, allergies, smoking and stress (29). In what way these environmental factors contribute to the development of AITD is not yet fully understood. Among the genetic factors, both common and unique susceptibility genes exist for HT and GD. Common genetic factors include HLA and CTLA-4. In contrast, a chromosomal region containing the CD40 gene was linked only to GD, but not to HT. Both GD and HT are characterized by lymphocytic infiltration and production of autoantibodies against thyroid-specific proteins. In HT, which is the most frequent form of AITD, the thyroid gland shows massive infiltration by lymphocytes and thyrocyte destruction through T cells. Clinically, HT is characterized by manifestations of hypothyroidism, which are fatigue, hair loss, dry skin, muscle cramps, weight gain, depression, constipation and bradycardia.

Patients suffering from Graves' disease produce autoantibodies against the TSH receptor, which is found on thyrocytes. Binding of these autoantibodies to the receptor mimics the normal function of TSH, resulting in production and release of T3 and T4. As the stimulation of the TSH receptor by autoantibodies is permanent and not regulated in a feed-back-loop, patients have symptoms of hyperthyroidism. These symptoms mainly reflect an increased metabolism, including hyperactivity, tremor, insomnia, sweating, palpitations, arrhythmia, weight loss and diarrhea.

Another disorder of the thyroid gland is the toxic multinodular goiter, in which the thyroid gland is enlarged and contains multiple nodules that are producing excess thyroid hormone, leading to hyperthyroidism. Symptoms are mainly as described above for Graves' disease, but there is an increased risk of developing thyroid cancer with time.

Sera of 63 patients suffering from AITDs were analyzed for their hBAFF concentration. Within these 63 patients, 57 patients were of female gender and only six of male gender. 13 patients were diagnosed with Graves' disease, with eleven female and two male patients. Hashimoto's thyroiditis was found in 34 patients, of which 31 were of female and three of male gender. 16 patients presented with a toxic multinodular goiter, including 15 female and one male patient. Twelve of the 13 patients with GD showed hBAFF concentration values below 5 ng/ml, and one patient had a value of about 8.5 ng/ml. Thus, all values are within the normal range (fig. 9). From the 34 patients with HT, 62% or 21 patients had hBAFF

concentration values below 5 ng/ml and two other patients were within the range of 5-10 ng/ml. Four patients presented with slightly elevated hBAFF levels which were within the 10-20 ng/ml range, and seven patients showed markedly increased levels above 20 ng/ml. Thus, the majority of patients with HT have hBAFF serum levels that are within the normal range, while 20% of patients had severely increased values.

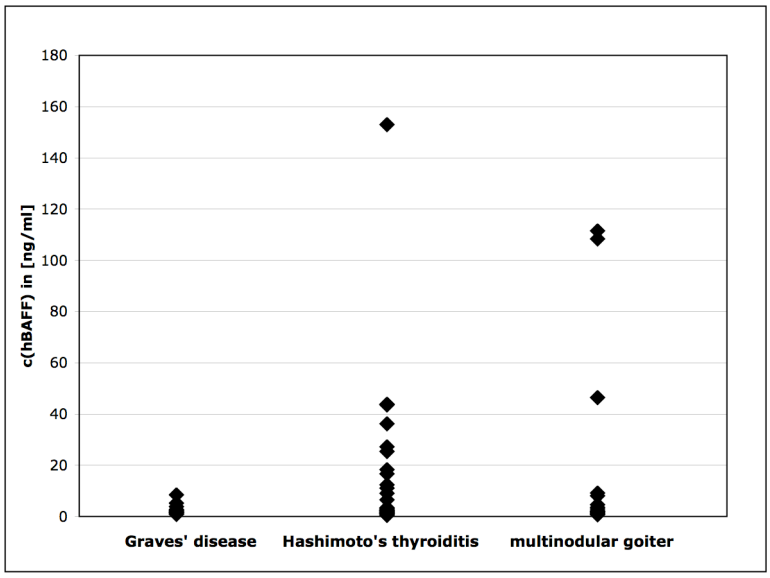

Figure 9. hBAFF concentration levels in patients with different forms of AITD. *Serum samples of 13 patients with Graves' disease, 34 patients with Hashimoto's thyreoditis and 16 patients with toxic multinodular goiter were analyzed for their hBAFF levels. All samples were analyzed in duplicates. Standard deviation values were too small to be depicted.*

Of the 16 patients with a toxic multinodular goiter, eleven patients had hBAFF levels below 5 ng/ml and two patients were within the range of 5-10 ng/ml, thus 81% of patients had hBAFF values within the normal range. Extremely elevated hBAFF levels with values between 40 and 120 ng/ml were found in three patients or 19%. Several of the patients diagnosed with HT were suffering from other autoimmune diseases including celiac disease or type 1 diabetes, but no correlation between serum hBAFF concentration and the presence of further autoimmune diseases could be found (data not shown). In all AITDs analyzed, neither age nor gender correlated with hBAFF values (data not shown). Moreover, no correlation could be found between hBAFF concentration and levels of IgG, IgA and IgM (data not shown). In addition, from some of the patients, serum levels of several cytokines, including TNF-α, IFN-γ, IL-2, IL-4, IL-10 and IL-12 had been determined. Patients affected

with GD or HT showed no correlation between hBAFF and any of these cytokines (data not shown). Interestingly, patients suffering from toxic multinodular goiter showed an inverse correlation between hBAFF and TNF-α, IL-2 or IFN-γ values, respectively. As depicted in figure 10, the three patients with increased hBAFF levels showed low values for TNF-α, IL-2 and IFN-γ, and all patients with increased levels of TNF-α, IL-2 and IFN-γ had relatively low hBAFF values of 10 ng/ml or below. No correlation between hBAFF concentration and levels of IL-4, IL-10 and IL-12 could be found in patients suffering from toxic multinodular goiter (data not shown). TNF-α, IL-2 and IFN-γ are cytokines that usually are elevated in T_H1 immune responses, while serum levels of IL-4, IL-10 and IL-12 are found to be increased in T_H2 immune responses. Thus, hBAFF levels were found to be low in patients with a strong T_H1 immune response. Nevertheless, as among the patients with toxic multinodular goiter only three patients with increased hBAFF levels were present, the statistical significance of this observation is questionable and further research is necessary.

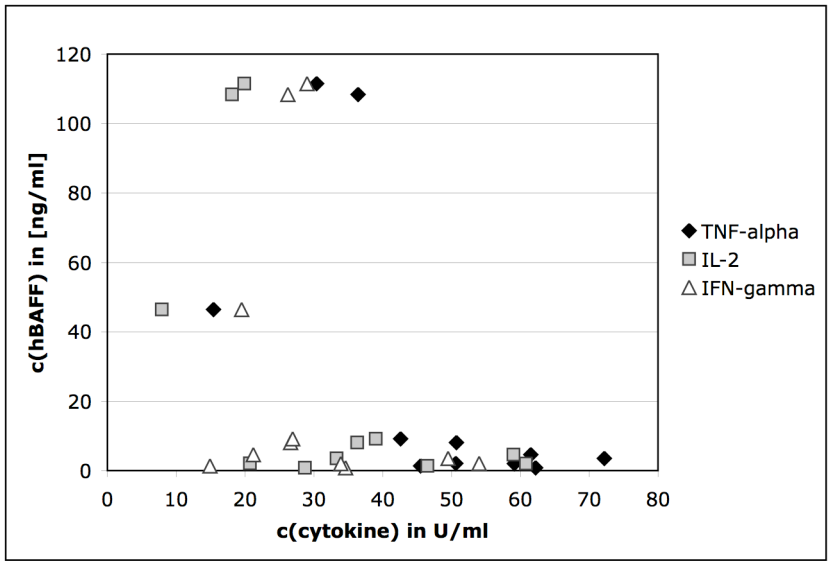

Figure 10. Correlation between concentration levels of hBAFF and certain cytokines in patients with a toxic multinodular goiter. *Serum levels of hBAFF, TNF-α (◆), IL-2 (■) and IFN-γ (△) from eleven patients with a toxic multinodular goiter were measured and cytokine levels were plotted against hBAFF values.*

7.2.4 Serum hBAFF concentrations in patients suffering from Sjögren's Syndrome

Sjögren's syndrome (SS) is a common, chronic autoimmune disorder affecting exocrine glands (for a review see (30, 31)). Characteristically, patients have diminished lacrimal and salivary gland functions, resulting in dryness of the eyes (keratoconjunctivitis) and mouth (xerostomia). This can severely affect the daily life of patients, interfering with functions such as eating and speaking. Furthermore, decreased mucus gland secretion of the upper and lower respiratory tract may produce dryness of the nose, throat and trachea, sometimes leading to a chronic dry cough. Besides this, other organs including the kidneys, lungs, blood vessels, liver, pancreas and brain may be involved. All these symptoms may be attributed to the presence of organ-specific autoantibodies against cellular antigens of salivary ducts, the thyroid gland, the gastric mucosa, the pancreas, the prostate, erythrocytes and nerve cells. As for many systemic diseases, the clinical picture of Sjögren's syndrome is quite heterogeneous. Some patients experience only the mild symptoms of dry eyes and mouth, whereas others have more severe symptoms, like recurrent mouth infections, joint pain, fatigue or renal problems. In primary Sjögren's syndrome, the exocrinopathy occurs alone, whereas in secondary Sjögren's syndrome there is an association with a systemic autoimmune rheumatic disease like rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) or systemic sclerosis. The heterogeneity of the clinical manifestations hampers diagnosis of Sjögren's syndrome and often leads to misdiagnosis. Until now, there is no specific treatment for Sjögren's syndrome available and treatment of patients is only symptomatic.

In Sjögren's syndrome, a chronic stimulation of the immune system is observed. In lymphocytic infiltrates, found in salivary and lacrimal glands and other exocrine glands of the respiratory and gastrointestinal tracts, T cells, B cells and plasma cells are present. Among these, activated $CD4^+$ T helper cells are predominant, producing IL-2, IL-4, IL-6, IL-1β and TNF-α. B-cell hyperreactivity is obvious from polyclonal B cell activation, hypergammaglobulinemia and formation of ectopic germinal centers in the exocrine glands, thus it is assumed that also Sjögren's syndrome is a B-cell mediated autoimmune disease.

Sera from 17 patients suffering from Sjögren's syndrome were analyzed. As shown in figure 11, eleven patients (65%) had extremely low hBAFF values, with a serum concentration of less than 2 ng/ml, and three patients showed values between 10 and 15 ng/ml. Thus, 82% or 14 patients had a hBAFF serum concentration within or slightly above the normal range found for healthy people. The other three patients showed in part extremely elevated hBAFF values between 30 and 290 ng/ml. No additional clinical data were available from any of these patients, thus it can not be ruled out that the increased hBAFF values observed might be correlated with another autoimmune disease, as Sjögren's syndrome often appears in combination with other autoimmune diseases like RA or SLE (secondary SS), as mentioned above.

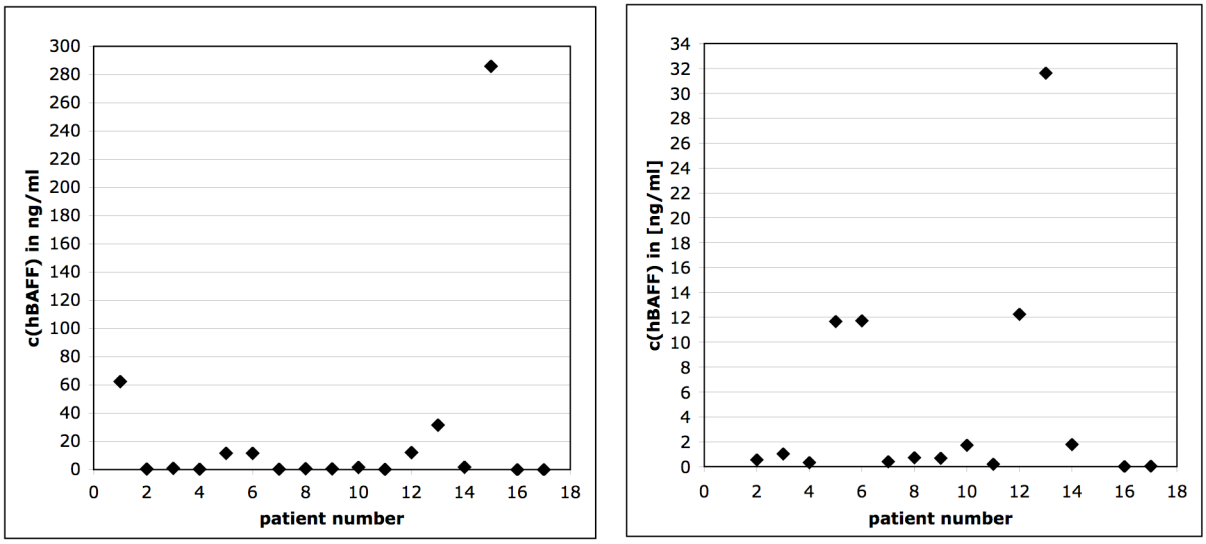

Figure 11. hBAFF concentration in serum of patients suffering from Sjögren's syndrome. *Samples were analyzed in duplicates. In the left picture, all samples tested are shown. The right picture shows only the patients for which a value of less than 35 ng/ml was measured, meaning that patients number 1 and 15 were omitted. Standard deviation values were too small to be depicted.*

7.2.5 Serum hBAFF concentrations in patients having lymphomas

Lymphomas are, as leukemias, malignant tumors of hematopoietic cells. In leukemias, the neoplastic cells proliferate as single cells and can be found in the blood, whereas lymphomas grow as solid tumors and are located in lymphoid tissues, such as the bone marrow, lymph nodes, thymus or MALT. As observed for cancer development in general,

the mechanisms leading to a neoplastic transformation of B- and T-cells is a multistep process, which is to date only partially understood.

Lymphomas are classified according to the WHO classification as Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL) (32). The hallmark of Hodgkin's lymphoma is the presence of multinucleated large cells, called Reed-Sternberg cells (RSCs), and Hodgkin cells, which are atypical mononuclear RSCs. Hodgkin's lymphoma are further subdivided, based on differences in morphology and phenotype of the lymphoma cells and the composition of cellular infiltrates, into classical HL and nodular lymphocyte-predominant HL. The later accounts only for about 5% of all cases. Classical HL itself is further subdivided into four specific forms. All lymphomas not characterized as HLs fall into the group of NHLs. Non-Hodgkin's lymphomas account for around 90% of all malignant lymphomas. According to the WHO classification, more than 35 subtypes of both B- and T-cell origin can be distinguished, with B-cell type lymphomas being more prevalent. Thus, the part of lymphomas classified as NHLs represents an extremely diverse group. Several inherited disorders of the immune system and infectious agents like HIV and EBV are associated with lymphoma development. Recent evidence suggests a role for hepatitis C virus (HCV) in lymphoma development, whereas the role of simian virus 40 is still uncertain. Also, some bacteria are brought in context with lymphoma development, including *Helicobacter pylori* and maybe also *Borrelia burgdorferi*. In addition, in autoimmune and chronic inflammatory disorders an excess risk of lymphoma development has been reported. This includes RA, SLE, SS, celiac disease and chronic thyroiditis (33). The mechanisms leading to lymphoma development in these disorders is to date not completely clear. Chronic inflammation and antigen activation is discussed in this context, besides other factors like treatment of patients with immunosuppressants and TNF-blocking reagents.

The sera of nine patients diagnosed as Morbus Hodgkin (MH) and 14 patients suffering from NHL were analyzed. From the patients, only gender and age were known. As can be seen in figure 12, six of the nine patients suffering from MH showed hBAFF values below 10 ng/ml. One patient had a slightly increased value in the range of 10-15 ng/ml and two patients showed markedly increased values between 15 and 25 ng/ml. From one patient (termed patient number 9) another blood sample which was taken several weeks later was available (termed patient numer 10). This second sample showed an extremely high hBAFF value of 285 ng/ml. Interestingly, at the time point of the first blood take this patient showed a relatively low value for the hBAFF concentration of 1.2 ng/ml (see fig. 12, patient number

9), thus, the patient had an increase in serum hBAFF concentration of more than 200 times at the second time point of blood take. This increase might be due to medical treatment of the lymphoma, but this could not be addressed further as additional patient data was not available. Furthermore, no difference in hBAFF levels between female and male patients could be observed.

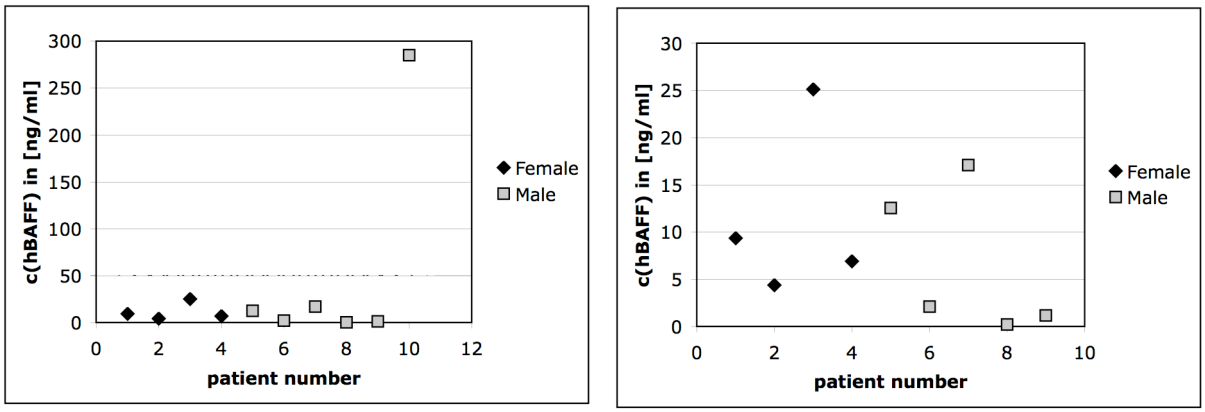

Figure 12. hBAFF concentration in serum of patients suffering from Morbus Hodgkin (MH). *Samples from female (◆) and male patients (■) suffering from MH were analyzed in duplicates. In the left picture, all samples tested are shown. The right picture shows only patients for which a value of less than 30 ng/ml was measured, meaning that sample number 10 was omitted. Note that samples 9 and 10 are from the same patient, with sample 10 taken at a later time point than sample 9. Standard deviation values were too small to be depicted.*

Of the 14 patients with NHL, only three patients (21%) showed serum hBAFF values below 5 ng/ml (fig. 13). Seven patients had hBAFF values in the range of 5-10 ng/ml. One patient had a value in the range of 10-15 ng/ml and the remaining three patients showed increased values between 15 and 30 ng/ml. Between female and male patients, no significant differences in hBAFF values could be found. The low percentage of patients with a serum hBAFF concentration below 5 ng/ml is interesting, as this was found to be the normal range for the majority of healthy persons. As the number of NHL patients was quite small, further investigations are necessary to confirm this observation.

Altogether, 22 patients suffering from Hodgkin or non-Hodgkin lymphomas have been analyzed for serum hBAFF concentrations. Most of the patients with NHL had increased hBAFF levels, while elevated hBAFF levels in patients with MH could be observed only in about half of the patients.

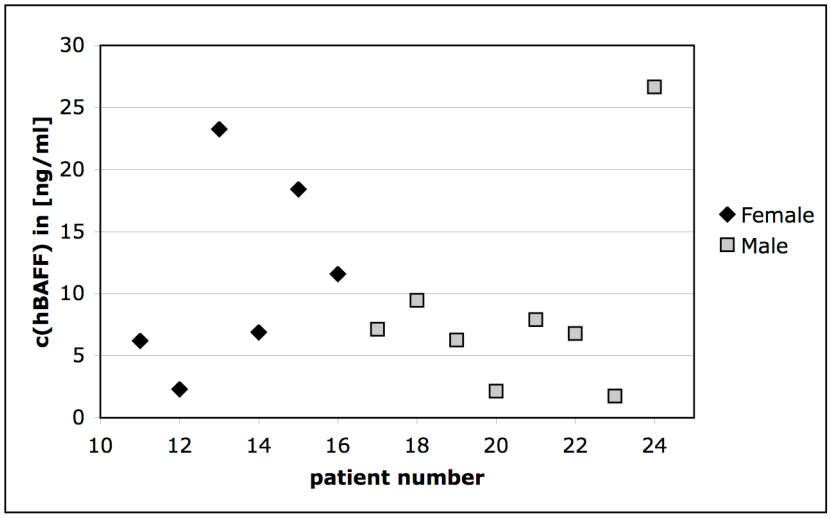

Figure 13. hBAFF concentration in serum of patients suffering from NHL. *Samples of female (◆) and male (■) patients suffering from NHL were analyzed in duplicates. Standard deviation values were too small to be depicted.*

7.2.6 Serum hBAFF concentration in patients suffering from chronic hepatitis C

Hepatitis C is caused by one of the six identified hepatitis viruses, named A-G. Among these viruses, hepatitis C virus (HCV) is considered to be the most serious one. Acute infections with hepatitis C are often unnoticed, as they commonly produce no signs or only mild flu-like symptoms. In 60-80% of cases, acute hepatitis C infections become chronic without the development of any symptoms in the beginning. After 10 to 30 years, 20-30% of the patients develop liver cirrhosis, leading to liver failure. In addition, 1-4% of these patients develop hepatocellular carcinoma. Furthermore, an increased risk exists of developing extra-hepatic diseases like cryoglobulinemia, NHL, arthralgia, SS or Raynaud's syndrome. All of these disorders are connected to either a disturbed B cell homeostasis or to autoimmunity, or both. As hepatitis C virus is known to be not only a hepatotropic but also a lymphotropic virus, it might be that hBAFF regulation is disturbed in patients with chronic hepatitis C (for reviews see (34, 35)).

Serum samples from 41 patients suffering from hepatitis C were analyzed. 24 or 58% of these patients showed serum hBAFF levels below 5 ng/ml and five patients had a value in the range of 5-10 ng/ml (fig. 14, right). Seven patients had increased serum levels of hBAFF in the range of 10-20 ng/ml (fig. 14, right), two patients showed markedly elevated hBAFF

levels between 25 and 40 ng/ml, and three patients showed extremely increased hBAFF levels above 50 ng/ml (fig. 14, left). From all patients, several data and blood test results were available, including liver enzymes (AST, ALT, GGT and alkaline phosphatase), creatinine, bilirubin, CRP, albumin, globulin as well as leucocyte and thrombocyte numbers. No correlation between increased hBAFF concentrations and age or gender, respectively, could be found (data not shown). Furthermore, neither the values for the liver enzymes nor any other of the aforementioned blood parameters correlated with an elevated hBAFF concentration. Especially, CRP values were relatively low in the three patients with the highest hBAFF values, indicating that these patients had no acute infections at the time blood was taken (data not shown). Taken together, the majority of patients with chronic hepatitis C infection did not show increased hBAFF levels, albeit about 40% of the patients had considerably elevated hBAFF levels. Thus, elevated hBAFF levels were found only in a subgroup of hepatitis C patients.

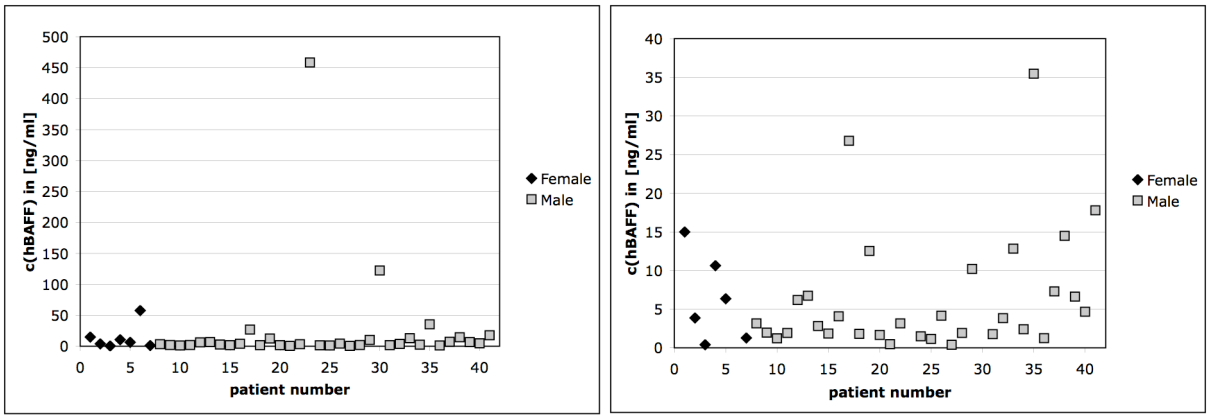

Figure 14. hBAFF concentration in serum of patients suffering from chronic hepatitis C. *Samples of 41 patients were analyzed in duplicates. The right image shows a detail of the left image, including only patients showing hBAFF concentrations up to 40 ng/ml. Standard deviation values were to small to be depicted.*

7.3 Discussion

In this study we found that within the analyzed autoimmune diseases, only a subgroup of patients show increased levels of serum hBAFF, while the majority of patients have hBAFF levels within the normal range. In SLE, 17% of all patients have elevated hBAFF concentration levels, and there is no correlation between hBAFF levels and disease activity or anti-dsDNA antibody titer. Of the 17 patients suffering from SS, 65% have amazingly

low levels of serum hBAFF, while the remaining 35% show increased levels, which partially are extremely elevated. Several groups report a general increase in serum hBAFF levels in SLE and SS patients and a correlation between hBAFF values and autoantibody titers (36-40), which is in contrast to the findings presented here. Nonetheless, one of the first studies analyzing hBAFF levels in patients with SLE or SS found elevated levels in 23% of the SLE patients and in 36% of the SS patients (23). Furthermore, in this study no correlation between hBAFF levels and titer of total IgG or RF was found. Moreover, a closer look on the results presented by one group revealed that the majority of patients with SLE analyzed had serum hBAFF levels comparable to the healthy control group, and only a subgroup of the analyzed patients with SLE showed increased hBAFF levels (40). Furthermore, in an analysis of SLE patients over a period of one year, no effect of serum hBAFF levels on disease activity was found (36). Thus, for SLE and SS, the findings of several different groups are consistent with the results presented in this thesis.

Within the AITDs we found that all 13 patients with Graves' disease have normal or only marginally elevated serum hBAFF levels, while about one third of the 34 patients with Hashimoto's thyroiditis and approximately one fifth of the 16 patients with multinodular goiter show markedly increased hBAFF levels. In all AITDs, no correlation could be found between hBAFF levels and autoantibody titers. Until now there is no analysis on hBAFF levels in human AITDs reported, thus the results obtained in our group can not be discussed further.

In contrast to the findings for autoimmune diseases, we found that the majority of patients suffering from one of the two types of lymphomas analyzed, have increased levels of serum hBAFF. In MH, 55% of the nine patients show elevated levels and of the 14 patients with NHL, nearly 80% have elevated levels. Several groups have analyzed hBAFF levels in patients with different types of lymphomas. Elevated levels of serum hBAFF were found in patients with NHL, B-CLL and Waldenström's macroglobulinemia (24, 41, 42). In this thesis, elevated levels in the majority of patients with NHL could be confirmed and in addition were also found in more than half of the patients with MH.

Furthermore, we found that serum hBAFF levels are elevated in about 40% of the patients with a chronic hepatitis C infection. Other groups also reported elevated hBAFF levels in a subgroup of patients with chronic hepatitis C infection and it seems as hBAFF levels are only weakly associated with the presence of cryoglobulins, RF and arthritis or vasculitis (43, 44), thus confirming our results.

Even though most of the results presented in this thesis have been confirmed by the findings of other groups, there are still some inconsistencies. First of all, the serum hBAFF value for healthy individuals varies quite a lot in all the reports published so far. Some groups found as a normal hBAFF level a value of around 5 ng/ml (37, 39, 40), which is consistent with our result, whereas other groups found both higher mean values of about 10 ng/ml (23) and lower mean values around 1 to 2.5 ng/ml (38, 43, 44). These differences can most likely be ascribed to the different antibodies used in the ELISA assays and the different sensitivity of the assays. Furthermore, in most of the reports, the distribution of serum hBAFF values for healthy donors is quite homogeneous, while in our study we observed a more heterogeneous picture. While around 60% of the healthy controls had hBAFF values of 5 ng/ml or below, around 10% of donors were found both in the range of 5-10 ng/ml and 10-15 ng/ml. In addition, around 10% of the patients had extremely elevated hBAFF levels between 40 and 160 ng/ml. There are a few reports published were also some control patients had markedly increased hBAFF levels (23, 39). It might be that the blood donors, who were supposed to be healthy on the basis of a short medical examination preceding the blood donation, suffer from a yet undiscovered or a harmless chronic disease like an allergy. To date, serum hBAFF levels during a normal immune response or in patients with allergic disorders have not been analyzed. As further medical data from the healthy blood donors analyzed by our group was not available, the reason for the elevated hBAFF concentration levels observed in allegedly healthy individuals remains to be determined.

Another interesting observation made by us and other groups is the heterogeneity of the hBAFF levels in patient samples. In almost all disease types analyzed, there were patients with extremely low or high levels. Incorrect levels of hBAFF might be meassured in patient serum containing BAFF - APRIL heterotrimers. Depending on the epitopes recognized by the antibodies used in the ELISA assay, these heterotrimers do not influence the assay if they are not recognized by the capture antibody, or lead to lower levels, if recognized by the capture antibody alone, and to higher levels, if recognized by both the capture and detection antibody. The presence and serum level of BAFF - APRIL heterotrimers could have been meassured using an ELISA with a combination of an anti-APRIL and an anti-BAFF antibody. As no anti-APRIL antibody was at hand, this issue could not be addressed. The high hBAFF values might reflect interference in the hBAFF ELISA. It is known that the presence of rheumatoid factor (RF) can lead to high values in ELISA assays due to unspecific binding to the antibodies used. In order to determine whether the observed high hBAFF levels result from the presence of RF and its interference in the ELISA assay, the

serum samples could have been depleted from antibodies by the use of commercially available resins, for instance, or the ELISA could have been performed using an isotype matched antibody of an irrelevant specificity for coating. Due to shortness in the amount of patient serum available, this issue could not be pursued. Another possibility is the change of hBAFF levels due to medical treatment of the actual disease. Hepatitis C, for instance, is usually treated with IFN-α, which has been shown to increase the amount of hBAFF secreted from monocytes *in vitro* (45) and treatment of patients suffering from autoimmune hepatitis or chronic graft-versus-host disease with corticosteroids lead to a reduction in serum hBAFF levels (46, 47). Unfortunately, from none of the patients could we analyze samples collected both before and after starting of medication. Thus, this aspect remains to be elucidated in future experiments. Furthermore, the number of patient samples was quite limited in some disease types, thus the obtained results might not reflect the actual situation. Moreover, within most of the analyzed disease types, the clinical picture differs in part substantially from patient to patient. SLE and SS are systemic diseases affecting a variety of different organs, and the serum hBAFF level might be dependent on the clinical picture. In this respect it would have been interesting to analyze the data of the SS patients with respect to the presence of other diseases. As already mentioned, SS can appear alone (primary SS) or in combination with other autoimmune diseases like RA or SLE (secondary SS). This subject could not be addressed due to the lack of further patient data. Also, it might be that in inflammations which are limited to a specific organ, hBAFF levels are only locally elevated and this increase is not reflected in the serum levels. Indeed, local production of hBAFF was observed for instance in patients with RA, where hBAFF is produced by synoviocytes, leading to high hBAFF levels in the synovial fluid (48).

Another aspect that has until to date not been investigated thoroughly is the possible involvement of APRIL and BAFF - APRIL heterotrimers in formation or maintenance of different diseases. Also APRIL levels have been shown to be elevated in patients with different types of lymphomas, systemic sclerosis or SS (49-53), indicating that in some diseases both BAFF and APRIL levels might be of importance. Furthermore, nothing is known about the function of the BAFF - APRIL heterotrimer and its potential implication in diseases. Also, the function of membrane bound BAFF, which might be different from that of soluble BAFF, has not yet been explored, as well as the function of the splice variant deltaBAFF and the fusion protein TWE-PRIL.

Several different BAFF antagonists have been developed for the treatment of various human disorders (54), including monoclonal antibodies against hBAFF (belimumab) and BAFF-R,

and fusion proteins of the extracellular domain of BAFF-R and TACI, respectively, with the Fc part of human IgG1, termed BR3-Fc and TACI-Ig. All reagents have been tested in cynomolgus monkeys, where a reduction in serum Ig and numbers of circulating B cell was observed, but plasma cells were not affected. All reagents showed a similar effect on B cells, but serum IgM and IgA levels were more profoundly decreased when TACI-Ig was used, which binds both BAFF and APRIL. At the moment, all reagents are in clinical trials, and the outcome of these studies will shed more light on the question, how BAFF and APRIL are involved in autoimmune and other diseases with disturbed B cell homeostasis.

7.4 References

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8 GENERAL DISCUSSION

The regulation of lymphocyte numbers and activation is a critical point, necessary to both keep the immune system functioning and to prevent diseases. The maintenance of B cell homeostasis is quite challenging, as mature B cells are distributed throughout the body. B cell homeostasis is regulated at numerous different stages of B lymphocyte development and it has become clear in the last years that this is mainly achieved by cell extrinsic signals. Through the combination of these signals, a cell fate decision is produced, resulting in survival, proliferation or apoptosis of a specific B cell. When the normal mechanisms of homeostatic regulation get out of control, the outcome of this is increased cell death or inappropriate cell activation and proliferation, leading to diseases such as immunodeficiency, autoimmunity and cancer.

The control of B lymphocyte homeostasis by mainly extrinsic signals is an elegant way of regulating such a complex system. The approach of making the decision for a certain cell-fate not dependent on an individual cell but on other cells allows the control over cell numbers and cell activation by regulating microenvironmental conditions and costimulatory signals. Furthermore, B lymphocytes at different stages of development are dependent on different extracellular stimuli, thus allowing a subset-specific control.

A wide variety of receptors are known to be responsible for controlling B cell homeostasis at different developmental stages. One of the earliest receptors expressed on multi-lineage precursor cells is FLT3. Signaling via FLT3 is necessary for the maintenance of these precursors, and the level of FLT3L seems to control the numbers of precursor cells, as mice deficient for FLT3L have reduced numbers of CLPs, and treatment of mice with FLT3 leads to an increase in progenitor cells, including EPLMs (this thesis). The downregulation of FLT3 has been shown to be important for the comittment of precursor cells to the B cell lineage, and maintained or increased signaling via FLT3 results in the preference of the myeloid developmental pathway. Thus, FLT3L does not only control the pool of hematopoietic progenitor cells but also drives the induction of a specific cell fate.

Another important factor in murine B cell development is IL-7. Mice lacking either IL-7 or its receptor have reduced numbers of mature B cells and B cell precursors. Signaling via the IL-7R influences not only survival of the B cell precursors, but also acts in a synergistic way with PAX5 and the pre-BCR to control and maintain B cell lineage comittment. Immature B cells that express a complete BCR downregulate the IL-7R, but start expression

of BAFF-R (this thesis). Thus, IL-7 is an important regulator of the immature B cell pool in the BM, and regulation of B cell homeostasis is switched to BAFF at the time the cells leave the BM.

Survival of peripheral B cell subsets is dependent on signaling via the BAFF - BAFF-R axis. This includes splenic transitional and mature Fol and MZ B cells, but not B1 B cells. Furthermore, the survival of long-lived plasma cells is dependent on the presence of either BAFF or APRIL, while survival of memory B cells seems to be independent of both ligands (1). The number of immature B cells that survive the selection process in the spleen is dependent on the amount of BAFF, and an increase in BAFF allows autoreactive B cells to survive. Another receptor important for the survival of peripheral B cells is the BCR. Cells that are unable to produce a signaling competent BCR die by neglect and mature B cells fail to survive upon elimination of the BCR by conditional gene targeting (2). To date, it is still unclear how the basal BCR signals that are necessary for B cell survival are generated. In this respect, constant low-level interactions with self or environmental antigens that stimulate BCR signaling, or ligand independent, tonic signaling are discussed.

Taken together, homeostasis of B cells is achieved by the spatio-temporal control of extrinsic survival and differentiation signals. Although many of these signaling factors have been uncovered, some open questions remain. Most interesting are the differences between the human and the mouse system in the requirement for IL-7 and BAFF. In contrast to mice, humans deficient for IL-7Rα or the γ_c chain (X-SCID) have normal numbers of B cells. *In vitro*, human HSCs are able to develop into B cells without IL-7. To date, the human factor mediating survival of lymphocyte progenitor cells has not been identified. In the BAFF-APRIL system, the differences to the mouse system have just begun to be discovered. In humans, the ligands show a different affinity for their receptors, and humans deficient for TACI, which was found to be a negative regulator in the murine system, suffer from immunodeficencies. The analysis of patients with mutations in BAFF-R, BCMA or one of the ligands will allow to identify the functions of these molecules in the human system.

8.1 References

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Abbreviations

aa	amino acid
APRIL	a proliferation inducing ligand
bp	base pair
BCMA	B cell maturation antigen
BAFF	B cell activating factor of the TNF family
BAFF-R	BAFF receptor
BCR	B cell receptor
BM	bone marrow
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumine
CAML	calcium modulator and cyclophilin ligand
CD	cluster of differentiation
CSR	class switch recombination
DMSO	dimethylsulfoxid
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetate
FCS	fetal calf serum
FITC	fluorescein-isothiocyanate
Fol B	follicular B
FLT3	FMS-like tyrosine kinase
FLT3L	FLT3 ligand
GC	germinal center
Ig	immunoglobulin
H chain	heavy chain
kb	kilo bases
L chain	light chain
MALT	mucosal associated lymphoid tissue
min	minutes
MHC	major histocompatibility complex
MZ	marginal zone
NIK	NF-κB inducing kinase

NIP	4-Hydroxy-3-nitro-5-iodo-phenylacetyl
OD	optical density
O/N	over night
PCR	polymerase chain reaction
PI	propidium iodide
preBCR	pre-B cell receptor
RAG	recombination activating gene
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecylsulfate
SL chain	surrogate light chain
SLE	systemic lupus erythematosus
T1 (T2, T3)	transitional type 1
TACI	transmembrane activator and CAML interactor
TD	T cell dependent
TdT	terminal deoxynucleotidyl transferase
TI	T cell independent
TRAF	TNF receptor associated factor
UV	ultra violett
WT	wild type

Curriculum vitae

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