# REGULATION OF MURINE B CELL DEVELOPMENT AND FUNCTION

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# Summary

Vertebrates have developed an effective and protective, yet complex and dynamic immune system to defend themselves against the many threats from microorganisms. Although considerable advance in our understanding of B lymphocyte development have been made in recent years, still many questions are in debate and new questions have emerged. In this thesis some of the questions regarding the regulation of murine B cell development and function have been addressed.

In the first part (chapter 3) it was investigated whether the quality of the pairing interaction between the μH chain and the surrogate light (SL) chain of a given pre-B cell receptor may determine the size of the clonal expansion at the large pre-B II cell stage in the bone marrow (BM). $V_HD_HJ_H$-rearrangements derived from pre-B cells having proliferated to different extent *in vitro* in the absence of any added cytokines were isolated and analysed for their sequences and pairing capacities. It could be shown that the capacity of μH-SLC pairings can be measured not only as an all-or-nothing event, but at least also semi-quantitatively. To this end, the present study shows a tendency of a better pairing capacity for $V_HD_HJ_H$-rearrangements derived from cells, which have undergone more cell divisions.

In the second part (chapter 4) the effect of TSLP in lymphocyte development was investigated. It could be shown that increased TSLP availability through transgene expression fully restored lymphopoiesis in IL-7 deficient mice. It rescued B-cell development, increased the thymic cellularity, and rescued the thymic architecture in IL-$7^{-/-}$ mice. Adult wt bone marrow cells differentiated normally into B and T lineages and restored peripheral compartments when adoptively transferred into lethally irradiated IL-$7^{-/-}$ TSLP Tg recipients. Moreover, it could be shown that B cells generated in IL-$7^{-/-}$ TSLP Tg mice originated from adult precursors as these B cells contained N nucleotide additions in their IgH junctions.

In the third part (chapter 5) the expression of BAFF-R on murine BM B cells was investigated with the use of novel monoclonal anti-mBAFF-R antibodies. We found that expression of BAFF-R is first detectable by flow cytometry (FACS) on a fraction of $CD19^+$ $CD93^+$ $IgM^+$ $CD23^-$ 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 upregulation of RAG-2 and 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 fourth part (chapter 6) a putative new B cell population was phenotypically and functionally characterized. These cells, in this study termed 'newB' cells, were defined by the expression of $CD19^{+}$ $CD93^{-}$ $CD21^{-/lo}$ $CD23^{-/lo}$ $CD5^{-}$. They are small in size, comparable to follicular B (FOB) cells, and represent 2% of $CD93^{-}$, mature B cells in spleens of wild-type C57BL/6 mice. In addition, newB cells were detected in lymph nodes. However, the analysis of surface markers suggests that newB cells have a less mature phenotype. In addition, they showed a high turn-over *in vivo* as compared to other mature B cell populations, but lower as for transitional B cells. In functional assays newB cells showed only a limited response to LPS, α-IgM and α-CD40 as compared to FOB and marginal zone B (MZB) cells. The findings of the analysis of λ1 light chain sequences demonstrated that newB cells are not subjected to somatic hypermutations and hence, most probably, do not belong to germinal center B cells and B cell memory. The collected data so far supports the idea that newB cells could represent an additional intermediate cell population in the transition of immature to mature B cells. However, this is in conflict with the detection of these cells in lymph nodes. Alternatively, newB cells could represent cells, which for as yet, unknown reasons get selected out from the pool of mature, reactive B cells later, because they do not fulfill criteria to remain and therefore are rendered anergic and regain a more immature phenotype.

In the last part (chapter 7) the differential response of splenic mature B cells to the mitogen lipopolysaccharide (LPS) was investigated. It was known that frequencies of LPS-reactive B cells in C57BL/6 mice is higher than in BALB/c mice. In this study, it could be shown that actually the FOB cells of C57BL/6 respond stronger to LPS *in vitro* than FOB cells of the BALB/c strain. In this study, MZB cells of both mouse strains showed a stronger response to LPS than the FOB cells. However, in contrast to the observation in FOB cells, MZB cells of both tested strains responded equally strong. A genetic approach did not lead to the identification of a responsible locus for the observed differential response in FOB cells, but indicated that most probably multiple genes control the response in a differential fashion in FOB cells in the tested mouse strains. Furthermore, the results suggest that the stronger response of FOB cells from C57BL/6 mice either involves components within the MyD88-dependent pathway or components, which can modulate the

MyD88-dependent signaling pathway. In addition and in contrast to another study, we could show that the observed differential response is not determined by the MHC class II haplotype in these strains.

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# 1 GENERAL INTRODUCTION

During evolution organisms have always been in competition with other organisms. Numerous mechanisms have evolved to attack and to defend against various other competitors. Thus, highly developed vertebrates are constantly exposed to potentially pathogenic threats from microorganisms, like bacteria, viruses, fungi and nematodes. Vertebrates have developed an immune system, an effective and protective, yet complex and dynamic system to defend themselves against these threats from microorganisms. This potent system can be separated into two interacting branches. The innate immune system is more unspecific, relying on barriers involving anatomic, physiologic, endocytic and inflammatory mechanisms against a wide variety of pathogenic threats. The other is the adaptive immune system, which consists of B and T cells. Each of these lymphocytes carries unique cell surface receptors, which distinguishes self from nonself structures (called antigens) and which will therefore bind to invading pathogens or molecules produced by them. Following binding, B and T cells of the adaptive immune system respond by proliferation and differentiation into effector cells as well as memory cells with increased lifespan. Because of the large diversity of their antigen specific receptors, B cells and T cells can respond to virtually any kind of foreign antigen.

## 1.1 B lymphocytes

B cells recognize antigens via their B cell receptor (BCR). After interacting with antigen they can differentiate into plasma B cells, which then secrete the soluble form of the B cell receptor as an antibody. These secreted immunoglobulin (Ig) molecules can bind to free antigen and thereby mediating the clearance of the antigen from the organisms. Ig molecules binding to antigens on surfaces of invaders can activate the complement system resulting in the formation of pore forming complexes at the surface of these invaders, which results in their destruction by lysis. Ig molecules may also bind with their Fc moieties to Fc receptors on cells of the innate immune system, namely monocytes, macrophages, granulocytes, mast cells and natural killer cells. Thereby, the high diversity of the Ig molecules mediates more specificity to, for example, NK cells of the innate immune system in the antibody dependent cellular cytotoxicity (ADCC) reaction. The diversity and specificity are properties of the adaptive immune system and are generated during the development of B and T cells.

## 1.2 The B cell receptor (BCR)

### 1.2.1 Organization of the B cell receptor complex and its genomic locus

The basic structure of a B cell receptor and its secreted form, termed antibody, consists of two identical heavy and two identical light chains. Each of the two light chains are covalently linked by a disulfide bond to one heavy chain, which in turn are also covalently linked by a variable number of disulfide bonds, depending on the isotype of the heavy chain. Heavy and light chains contain several protein domains. Each of the domains has a characteristic tertiary structure called the immunoglobulin fold. The immunoglobulin fold consists of two β-pleated sheets folded into a globular domain, therefore the resulting molecules are so-called immunoglobulins (Igs). The amino-terminal region (110 aa), called variable (V) region, in each heavy- and light chain contains the antigen-binding site and varies greatly among antibodies of different specificities. The variability in the V regions is not uniformly distributed, but is concentrated into three hypervariable regions, comprising loops of the β-pleated sheets. These regions are called complementarity-determining regions (CDRs) and form most of the antigen-binding site. The remainder of each heavy chain contains three or four constant domains ($C_{H1\text{-}3(4)}$), while the remainder of each light chain consists of only one constant domain ($C_L$).

Immunoglobulins are expressed in two forms: membrane-bound as a B cell receptor (BCR) on resting B cells and secreted as an antibody from plasma cells. This variation depends on alternative splicing of the primary mRNA including the trans-membrane region encoding exon or a nucleotide sequence coding for the hydrophilic tail of the secreted form (1, 2). For successful surface deposition of the BCR, the molecule has to associate with the heterodimeric membrane protein Ig-α/Ig-β (encoded by the *mb-1*/*B29* genes) (3).

A complete heavy chain is encoded by a variable region gene segment ($V_H$), a diversity gene segment ($D_H$), a joining gene segment ($J_H$) and the constant gene segments ($C_{H1-3(4)}$), the last expressing the constant region while the others form the variable region. In contrast to the heavy chain variable region, the light chain variable region ($V_L$) is encoded only by a variable and a joining gene segment (4). Each gene segment is a member of a cluster of gene segments located in three different genomic loci.

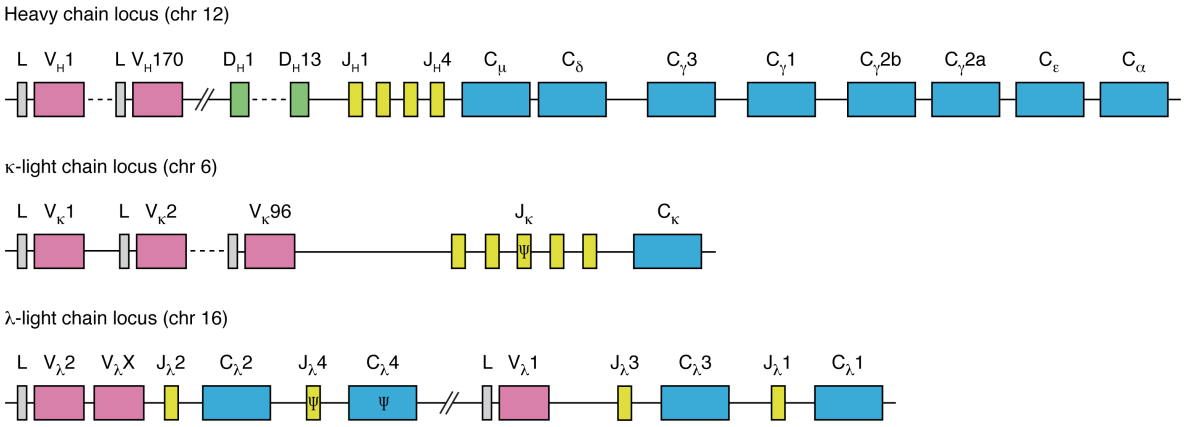


**Figure 1. Schematic representation of mouse Ig genomic loci.**

Genomic organization Ig of heavy and light chain loci containing variable (V), diversity (D), joining (J) and constant region (C) gene elements; pseudogenes (Ψ).

In mice, the Ig heavy (IgH) chain locus is found on chromosome 12. The locus spans over 2.4 Mb and contains the clusters coding for 170 variable ($V_{1-170}$) in 15 $V_H$ families, 13 diversity ($D_{1-12}$), 4 joining ($J_{1-4}$) and 8 constant (Cμ, δ, γ3, γ1, γ2b, γ2a, ε, α) gene segments (4, 5). Based on the coding sequence homology, $V_H$ gene segments in mice are grouped into 15 families. Family members can be clustered but some members from various families are interspersed (6-11).

The κ light chain locus is located on chromosome 6 containing 140 (of which 96 are functional) $V_\kappa$ genes (12), 5 (4 functional) $J_\kappa$ genes and 1 $C_\kappa$ gene segment. The λ light chain locus, located on chromosome 16, consists of only 3 $V_\lambda$, 3 $J_\lambda$ and 3 $C_\lambda$ gene segments.

Both, the heavy- and the light-chain loci include also pseudogene segments. It could be shown for the V regions of the heavy-chain locus that these pseudogenes are not evenly distributed over the locus but rather form clusters (13) (Figure 1).

### 1.2.2 V(D)J recombination process

The diversity of antigen receptors on B cells is generated by a somatic gene rearrangement process, where single variable (V), in the case of the heavy chain also diversity (D) and joining (J) gene segments are recombined. This mechanism is referred to as V(D)J recombination (4). Developing B lymphocytes rearrange their V, (D) and J gene segments in an orderly and stepwise fashion. Thus, the H chain locus is always rearranged before the L chain locus. In case of the heavy chain gene rearrangement, a D element is first rearranged to a J gene segment, followed by a subsequent V to DJ rearrangement (4, 14). In the light chain gene rearrangement V and J gene elements are directly joined to form the complete V segment. The order of the V(D)J recombination process is determined by the temporal accessibility of the different loci to the recombination machinery by mechanisms including subnuclear relocation, chromatin remodeling, DNA methylation, histon acetylation and germline transcription (15-20).

The recombination machinery recognizes DNA sequences called recombination signal sequences (RSS), adjacent to each V(D)J gene segment. Each RSS consists of a conserved palindromic heptamer and an AT-rich nonamer sequence separated by either a 12- or a 23-base pair spacer. Recombination occurs only between two segments flanked by RSS with different spacer length: the so-called 12/23bp spacer rule (21). The recombinase is made up of a complex consisting of recombinase activating gene-1 (RAG-1) and RAG-2, which, in conjunction with the non-specific DNA-bending proteins, High mobility group protein 1 and 2 (HMG-1 and HMG-2), recognize the RSS, where a site-specific DNA cleavage is catalyzed (22, 23). The RAG proteins introduce a double-strand DNA break precisely between the V, D or J coding sequences and the RSS. Initially RAG introduces a single strand nick. The released 3'-OH group of the coding gene segment attacks via transesterification the phosphodiester bond on the opposite DNA strand leading to blunt signal ends and hairpins at the coding ends (23). General DNA repair factors of the nonhomologous end joining (NHEJ) DNA repair pathway are then recruited to perform processing and ligation of the

DNA ends (24-26). Prior to joining the coding ends, the hairpins must be opened. The opening of the hairpins frequently occurs off-center, leading to palindromic single-stranded tails. 3' overhangs have to be trimmed before ligation, unless both coding ends have complementary overhangs. 5' overhangs can be filled in, giving rise to short palindromic (P nucleotide) insertions (27). During the rearrangement of the heavy-chain gene segments occasionally up to 15 nontemplated nucleotides (N nucleotides) are added to the coding ends by the terminal deoxynucleotidyl transferase (TdT).

Combinatorial recombination of V, D and J gene segments, junctional diversification due to imprecise processing of the coding ends and N-nucleotide additions, as well as combinatorial heavy-light chain pairing can produce a giant repertoire ($>10^8$) of possible antigen receptors.

## 1.3 B cell development

In the mouse, B cells develop from pluripotent hematopoietic stem cells in the liver during mid-to-late fetal development and in the bone marrow after birth (28). The differentiation of precursor cells along the developmental pathway from the B lineage-committed progenitor to the mature antigen-sensitive BCR-expressing B cell is characterized by changes in the expression pattern of molecular markers. Along this pathway different checkpoints guarantee that competent B cells develop. This includes elimination or silencing (anergy) of cells with a non-functional BCR or cells, which bear a potentially harmful receptor recognizing autoantigenes.

### 1.3.1 B cell development in the bone marrow

Hematopoetic stem cells are pluripotent and can develop into cells of the lymphoid, myeloid or megakaryocyte-platelet or erythrocyte pathways.

A common lymphoid progenitor (CLP) subset, which has lost the myeloid potential, has been characterized by lack of the lineage markers CD45R/B220, CD11b/Mac-1 and GR, but expression of CD117 (c-kit) and the IL-7R $\alpha$-chain (29, 30).

This model is now being questioned and current findings (31) support the model (32) that development proceeds rather through separate B and T cell progenitors retaining myeloid differentiation potential than through the postulated CLP, which has lost the myeloid potential.

The different stages of B cell development have been characterized by their marker expression and rearrangement status at the IgH and IgL loci. Different nomenclatures for the different stages of B cell development have emerged from various research groups (33-35). In this thesis the nomenclature from Rolink and Melchers will be used (Figure 2).

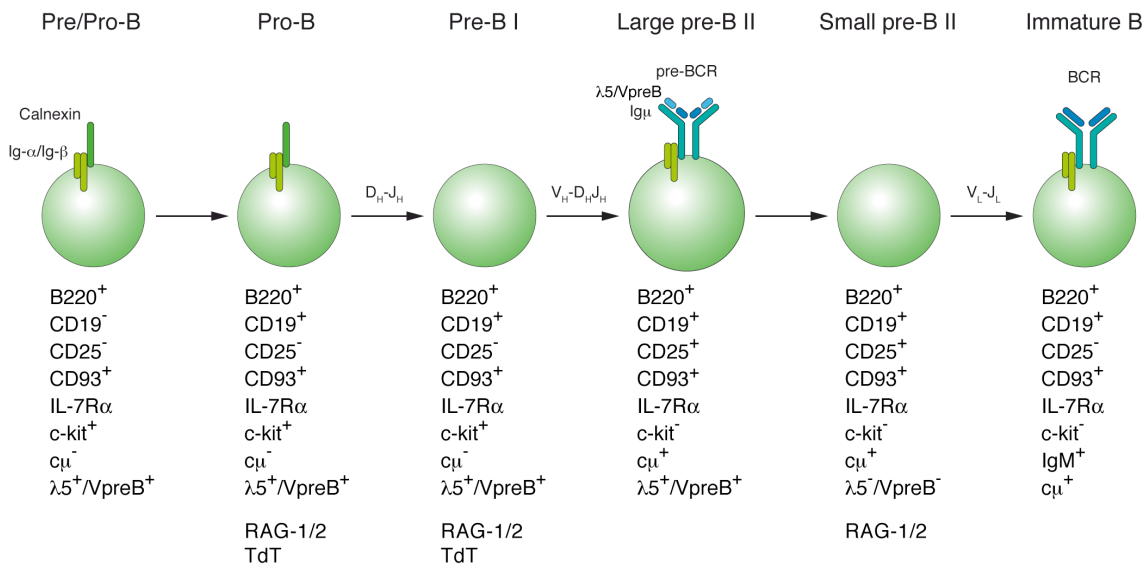


**Figure 2. Schematic representation of B cell development in the bone marrow.**

B cell development in mice after birth starts from hematopoietic precursors in the BM. The different stages of development have been characterized by their marker expression and rearrangement status at the IgH and IgL loci. Nomenclature used is according to Rolink and Melchers ((35)).

The earliest B cell progenitor, the pre/pro-B cell, is phenotypically defined by the pan B cell marker B220 (CD45R), IL-7Rα (CD127) chain and c-kit (CD117) (36-38). At the pro-B cell stage, cells start to express the BCR complex component CD19, which is then expressed on all later B cell stages, except the plasma cells. The CD19 gene is a direct target for the Pax-5 (BSAP) transcription factor. Pax-5 is crucial for maintaining B cell lineage commitment (see later) (39, 40). Pro-B cells start to rearrange their $D_H$ and $J_H$ gene elements in their IgH loci and develop into preB-I cells, which have $DJ_H$ rearrangements, often on both IgH alleles. Furthermore, the pre-B cell receptor (pre-BCR) components VpreB and λ5 are expressed. Pre-B I cells are similar to pro-B cells with respect to intracellular and surface marker expression and cell cycle status. They maintain the capacity to proliferate on stem cell factor expressing stromal cells in the presence of the cytokines IL-3 or IL-7.

Pre-B I cells start to rearrange a $V_H$ chain gene element to the $DJ_H$ segments, thus finalizing the rearrangement of a μH chain. Initially only one allele is rearranged. If the rearrangement is successful, i.e. in-frame, RAG-1/2 and TdT expression are downregulated (41) and the cells start to express the μH chain protein, a hallmark of pre-B II cells. At this stage pre-B II cells gain IL-2R α chain (CD25) expression (35) but have lost c-kit expression and are therefore not expandable any longer in stromal cell cultures. Pre-B II cells can be further subdivided into cycling large pre-B II and resting small pre-B II cells (35). In approx. 25% of large pre-B II cells the μH chain is expressed together with the surrogate light chain (SL chain) at the cell surface as a pre-BCR. This surface expression is dependent on a successful pairing interaction with the VpreB and λ5 components of the SL chain, components of the pre-BCR, which together resemble the λ light chain, and mounts an important checkpoint in pre-B cell development. Successful pairing leads to the clonal expansion of the large pre-B II cells through 2-5 rounds of division and the cells then become resting small pre B II cells, which have lost pre-BCR expression (42). Mutations affecting the rearrangement, the μH chain expression or the SL chain expression (35, 43-49), all lead to a severe reduction in pre-B II cells, and none of them are cycling, stressing the importance of pre-BCR expression in driving the clonal expansion.

If rearrangement at the first allele did not succeed in producing a functional pre-BCR, the cell may retry a $V_H$ to $DJ_H$ rearrangement at the other allele, or else go into apoptosis. It is estimated, that, at least, 40% of emerging pre-B cells die during this process.

Upon re-expression of the recombination machinery, small pre-B II cells start to rearrange their IgL chain gene loci. In mice, the majority of rearrangements are found in the κL chain loci, resulting in a 10:1 κL versus λL chain ratio among BCRs. This ratio remains stable also in the peripheral B cell pool (50) and among serum immunoglobulins.

Cells with a successful light chain rearrangement become surface IgM (sIgM) positive immature B cells expressing CD19, intermediate levels of B220 and low to high levels of sIgM.

Some of the immature B cells expressing a BCR with affinity for autoantigens may become negatively selected and die in the bone marrow. The interaction with antigen results in down-regulation of sIgM and B220 (51). As apoptosis is not a sudden death event and since these cells still express and may even up-regulate the RAG-1/2 enzymes (52) they have been reported to undergo receptor editing through a secondary light chain gene rearrangement, trying to escape the negative selection (53, 54).

In adult mice (6-8 weeks) $2x10^7$ of these immature cells are produced daily (55, 56), and 10-20% of them (57) will enter the spleen but only 5-10% will enter the pool of long-lived mature B cells (58) after final maturation in the spleen.

### 1.3.2 The pre-B cell receptor complex

The pre-B cell receptor complex (pre-BCR) at the B cell surface comprises the μH chain, the surrogate light chain with its components VpreB and λ5, and the transmembrane heterodimer Ig-α/Ig-β (Figure 3).

The genes coding for two functional VpreB proteins (VpreB1 and VpreB2) and λ5 are located on chromosome 16 in mice (59, 60). The VpreB proteins are highly homologous and seem to form a variable region-like structure (61, 62). The constant region-like λ5 protein has a C-terminal Ig domain, followed by the seventh β-strand of a V domain encoded by a J-like region of the gene and an N-terminal non-immunoglobulin domain. Only when λ5 is non-covalently associated with VpreB will the λ5 protein form a disulphide bond with the first constant region ($C_H1$) of a μH chain capable of pairing. The Ig domains of λ5 and VpreB are responsible for their non-covalent association (63, 64). The genes encoding Ig-α and Ig-β glycoproteins proteins, *mb-1* and *B29*, are located on chromosome 7 and 11, respectively. The disulfide-linked transmembrane heterodimer Ig-α/Ig-β associates with the μH chain of the membrane-bound pre-BCR. The cytoplasmic tails of both Ig-α and Ig-β proteins contain tyrosine-based activation motifs (ITAM), which couple the pre-BCR complex to intracellular signal transducers (3, 65, 66).

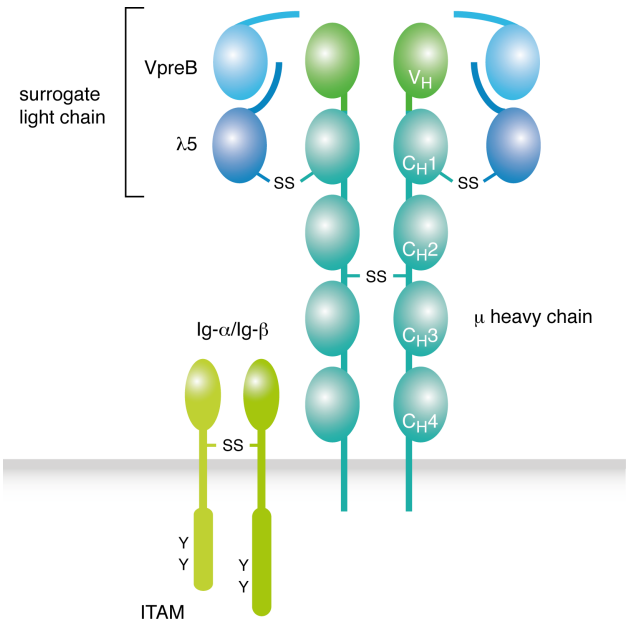


**Figure 3. Pre-B cell receptor complex.**

Pre-BCR complex comprises the μ heavy chain (μH), surrogate light chain (SLC) components λ5 and VpreB and heterodimeric Ig-α/Ig-β.

### 1.3.3 Pre-B cell receptor signaling

After an in-frame $V_HD_HJ_H$ rearrangement, a μH chain protein is produced. Initially the translated μH chain is associated with the endoplasmatic reticulum-resident chaperon protein BiP (67, 68). Later, the BiP can be replaced by a successful pairing of the μH chain with the surrogate light chain (SL chain) (62), which leads to the translocation of the pre-BCR to the cell membrane where it forms the pre-BCR complex together with the transmembrane heterodimer Ig-α/Ig-β. As the pre-BCR itself lacks an intrinsic signaling capacity, the non-covalent pairing with the signaling molecule Ig-α/Ig-β to form the pre-BCR complex is required. Indeed, the expression of Ig-α/Ig-β in the absence of a μH-chain is sufficient to deliver a ligand-independent tonic signal in pre-B cells that can trigger early bone marrow and limited peripheral B cell maturation (69).

### 1.3.4 Allelic exclusion and pre-BCR signaling

Recombination of V, (D) and J gene segments occurs in a lineage- and stage specific manner.
At the pre-BCR stage, developing B cells are monitored for the production of a heavy-chain which is competent to pair with a light-chain giving rise to a single specific antigen receptor. The stepwise process of first rearrangement on only one IgH locus together with functional testing for pairing with the SL chain, as the pre-BCR ensures that a given B cell will finally express only one functional antigenic receptor, thus avoiding cells with multiple specificities, which, in theory, could result in more than 25 different specificities expressed on a single B cell. This mechanism has been termed allelic exclusion (70, 71). In 2-4% of all B cells, the cell contains two productively $V_HD_HJ_H$-rearranged H chain alleles. However, only one of the μH chains is capable of pairing with the SL chain (72). The mechanism of allelic exclusion is as yet not fully understood. It was suggested that the SL chain is involved and therefore that a defective SL chain might prevent allelic exclusion. However, the absence of the λ5 component does not abolish allelic exclusion, but rather prevents the proliferative expansion at the large pre-B II stage (42, 72). A similar phenotype has been described for the double-deficient $VpreB1^{-/-}VpreB2^{-/-}$ mice (46). In contrast, the absence of the transmembrane region of the μH chain in μMT mice results in a loss of allelic exclusion. Furthermore, in transgenic mice, the membrane form, but not the secreted, of a μH chain leads to allelic exclusion of the endogenous IgH chain loci indicating that the transmembrane form is necessary to maintain allelic exclusion (44, 70, 73).

It has been shown that pre-BCR signaling leads to a rapid loss of RAG protein expression and therefore the inactivation of the recombination machinery, which results in the inhibition of further V(D)J recombination and, therefore, establishes the basis for allelic exclusion (41). In addition, pre-BCR signaling in large pre-B cells induces rapid decontraction of the genome and recruitment to repressive centromeric domains of the Ig heavy-chain locus, which aids in the prevention of further $V_H$-$DJ_H$ rearrangement at the second allele during the course of Ig light-chain rearrangements later in small pre-B cells. However, the four most proximal $V_H$ genes can escape allelic exclusion as has been shown in μ-transgenic B cells, since the rearrangements of these genes do not depend on locus contraction (74). This is in accordance with data, which showed that the rare allelically included B cells, which express two different μH proteins, have a disproportionately high frequency of proximal $V_HQ52$-$D_HJ_H$ and $V_H7183$-$D_HJ_H$ rearrangements (75).

### 1.3.5 B cell maturation in the spleen

$IgM^{hi}$ immature B cells transit from bone marrow via blood into the spleen through the terminal branches of central arterioles and marginal sinuses into the outer zone of the periarteriolar lymphoid sheath (PALS) (76, 77). In the spleen the immature B-cell compartment can be further subdivided into three sequential, transitional B cell stages, of short-lived ($t_{1/2}$ = 2-4 days) transitional 1 (T1), T2 and T3 cells (57, 78, 79). The transitional compartment is characterized by the expression of the complement component C1q-like receptor C1qRp (CD93) recognized by the 493 (57) or the AA4.1 antibody (78). T1 cells are $CD19^+$ $CD93^+$ $CD21^{-/lo}$ $CD23^-$ $IgM^{hi}$ $IgD^{lo}$ and appear to be localized at the outer limits of the PALS. T2 cells become $CD21^+$ $CD23^+$ $IgD^{hi}$ and migrate to the B cell follicles (76, 79). In T3 cells, IgM expression is lower as in cells of the T2 stage ($IgM^{int}$). Functional analysis showed that BCR crosslinking induced apoptosis in T1 and T2 cells whereas T3 cells responded with proliferation (78, 80). Nevertheless, there is a disagreement in the literature, whether the change in responsiveness takes place or not already at the T2 stage (81). In normal mice the ratio of the different transitional cell subcompartments (1:1:1) remains stable with age while the relative size of the immature compartment decreases with respect to all B cells in the spleen (80) (Figure 4).

Mature B cells can be discriminated from all the different immature B cell populations by their CD93 negative phenotype (Figure 4). The largest population (~90%) of mature B cells in the spleen comprises the relatively long-lived ($t_{1/2}$ approx. 4.5 months) follicular B cells (FOB) (82). As their name implies, they are located inside follicles and are mainly involved in T cell dependent

(TD) immune responses. FOB cells are characterized by the $CD19^{+}$ $CD93^{-}$ $CD21^{+}$ $CD23^{+}$ $IgM^{+}$ $IgD^{hi}$ set of surface markers. Normally resting, upon activation they enter germinal centers, where, with the support of T cells, their antigen receptor genes undergo somatic hypermutations, which eventually leads to cells bearing receptor molecules with a higher affinity for antigen and the production of highly specific antibodies. FOB cells can recirculate to other lymphoid tissues including lymph nodes and the bone marrow.

Another type of mature B cells in the spleen, although less abundant (~5%) than the FOB cell population, are the marginal zone B (MZB) cells (Figure 4). They are located in the marginal zone, which is in close proximity to the marginal sinus, making them particularly well positioned to respond rapidly to blood-borne antigens. In contrast to the recirculating FOB cells, MZB cells are relative sessile and not part of the recirculating pool of lymphocytes (83). MZB cells have a $CD19^{+}$ $CD93^{-}$ $CD21^{hi}$ $CD23^{-/lo}$ $IgM^{hi}$ $IgD^{lo}$ phenotype. It has been suggested that MZB cells are mainly involved in T cell independent type 2 (TI-2) responses against multivalent antigens (84-90), since splenectomy as well as mutations in certain signaling pathways resulting in the absence of MZB cells correlated with an increased susceptibility to encapsulated bacteria. MZB may mature rapidly into plasmablasts. Thus, within the first three days following exposure to blood-borne pathogens, antigen-specific MZB cells proliferate concomitantly with their differentiation into IgM-secreting plasmablasts (91, 92). In *in vitro* stimulation assays, MZB cells proliferated stronger than follicular B cells when stimulated with lipopolysaccharide (LPS), CD38 cross-linking or CD40 ligation and secreted more IgM and IgG3 molecules (93).

There is growing evidence that MZB cells are also involved in T-dependent immune responses. MZB may also become potent antigen presenting cells (APCs) since they express higher levels of MHC class II and CD80/86 molecules than FOB cells and are thus, potent activators of naïve $CD4^{+}$ T cells (92, 94).

Both, FOB and MZB cells belong to the B-2 subset of mature B cells.

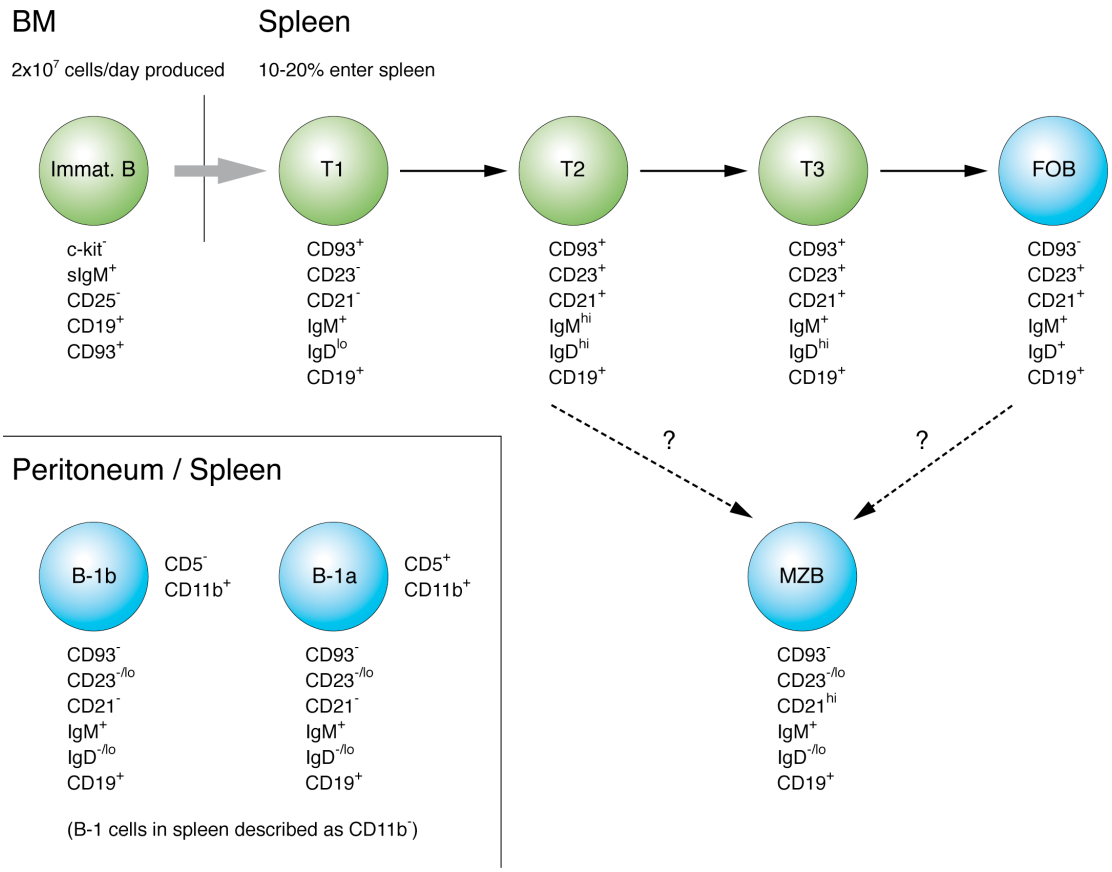


**Figure 4. Schematic representation of B cell development in the spleen.**

BM: bone marrow, T: transitional type B cells, FOB: follicular B cell, MZB: marginal B cell, B1-a/b: type 1 B cells. Immature stages (green), mature stages (blue) defined by CD93 expression.

The B-1 B cells, which represent only a minor fraction of B cells in the spleen, but represent the major B cell population in the peritoneal and pleural cavities were originally characterized by their $B220^{lo}$ $IgM^{hi}$ $CD23^{-}$ $CD43^{+}$ $IgD^{lo}$ phenotype. They also have a larger size and exhibit more side scatter than do B-2 B cells. Furthermore, they can be subdivided into $CD5^{+}$ B-1a and $CD5^{-}$ B-1b subpopulations. In addition, B-1 cells in the peritoneal and pleural cavities are Mac-$I^{+}$ (CD11b), while they are Mac-$I^{-}$ in the spleen (Figure 4). In contrast to the FOB cells, B-1 B cells are rarely found in lymph nodes. Furthermore, for B-1a B cells it has been shown that they require the spleen for their generation and maintenance (95).

These cells mainly participate in responses to T-independent type 2 (TI-2) antigens. They are also the major producers of polyreactive, weakly autoreactive, naturally occurring serum IgM antibodies. Such antibodies seem to be produced in the apparent absence of exogenous antigenic stimulation (91, 96-98). B-1 B cells activated in the peritoneal and pleural cavities rapidly migrate

into the spleen and gut lymphoid tissues, where they lose CD5 expression and differentiate into antibody-secreting plasma cells (99, 100).

Compared to B-2 B cells, the B-1 cell compartment contains a high frequency of cells with specificities towards self-antigens, like phosphatidyl choline (PtC) and both single and double stranded DNA, as well as common bacterial carbohydrate antigens such as phosphorylcholine (PC) (97, 98). It has been suggested that the development of B-1 B cells reflects differences in fetal and adult B cell development due to the fetal origin of the majority of $CD5^+$ B cells (101) and that BCR specificity plays an important role (102, 103). Originally, it was proposed that B-1 B cells are derived from different, committed, precursors than the B-2 B cells. This was based on transfer studies where fetal liver reconstituted the B-1 and B-2 compartments in irradiated recipient mice, while adult bone marrow was limited to the generation of B-2 B cells (104, 105). Other experiments suggest an "induced-differentiation" model where encounters with naturally occurring TI2 antigens account for the appearance of B-1 B cells *in vivo* (106, 107). The fetal and neonatal repertoire is skewed towards the expression of immunoglobulins that frequently bind encountered TI-2 antigens while the adult repertoire generates less of these specificities and therefore generates fewer B-1 B cells (108, 109). Further support for the "induced-differentiation" model comes from studies showing that the quality of B cell receptor signaling is essential for B-1 B cell development. Mutations that disrupt BCR signaling result in a strong reduction of the B-1 B cell compartment, while enhancing BCR signaling results in an expanded B-1 compartment (110, 111). Studies with mice transgenic for a single Ig molecule with B-1 specificity, showed a reduction of the B cell receptor surface density by a factor of only two, but significantly reduced the number of transgene expressing B-1 B cells. (112, 113).

Nevertheless, recently a B-1 B cell-restricted progenitor has been described, which numbers are highest in fetal bone marrow, but were lower in adult bone marrow and supports the model that B-1 cells develop from unique progenitors (114).

### 1.3.6 Factors regulating B cell maturation and lineage commitment

Many factors influencing B cell maturation and lineage commitment are known but the exact mechanism of action remains to be elucidated. Naturally occurring and genetically engineered mutations are valuable tools helping to shed light on the factors and mechanisms involved in lineage commitment.

### *1.3.6.1 Transcription factors*

Differentiation of the common lymphoid precursor (CLP) to committed pro-B cells depends on the transcription factors E2A, EBF, and Pax5 (115).

#### *1.3.6.1.1 E2A and EBF (Early B cell Factor)*

E2A, a basic helix-loop-helix (bHLH) protein with two splice forms E12 and E47 (116), is widely expressed in tissue as a heterodimer with tissue-restricted other bHLH proteins, but specifically functions as a homodimer in the B cell lineage (117). Together with EBF, which also is expressed as a homodimer and recognizes DNA via the N-terminal domain containing a zinc finger motif (118, 119). E2A controls the earliest phases of B cell development. Studies with *E2A* or *EBF* mutant mice showed a similar arrest of early B cell development in both mice with the Ig heavy-chain locus remaining in germline configuration (120). This suggests that the two factors act in concert to regulate the expression of multiple B-lymphoid genes including the *RAG1* and *RAG2* genes (121). Moreover, it could be shown that the E2A and EBF proteins bind to and cooperatively activate the promoters of the SL chain-components λ5, VpreB, and the pre-BCR complex component Ig-α (122-125). EBF was in addition shown to control the *Ig-β* promoter (126).

#### *1.3.6.1.2 Pax-5 (BSAP; B-cell-specific activator protein)*

The *Pax5* gene is located at chromosome 4 and encodes a member of the paired box (PAX) family of transcription factors. It contains a highly conserved DNA-binding domain, known as the paired box, a repression, and a transactivation domain. The paired domain is composed of two subdomains that bind to the two half-sites in adjacent major grooves at the same side of the DNA helix (127-129).

Pax5 is crucial for maintaining the B cell lineage commitment (39) since Pax5-deficiency blocks the B lymphopoiesis at the pre-B I cell stage (130, 131). In addition, in mice where the Pax5 gene can be conditionally removed, the inactivation of *Pax5* leads to the loss of phenotypes and functions of mature B cells (132). However, transferring cultured Pax5-deficient pre-B cells into irradiated recipients gives rise to long-term *in vivo* reconstitution of T cells (40). The Pax5 protein supports the B-cell commitment by activating lineage-specific genes, i.e. *CD19*, and repressing lineage inappropriate genes, such as *M-CSR, Notch1, Flt3* (133-135). Furthermore, Pax-5 has an important role in regulating the contraction of the Ig heavy-chain locus (136). Thus, Pax-$5^{-/-}$ pro-B

cells fail to rearrange the central and distal $V_H$ gene elements in an otherwise fully accessible and active chromatin (137).

### *1.3.6.2 Role of the BCR and BCR signaling*

Expression of the BCR is required for the survival of all B cells. In mice where the BCR can be conditionally removed from B cells, such modified cells rapidly disappear from the circulation and from lymphoid organs (138). Both the affinity of a given BCR to its antigen and the surface density of BCR may also determine the lineage-fate of B cells (112, 113, 139). Furthermore, the specificity of the expressed BCR may influence the lineage decision. In contrast to FOB cells, the differentiation into MZB and B-1 cells is dependent on self or environmental antigens, such as phosphorylcholine and phosphatidylcholine, suggesting that these compartments require a positive selection mediated by interactions between BCR and antigen (140-143). However, in a recent study, it could be shown that a ligand-independent tonic signal may be sufficient to form FOB cells but not MZB or B-1 cells (69).

There is ongoing discussion whether weak signals drive cells in the direction of FOB differentiation while strong signals preferentially result in the formation of MZB cells or that MZB cell development is driven by weaker BCR signals than those leading to FOB cell development.

Deletion of the CD19 BCR complex component, a positive regulator of BCR signaling, leads to absence of MZB development and a reduction of the development of B-1 cells (110, 144), while over-expression of this complex results in an increase in the number of B-1b B cells as well as the breakdown of peripheral tolerance (145). Mice lacking the negative regulator CD22 are deficient in MZB cells (146) and also show an increased number of B-1b cells together with increased serum titers of autoantibody (147). In comparison, mice lacking the CD21 BCR complex component, show an enlarged MZB cell compartment and a decreased number of FOB cells.

Conversely, the absence of the Aiolos transcription factor enhances the signal delivered by the BCR resulting in an increased FOB compartment and absence of MZB cells (148).

And furthermore, CD5 expression in B-1a cells seems to be implicated in the negative regulation of B1 cells (100, 149).

However, overall, the literature suggests that the lineage decision is driven primarily by differences in BCR signal strength.

### *1.3.6.3 Chemokines affecting the rate of B cell generation and survival may also affect lineage decision*

#### *1.3.6.3.1 Interleukin-7 (IL-7)*

Interleukin-7 (IL-7) is produced by stromal cells in lymphoid tissues such as the bone marrow and is required for the normal development of lymphocytes.

Early stages of B-cell development in adult mice depend upon IL-7 for growth, survival, and maturation. In the absence of interleukin 7 (IL-7), B lymphocytes are generated exclusively during fetal and perinatal life, but persist throughout adult life and consist only of cells that belong to the B1 and MZB compartments (150, 151). In contrast, mice overexpressing IL-7 as a transgene have an increased B cell generation in the bone marrow with a massive influx into the spleen, also of early B cell precursors, leading to splenomegaly. Analysis of mature B cells in the spleen of these IL-7Tg animals show a massive expansion of the FOB cell compartment without altering the MZB cell compartment. In mixed radiation bone marrow chimeras MZB cells developed to the same extent from wt and IL-7Tg adult bone marrow showing that IL-7 does not directly influence the lineage decision. The authors conclude that the number of cells in the FOB and MZB cell compartments may be independently regulated (152). These data support the model that the influx from the bone marrow to the spleen influences the lineage decision. While in a lymphopenic situation, the system preferentially propagates and maintains the B-1 and MZB compartments, an increased influx of progenitors from the bone marrow favors the FOB lineage decision.

#### *1.3.6.3.2 Interleukin-7 receptor (IL-7R)*

The interleukin-7 receptor (IL-7R) is expressed throughout B-cell development from the IL-7-indepent CLP stage to the early surface $IgM^+$ stage, but is not expressed in mature B cells. As for IL-7, an IL-7R-deficiency in adult mice blocks early B cell development already at the transition from CLP to pro-B cells (153-156).

#### *1.3.6.3.3 Thymic stromal lymphopoietin (TSLP) and its receptor (TSLP-R)*

The hemopoietic cytokine thymic stromal lymphopoietin (TSLP) signals through a heterodimeric receptor composed of the IL-7Rα and the thymic stromal lymphopoetin receptor (TSLPR) chain leading to the translocation of functional STAT5 to the nucleus. TSLP was originally cloned from a murine thymic stromal cell line. It promotes the homeostatic expansion of $CD4^+$ T cells, enhances

dendritic cell maturation and triggers inflammatory allergic responses. The role in B cell development is less clear. TSLP was shown to support B-cell development *in vitro* (157), however, its role in B-cell development *in vivo* is controversial and some studies have suggested that TSLP might be limited to fetal rather than adult B-cell development (158-160). It is unknown whether these differences reflect distinctive TSLP responsiveness or availability of the cytokine in fetal versus adult hematopoiesis.

#### *1.3.6.3.4 Components of the Notch signaling pathway*

Notch signaling affects lineage decisions through the activation of a number of genes involved in the regulation of cell differentiation.

The Notch family comprises four highly conserved transmembrane receptors that regulate differentiation in various tissues. Upon ligand binding, the intracellular domain is released through a proteolytic cleavage within the transmembrane domain. The Notch intracellular domain is then translocated into the nucleus, where it acts as a transcriptional activator.

While signaling mediated by the Delta-like1 ligand and Notch1 blocks B cell differentiation but allows T cell development (161, 162), signaling through Notch2 influences the MZB versus FOB cell fate within the spleen. Notch2 is highly expressed in mature B cells, and conditional deletion of Notch2 results in a decreased number of MZB cells, whereas the FOB and B-1 cell compartments developed normally (163).

In a conditional knockout of the Notch signaling key mediator RBP-J selectively in CD19 expressing cells only, MZB cells were lost with a concomitant increase in FOB cells, while the B1 compartment was not affected. (90). These results were further supported by the finding of the negative regulator of Notch signaling, Msx2-interacting nuclear target protein (MINT), which competes with Notch for the binding to RBP-J and is more abundant in FOB than MZB cells. Splenic B cells derived from $RAG2^{-/-}$ chimeric mice reconstituted with MINT-deficient fetal liver cells differentiated more efficiently into MZB cells with a concomitant reduction in FOB cells (164).

#### *1.3.6.3.5 BAFF and BAFF-R*

Interactions of the TNF-family member BAFF (B-cell activating factor) with its receptor BAFF-R on B cells is crucial for the transition from the T1 to the T2 stage during B cell maturation in the spleen and later for mature B cells.

Deficiency for either component results in a strong reduction of late transitional (T2, T3) as well as mature B cells. In *Baff-r*$^{-/-}$ mice, MZB cells are hardly detectable. Absence of BAFF prevents the development of B-2 B cells beyond the T1 stage such that virtually no mature B cells are formed. Interestingly, the B-1 B cell compartment seems not to be affected by this mutation (165-167). These findings support the theory that B-1 cells develop independently from B-2 B cells, which was already suggested through findings with $\lambda5^{-/-}$ mice, where B-2 cells develop only to low numbers, while B-1 cells reach normal numbers (168). However, it does not absolutely exclude the possibility of an activation-dependent generation of B-1 B cells out of the B-2 B cell compartment.

BAFF over-expression in transgenic mice leads to enlarged T2 and mature B cell compartments with B cell hyperplasia and hyperglobulinemia. It is especially the number of MZB cells, which become increased, and autoimmunity is manifested through elevated serum autoantibody titers. The pathology exhibited by these mice resembles that of the human autoimmune disorder systemic lupus erythematosus (SLE), and later in life of the Sjögren's syndrome (169). *In vitro* experiments showed that BAFF increased survival of immature and mature B cells. Transitional B cells cultured in the presence of BAFF also acquired mature B cell markers, indicating that BAFF may also be involved in B cell maturation (170). However, the precise mechanism how BAFF/BAFF-R mediates survival is not yet fully understood. *In vivo* and *in vitro* studies indicated that BAFF/BAFF-R signaling is coupled to the Bcl family members of oncogenes. Transgenic expression of Bcl-2 in B cells, or the absence of the Bcl-2 antagonist Bim results in phenotypes comparable to BAFF-Tg mice and increased Bcl-2 levels were also measured in B cells of BAFF-Tg mice. Furthermore, introduction of a Bcl-2 transgene into *Baff-r*$^{-/-}$ mice rescued the development of mature B cells and brought the splenic compartment to normal wild type levels (166, 169, 171, 172). However, a more recent study suggests that BAFF prevents BCR-induced apoptosis not by upregulating Bcl-2 expression but rather through down-regulation of the Bcl-2 antagonist Bim (173).

It has also been suggested that BAFF may exert other, non-survival functions. It seems to be involved in MZB cell differentiation and may regulate the CD21 and CD23 expression. In contrast to the role in B cell survival these functions are independent of Bcl-2 (174, 175).

Compared to the important role of BAFF and BAFF-R in the transitional B cell compartment for the generation of T2 B cells and subsequent stages of B cell maturation, results from genetically modified mice for gain and loss of BAFF/BAFF-R did not show any role for these molecules during the early B cell development in the bone marrow, nor the survival of immature bone marrow or splenic T1 B cells.

#### *1.3.6.4 Integrins affecting splenic homing*

MZB cells express higher levels of the integrins LFA-1 and $\alpha 4\beta 1$ than FOB cells. These integrins interact with ICAM-1 and VCAM-1. Inhibition of these integrins causes a rapid and selective release of B cells from the marginal zone (176). Additionally, MZB cells may be retained in the marginal zone by a direct interaction with marginal zone macrophages (MZM) via the Macrophage receptor with collagenous structure (MARCO) scavenger receptor on MZMs (177).

Taken together; in a lymphopenic situation, the proportion of MZB and B-1 cells compared to FOB cells is increased suggesting that the system is trying to maintain a first line of defense against blood-borne and T-independent antigens through the MZB cells in the spleen and the B-1 cells of the body cavities such as the peritoneum and the pleural cavity. The lineage decision is further dependent on the signal strength of the BCR and the signals provided by the environment of the cell.

## 1.4 Aim of thesis

Although considerable advance in our understanding of B cell development have been made in recent years, still many questions are in debate and new questions have emerged. In this thesis some of these questions have been addressed.

Thus, it is known that during pre-B cell development, successful pairing of a μH chain with the SL chain results in surface expression of the pre-BCR and leads to clonal expansion of large pre-B II cells. But, since not all cells with a successful pairing expand to the same extent, it was investigated whether the quality of the pairing interaction may determine the size of the clonal expansion.

Furthermore, it is known that the interaction of BAFF with BAFF-R is vital for the transition of immature to mature B cells. *In vivo*, the transition from the T1 to T2 stage in the spleen was the first stage at which BAFF has been shown to be important, as both BAFF and BAFF-R deficient mice show a block at that stage. However, it was not known in detail at which stage during B cell development the BAFF-R is first expressed. Using novel monoclonal antibodies specific for mouse BAFF-R, we were able to determine the surface expression of this receptor during B cell development in the bone marrow. Furthermore, BAFF-R expression was assessed in the context of B cell receptor editing and RAG protein expression.

In a study, testing the potential of TSLP to rescue lymphopoiesis in the absence of IL-7, the question was addressed whether B lymphocyte development in IL-7 deficient mice can occur from the differentiation of adult BM progenitors in a setting, where TSLP is over-expressed. Using the fact that only B cells from adult BM precursors display N nucleotides in the IgH junction of the B cell receptor allows distinguishing adult from fetal B cell development.

Furthermore, a putative new splenic B cell population was characterized. The mature B cell compartment in the spleen can be subdivided into $CD23^{+}$ cells comprising follicular B cells and into $CD23^{-/lo}$ cells, which include $CD21^{hi}$ expressing marginal zone B cells. A fraction, which we call “newB cells”, of this $CD23^{-/lo}$ cells, however, expresses low levels of CD21. A series of experiments, including phenotypic and functional analysis, were designed to characterize these cells more closely.

Using *in vitro* stimulation assays it was shown that splenocytes from the different mouse strains C57BL/6 and BALB/c responded differently to the mitogen lipopolysaccharide (LPS). Additionally, it was known that marginal zone B cells respond faster and more vigorously to LPS than follicular B cells. Now, in a series of experiments using sorted splenic B cell subpopulations, it was attempted to elucidate why the two mouse strains displayed different responses to LPS.

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# 2 MATERIALS AND METHODS

## 2.1 General buffers and reagents

| | |
|---|---|
| ABI sequencing loading buffer | 25 mM EDTA, pH 8.0<br>50 mg/ml blue dextran<br>in deionized formamide |
| ACK buffer (erythrocyte lysis buffer) | 0.15 M $NH_4Cl$<br>1.0 mM $KHCO_3$<br>0.1 mM EDTA<br>in $H_2O$, sterile filtered (0.2µm filter) |
| DNA loading buffer (6x) | 0.25% bromophenol blue<br>0.25% xylencyanol<br>1 mM EDTA<br>30% glycerol |
| dNTPs | 2.5 mM and 10 mM / nucleotide |
| ELISA buffer | 4% BSA<br>0.2% Tween 20<br>5 mM $NaN_3$<br>in PBS |
| FACS buffer | 2% FCS<br>5 mM $NaN_3$<br>in PBS |
| FACS sorting buffer | 2% FCS in PBS<br>sterile filtered (0.2 µm filter) |

| | |
|---|---|
| MACS buffer | 2% FCS in PBS<br>sterile filtered (0.2 µm filter) |
| Oligo(dT) | Oligo(dT)$_{12-18}$ (0.5 µg/µl) |
| PCR buffer (10x) | 100 mM Tris-HCl, pH 8.3<br>500 mM KCl<br>15 mM $MgCl_2$ |
| Protein K solution | 10 mM Tris / HCl, pH 8.0<br>5 mM EDTA, pH 8.0<br>1 % SDS<br>300 mM sodium acetate pH 8.0<br>add fresh 200 µg/ml Proteinase K |
| RNA isolation solution | Tri Reagent (MRC, Inc., Cincinnati) |
| TAE | 40 mM Tris-acetate, pH 8.0<br>1 mM EDTA |
| TBE (10x) | 21.6 g Tris<br>11 g boric acid<br>8 ml EDTA (0.5M, pH 8.0)<br>fill up to 200 ml with $ddH_2O$ |
| TE | 10 mM Tris / HCl, pH 8.0<br>1 mM EDTA |

## 2.2 Bacterial media and supplements

| | |
|---|---|
| LB | 10 g tryptone<br>5 g yeast extract<br>10 g NaCl<br>dissolved in 1 l $H_2O$, autoclaved |
| LB agar | 15 g agar<br>10 g tryptone<br>5 g yeast extract<br>10 g NaCl |
| TBS | 10 mM $MgCl_2$<br>10 mg $MgSO_4$<br>10% PEG 4000<br>5% DMSO<br>15% glycerol<br>in LB medium<br>sterile filtered (0.2µm filter) |
| Ampicillin | sodium salt in $H_2O$<br>stock 100 mg/ml<br>used at 100 µg/ml |
| Kanamycin | stock 20 mg/ml in $H_2O$<br>used at 50 µg/ml |

## 2.3 Plasmids

| Vector | Insert | Application | Reference |
|---|---|---|---|
| pCR2.1 TA-cloning | PCR amplificates | Subcloning of PCR products with TA-overhang | Invitrogen |
| pELVC | PCR amplified V(D)J-rearrangements | $V_HD_HJ_H$ cloning, expression of H-chains in preB cell lines | ten Boekel et al., 1997 (1) |

## 2.4 Primers for PCR and RT-PCR

| Primer | Sequence (5'-3') | Application |
|---|---|---|
| VHJ558 | GCG AAG CTT ARG CCT GGG RCT TCA GTG AAG | $V_HD_HJ_H$ rearrangement |
| VHQ52 | GCG AAG CTT CTC ACA GAG CCT GTC CAT CAC | $V_HD_HJ_H$ rearrangement |
| VH7183 | GCG AAG CTT GTG GAG TCT GGG GGA GGC TTA | $V_HD_HJ_H$ rearrangement |
| VHJ606, S107, X24 | GCG AAG CTT WCT GGA GGA GGC TTG GTG CAA | $V_HD_HJ_H$ rearrangement |
| VHGAM308 | GCG AAG CTT GGA GAG ACA GTC AAG ATC TCC | $V_HD_HJ_H$ rearrangement |
| VHJ558 A.A. #1 (Sal I) | TGC GAG *GTC GAC* CTG CAA CAR TCT GGA CCT | $V_HD_HJ_H$ rearrangement |
| VHJ558 overlap | TGC AAC ART CTG GAC CTG AGC TGG TGA AGC CTG GGG CTT CAG | $V_HD_HJ_H$ rearrangement |
| VHQ52 A.A. #1 (Sal I) | TGC CAG *GTC GAC* CTG AAG SAG TCA GGA CCT | $V_HD_HJ_H$ rearrangement |

| | | |
|---|---|---|
| VHQ52 overlap | TGA AGS AGT CAG GAC CTG GCC TGG TGG CGC CCT CAC AGA GCC TGT CC | $V_HD_HJ_H$ rearrangement |
| VH7183 A.A. #1 (Sal I) | TGC GAG *GTC GAC* CTG GTG GAG TCT GGG | $V_HD_HJ_H$ rearrangement |
| VHGAM308 A.A. #1 (Sal I) | TCT CAG *GTC GAC* TTG GTG CAG TCT GGA CCT GAG | $V_HD_HJ_H$ rearrangement |
| VHGAM308 overlap | GTG CAG TCT GGA CCT GAG CTT AAG AAG CCT GGA GAG ACA GTC AAG AT | $V_HD_HJ_H$ rearrangement |
| leader of SP6 (EcoRI) | TGA *GAA TTC* TGA CTC TAA CCA TGG GAT G | Sequencing of $V_HD_HJ_H$ rearrangements |
| JH2 | TGA GGA TGT CTG TCT GCG TCA GCC AG | $V_HD_HJ_H$ rearrangement |
| JH4 | AGG CTC TGA GAT CCC TAG ACA G | $V_HD_HJ_H$ rearrangement |
| JH1A | GCA GAG TGT GGC AGA TGG CC | $V_HD_HJ_H$ rearrangement |
| JH2A | AGG TGT CCC TAG TCC TTC ATG ACC TG | $V_HD_HJ_H$ rearrangement |
| JH3A | CCA GAC CCA TGT CTC AAC TTT GGG AC | $V_HD_HJ_H$ rearrangement |
| JH4A | GGG TCT AGA CTC TCA GCC GGC TCC CTC AGG G | $V_HD_HJ_H$ rearrangement |
| JH1,2,4-Cμ (Hind III) | TTT GGG *AAG CTT* TGA CTC TCT GAG GAG ACK GTG | $V_HD_HJ_H$ rearrangement |
| JH3-Cμ (Hind III) | TGG G*AA GCT T*TG ACT CTC TGC AGA GAC AGT GAC CA | $V_HD_HJ_H$ rearrangement |
| Cμ (CH1) | AGG GGG CTC TCG CAG GAG ACG AGG | Sequencing of $V_HD_HJ_H$ rearrangements |
| RAG-2fwd | CAC ATC CAC AAG CAG GAA GTA CAC | RAG-2 transcripts in immature B cells |
| RAG-2rev | GGT TCA GGG ACA TCT CCT ACT AAG | RAG-2 transcripts in immature B cells |

| | | |
|---|---|---|
| mMD1_S | GTG GAT TCT GAC TTC TCC GAG | MD-1 transcripts in FOB cells |
| mMD1_AS | AGC CAC AGT AGC ACG GTT T | MD-1 transcripts in FOB cells |
| RP105_S | AGT CTC CTC CCC ATC TTG TCC | RP105 transcripts in FOB cells |
| RP105_AS | GAT AGC GTC ACA TCG GAG AGC | RP105 transcripts in FOB cells |
| mCD14-S317 | GCG GAT TCC TAG TCG GAT TC | CD14 transcripts in FOB cells |
| mCD14-AS951 | GGT TCC TAT CCA GCC TGT TG | CD14 transcripts in FOB cells |
| 5'TLR4 | ACA CCA GGA AGC TTG AAT CC | TLR4 transcripts in FOB cells |
| 3'TLR4 | GCA CTC ATA ATG ATG GCA CC | TLR4 transcripts in FOB cells |
| 5'TLR9 | AAG GTC TGG TCA ACC TCT CG | TLR9 transcripts in FOB cells |
| 3'TLR9 | GAA TGT CAT TGT GTG CCA GG | TLR9 transcripts in FOB cells |
| 5_Vlambda1 | GGG TAT GCA ACA ATG CGC ATC TTG TC | λ1 light chain hypermutations |
| 3_Vlambda1 | CAC GGA CAG GAT CCT AGG ACA GTC AGT TTG GT | λ1 light chain hypermutations |
| 5_Vlambda1_nested | GCG AAG AGA AGC TTG TGA CTC AGG AAT CTG CA | λ1 light chain hypermutations |
| M13/pUC(-40) | GTT TTC CCA GTC ACG AC | Sequencing |
| M13/pUCrev(-24) | AAC AGC TAT GAC CAT G | Sequencing |

Primers were obtained from Microsynth GmbH, Switzerland or from QIAGEN, Germany. Working concentration: 10 μM; restriction sites in *italics*.

## 2.5 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 |
| QIAfilter Plasmid Midi Kit | QIAGEN |
| QIAprep Spin Miniprep Kit | QIAGEN |
| QIAquick Gel Extraction Kit | QIAGEN |
| QIAquick PCR Purification Kit | QIAGEN |
| Roche Extended PCR kit | Roche |
| SuperScript First-Strand Synthesis System for RT-PCR | Invitrogen |

## 2.6 Cell culture media and supplements

| | | |
|---|---|---|
| SF-IMDM (10 l) | 176.6 g IMDM powder<br>30.24 g $NaHCO_3$<br>100 ml Penicilin Streptomycin Solution<br>100 ml MEM Non essential amino acids<br>10 ml Insulin [5µg/ml final conc.]<br>10 ml 2-mercapto-Ethanol [50µg/ml final conc.]<br>30 ml Primatone RL [0.03% final conc.]<br>fill up to 10 l with double-quartz dist. $H_2O$ (25°C)<br>adjust to pH 7.0 (NaOH)<br>sterile filtered (0.2µm filter) | Gibco BRL<br>Fluka<br>Gibco BRL<br>Gibco BRL<br>Sigma<br>Fluka<br>Quest |
| BrdU | 5-bromo-2-deoxyuridine | Sigma |
| CFSE | 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester | Molecular Probes |
| Ciproxin | Ciprofloxacinum 0.2g/100ml | Bayer |

| | | |
|---|---|---|
| CpG | CpG1826<br>T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*<br>G*T*T (* thiolate modification) | Microsynth GmbH |
| DMEM | Dulbecco's Modified Eagle's Medium | Gibco BRL |
| FCS | Fetal calf serum, mycoplasma and virus screened | Amimed |
| Freezing medium | 10% DMSO in FCS | |
| HAT medium | 1 l SF-IMDM medium<br>20 ml FCS<br>5 ml Ciproxin<br>2 x HAT Media Supplement (50x) (Sigma)<br>10 ml IL-6 supernatant (in-house)<br>sterile filtered (0.2µm filter) | |
| IL-4 | Interleukin-4 (IL-4 producing X63 cells) 0.2 mM | In-house |
| IL-6 | Interleukin-6<br>IL-6 producing X63 cells (X63-IL6) cultured in SF-IMDM 2% FCS medium for 5-7 d, suspension sterile filtered through diamotaceous earth (Highflow Super Cell Medium, Fluka). Resulting supernatant stored at 4°C. | In-house |
| IMDM | Iscove's Modified Dubecco's Medium | Sigma |
| Lipofectamine + PLUS | Lipofectamine and PLUS reagents | Invitrogene |
| LPS | Phenol-extracted lipopolysaccharide | Sigma |
| PBS (10x) | 80 g NaCl<br>27.1 g $Na_2HPO_4$ dihydrate<br>2 g $KH_2PO_4$<br>2 g KCl<br>dissolved in 1 l $H_2O$, adjust to pH 7.3 (HCl) | University Hospital Basel |

| | | |
|---|---|---|
| PEG 1500 | PEG 1500, 50%, sterile solution | Boehringer Mannh. |
| Puromycin | Aminonucleodise antibiotic | Gibco BRL |
| RPMI | 'Roswell Park Memorial Institute' Medium | Sigma |
| Trypsin solution | 1x Trypsin-EDTA in HBSS | Gibco BRL |

## 2.7 Bacterial strains

| **Host strain** | **Genotype** | **Reference** |
|---|---|---|
| E. coli XL2-Blue | endA1 gyrA96(nal$^R$) thi-1 recA1 relA1 lac glnV44 F'[ ::Tn10 proAB$^+$ lacI$^q$ Δ(lacZ)M15 Amy Cm$^R$] hsdR17($r_K^-$ $m_K^+$) | Stratagene |
| TOP10 | F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str$^R$) endA1 λ$^-$ | Invitrogen; Grant et al., 1990 (2) |

## 2.8 Mouse strains

| **Strain** | **Description** | **Source** |
|---|---|---|
| BALB/b | H2$^b$ | In-house |
| BALB/c | H2$^d$ | RCC Ltd., Füllinsdorf |
| C57BL/6 | H2$^b$ wild type mice | RCC Ltd., Füllinsdorf |
| F1 (BALB/c x C57BL/6) | | In-house |
| F2 BALB/c x (BALB/c x C57BL/6) | | In-house |
| F2 C57BL/6 x (BALB/c x C57BL/6) | | In-house |
| BAFF tg | transgenic for BAFF gene under CMV promoter | In-house |
| Bcl-2 tg | transgenic for Bcl-2 gene under H2K$^b$ promoter | Dr. A. Trump, ISREC, Lausanne |

| | | |
|---|---|---|
| $CD40^{-/-}$ | deficient for CD40 | In-house |
| $\lambda5^{-/-}$ | $F_2$16B/B5T$^{+/+}$F3 Homo, deficient for surrogate light chain protein λ5 | In-house |
| Balb/c $MyD88^{-/-}$ | deficient for TLR-signalling pathway protein MyD88 | Prof. R. Landmann, University Hospital, Basel |
| C57BL/6 $MyD88^{-/-}$ | deficient for TLR-signalling pathway protein MyD88 | Prof. R. Landmann, University Hospital, Basel |
| B1-8Hi; 3-83κi | knock-in mouse for B1-8 HC and 3-83 κ-LC | In-house |

Inbred strains were provided by RCC Ltd. Füllinsdorf. Transgenic, inter- and backcrossed animals were bred and kept in the animal facilities of the Biocenter or the Center for Biomedical Research, University of Basel, Basel. The $MyD88^{-/-}$ -deficient animals were kindly provided by the group of Prof. Regine Landmann, University Hospital, Basel.

## 2.9 Cell lines

| **Cell line** | **Properties** | **Reference** |
|---|---|---|
| 40E1 | Abelson virus-transformed pre-B cell line | Warner et al., 1979 (3) |
| Phoenix-E | ecotropic retroviral packaging cell line | Pear et al., 1993 (4) |
| Sp2/0 (Sp2/0-Ag14) | mouse myeloma ATCC CRL-1581 | Shulman et al., 1978 (5) |

## 2.10 Antibodies and recombinant proteins

### 2.10.1 Antibodies for FACS analysis and sorting

| Antigen (mouse) | Clone | Provider | Reference |
|---|---|---|---|
| CD4 | RM4-5 | BD Pharmingen | |
| CD5 | 53-7.3 | BD Pharmingen | Ledbetter et al., 1980 (6) |
| CD8 (α-chain) | 53-6.7 | BD Pharmingen | |
| CD11b (Mac-I) | M1/70 | BD Pharmingen | Springer et al., 1978 (7) |
| CD14 | Sa2-8 | eBioscience | Akashi et al., 2003 (8) |
| CD19 | 1d3 | In-house<br>BD Pharmingen | Krop et al., 1996 (9) |
| CD21 (CR2/CR1) | 7G6 | BD Pharmingen | Kinoshita et al., 1988 (10) |
| CD23 (FcεRII) | B3B4 | In-house<br>BD Pharmingen | Rao et al., 1987 (11) |
| CD24 (HSA) | M1/69 | BD Biosciences | Alterman et al., 1990 (12) |
| CD25 (α-chain) | 7D4 | BD Pharmingen | Dantal et al., 1991 (13) |
| CD45R (B220) | RA3-6B2 | BD Pharmingen | Coffman et al., 1982 (14) |
| CD44 | IM7 | BD Biosciences | Trowbridge et al., 1982 (15) |
| CD62L | MEL-14 | BD Pharmingen | Gallatin et al., 1983 (16) |
| CD69 | H1.2F3 | BD Pharmingen | Yokoyama et al., 1988 (17) |
| CD93 (C1qRp) | PB493 | In-house | Rolink et al., 1998 (18) |
| CD117 (c-kit) | ACK4 | In-house | Ogawa et al., 1991 (19) |
| IgM | M41 | In-house | Leptin et al., 1985 (20) |
| IgM<br>(Cμ-chain specific) | | Jackson | Jackson ImmunoResearch |
| IgD | 1.19 | In-house | Parkhouse et al., 1992 (21) |
| BAFF-R | 7A5, B9B | In-house | Thesis M. Rauch, 2009 |
| BrdU | B44 | BD Pharmingen | Gratzner et al., 1982 (22) |

| | | | |
|---|---|---|---|
| κ-LC | 187.1 | In-house | Yelton et al., 1981 (23) |
| $\lambda_{1+2}$-LC | R26-46 | BD Pharmingen | |
| TLR4 / MD-2 | MTS510 | eBioscience | Akashi et al., 2000 (24) |
| CD180 (RP105) | RP/14 | eBioscience | Miyake et al., 1994 (25) |
| MD-1 | MD113 | eBioscience | Nagai et al., 2002 (26) |
| NK1.1 | PK 136 | BD Pharmingen | Koo et al., 1984 (27) |

In-house produced antibodies were individually titrated before use. Commercial antibodies were used at a final dilution of 1:200 (α-BrdU 1:30).

### 2.10.2 Secondary and other FACS reagents

| **Reagent** | **Dilution** | **Provider** |
|---|---|---|
| Streptavidin-PE | 1:1000 | Southern Biotech |
| Streptavidin-APC | 1:400 | BD Biosciences |
| Streptavidin-PE-Cy7 | 1:400 | BD Biosciences |
| Propidium iodide | 0.2 µg/ml | BD Biosciences |

### 2.10.3 Antibodies for functional assays

| **Antigen** | **Clone** | **Properties** | **Provider** | **Reference** |
|---|---|---|---|---|
| CD3 | 142-2c11 | | BD Pharmingen | |
| CD40 | FGK45.5 | | In-house | Rolink et al., 1996 (28) |
| IgM | M41 | Rat anti-mouse, µ-chain specific, biotin | In-house | Leptin et al., 1985 (20) |
| IgM | | Cµ-chain specific, FITC | Jackson ImmunoResearch | |
| κ-LC | 187.1 | | In-house | |

| | | | | |
|---|---|---|---|---|
| rat-IgG | | | Jackson ImmunoResearch | |

### 2.10.4 MACS beads

| **Antigen** | **Dilution** | **Provider** |
|---|---|---|
| B220 | 1:100 | Miltenyi Biotec |
| Phycoerythrin (PE) | 1:100 | Miltenyi Biotec |

## 2.11 Molecular biology methods

### 2.11.1 Agarose gel electrophoresis of DNA fragments

Linearized plasmid DNA and DNA fragments were separated for analytical and preparative purposes by gel electrophoresis using 0.75% - 1.5% (w/v) agarose gels depending on fragment size range in 1 x TAE buffer. For DNA staining the gels contained 0.1µg/ml ethidium bromide. Samples were loaded in 6x DNA loading buffer. Electrophoresis was performed at 3-5 V/cm.

### 2.11.2 Preparation of chemical-component *E. coli* strains

100 ml of LB medium were inoculated with 1 ml of a fresh overnight culture of bacteria. Bacteria were grown to early-log phase ($OD_{600}$ = 0.3 – 0.6) at 37°C shaking. The culture was centrifuged for 20 minutes (1500 g, 4°C). The bacteria pellet was then resuspended in 10 ml cold, sterile TBS medium, aliquoted and immediately shock frozen in liquid nitrogen. Chemical-competent bacteria were stored at -80°C.

### 2.11.3 Transformation of chemical-competent *E.coli* strains

Chemical-competent *E. coli* bacteria were thawed on ice. For each transformation 100 µl of bacteria were mixed with 1 µl of mini-prep plasmid DNA or 1 – 10 µl of ligation reaction and incubated for 30 minutes on ice. The heat-shock reaction was done for 90 second at 42°C and followed by chilling for 2 minutes on ice. Then 900 µl LB was added and the bacteria incubated for 1 h at 37°C. Afterwards 100 µl of bacteria were plated on selecting agar plates. After overnight growth at 37°C, colonies were picked and expanded in selecting LB medium. Mini-prep DNA was prepared and digested with appropriate restriction enzymes to test for correct products.

### 2.11.4 Restriction enzyme digestion of DNA

Restriction enzyme digest was carried out in 10 µl (analytical digests) or in 20 - 50 µl volume (preparative digests) with enzyme buffers and conditions recommended by the manufacturer (NEB-

Biolabs, Sure Roche Diagnostics). Preparative digests were performed for 2 hours and analytical digests were done for at least 1 hour.

### 2.11.5 Purification of DNA from agarose gels

Agarose gels were examined under UV light (366 nm) and the bands with size of interest excised. UV exposure was kept short do avoid damage to the DNA. Purification was then done with the QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's protocol.

### 2.11.6 Phosphatase treatment of linearized vector prior to insert ligation

To prevent self-religation of linearized vectors, the plasmid DNA was treated with 1 U of calf intestine alkaline phosphatase for 30 minutes at 37°C to remove 5' phosphates. The alkaline phosphatase was inactivated by heating the reaction mix to 75°C for 10 minutes and removed by gel electrophoresis and thereby the dephosphorylated vector was gel-purified.

### 2.11.7 DNA ethanol precipitation

Plasmid and genomic DNA were precipitated by adding 1/10 volume 3 M sodium acetate (ph 4.8) and 2 volumes of 100% ethanol. The mixed DNA solution was then incubated for 20 minutes at -20 °C followed by 15 minutes centrifugation (21'000 g, 4°C). The precipitated DNA was then washed with ice-cold 70% ethanol and air-dried. DNA was dissolved in 1 x TE buffer.

### 2.11.8 Ligation of DNA fragments into vectors

#### *2.11.8.1 Restriction enzyme fragments*

Ligation of restriction enzyme fragments with cohesive ends were performed at a molecular ratio between vector and insert of 1:2-4 in a volume of 20 µl using 1 µl of T4 DNA ligase (400U/µl). The reaction was done overnight at 16°C. 1 -10 µl of the ligation reaction was used to transform chemical-competent bacteria.

#### *2.11.8.2 PCR fragments*

PCR fragments amplified by use of the Taq DNA polymerase were directly cloned into the pCR2.1-Vector or TOPO-TA vector (TA Cloning kit, TOPO-TA Cloning kit, Invitrogen) according to the manufacturer's protocol.

### 2.11.9 DNA phenol-chloroform extraction

To remove protein and other impurities from DNA a phenol-chloroform extraction was performed. For this 1 volume pure phenol (TE saturated) was added, the sample mixed and spun for 10 minutes (21'000 g, 4°C). The DNA-containing water-phase was transferred into a new tube and extracted with 1 volume of 1:1 mixture of phenol and chloroform followed by a chloroform extraction at conditions as in the phenol extraction step. The DNA was precipitated by adding 1/10 volume 3 M sodium acetate (pH 4.8) and 1 volume isopropanol and let incubate at -20°C for 20 minutes followed by centrifugation for 20 minutes (21'000 g, 4°C). The precipitated DNA was then washed with ice-cold 70% ethanol and air-dried. DNA was dissolved in 1 x TE buffer.

### 2.11.10 Polymerase chain reaction (PCR)

#### *2.11.10.1 Analytical PCR*

For analytical purposes, PCR reactions were carried out with *Taq* DNA polymerase based on the following standard protocol:

PCR reaction mix:

| | |
|---|---|
| 1-5 µl | DNA template |
| 1 µl | forward primer |
| 1 µl | reverse primer |
| 2 µl | 10x PCR buffer |
| 3 µl | dNTPs (2.5 mM stock conc.) |
| 0.5 µl | *Taq* DNA polymerase |

filled up to 20 µl with $ddH_2O$

PCR program:

| | | | |
|---|---|---|---|
| initial denaturation | 94°C | 90” | |
| denaturation | 94°C | 30” | 38 cycles |
| annealing | $T_m$-3°C | 30” | |
| elongation | 72°C | 1 min/kb | |
| final elongation | 72°C | 10’ | |

($T_m$: primer melting temperature)

### *2.11.10.2 PCR for the amplification of $VJ558_HD_HJ_H$- and $V7183_HD_HJ_H$ rearrangements*

$V_HD_HJ_H$-rearrangements were amplified from purified genomic DNA of immature and mature B cells by a 2-step PCR strategy. In the first round of amplification a $V_H$-family specific primer was used together with a 3’ primer hybridizing immediately downstream to a $J_H$ gene element. In the second round of amplification the PCR product was elongated at the 5’ end with a second $V_H$-family-specific primer including a *SalI* restriction site and a nested 3’ primer containing the beginning of the Cμ gene element including a silent *HindIII* restriction site in the third and fourth codons of the Cμ region.

**1. round PCR:**

Primers:

forward: VHJ558 or VH7183

reverse: JH2

PCR reaction mix:

| | |
|---|---|
| 1 µl | DNA template (purified by phenol-chloroform extraction) |
| 1 µl | 5’ primer |
| 1 µl | 3’ primer |
| 3 µl | 10x Reaction buffer 1 (Roche Extended PCR kit) |
| 6 µl | dNTPs (2.5 mM stock conc.) |
| 0.5 µl | DNA Polymerase mix (Roche Extended PCR kit) |

filled up to 30 µl with $ddH_2O$

PCR program:

| | | | |
|---|---|---|---|
| initial denaturation | 94°C | 90” | |
| denaturation | 94°C | 30” | 38 cycles |
| annealing | 65°C | 30” | |
| elongation | 68°C | 2’ | |
| final elongation | 68°C | 10’ | |

## 2. round PCR:

Primers:

forward: VHJ558 A.A. #1 (Sal I) + VHJ558 overlap ratio 10:1 or
VH7183 A.A. #1 (Sal I)

reverse: JH1,2,4-Cμ (Hind III)

PCR reaction mix:

| | |
|---|---|
| 1 µl | DNA template (gel-purified from 1. round PCR) |
| 1 µl | 5’ primer |
| 1 µl | 3’ primer |
| 5 µl | 10x Reaction buffer 1 (Roche Extended PCR kit) |
| 7 µl | dNTPs (2.5 mM) |
| 0.75 µl | DNA Polymerase mix (Roche Extended PCR kit) |

filled up to 50 µl with $ddH_2O$

PCR program:

| | | | |
|---|---|---|---|
| initial denaturation | 94°C | 60” | |
| denaturation | 94°C | 30” | 5 cycles |
| annealing | 50°C | 30” | |
| elongation | 68°C | 45” | |
| denaturation | 94°C | 30” | 10 cycles |
| annealing | 55°C | 30” | |
| elongation | 68°C | 45” | |
| denaturation | 94°C | 30” | 20 cycles |
| annealing | 60°C | 30” | |
| elongation | 68°C | 45” | |

### *2.11.10.3 PCR for the amplification of λ1 light chain-rearrangements*

λ1 light chain-rearrangements were amplified from genomic DNA of sorted cells by a semi-nested PCR as described by Jacobs et al. (29).

**1. round PCR:**

Primers:

forward: 5'_Vλ1

reverse: 3'_Jλ1

PCR reaction mix:

| | |
|---|---|
| 1 µl | genomic DNA |
| 1 µl | 5' primer |
| 1 µl | 3' primer |
| 3 µl | 10x PCR buffer |
| 5 µl | dNTPs (2.5 mM) |
| 0.5 µl | *Taq* DNA Polymerase |

filled up to 30 µl with $ddH_2O$

PCR program:

| | | | |
|---|---|---|---|
| initial denaturation | 95°C | 2' | |
| | 88°C | 3' | |
| | 60°C | 2' | |
| | 72°C | 2' | |
| denaturation | 94°C | 1' | 29 cycles |
| annealing | 60°C | 1' | |
| elongation | 72°C | 1'30" | |
| final elongation | 72°C | 10' | |

**2. round PCR:**

Primers:

forward: 5'_Vλ1_nested

reverse: 3'_Vλ1

PCR reaction mix:

| | |
|---|---|
| 1 µl | Template DNA (gel-purified from 1. round PCR) |
| 1 µl | 5' primer |
| 1 µl | 3' primer |
| 3 µl | 10x PCR buffer |
| 5 µl | dNTPs (2.5 mM) |
| 0.5 µl | *Taq* DNA Polymerase |

filled up to 30 µl with $ddH_2O$

PCR program:

| | | | |
|---|---|---|---|
| initial denaturation | 92°C | 1' | |
| denaturation | 92°C | 1' | 40 cycles |
| annealing | 63°C | 1' | |
| elongation | 72°C | 1'30" | |
| final elongation | 72°C | 10' | |

## 2.11.11 Sequencing of plasmid DNA

Sequencing of plasmid DNA was performed by dye-terminator sequencing on an automated gel electrophoresis sequencer (ABI PRISM 377 DNA Sequencer).

The sequencing gel was prepared by dissolving 18 g urea and 7.5 ml Acrylamid/Bis (30%, 37.5:1) in 23 ml $ddH_2O$. The gel mix was then de-ionized by adding a spoon of amberlite and stirring it for 20 min at RT. 200 ml of freshly prepared 10x TBE buffer were sterile filtered (22 µm filter). The gel mix was filtered through the same filter, and 6 ml of the 10x TBE buffer were added. The gel mix was then degassed for 5 min and cooled on ice. The glass plates of the sequencer were washed with Biorad cleaning solution and rinsed with a 9:1 isopropanol-water mixture. The readily cleaned glass plates were assembled with 0.2 mm spacers defining the pouring chamber. The polymerization of the gel was initiated by adding 200 µl APS (10%) and 20 µl TEMED to the gel mix. Immediately afterwards, the gel was poured. Polymerisation was allowed for 2 h at RT. Subsequently, the gel was mounted into the sequencer and the rest of the 10x TBE buffer diluted to 1x TBE and added to the corresponding tanks of the sequencer as running buffer.

The necessary sequencing product was obtained by PCR from sample DNA incorporating fluorescent terminator dideoxynucleotides (Big Dye, Perkin-Elmer). The reaction was performed according to the following standard protocol:

Sequencing reaction mix:

| | |
|---|---|
| 3 µl | Sample DNA (0.5-1 µg, mini-prep) |
| 0.35 µl | primer (3.5 pmol/µl) |
| 4 µl | Big Dye sequencing mix |
| filled up to 20 µl with $ddH_2O$ | |

Sequencing reaction standard program:

| | | | |
|---|---|---|---|
| initial denaturation | 96°C | 1’ | |
| denaturation | 96°C | 30” | 35 cycles |
| annealing | 50°C | 20” | |
| elongation | 60°C | 4’ | |
| final elongation | 60°C | 5’ | |

The samples were precipitated by adding 2 µl 3 M sodium acetate and 50 µl 100% ethanol. After incubation at RT for 20 minutes, the samples were centrifuged for 20 minutes (21’000 g, RT). The precipitate was washed with 150µl 70% ethanol, centrifuged, dried for 5 minutes in a Speed-Vac at RT and resuspended in 3.5 µl ABI loading buffer. Prior to loading on the gel, the samples were denatured at 95°C for 3 minutes. 1 µl of each sample was loaded on the gel. Sequencing runs were done for 10 h at 2400 V. Sequence readout was performed using the ABI PRISM 377XL Data Collection software (version 2.6).

## 2.11.12 Isolation of genomic DNA

### *2.11.12.1 <100’000 cells / sample*

Cells were resuspended in 50 µl PBS, snap freezed and stored at -70°C prior to genomic DNA preparation. The frozen cells were heated for 1 minute to 68°C and immediately chilled on ice. Then 5 µl proteinase K (10 µg/µl) were added and the cells incubated for 2 hours at 55°C followed

by a heat-inactivation of the proteinase K for 10 minutes at 94°C. The lysate was directly used for PCR or stored at -20°C.

#### *2.11.12.2 >100'000 cells / sample*

Cells were incubated in 400 µl of proteinase K solution overnight at 55°C shaking. To remove protein and other impurities from DNA a phenol-chloroform extraction was performed. 400 µl pure Phenol (TE saturated) was added to the cell lysate, the sample mixed and spun for 10 minutes (21'000 g, 4°C). The DNA-containing water-phase was transferred into a new tube and extracted with 200 µl phenol and 200 µl chloroform followed by an extraction with 400 µl chloroform at conditions as in the phenol extraction step. The DNA was precipitated by adding 400 µl isopropanol and let incubate at -20°C for 20 minutes followed by centrifugation for 20 minutes (21'000 g, 4°C). The precipitated DNA was then washed with ice-cold 70% ethanol and air-dried. DNA was dissolved in 50 µl 1 x TE buffer for 30 minutes at 55°C.

### 2.11.13 Isolation of total RNA

Sorted cells up to 3 x $10^6$ cells where resuspended in 500 µl Trizol and incubated for 5 minutes at RT. Then 100 µl $CHCl_3$ were added, the sample vigorously shaken during 15 seconds and let stand for 10 minutes at RT. After centrifugation for 15 minutes (12000 g, 4°C) the aequous phase was transferred into a fresh tube. Then 1 µl of linearized polyacrylamide and 250 µl isopropanol were added and let the total RNA precipitate for 10 minutes. After that the sample was centrifuged for 8 minutes (12000g, 4°C), the pellet washed with 500 µl 75% ethanol, again centrifuged for 5 minutes (12000 g, 4°C). Finally, the pellet was air-dryed for 5 minutes and resuspended in 10 µl DEPC-treated dd$H_2O$. The RNA was stored at -80°C.

### 2.11.14 cDNA by reverse transcription for semi-quantitative PCR

Normally, half of the isolated RNA was used for reverse transcription. The reverse transcription reaction mix was incubated for 30 minutes at 37°C and then the RQ DNase 1 was inactivated for 10 minutes at 65°C. Before adding the reverse transcriptase, the sample was quickly chilled on ice

and 20 µl removed for a negative control. For the reverse transcription 0.5 µl RNase OUT and 1 µl Superscript II reverse transcriptase (200U/µl) were added.

Reverse transcription reaction mix:

| | |
|---|---|
| 5 µl | RNA in DEPC-treated $ddH_2O$ |
| 6 µl | 10 x reverse transcriptase buffer |
| 12 µl | $MgCl_2$ (25 mM) |
| 4 µl | dNTPs (10 mM) |
| 4 µl | DTT (0.1 M) |
| 2 µl | $Oligo(dT)_{12-18}$ (0.5 µg/µl) |
| 0.4 µl | random hexamers (50 ng/µl) |
| 2 µl | RQ DNase 1 |
| 21.6 µl | DEPC-treated $ddH_2O$ |

Reverse transcription reaction:

Add 1 µl Superscript II reverse transcriptase (200 U/µl) and 0.5 µl RNase OUT

| | |
|---|---|
| 25°C | 8’ |
| 65°C | 1h 30’ |
| 70°C | 15’ |

The cDNA was stored at -20°C. The cDNA was tested in a PCR reaction for β-actin transcripts and the negative control was used to test for the absence of genomic DNA contaminations.

## 2.12 Cell culture

### 2.12.1 Cryopreservation of cells

For cryopreservation pelleted cells were resuspended in cold (+4°C) freezing medium at a cell density dependent on the cells. 1 ml aliquots in 2 ml cryotubes (Nunc) were cooled down on ice and incubated at -80°C for >12 h. For longterm storage the aliquots were stored in a liquid nitrogen tank.

For thawing, cells were warmed up in a 37°C water bath or at RT and transferred immediately to 10 ml of the appropriate cell culture medium. After centrifugation at 1200 rpm at 4°C for 10 min, the cells were gently resuspended in the appropriate cell culture medium and transferred into a tissue culture dish.

### 2.12.2 Determination of cell numbers

Cell numbers were determined by manually counting cells in an improved Neubauer haemocytometer with 0.1 mm depth. An aliquot of the cell suspension was diluted 1:5-1:10 in trypan blue solution and loaded into the chamber. Cells in at least two of the outer squares, consisting of 16 small squares each, were counted. Debris and dead cell were excluded by the trypan blue dye. The cell number per ml was calculated from the average cell number in an outer square times $10^4$.

### 2.12.3 Isolation of mouse bone marrow cells

Bone marrow cells were collected from femur and tibea. Bones were separated from muscle tissue and opened on both ends followed by flushing out the bone marrow with cell culture medium and a siringe with a 27G needle. Cell suspensions were achieved by carefully pipetting the flushed out marrow up and down.

### 2.12.4 Lysis of red blood cells

Cell suspensions containing erythrocytes were centrifuged for 10 minutes at 250 g, 4°C. Cells were resuspended in 1-2 ml ACK buffer and incubated for 1-2 minutes at RT. The lysis reaction was stopped by adding 10 ml cell culture medium to the cells. After that the cells were centrifuged again and then resuspended in cell culture medium.

### 2.12.5 Surface staining of cells for FACS analysis

Normally cell suspensions of 10-20 x $10^6$ cells/ml were prepared. The antibodies used were either directly labeled with a fluorescent dye or the antibodies were biotinylated and revealed by a fluorescent dye-labeled streptavidin reagent. The appropriate antibody dilutions had been titrated on appropriate cells beforehand. Commercial antibodies were mostly used at a final dilution of 1:200 in FACS buffer for FACS analysis or in cell culture media or Sorting buffer, respectively, for FACS sorting.

For analytical staining 50 µl of cell suspensions were mixed with 50 µl of prediluted antibodies in FACS buffer in U-bottom 96-well plates. The cell suspensions were incubated for at least 30 minutes on ice or at 4°C, protected from light. After the incubation the cells were washed with FACS buffer, resuspended in 200 µ l FACS buffer and analysed on a FACS Calibur (BD Biosciences). If possible (FL3 not used for staining) dead cells were excluded from the analysis using propidium iodide [0.2 µg/ml]. The collected data was either analysed in CellQuest Pro v3.4 (BD Biosciences) or in FlowJo v6.4 (Tree Star) software.

For preparative cell sorts fluorescent dye-coupled or biotinylated antibodies were directly diluted in single cell suspensions. Sorting was performed on a 2-Laser FACS Aria cell sorter with FACSDiva Software (BD Biosciences).

### 2.12.6 Surface staining for pre-BCR expression on 40E1 cells (pairing assay)

Due to the relatively low density of pre-BCR expression on the cell surface a FACS staining was established using an amplifying staining protocol by means of α-$IgM^{bio}$ (1:800) in combination with the anti-isotype antibody mouse-α-rat $IgG^{bio}$ (1:800) and strep-PE (1:1000). Incubation and washes were otherwise done as for normal surface stainings. Dead cells were excluded from the analysis using propidium iodide [0.2 µg/ml].

### 2.12.7 Intracellular staining of cells for FACS analysis

1-2 x $10^6$ cells were fixed in 600 µl of 2% paraformaldehyde in 1xPBS for 30 minutes at 4°C. After fixation the cells were washed twice with 1xPBS (centrifugation 10 minutes at 290 g) and resuspended in 200 µl FACS buffer + 2% saponin. The cells were distributed in half and to one

tube directly labeled antibody in FACS buffer + 2% saponin added (final conc.: 1:200 in 200 µl). After incubation for 45 minutes at 4°C the cells were washed once with FACS buffer + 0.2% saponin and once with FACS buffer, resuspended in 200 µl FACS buffer and analysed on a FACS Calibur (BD Biosciences).

### 2.12.8 Purification of newB cells from mouse spleens

Single cell suspensions from spleens were made. After erythrocyte-lysis splenocytes were stained with α-NK1.1$^{PE}$, α-CD93$^{bio}$ and α-CD5$^{PE}$ for 30' at 4°C. Cells were washed and resuspended in FACS-sort buffer and incubated 15' at 4°C with streptavidin-coupled phyco-erythrin (strep-PE 1:2000). Again the cells were washed and resuspended in FACS-sort buffer with anti-PE MACS beads (final conc.: 1:10) and incubated for 45' at 4°C. After washing the cells were resuspended in FACS-sort buffer, filtered and loaded twice on pre-equilibrated MACS columns (model LS). The columns were washed with FACS-sort buffer and the total efflux collected and stained with α-B220$^{PE-Cy7}$, α-CD21$^{FITC}$ and α-CD23$^{PE}$ for 30' at 4°C. After washing and resuspending in FACS-sort buffer the cells were ready to be sorted by FACS. NewB cells were sorted as B220$^{+}$ CD21$^{-/low}$ CD23$^{-/low}$ cells.

### 2.12.9 $Ca^{2+}$ flux measurement

Single cell suspensions from spleens were made. After erythrocyte-lysis the cells were resuspended in IMDM+2% FCS to 1 x $10^7$ cells/ml. Then the cells were loaded with 5 µg/ml of the $Ca^{2+}$-sensitive Indo-1 dye for 45 minutes at 37°C. After the Indo-1 loading the cells were stained in IMDM+2% FCS with fluorescent dye-labeled antibodies for surface markers for 45 minutes on ice. For labeling with fluorescent dye-labeled streptavidin reagent the cells were washed and labeled in D-PBS ($CaCl_2$ / $MgCl_2$). After the surface labeling the cells were resuspended in 1 ml IMDM+2% FCS. The $Ca^{2+}$ flux measurement was done on a 4-Laser FACS LSRII (BD Biosciences). After establishing of the baseline fluorescence, cells were triggered with 50 µg/ml α-IgM (µ-chain specific) or 7.5 µg/ml α-CD3 (clone 2c11). As a positive control of Indo-1-loading cells were triggered with 5 µg/ml of the ionophore ionomycin.

### 2.12.10 Intracellular anti-BrdU staining

Cells sorted for the appropriate surface phenotype were fixed ON in 70% ethanol at 4°C. After the fixation the cells were washed 3 times in 1xPBS (centrifugation 6 minutes at 2500 g) and then resuspended in 500 µl 3 M HCl 0.5% Tween20 for 20 minutes at RT. Then the cells were spun down (6 minutes at 2500 g) and resuspended in neutralizing 250 µl 0.01 M sodium tetraborat for 3 minutes at RT. After two washes with 1xPBS 0.5% Tween20 (6 minutes at 2500 g) the cells were resuspended in 200 µl with 1xPBS 0.5% Tween20, distributed in half and to one tube α-BrdU-FITC antibody added (final conc.: 1:10). After incubation for 20 minutes at 4°C the cells were washed with 1xPBS (6 minutes 2500 g) and resuspended in 250 µl PBS for FACS analysis.

### 2.12.11 *In vitro* CFSE-labeling of sorted cells

Sorted cells from bone marrow or spleen were washed with 1xPBS to get rid of proteins and resuspended in 500 µl PBS with CFSE (Carboxyfluorescein succinimidyl ester). The cells were incubated for 10 minutes at RT and then washed with cell culture medium. After that the cells were resuspended in cell culture medium at the appropriate concentration and used for *in vitro* cell culture assays. At the end of the assay the cells were analysed on a FACS Calibur or sorted on a FACS Aria cell sorter (BD Biosciences).

### 2.12.12 Transfection and transduction

#### *2.12.12.1 Transfection of packaging cell line Phoenix-E*

Cells of the packaging cell line Phoenix-E were seeded at 2.5 x $10^5$ cell/6-well in RPMI + 10% FCS and incubated at 37°C 5% $CO_2$ for two days before the transfection (semi-confluent). For the transfection, 2µg of plasmid DNA with 10 µl PLUS reagent and 100 µl DMEM medium were mixed and incubated for 15 minutes at RT and then 2.5 µl Lipofectamine and 100 µl DMEM medium were added, mixed and incubated for further 15 minutes. Immediately before the transfection the cells were washed twice with serum-free DMEM medium and kept in 1 ml serum-free DMEM medium. Then the transfection mix was drop-wise added to the cells. The cells were incubated with the transfection mix for 5–6 hours at 37°C 10% $CO_2$. After that, the supernatant was replaced with 3 ml complete RPMI medium with 10% FCS and incubated for 24 hours at 37°C 5%

$CO_2$. To remove incomplete viral particles, which are generated within the first hours after the transfection, the supernatant was carefully aspirated and replaced with fresh 3 ml complete RPMI + 10% FCS and the cultures incubated for a further 24 hours at 37°C 5% $CO_2$. The supernatant was finally collected and used for the retroviral transduction of the pre-B cell line 40E1 by spin-infection.

#### *2.12.12.2 Retroviral transduction of the pre-B cell line 40E1 by spin-infection*

Collected virus-containing supernatant was shortly spun down in order to remove cell debris. Semi-confluent cells of the pre-B cell line were harvested and counted. 5 x $10^4$ cells were transfered into 2 ml Eppendorf-tubes and 1 ml retroviral supernatant added. The spin-infection was then carried out in a table-top Eppendorf centrifuge for 3–4 hours at 1160 g and 30°C. After that, the supernatant was carefully aspirated and the cells plated in 2 ml SF-IMDM + 2% FCS in a 6-well plate. The cells were incubated at 37°C 10% $CO_2$ for 1-2 days. Then the cells were selected for successful transduction in 6 ml cultures with 2 µg/ml puromycin and expanded.

### 2.12.13 *In vitro* proliferation assays

Cells sorted for the appropriate surface phenotype were cultured in flat-bottom 96-well plates at 1 x $10^5$ cells/well in SF-IMDM + 2% FCS. The cultures were supplemented either with a single stimulus or a combination of 25 µg/ml α-IgM (M41), 20 µg/ml α-CD40 (clone FGK45.5), 20 µM IL-4 (clone X63), 1-50 µg LPS (phenol-extracted) and 0.5 µg/ml CpG (CpG1826). After 63 hours cultures were pulsed with 1 µCi [$^3$H]thymidine for 9 hours before the cells were harvested onto Filtermat A filters and analyzed either on a BetaPlate 1205 or Microbeta Trilux scintillation counter (Perkin Elmer).

### 2.12.14 Hybridoma from sorted splenic B cells

Sorted FOB ($CD19^+$ $CD93^-$ $CD21^+$ $CD23^+$), MZB ($CD19^+$ $CD93^-$ $CD21^{hi}$ $CD23^{-/lo}$) or newB ($CD19^+$ $CD93^-$ $CD21^{-/lo}$ $CD23^{-/lo}$ $CD5^-$) cells were cultured under activating conditions (5x$10^5$ cells/24-well in 1 ml SF- IMDM + 2%FCS and 10µg/ml LPS) for 3 days. Then the cultures were washed twice with SF-DMEM and transferred into 50 ml tubes. In parallel, in log-phase growing

Sp2/0 cells were harvested by centrifugation at 1200 rpm and 4°C for 10 min and then washed twice with SF-DMEM. Then $2x10^6$ Sp2/0 cells were added to the FOB, MZB or newB cells, and centrifuged again. After careful and complete removal of the supernatant the tube was shaken in a 37°C water bath, while 1 ml of pre-warmed 50% PEG solution was added drop-wise during 1 min to the cell pellet. The dissolved pellet was shaken for 1 min at 37°C, and then 10 ml of pre-warmed, alkaline SF-DMEM were added drop-wise to the cells while shaking the tube. Subsequently, the cell suspension was centrifuged at 800 rpm at RT for 5 min. The cell pellet was resuspended carefully in 5000 ml pre-warmed HAT medium and plated out into 96-well F-bottom plates (200 µl/well). Cultures were incubated at 37°C for 10-14 days until cell clusters were visible by the naked eye. Clones tested positively for Ig production in ELISA were sub-cloned, its supernatants harvested and cells frozen.

## 2.13 Animal work

### 2.13.1 *In vivo* labeling of proliferating cells with BrdU in mice

Initially, adult mice received an intraperitoneal injection of 1.0 mg of the thymidine analog BrdU in 1xPBS. In addition, mice received 1.0 mg/ml BrdU in the drinking water, which was exchanged every 3 days with a fresh preparation during the time of labeling (pulse). The BrdU-containing drinking-water bottles were shielded with aluminum foil to prevent light-mediated degradation of the BrdU. At the end of the experiment cell suspensions were prepared from lymphoid organs and stained for intracellular BrdU.

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# 3 ANALYSES OF THE PRE-BCR QUALITY IN PRE-B I CELLS WITH DIFFERENT PROLIFERATIVE CAPACITIES

## 3.1 Introduction

The maturation of B cells from B lineage-committed progenitors starts in the fetal liver during perinatal life and continues in the bone marrow after birth. Antigen-sensitive, immature B cells leave these primary lymphoid organs, migrating to the spleen, where further development forms the mature B cell pool. Along this pathway, checkpoints exist to assure the development of competent, B cell receptor (BCR)-expressing B cells. This pathway can be characterized by various changes in intracellular and surface-expressed molecular markers (1). At the pre-B I cell stage, cells start to rearrange a $V_H$ chain gene element to the already rearranged $D_HJ_H$ segments, thus, finalizing the rearrangement of a complete μH chain. When the rearrangement is in-frame, the cells start to express the μH chain protein in the cytoplasm ($c\mu^+$). However, producing a μH chain protein is not sufficient for the further development of the pre-B cell into later stages of more mature B cells. In approximately 25% of the pre-B cells, the μH chain is expressed together with the surrogate light (SL) chain at the cell surface as a pre-BCR. This surface expression is dependent on a pairing interaction of the μH chain with the VpreB- and λ5-components of the SL chain and, hence, establishes an important checkpoint in pre-B cell development. In addition, this pairing interaction seems to be involved in maintaining IgH allelic exclusion. It could be shown for the few pre-B II cells that productively rearrange both IgH alleles, that only one of the two μH chain proteins is pairing with the SL chain and gives rise to a pre-BCR (2). Successful pairing leads to the clonal expansion of these cells through 2-5 rounds of division. Mutations affecting the pairing between the μH chain and the SL chain or absence of one of the SL chain components abrogate the clonal expansion and therefore lead to much reduced output of B cells from the bone marrow and, hence, resulting in B lymphopenia (3).

Pre-B I cells cultured on stromal cells together with IL-7 prevents the cells from further differentiation and they can be cultured for extended times with a cell cycle time of 18 hours. Withdrawal of IL-7 results in the arrest of the cell cycle in early G1 and induces differentiation to surface Ig-expressing ($sIg^+$) immature B cells (4).

Pre-B I cells isolated from bone marrow of wild-type mice may be cultured, for a limited time, in the absence of stromal cell support or any added cytokines. These *in vitro* bulk cultures of pre-B I

cells show proliferation and differentiation into $sIg^+$ immature B cells. However, pre-B I cells of SL chain-deficient $\lambda5^{-/-}$ mice do not show that proliferative expansion. As the proliferation of pre-B I cells depends on pre-BCR expression it could still be argued, that a ligand is required to trigger signaling through the pre-BCR resulting in proliferation. This ligand could for example, be provided by a neighboring cell. To limit this possibility, *ex vivo* isolated single pre-B I cells were cultured in the absence of stromal cells and cytokines. Still, these cells were able to proliferate with a frequency and efficiency similar to pre-B I cells in bulk cultures (5).
However, of the 15% *ex vivo* isolated pre-B I cells, which go through a clonal expansion, not all of them proliferate to the same extent, giving rise to clones of different size of 4, 8, 16, 32 and 64 cells (2-5 rounds of cell division). Taking into account that these cells proliferate in the absence of any cytokine, other ligand or cell-cell contact, and that SL chain-deficient pre-B I cells do not show this clonal expansion, it is, therefore, possible that the "fitness" of the pre-BCR complex itself plays a major role in this proliferative expansion. We define this "fitness" as the avidity of any given µH chain in its pairing with the invariant SL chain and, therefore, its cell surface expression as a pre-BCR. According to this hypothesis, well pairing µH-SL complexes would give rise to larger clones and loosely pairing to smaller clones, while a non-pairing µH chain abrogates clonal expansion and can block the further development of the pre-B cell (4, 5).

The aim of this study is to verify the hypothesis, that the avidity of a given µH chain to pair with the SL chain can determine the efficiency by which pre-B I cells go through a proliferative burst. To that end, we isolated pre-B I cells from bone marrow of mice, labeled them with CFSE and cultured them in the absence of stromal cell support and any added cytokines. After 5 days of culture, cells were isolated according to their proliferative history. The µH chain gene rearrangements were cloned and sequenced and used in an *in vitro* pairing assay to test the ability of individual µH chains to pair with the SL chain and consequently being expressed as a pre-BCR on the cell surface.
Finally, the efficiency of cell surface expression of these individually cloned µH chains were compared to the proliferative history of the cells, from which they were derived. Thus, we would test for a correlation between the efficiency of the pre-B cell to proliferate and the "fitness" of the pre-BCR.

## 3.2 Results

### 3.2.1 *In vitro* proliferation of sorted pre-B I cells in absence of stromal cell layer and cytokines

Pre-B I cells ($B220^+$ $c\text{-}kit^+$ $CD19^+$) were isolated by FACS from bone marrow (BM) of young (3 weeks old) C57BL/6 mice (5). The sorted pre-B I cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) to monitor the proliferative expansion during the *in vitro* culture in normal tissue culture medium (SF-IMDM + 2% FCS) without stromal cell support and any added cytokines.

After 5 days, cultured cells were analysed by FACS for CFSE fluorescence intensity. The data indicate, that the initially seeded cells have undergone proliferation to different extent. According to the CFSE-profile, the cells segregated into roughly four major populations, which allowed sorting four subpopulation of cells comprising of (non-) or low, lower-intermediate, higher-intermediate or highly proliferated cells (Figure 1).

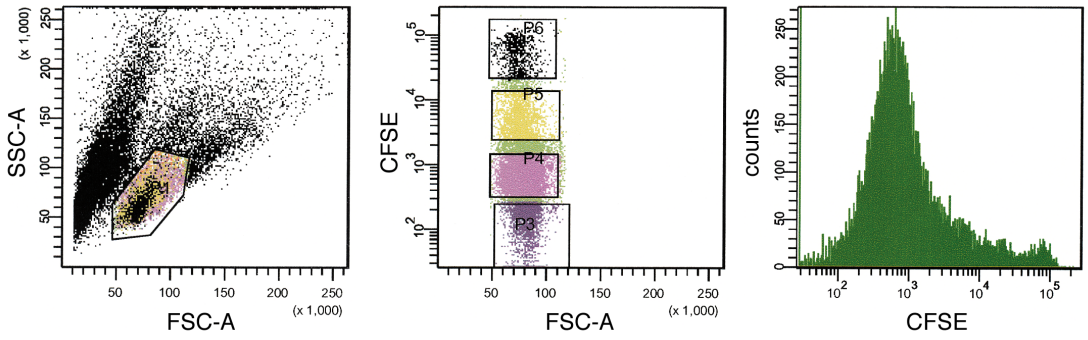


**Figure 1. Proliferative expansion of *in vitro* cultured pre-B I cells.**

Sorted pre-B I cells from bone marrow of young (3 weeks) C57BL/6 mice were labeled with the fluorescent dye CFSE and cultured *in vitro* for 5 d. Subsequently cells were sorted according to their CFSE content separating them into four subpopulations of cells, which have undergone increasing rounds of division.

### 3.2.2 Isolation and cloning of $V_HD_HJ_H$-rearrangements from low to highly proliferated pre-B cells

$V_HD_HJ_H$-rearrangements were isolated from each pool of cells sorted according to their CFSE content using a $V_H$ gene family-specific PCR. The PCR utilized 5' primers specific for sequences

of the proximal $V_H$7183 and distal $V_H$J558 $V_H$ gene families, which together represent the majority of $V_H$ gene elements expressed in the BM (6). These primers were combined with a 3' primer representing sequences downstream of the $J_H$2 (Figure 2) limiting the isolation and cloning to $V_HD_HJ_H$-rearrangements containing the $J_H$2 gene element.

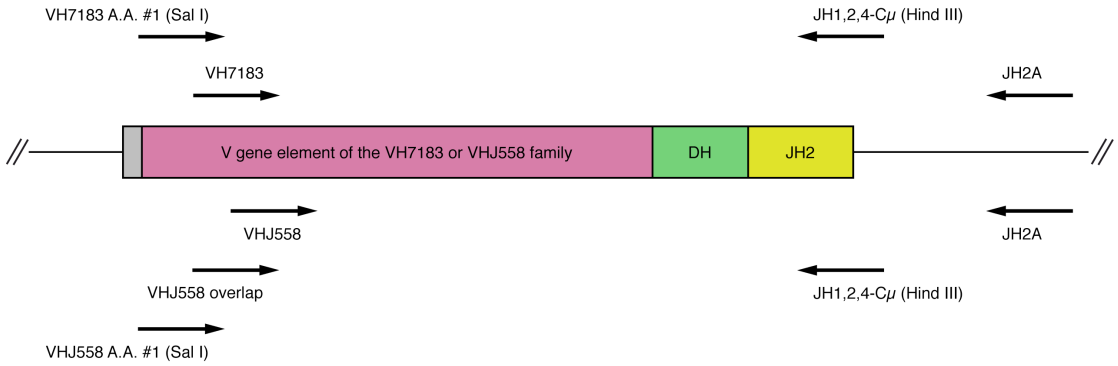


**Figure 2. Genomic PCR for the isolation of rearranged $V_HD_HJ_H$ genes.**

PCR strategy for the amplification of $V_HD_HJ_H$-rearrangements containing (top) a VH7183 gene element or a VHJ558 gene element (bottom).

The PCR products with the correct size for either $V_H$7183$D_HJ_H$2 or $V_H$J558$D_HJ_H$2 rearrangements were cloned into the retroviral expression vector pELVC, thus generating $V_H$7183 and $V_H$J558 gene family-specific libraries of $V_HD_HJ_H$-rearrangements for each sorted population. The retroviral expression vector pELVC allows the expression of each cloned productive $V_HD_HJ_H$-rearrangement in a pre-B cell line to test the cytoplasmic and surface expression capabilities of the isolated heavy chain. The pELVC vector contains the leader (L) exon/intron sequence of the SP6 μ gene and the membrane form of the μH chain constant-region ($C\mu_m$). Particular $V_HD_HJ_H$-rearranged fragments were cloned into the expression cassette using the restriction sites *SalI* in the framework-region 1 of $V_H$ and the silent *HindIII* site in the third and fourth codons of the constant region. Both restriction sites were introduced during the second PCR amplification step (Figure 2 and Methods). The expression of the cloned μH chain is controlled by the μ-core enhancer (Eμ-core), which is located upstream of the leader sequence (Figure 3) (6, 7).

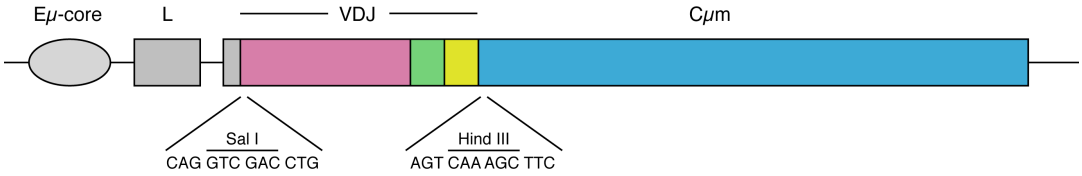


**Figure 3. pELVC vector.**

Part of the vector that contains the leader (L) of the SP6 μ gene (7), a $V_HD_HJ_H$-rearrangement, followed by the membrane form of the μH chain constant-region ($C\mu_m$). Expression is under the control of the μ-core enhancer (Eμ-core) located upstream of the leader sequence.

Cloned $V_HD_HJ_H$-rearrangements obtained from each sorted pre-B cell population and representing both the $V_H$7183 and the $V_H$J558 gene families were fully sequenced. The obtained sequences were analysed for productive, in-frame rearrangements. Furthermore, the $V_H$ gene family members were determined and the junction defining the CDR3 region was analysed. From a total of 71 unique $V_HD_HJ_H$-rearrangements sequenced, 31 productive $V_HD_HJ_H$-rearrangements were obtained.

**A**

| # | Proliferation | $V_H$ gene | $D_H$ gene | CDR1 (1–26) |
|---|---|---|---|---|
| 1 | high | IGHV5-16 | IGHD2-4 | QVDLVESGGGLVQPGSSMKLSCTAS**GFTFSDYY**MAWVRQV |
| 2 | high | IGHV5-9-1 | IGHD1-1 | QVDLVESGGGLVKPGGSLKLSCAAS**GFTFSSYA**MSWVRQT |
| 3 | high | IGHV5-9-1 | IGHD1-1 | QVDLVESGGGLVKPGGSLKLSCAAS**GFTFSSYA**MSWVRQT |
| 4 | high | IGHV5-4 | IGHD2-3 | QVDLVESGGGLMQPGGSLKLSCAAS**GFTFSSYA**MSWVRQT |
| 5 | high | IGHV5-17 | IGHD2-4 | QVDLVESGGGLVKPGGSLKLSCAAS**GFTFSDYG**MHWVRQA |
| 6 | higher intermediate | IGHV5-9-1 | IGHD1-1 | QVDLAESGGGLVKPGGSLKLSCAAS**GFTFSSYA**MSWVRQT |
| 7 | higher intermediate | IGHV7-3* | IGHD2-8 | QVDLVEAGGGLVQPGGSLSLSCAAS**GFTFTDYY**MSWVRQP |
| 8 | higher intermediate | IGHV5-2 | IGHD2-5 | QVDLVESGGGLVQPGESLKLSCESN**EYEFPSHD**MSWVRKT |
| 9 | higher intermediate | IGHV5-6 | IGHD1-1 | QVDLVESGGGLVKPGGSLKLSCAAS**GFTFSSYG**MSWVRQT |
| 10 | low | IGHV5-9-1 | IGHD2-1 | QVDLVESGGGLVQPGGSLKLSCAAS**GFTFSSYA**MSWVRQT |

CDR2 (55) CDR3 (104)

PEKGLEWVAN**INYDGSST**--YYLDSLKSRFIISRDNAKNILYLQMSSLKSEDTATYYC**AREDYDYNFDY**-----WGQGTTLTVSS
PEKRLEWVAY**ISSGGDYI**--YYADTVKGRFTISRDNARNTLYLQMSSLKSEDTAMYYC**TPRRSLDYGSNLDFDY**WGQGTTLTVSS
PEKRLEWVAY**ISSGGDYI**--YYADTVKGRFTISRDNARNTLYLQMSSLKSEDTAMYYC**TRGYGSSYIDY**-----WGQGTTLTVSS
PEKRLEWVAT**ISDGGSYT**--YYPDNVKGRFTISRDNAKNNLYLQMSHLKSEDTAMYYC**ARDQDGYSVDY**-----WGQGTTLTVSS
PEKGLEWVAY**ISSGSSTI**--YYADTVKGRFTISRDNAKNTLFLQMTSLRSEDTAMYYC**ARNYDYDALDY**-----WGQGTTLTVSS
PEKRLEWVAY**ISSGGDYI**--YYADTVKGRFTISRDNARNTLYLQMSSLKSEDTAMYYC**TREGITTVVDVPFGY**-WGQGTTLTVSS
PGKALEWLGF**IRNKANGYTT**EYSASVKGRFTISRDNSQSILYLQMNALRAEDSATYYC**ARYEIYYGNYDY**----WGQGTTLTVSS
PEKRLELVAA**INSDGGST**--YYPDTMERRFIISRDNTKKTLYLQMSSLRSEDTALYYC**ARPTIVTIDY**------WGQGTTLTVSS
PDKRLEWVAT**ISSGGSYT**--YYPDSVKGRFTISRDNAKNTLYLQMSSLKSEDTAMYYC**AVEG**----------WGQGTTLTVSS
PEKRLEWVAY**ISSGGDYI**--YYADTVKGRFTISRDNARNTLYLQMSSLKSEDTAMYYC**TRAVLLGDY**-------WGQGTTLTVSS

**Figure 4. Protein sequences of isolated, in-frame (A) $V_H$7183$D_HJ_H$2 or (B,C) $V_H$J558$D_HJ_H$2 rearrangements with junctional analysis.**

For panels B, C and description see next page.

**B**

| # | Proliferation | $V_H$ gene | $D_H$ gene | CDR1 (1–26) | CDR2 (55) | CDR3 (104) |
|---|---|---|---|---|---|---|
| 11 | high | IGHV1-26 | n.a. | QVDLQQSGPELVKPGASVKISCKAS**GYTFTDYY**MNWVKQS | HGKSLEWIGD**INPNNGGT**SYNQKFKGKATLTVDKSSSTAYMELRSLTSEDSAVYYC | **ARHFFDY**--------WGQGTTLTVSS |
| 12 | high | IGHV1-19 | IGHD2-1 | QVDLQQSGPELVKPGASVKMSCKAF**GYTFTDYY**MNWVKQS | HGKSLEWIGV**INPYNGGT**SYNQKFKGKATLTVDKSSSTAYMELNCLTSEDSAVYYC | **ARGIYYGNPYYFDY**-WGQGTTLTVSS |
| 13 | high | IGHV1-72 | IGHD2-5 | QVDLQQSGPELVKPGASVKLSCKAS**GYTFTSYW**MHWVKQR | PGRGLEWIGR**IDPNSGGT**KYNEKFKSKATLTVDKPSSTAYMQLSSLTSEDSAVYYC | **ARWGSNYVPLDY**---WGQGTTLTVSS |
| 14 | high | IGHV1-26 | IGHD2-8 | QVDLQQSGPELVKPGASVKISCKAS**GYTFTDYY**MNWVKQS | HGKSLEWIGD**INPNNGGT**SYNQKFKGKATLTVDKSSSTAYMELRSLTSEDSAVYYC | **ASEDGNYLY**------WGQGTTLTVSS |
| 15 | high | IGHV1-53 | IGHD3-2 | QVDLQQSGPELVKPGASVRLSCKAS**GYTFTSYW**MHWVKQR | PGQGLEWIGN**INPSNGGT**NYNEKFKSKATLTVDKSSSTAYMQLSSLTSEDSAVYYC | **ARGQLRLRDFDY**---WGPKAPLSPSP |
| 16 | high | IGHV1-39 | IGHD1-3 | QVDLQQSGLELVKPGTSVKISCKAS**GYTFTDYY**MNWVKQS | NGKSLEWIGV**INPNYGTT**SYNQKFKGKATLTVDQSSSIAYMQLNSLTSEDSAVYYC | **ARSTSFKRFDY**----WGQGTTLTVSS |
| 17 | high | IGHV1-81 | IGHD2-1 | QVDLQQSGPELVKPGASVRMSCKAS**GYTFTSYG**ISWVKQR | TGQCLEWIGE**IYPRSGNT**YYNEKFKGKATLTADKSSSTAYMELRSLTSEDSAVYFC | **ARQGPDLLWTVTFDY**WGQGTTLTVSS |
| 18 | high | IGHV1-50 | IGHD2-4 | QVDLQQSGPELVKPGASVKLSCKAS**GYTFTSYW**MQWVKQR | PGQGLEWIGE**IDPSDSYT**NYNQKFKGKATLTVDTSSSTAYMQLSSLTSEDSAVYYC | **ARYCYYDYDVDY**---WGQGTTLTVSS |
| 19 | high | IGHV1-19 | IGHD1-1 | QVDLQQSGPELVKPGASVRMSCKAS**GYTFTDYY**MNWVKQS | HGKSLEWIGV**INPYNGGT**SYNQKFKGKATLTVDKSSSTAYMELNSLTSEDSAVYYC | **ARGCGRYYFDY**----WGQGTTLTVSS |
| 20 | high | IGHV1-72 | IGHD4-1 | QVDLQQSGPELVKPGASVRLSCKAS**GYTFTSYW**MHWVKQR | PGRGLEWIGR**IDPNSGGT**KYNEKFKSKATLTVDKPSSTAYMQLSSLTSEDSAVYYC | **ARGGALGLDY**-----WGQGTTLTVSS |
| 21 | higher intermediate | IGHV1-53 | IGHD3-2 | QVDLQQSGPELVKPGASVKLSCKAS**GYTFTSYW**MHWVKQR | PGQGLEWIGN**INPSNGGT**NYNEKFKSKATLTVDKSSSTAYMQLSSLTSEDSAVYYC | **ARRGAAQAHFDY**---WGQGTTLTVSS |
| 22 | higher intermediate | IGHV1-26 | IGHD2-4 | QVDLQQSGPELVKPGASVKISCKAS**GYTFTDYY**MNWVKQS | HGKSLEWIGD**INPNNGGT**SYNQKFKGKATLTVDKSSSTAYMQFSSLTSEDSAVYFC | **ARSYDYLDY**------WGQGTTLTVSS |
| 23 | higher intermediate | IGHV1-55 | IGHD2-3 | QVDLQQSGPELVKPGASVRMSCKAS**GYTFTSYW**ITWVKQR | PGQGLEWIGD**IYPGSGST**NYNEKFKGKATLTADKSSSTAYMQFSSLTSEDSAIYYC | **ARVLFYDFVDY**----WGQGTTLTVSS |
| 24 | higher intermediate | IGHV1-5 | IGHD1-1 | QVDLQQSGPELVKPGASVRMSCKTS**GYTFTSYW**MHWVKQR | PGQGLEWIGA**IYPGNSDT**NYNQKFKGKAKLTAITSASTAYMELNNLTNEDSAVYYC | **TVLPYPYYFDY**----WGQGTTLTVSS |
| 25 | higher intermediate | IGHV1-52 | IGHD1-1 | QVDLQQSGPELVKPGASVKLSCKAS**GYTFTSYW**MHWVKQR | PIQGLEWIGN**IDPSDSET**HYNQKFKDKATLTVDKSSSTAYMQLSSLTSEDSAVYYC | **ARRRFYGSSYYFDY**-WGQGTTLTVSS |
| 26 | higher intermediate | IGHV1-76 | IGHD1-1 | QVDLQQSGPELVKPGASVKLSCKAS**GYTFTDYY**INWVKQR | PGQGLEWIAR**IYPGSGNT**YYNEKFKGKATLTVDKSSSTAYMELRSLTSEDSAVYFC | **ARSGLLLRLYYFDY**-WGQGTTLTVSS |
| 27 | lower intermediate | IGHV1-59 | IGHD2-3 | QVDLQQSGLELVKPGASVKLSCKAS**GYTFTSYW**MHWVKQR | PGQGLEWIGV**IDPSDSYT**NYNQKFKGKATLTVDTSSSTAYMQLSSLTSEDSAVYYC | **ARRGWLLRVDY**----WGQGTTLTVSS |
| 28 | lower intermediate | IGHV1-18 | IGHD1-1 | QVDLQQSGPELVKPGASVKIPCKAS**GYTFTDYN**MDWVEQS | HGKSLEWIGD**INPNNGGT**SYNQKFKGKATLTVNKSSSTAYMELRSLTSEDTAVYYC | **ARRIYYGSTYYFDY**-WGQGTTLTVSS |
| 29 | lower intermediate | IGHV1-59 | IGHD2-7 | QVDLQQSGLELVKPGASVRLSCKAS**GYTFTSYW**MHWVKQR | PGQGLEWIGV**IDPSDSYT**NYNQKFKGKSTLTVDKSSSTAYMELRSLTSEDTAVYYC | **ARFPYGYDDY**-----WGQGTTLTVSS |
| 30 | lower intermediate | IGHV1-18 | IGHD1-1 | QVDLQQSGPELVKPGASVRIPCKAS**GYTLTDYN**MDWVKQS | HGKSLEWIGD**INPNSGGT**IYNQKFKGKATLTVDKSSSTAYMELRSLTSEDTAVYYC | **ARSPRSSYDY**-----WGQGTTLTVSS |
| 31 | low | IGHV1-22 | IGHD1-2 | QVDLQQSGPELVKPGASVRMSCKAS**GYTFTDYN**MHWVKQS | HGKSLEWIGY**INPNNGGT**SYNQKFKGKATLTVNKSSSTAYMELRSLTSEDSAVYYC | **AGGYYFDY**-------WGQGTTLTVSS |

**C**

| # | Proliferation | $V_H$ gene | $D_H$ gene | CDR1 (1–26) | CDR2 (55) | CDR3 (104) |
|---|---|---|---|---|---|---|
| 32 | high (control) | IGHV1-15 | IGHD2-13 | QVDLQQSGPELVKPGASVKLSCKAS**GYTFTDYE**MHWVKQT | PVHGLEWIGA**IDPETGGT**AYNQKFKGKATLTADKSSSTAYMELRSLTSEDSAVYYC | **TRSLVLFDY**--WGQGTTLTVSS |
| 33 | high (control) | IGHV1-15 | IGHD3-1 | QVDLQQSGPELVKPGASVKLSCKAS**GYTFTDYE**MHWVKQT | PVHGLEWIGA**IDPETGGT**AYNQKFKGKATLTADKSSSTAYMELRSLTSEDSAVYYC | **TKGAFDY**----WGQGTTLTVSS |
| 34 | high (control) | IGHV1-15 | IGHD5-5 | QVDLQQSGPELVKPGASVKLSCKAS**GYTFTDYE**MHWVKQT | PVHGLEWIGA**IDPETGGT**AYNQKFKGKATLTADKSSSTAYMELRSLTSEDSAVYYC | **TRTSLPYYFDY**WGQGTTLTVSS |

**Figure 4. Protein sequences of isolated, in-frame (A) $V_H$7183$D_HJ_H$2 or (B,C) $V_H$J558$D_HJ_H$2 rearrangements with junctional analysis.**

Unique, productive $V_HD_HJ_H$-rearrangements isolated and used in pairing assays to test their ability to pair with the SL-chain and as a consequence being expressed on the cell surface in a pre-BCR.

**(A,B)** µH chains derived from *in vitro* cultures. **(C)** µH chains derived from splenic B cells. In addition to the classical $V_H$ family names (A: VH7183, B: VHJ588) the $V_H$ and $D_H$ gene element names as well as the CDR annotations were assigned using the newer definitions according to IMGT unique naming and numbering (8).

The sequenced functional and non-functional rearrangements were not clustered within the $V_H$ gene family locus with regard to the proliferative history of the cells from which the rearrangements were obtained (Figure 4).

In addition to these pre-B cell-derived $V_HD_HJ_H$-rearrangements three functional $V_HJ558D_HJ_H2$ rearrangements were cloned from splenic B cells. These rearrangements served as positive controls in the pairing-assay assuming that these cells, by their origin, must express a μH chain capable of forming a pre-B cell receptor.

### 3.2.3 Pairing assay: testing the quality of the pre-BCR

The *in vitro* pairing assay allowed testing the ability of individual μH chains to pair with the surrogate light (SL) chain. Successful pairing of the μH chain with the Vpre-B and λ5 components of the SL chain leads to the surface expression of the pre-BCR (2).

The productively rearranged $V_H7183D_HJ_H2$ or $V_HJ558D_HJ_H2$ rearrangements originating from the low, lower-intermediate, higher-intermediate and highly proliferated cell populations and the $V_HJ558D_HJ_H2$ rearrangements derived from splenic B cells were used to transduce the Abelson virus-transformed, SL chain-expressing, $c\mu^-$ pre-B cell line 40E1 (9). After expansion of transduced cells under selection for puromycin-resistance the cells were tested for cytoplasmic and cell surface expression of the μH chain by FACS. Cytoplasmic expression was tested using a polyclonal α-Cμ antibody. Testing the surface expression of the pre-BCR was more difficult, due to the relatively low density of the pre-BCR on the cell surface. FACS analysis was therefore performed by an amplifying staining protocol using monoclonal $\alpha\text{-IgM}^{bio}$ in combination with the anti-isotype antibody mouse-α-rat $IgG^{bio}$ and streptavidin-PE (see Material and methods). Measuring the cytoplasmic expression of μH chain of different clones showed some level of variation, which could be at least in part be explained by the random integration of the transgene in the transduced cells. Additionally, the puromycin selection allowed many cells to be selected without expressing the μH chain (Figure 5A). Therefore in order to quantitate the pairing quality ("fitness") of a pre-BCR the surface μH chain expression was normalized against cytoplasmic μH chain expression by determining the mean fluorescence intensities (MFI) for both, surface-positive and cytoplasmic-positive μH chain expressing cells. The "fitness" of a pre-BCR was then calculated from the ratio of the surface-positive MFI versus the cytoplasmic MFI.

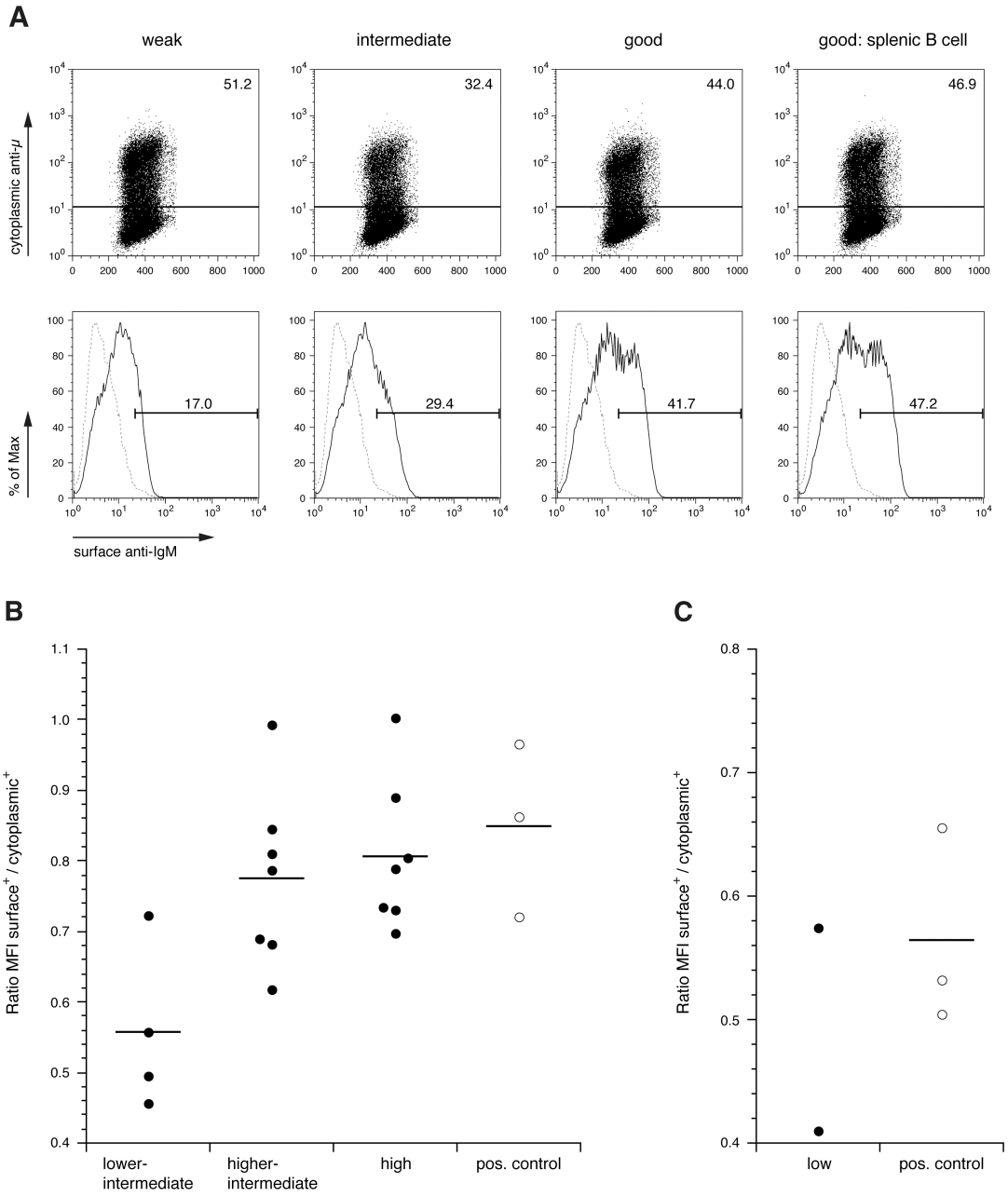


**Figure 5. Representative FACS profiles of cytoplasmic and surface μH chain expression and pairing quality graphs.**

**(A)** Representative FACS profiles for a weak, intermediate and good pairing. Expression of the μH chains in the cytoplasm and detection of cell surface expression of the pre-BCR after transduction in 40E1 cells. Indicated gates were used to calculate the mean-fluorescence intensity (MFI) of cytoplasmic and surface μH chain-expressing cells. Neg. control: non-productive $V_HD_HJ_H$-rearrangement (dashed line).

**(B, C)** Ratio MFI surface$^+$/cytoplasmic$^+$ μH chain expression ordered by the proliferative history of the originating cell from low, lower-intermediate, higher-intermediate to highly proliferated. Right column in each graph includes the pairing quality of the splenic B cell-derived $V_HD_HJ_H$-rearrangements (pos. control).

Although that due to the low density of pre-BCR on the cell surface and the varying cytoplasmic expression levels the resolution of this method is not high, the results obtained show that the pairing quality can be measured not only as an all-or-nothing event, but at least also semi-quantitatively. Additionally, this analysis shows a tendency of a better pairing capacity for rearrangements derived from cells, which have undergone more cell divisions during the 5 days in culture. However, more productive rearrangements would be needed to make a final conclusion (Figure 5B). Interestingly, from the only two functional rearrangements derived from the sorted subpopulation of low proliferated cells one rearrangement shows a good pairing as compared to the splenic B cell-derived rearrangements. The fact that pre B cells have the possibility to rearrange on both alleles, if the rearrangement on the first allele failed for a productive rearrangement one could assume that this pairing-competent rearrangement originates from the second allele and that rearrangement comes to late in time to let the cell substantially proliferate before reaching the next stage in differentiation.

## 3.3 Discussion

According to one hypothesis, well pairing μH-SL complexes (pre-BCR) on the cell surface of large pre-B II cells would allow them to undergo more rounds of cell division than if their μH-SL complex only loosely pairs, while a non-pairing μH chain abrogates the clonal expansion completely, as is demonstrated in SL-chain deficient pre-B cells (4, 5).

To this end, the present study shows a tendency of a better pairing capacity for $V_HD_HJ_H$-rearrangements derived from cells, which have undergone more cell divisions. However, many more productive μH chain rearrangements would have to be analysed for their pairing capacity in order to make a final conclusion. Nevertheless, this study revealed that indeed the capacity of μH-SLC pairings could be measured not only as an all-or-nothing event, but at least also semi-quantitatively.

The main problems of the "pairing-assay" in this study are to deal with the relatively low surface expression of pre-BCR and the normalization of these expression levels. The relatively low surface expression could be overcome, to some extent, by the use of an amplifying staining procedure using an α-IgM and an anti-isotype antibody directed against the first antibody, both biotinylated for obtaining a higher number of fluorochrome labeling-positions. For the second problem, alternatively, instead of a puromycin selection, which allows many cells to be selected without expressing the μH-chain in the cytoplasm, an IRES-driven GFP or other marker proteins could be used. GFP would have the advantage to be accessible for direct measurement independently of the surface expression of the μH-chain and serve as an internal standard. However, it is known, that depending on the integration site, IRES-driven GFP expression does not always correlate with the expression level of the first product situated 5' of the IRES sequence within the mRNA.

In a recent work, where a somewhat reversed approach was used, the authors received supporting results (10). Thus, these authors isolated $V_HD_HJ_H$-rearrangements from early pre-B cells and analysed their cytoplasmic and surface expression as pre-BCR in a pre-B cell line. They found, as well, that surface expression of the pre-BCR varied between individual clones independently of the level of cytoplasmic expression. In addition, they also found a correlation between the pairing quality of the analysed rearrangements and the capacity to drive proliferation of pre-B cells by introducing the μH chains into pre-B cells of μH-deficient μMT recipient cells and culture them in the absence of IL-7.

Pre-B cells with a non-functional $V_HD_HJ_H$-rearrangement on the first allele, or expressing an SL-chain non-pairing μH chain can be rescued by rearranging the second allele for an SL (or L)-chain pairing μH chain. It could be shown for the few pre-B II cells that productively rearrange both IgH

alleles, that only one of the two μH chain proteins actually is capable of pairing with the SL chain (2). However, this would lead to a delay in the further differentiation including the proliferative expansion. Alternatively these cells are excluded from the proliferative expansion because of that delay. The assay, as conducted in this study, might include such cells in the non- or low-proliferated fractions, although their productive rearrangement is well capable to pair with the SL-chain (found for 1 clone in the non-low proliferating fraction, which is well capable to pair). This process resembles that found in SL-chain deficient strains of mice where the proliferative expansion at the large pre-B II stage is abrogated and functional B cell receptors are only expressed on the cell surfaces after light-chain gene rearrangements, although the overall output of B cells from the bone marrow of such mice is strongly reduced.
It would be interesting to isolate and analyse the $V_HD_HJ_H$-rearrangements of individual cells sorted after 5 days in culture. The prediction would be, that productive $V_HD_HJ_H$ rearrangements from a secondary rearranged allele and which also show pairing capacity, would be derived from cells, which have undergone less proliferation. In this view, the rearrangements on the second allele would come too late for the time window during that the proliferative burst occurs.

Still, the outcome in terms of proliferation for an individual pre-B cell *in vivo* might depend on additional factors like the availability of IL-7. It has been demonstrated by others that there is a collaborative interaction between the pre-BCR- and the IL-7R-signaling pathway that promotes the proliferation of pre-B cells (11).
Therefore, taken together, an extended model can be proposed, where the quality of the pre-BCR, the availability of cytokines (e.g. IL-7) and time determines the amount of proliferation for a given B cell.

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# 4 TSLP IN ADULT LYMPHOPOIESIS

## 4.1 Increased TSLP availability restores T- and B-cell compartments in adult IL-7-deficient mice

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### Abstract

Interleukin 7 (IL-7) plays a crucial role in adult lymphopoiesis, while in fetal life its effect can be partially compensated by TSLP. Whether adult hematopoietic progenitor cells are unresponsive to TSLP or whether TSLP is less available in adult microenvironments is still a matter of debate. Here, we show that increased TSLP availability through transgene (Tg) expression fully restored lymphopoiesis in IL-7-deficient mice: it rescued B-cell development, increased thymic and splenic cellularities, and restored double-negative (DN) thymocytes, αβ and γδ T-cell generation, and all peripheral lymphoid compartments. Analysis of bone marrow chimeras demonstrated that hematopoietic progenitor cells from adult wild-type mice efficiently differentiated toward B- and T-cell lineages in lethally irradiated IL-7 deficient mice provided TSLP Tg was expressed in these mice. In vitro, TSLP promoted the differentiation of uncommitted adult bone marrow progenitors toward B and T lineages and the further differentiation of DN1 and DN2 thymocytes. Altogether, our results show that adult hematopoietic cells are TSLP responsive and that TSLP can sustain long-term adult lymphopoiesis.

HEMATOPOIESIS

# Increased TSLP availability restores T- and B-cell compartments in adult IL-7–deficient mice

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**Interleukin 7 (IL-7) plays a crucial role in adult lymphopoiesis, while in fetal life its effect can be partially compensated by TSLP. Whether adult hematopoietic progenitor cells are unresponsive to TSLP or whether TSLP is less available in adult microenvironments is still a matter of debate. Here, we show that increased TSLP availability through transgene (Tg) expression fully restored lymphopoiesis in IL-7–deficient mice: it rescued B-cell development, increased thymic and splenic cellularities, and restored double-negative (DN) thymocytes, αβ and γδ T-cell generation, and all peripheral lymphoid compartments. Analysis of bone marrow chimeras demonstrated that hematopoietic progenitor cells from adult wild-type mice efficiently differentiated toward B- and T-cell lineages in lethally irradiated IL-7 deficient mice provided TSLP Tg was expressed in these mice. In vitro, TSLP promoted the differentiation of uncommitted adult bone marrow progenitors toward B and T lineages and the further differentiation of DN1 and DN2 thymocytes. Altogether, our results show that adult hematopoietic cells are TSLP responsive and that TSLP can sustain long-term adult lymphopoiesis. (Blood. 2007;110:3862-3870)**



## Introduction

Lymphopoiesis is regulated by a number of cytokines that control the proliferation, differentiation, and survival of hematopoietic progenitor cells. Among these factors, IL-7 plays an essential role in B- and T-cell development.[1-3] IL-7 signals through the IL-7 receptor that is composed of the common γ-chain ($\gamma_c$ or CD132) and the IL-7Rα (CD127) chain.[4] IL-7Rα can also associate with the thymic stromal lymphopoietin receptor (TSLPR) chain to form the receptor for the cytokine TSLP.[5,6] TSLPR signaling occurs independently of $\gamma_c$[5-7] and does not rely on the phosphorylation of any known Janus family kinases but leads to the translocation of functional Stat5 to the nucleus.[7,8]

TSLP was originally cloned from a murine thymic stromal cell line.[9] While displaying only 43% protein identity,[10,11] human and mouse TSLP share similar biologic functions. Both promote homeostatic expansion of $CD4^+$ T cells,[12,13] trigger dendritic cell maturation,[14-16] induce TH2 differentiation,[14-18] and are important factors in triggering inflammatory allergic responses.[14-16,18,19]

Several studies have suggested that TSLP may play a role in fetal rather than adult B-cell development. For instance, at 4 weeks of age, $\gamma_c^{-/-}$ mice, which are responsive to TSLP but not to IL-7, showed residual B lymphopoiesis. In contrast, in 4-week-old IL-7Rα$^{-/-}$ mice, which are unresponsive to both TSLP and IL-7, B-cell development was absent. However, at 12 weeks of age, both mouse strains were devoid of B lymphopoiesis.[20] Moreover, although both adult IL-7$^{-/-}$ and IL-7Rα$^{-/-}$ mice lack γδ T cells, fetal IL-7$^{-/-}$ but not IL-7Rα$^{-/-}$ thymi contained γδ T cells.[3,21] It is currently unknown whether these differences reflect distinctive TSLP responsiveness of fetal versus adult hematopoietic cells, or if they are the result of decreased availability of a biologically active form of TSLP in adult mice.

TSLP was shown to support B-cell development in vitro.[7,9,22] However, there are conflicting results on the role of TSLP for B-cell development in vivo. TSLPR$^{-/-}$ mice had no defect in B-cell development,[12,23] while TSLP Tg expression either promoted[24] or inhibited[25] B lymphopoiesis.

Experimental data showing a role for TSLP in T lymphopoiesis are limited. TSLP could induce a moderate in vitro proliferation of adult double-negative (DN) thymocytes in synergy with IL-1, but failed to sustain the proliferation of fetal thymocytes.[26] While TSLPR$^{-/-}$ mice had no defect in T development, mice lacking both the $\gamma_c$ and the TSLPR chains showed lower thymic cellularity than $\gamma_c^{-/-}$ mice.[12] Moreover, the injection of recombinant TSLP could transiently increase the number of thymocytes in $\gamma_c^{-/-}$ mice.[12] Although these data suggest that TSLP may be involved in T lymphopoiesis, the developmental stage at which TSLP exerts its biologic function has not been clearly identified.

To understand the function of TSLP on hematopoiesis in vivo, TSLP-transgenic (Tg) mice were generated and backcrossed to an IL-7–deficient background. The TSLP Tg expression was driven by the keratin 14 (K14) promoter that targets gene expression to epithelial cells.[27]

In this study, we show that TSLP Tg expression rescued B-cell development, increased the thymic cellularity, and rescued the thymic architecture in IL-7$^{-/-}$ animals. DN1 and DN2 thymocytes but also γδ T cells developed in response to TSLP. Moreover, adult WT bone marrow (BM) cells differentiated normally into B and T lineages and restored peripheral compartments when adoptively transferred into lethally irradiated IL-7$^{-/-}$ K14-TSLP Tg recipients. In addition, we observed a strong effect of TSLP overexpression on peripheral myeloid cell expansion. In vitro, TSLP promoted

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the generation of B and T lineage cells from early lymphoid/myeloid BM progenitors and supported the differentiation of DN1 and DN2 thymocytes. Altogether, we show here that TSLP is a potent cytokine able to support adult B- and T-cell development and to expand both lymphoid and myeloid compartments in peripheral lymphoid organs.

## Materials and methods

### Mice

All mice were bred and maintained in our animal facility under specific pathogen-free conditions. The animal experiments received the approval of the Cantonal Veterinary Office of the city of Basel, Switzerland.

C57BL/6 mice were purchased from RCC (Itingen, Switzerland). IL-7$^{-/-}$ mice were previously described.[1] RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice on C57BL/6 background were kindly provided by Jörg Kirberg (MPI, Freiburg, Germany).

For generation of transgenic mice, the murine TSLP open reading frame[26] was inserted into the pK14pA construct.[28] The transgene DNA was microinjected into fertilized (C57BL/6 × DBA/2) F2 embryos to generate transgenic founders. Progenies of the founder mice were phenotypically identical. One line was backcrossed with C57BL/6 mice for at least 8 generations. The genotype of TSLP Tg mice was identified by polymerase chain reaction (PCR) from genomic DNA with the following primers: 5′-TGCAAGTACTAGTACGGATGGGGC-3′ from the 5′ coding region and 5′-GGACTTCTTGTGCCATTTCCTGAG-3′ from the 3′ coding region. PCR conditions were 94°C for 2 minutes followed by 34 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds, and finally 72°C for 5 minutes. PCR products were separated on a 1% agarose gel and detection of a 323-bp fragment positively identified TSLP Tg presence.

For BM chimera, IL-7$^{-/-}$ K14-TSLP Tg mice, IL-7$^{-/-}$ littermates, and C57BL/6 WT mice were lethally γ-irradiated (9 Gy) and intravenously injected with $10^7$ total BM cells from 8-week-old WT mice (CD45.1$^+$). Six weeks or 6 months after reconstitution, BM chimeric mice were analyzed.

### Immunization

NP stands for 4-hydroxy-3-nitrophenyl-acetyl. Ten- to 12-week-old IL-7$^{-/-}$ K14-TSLP, IL-7$^{-/-}$, and C57BL/6 mice were immunized intraperitoneally with 50 μg alum-precipitated NP-ovalbumin (NP-OVA) or intravenously with 100 μg NP-Ficoll. Sera were collected prior to immunization and 10 or 14 days after NP-Ficoll or NP-OVA immunization, respectively.

### ELISA

NUNC Immunoplate Maxisorb F96 plates (NUNC, Roskilde, Denmark) were coated with 5 μg/mL NP-BSA at 4°C. Plates were incubated with serial dilutions of sera for 2 hours at room temperature. After washing, alkaline phosphatase–conjugated rat anti–mouse IgM (R6–60.2; PharMingen, San Diego, CA) or goat anti–mouse IgG (Southern Biotechnology Associates, Birmingham, AL) was added to the plates. Plates were developed with FAST pNPP (*p*-nitrophenylphosphate; Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. Serum titers were determined using the end point titer procedure. TSLP enzyme-linked immunosorbent assay (ELISA) was performed with the DuoSet ELISA kit (R&D, Abington, United Kingdom) according to the manufacturer's instructions.

### Cell culture

OP9[29] and OP9-DL1 cells[30] were cultured as previously described.[31] Semiconfluent cultures of stromal cells were γ-irradiated (30 Gy) before adding sorted DN1, DN2, or early progenitors for lymphoid and myeloid progenitors (EPLMs). Sorted cells ($5 \times 10^3$)/well of a 24-well plate were cultured in supplemented IMDM on OP9 or OP9-DL1 cells in the presence of either 100 U/mL IL-7 or 500 U/mL TSLP or without additional cytokine. At day 7, cells were harvested, counted, stained, and analyzed by flow cytometry.

### Flow cytometry and cell sorting

FITC-, PE-, PE-Cy7–, APC-, or biotin-conjugated α-CD4 (GK1.5), α-CD8α (53–6.7), α-CD19 (1D3), α-CD21 (CR2/CR1), α-CD23 (B3B4), α-CD25 (7D4), α-CD44 (IM7), α-CD45.1 (A20), α-γδ TCR (GL3), α-Vγ3 TCR (536), and α-NK1.1 (PK 136) Abs were purchased from BD Biosciences (Basel, Switzerland). α-CD3 (145–2C11), α-CD11b (M1/70), α-CD24 (M1/69), α-CD62L (MEL-14), α-B220 (RA3–6B2), α-TER119 (TER-119), and α-TCRβ chain (H57–597) were from Biolegend (San Diego, CA). α-CD45 (30-F11), α-CD71 (R17217), α-CD117 (2B8), and α-Gr-1 (RB6–8C5) Abs were from eBioscience (San Diego, CA). α-Vγ1.1-PE (2.11) and α-Vγ2-PE (UC3–10A6) were obtained from A. Wilson (LICR, Epalinges, Switzerland). α-CD93 (PB493) Ab was produced in our laboratory and labeled with biotin by standard methods. As secondary reagent, streptavidin-PE and streptavidin-PE/Cy7 (Biolegend) were used. Flow cytometry acquisition was performed with a FACSCalibur (BD Biosciences) and data were analyzed using Flowjo software (Tree Star, Eugene, OR). EPLM sorting was done as previously described.[31] Briefly, erythrocyte-depleted BM cells were sorted as B220$^+$ CD19$^-$ CD117$^+$ CD93$^+$ NK1.1$^-$ cells. DN1 and DN2 thymocytes were sorted as CD117$^{high}$ CD25$^-$ CD44$^+$ and CD117$^{high}$ CD25$^+$ CD44$^+$ cells, respectively.[32] Cell sorting was done using a FACS Aria (BD Biosciences) and reanalysis of sorted cells indicated that they were more than 98% pure.

### V(D)J rearrangement analysis

B220$^+$ CD19$^+$ double-positive cells ($5 \times 10^5$) from IL-7$^{-/-}$ K14-TSLP Tg mice (8 weeks old) were fluorescence-activated cell sorting (FACS) sorted, and genomic DNA was isolated by standard protocols. DNA amplification was carried out in 2 rounds of PCR. The first round of PCR amplification contained 5 different 5′ $V_H$ primers recognizing the $V_H$ families $V_HJ558$, $V_H7183$, $V_HQ52$, $V_HJ606$, $V_HS107$, $V_HX24$, and $V_HGAM308$ together with a nested 3′ $J_H4$ primer. In the second round, 2 μL of the first PCR product was reamplified with a $V_H$ family–specific 5′ primer and the same nested 3′ $J_H4$ primer. PCR products were purified on a 1.5% agarose gel by cutting the band with the appropriate size of a V(D)J rearrangement followed by TA cloning. A set of clones was sequenced using the Big Dye Terminator method and the automated DNA sequencer 377 (Applied Biosystems, Weiterstadt, Germany). Sequence analysis was performed with 4Peaks (Apple Computer, http://mekentosj.com/4peaks/), IMGT/JunctionAnalysis (The International Immunogenetics Information System, http://imgt.cines.fr/cgi-bin/IMGTjcta.jv) and IgBLAST (http://www.ncbi.nlm.nih.gov/projects/igblast/).[33]

### Immunofluorescence confocal microscopy

Acetone-fixed thymic sections (8 μm) were incubated with polyclonal α-K5 (PRB-160B; Covalence, Princeton, NJ), α-CD3-biot (145–2C11; eBioscience), and α-K8-Cy5 Abs (TROMA-1; Developmental Studies Hybridoma Bank, University of Iowa) followed by incubation with streptavidin–Alexa 488 (Molecular Probes, Leiden, the Netherlands) and goat α-Rabbit–Alexa 555 (Molecular Probes), and finally embedded in Fluorsave (Calbiochem, San Diego, CA). Images were captured on a Zeiss LSM 510 Meta Laser Confocal Scanning Confocal Microscope System (Carl-Zeiss, Fedbach, Switzerland). Overlays of blue (Cy5), red (Alexa 455), and green (Alexa 488) stainings were colored by computer-assisted management of confocal generated data with Zeiss LSM 510 software version 3.2.

### Quantitative real-time PCR

RNA extraction was performed with the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) followed by DNase digestion with RQ1 RNase-Free DNase (Promega, Madison, WI). RNA (750 ng) was used to perform the Reverse Transcription with Oligo dT (Promega) and dNTPs (Roche, Rotkreuz, Switzerland) with the Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed with Sensimix (Quantace, Watford, United Kingdom) on a Rotor Gene RG-3000 (Corbett Research, Sydney, Australia). The following primers were used: TSLP FWD: AGGCTACCCTGAAACTGAG, TSLP RVS: GGAGATTGCATGAAGGAATACC, TBP FWD: CGTGAATCTTGGCTGTAAACT, TBP RVS: GTCCGTGGCTCTCTTATTCT. TSLP and TBP primer pairs had

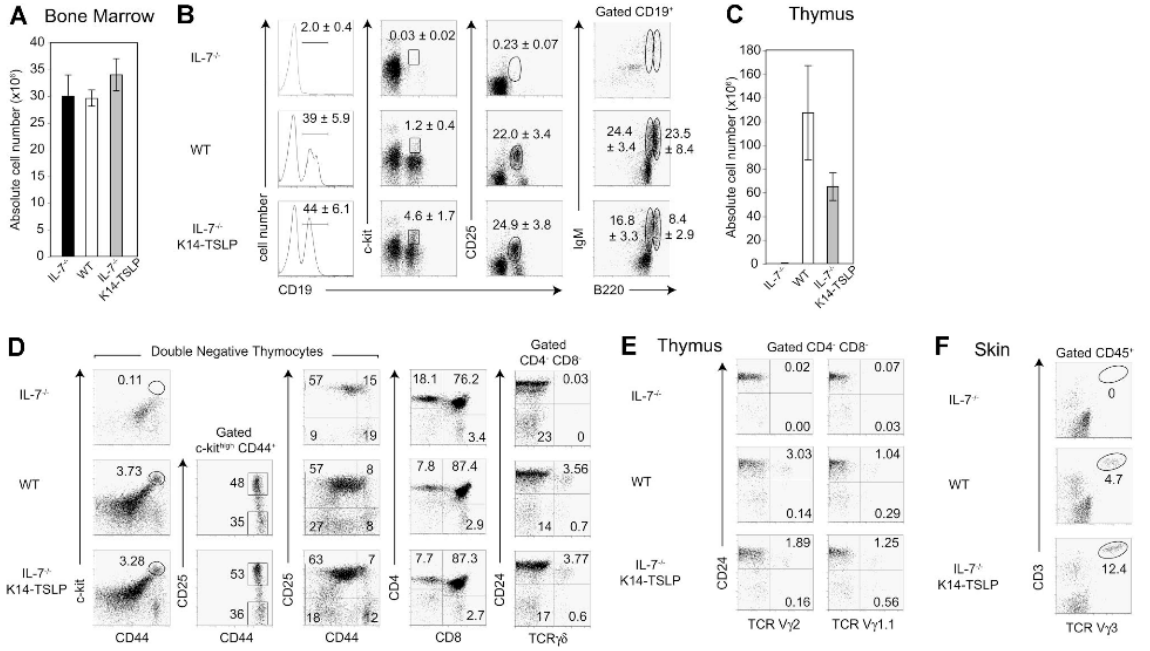


**Figure 1. TSLP Tg expression rescues B- and T-cell development in IL-7$^{-/-}$ mice.** IL-7$^{-/-}$, WT, and IL-7$^{-/-}$ K14-TSLP Tg mice were analyzed at 12 weeks of age. (A) Absolute cell number per 2 femurs. (B) Regions indicate the pro-B/pre-B-I (CD19$^+$ c-kit$^+$), pre-BII (CD19$^+$ CD25$^+$), mature (CD19$^+$ B220$^{high}$ IgM$^+$), and immature (CD19$^+$ B220$^+$ IgM$^+$) B cells in the BM. Numbers are mean and standard deviation of percentage (n = 5). (C) Absolute thymocyte number and (D) thymocyte profiles of DN1 (c-kit$^{high}$ CD44$^+$ CD25$^-$), DN2 (c-kit$^{high}$ CD44$^+$ CD25$^+$), DN3 (CD44$^-$ CD25$^+$), DN4 (CD44$^-$ CD25$^-$), DP (CD4$^+$ CD8$^+$), and SP (CD4$^+$ CD8$^-$, CD4$^-$CD8$^+$) thymocytes as well as of immature (CD24$^{lo/-}$ γδ$^+$) and mature (CD24$^+$ γδ$^+$) γδ T cells are shown. (E) Pregated on CD4$^-$ CD8$^-$ cells, percentages of Vγ2 and Vγ1.1 T cells in the thymus are shown. (F) Pregated on CD45$^+$ cells, percentages of Vγ3$^+$ CD3$^+$ cells in the skin are shown. Histograms represent the mean and standard deviation from analyzing 5 animals.

identical efficiency. The cycling conditions for both TSLP and TBP amplifications were 10 minutes at 95°C, followed by 40 cycles of 10 seconds at 95°C, 15 seconds at 60°C, and 20 seconds at 72°C. The relative expression of TSLP on TBP was calculated with the comparative $C_T$ ($\Delta\Delta C_T$) method.

### Isolation of skin-resident lymphocytes

Ears were digested with collagenase IV (Sigma-Aldrich) in HBSS containing 10 mM Hepes, 2.5 mM $CaCl_2$, and 2% FCS for 30 minutes at 37°C. Cell suspensions were filtered, stained, and analyzed by flow cytometry.

### Statistical analysis

Statistical significance between the individual groups was analyzed using the unpaired Student *t* test.

## Results

### The K14-TSLP Tg rescues B- and T-cell development in IL-7$^{-/-}$ mice

As previously reported,[1,2] B lymphopoiesis is dramatically impaired in adult IL-7$^{-/-}$ mice as shown by the absence of CD19$^+$ B-cell progenitors in the BM (Figure 1B). To test if increased TSLP availability could rescue B lymphopoiesis in IL-7$^{-/-}$ mice, K14-TSLP Tg mice were generated and backcrossed to an IL-7$^{-/-}$ background. Absolute numbers of cells in the BM of IL-7$^{-/-}$, WT, and IL-7$^{-/-}$ K14-TSLP Tg mice were comparable (Figure 1A). In the BM of IL-7$^{-/-}$ K14-TSLP Tg mice, the number of CD19$^+$ B cells was restored to WT numbers (Figure 1B). All B progenitor cell subsets were detectable in IL-7$^{-/-}$ mice overexpressing TSLP. The major effect of TSLP was found in the pro-B/pre-B-I (CD19$^+$ c-kit$^+$) cell compartment (4-fold increase in percentage compared with WT controls) and absolute numbers of pro-B/pre-B-I cells were 4-fold increased (S.C. and D.F., unpublished data, December 2006). Pre-B-II (CD19$^+$ CD25$^+$) cells were present at WT percentages in IL-7$^{-/-}$ K14-TSLP Tg mice. Immature (CD19$^+$ B220$^+$ IgM$^+$) and mature (CD19$^+$ B220$^{++}$ IgM$^+$) B cells in IL-7$^{-/-}$ K14-TSLP Tg mice were present in lower percentages than in WT mice. These results show that TSLP Tg expression was sufficient to rescue the block of B-cell development in the BM of IL-7$^{-/-}$ animals. In addition, these results suggest that the major target cells for TSLP were, similar to IL-7,[34] pro-B/pre-B-I cells.

IL-7$^{-/-}$ mice have reduced thymic cellularity and lack γδ T cells.[1,3] The thymus cellularity of IL-7$^{-/-}$ K14-TSLP Tg animals was 60-fold increased compared with IL-7$^{-/-}$ littermates but still remained 2-fold lower than controls (Figure 1C). Contrary to IL-7$^{-/-}$ mice, in which DN1 (c-kit$^{high}$ CD44$^+$ CD25$^-$) and DN2 (c-kit$^{high}$ CD44$^+$ CD25$^+$) thymocytes were absent, IL-7$^{-/-}$ K14-TSLP Tg mice showed normal percentages of the DN1 and DN2 subsets (Figure 1D). Moreover, the percentages of DN3 (CD44$^-$ CD25$^+$), CD4$^+$ SP, CD8$^+$ SP, and double-positive (DP) thymocyte cells in IL-7$^{-/-}$ K14-TSLP Tg mice were in the range of WT controls (Figure 1D). While γδ T-cell development was undetectable in IL-7$^{-/-}$ mice, IL-7$^{-/-}$ K14-TSLP Tg mice had normal percentages of both mature (CD24$^{lo/-}$) and immature (CD24$^{high}$) thymic γδ$^+$ T cells (Figure 1D). Indeed, Vγ2$^+$ T and Vγ1.1$^+$ T cells were generated in IL-7$^{-/-}$ K14-TSLP Tg mice (Figure 1E). Vγ3$^+$ T cells, which develop during fetal life and are later found exclusively in the adult skin,[35] were present in the skin of IL-7$^{-/-}$ K14-TSLP Tg but not in IL-7$^{-/-}$ mice (Figure 1F).

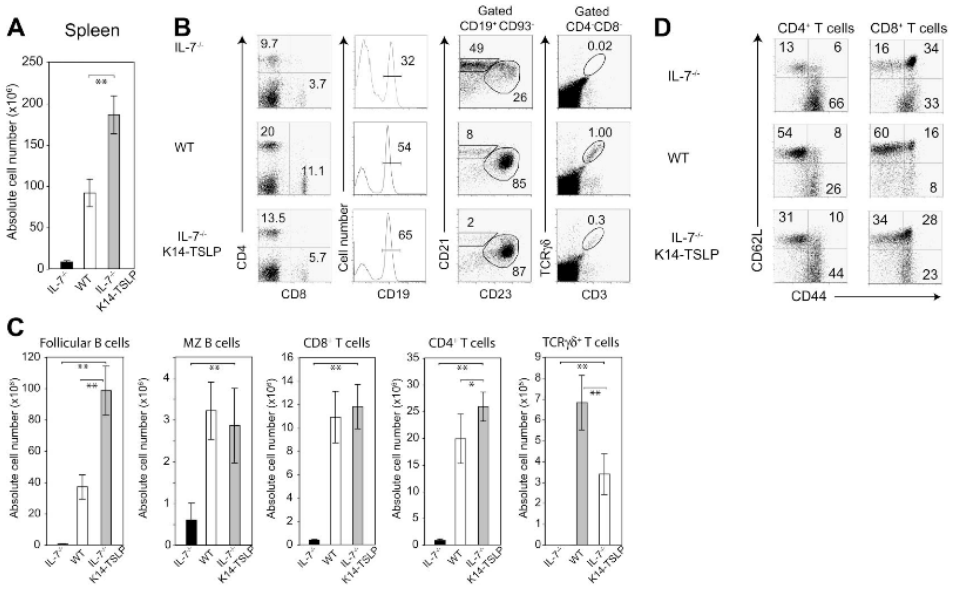


**Figure 2. TSLP Tg expression restores splenic lymphocyte compartments in IL-7$^{-/-}$ mice.** (A) Absolute splenocyte numbers of IL-7$^{-/-}$, WT, and IL-7$^{-/-}$ K14-TSLP Tg (12-week-old). (B) Percentages and (C) absolute cell number of CD4$^+$, CD8$^+$, and γδ$^+$CD3$^+$ T cells, and CD19$^+$CD93$^-$ mature B cells composed of follicular (CD23$^+$ CD21$^+$) and MZ (CD21$^{high}$ CD23$^-$) B cells are shown. Histograms represent the mean and standard deviation from analyzing 5 animals. *$P < .05$; **$P < .005$ (Student $t$ test). (D) Gated on splenic CD4$^+$ and CD8$^+$ T cells, CD62L and CD44 expression is shown.

Mouse strains in which thymocyte development is impaired beyond the DN1 stage display an abnormal cortex with cortical thymic epithelial cells (cTECs) coexpressing keratin 5 (K5) and keratin 8 (K8).[36,37] Consistent with this, the majority of the cTECs in IL-7$^{-/-}$ thymi retained an immature K8$^+$ K5$^+$ phenotype and numerous cysts were found in IL-7$^{-/-}$ thymi (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). In contrast, IL-7$^{-/-}$ K14-TSLP and WT thymi were devoid of cysts and showed a normal segregation into K8$^+$ K5$^-$ cTECs and K8$^-$ K5$^+$ mTECs. SP thymocytes, which express high levels of CD3, were localized in the thymic medulla of IL-7$^{-/-}$ K14-TSLP mice. Hence, TSLP overexpression substantially increased thymus cellularity, rescued the generation of αβ and γδ T cells, and corrected the aberrant thymic architecture in IL-7$^{-/-}$ mice.

### Peripheral B- and T-cell compartments are normal in IL-7$^{-/-}$ K14-TSLP Tg mice

IL-7$^{-/-}$ mice are lymphopenic[1] and their spleen cell numbers were 10-fold less than WT controls (Figure 2A). TSLP Tg expression in IL-7$^{-/-}$ mice increased splenocyte numbers by an average of 20-fold. The percentages of splenic CD4$^+$ and CD8$^+$ T cells in IL-7$^{-/-}$ K14-TSLP Tg mice were below WT percentages. However, absolute T-cell numbers in IL-7$^{-/-}$ K14-TSLP Tg mice were normal or, for CD4$^+$ T cells, even beyond WT numbers (Figure 2C). To test if the restoration of peripheral T cells was a result of egress of thymic emigrants or peripheral expansion, we tested CD62L and CD44 expression by splenic CD4$^+$ and CD8$^+$ T cells. The percentage of naive (CD62L$^{high}$ CD44$^{low}$) T cells was clearly higher in IL-7$^{-/-}$ K14-TSLP Tg mice compared with IL-7$^{-/-}$ percentages but was lower than WT percentages (Figure 2D). These results suggest that the restoration of peripheral T cells in IL-7$^{-/-}$ K14-TSLP Tg mice was a result of both restoration of thymic development and peripheral expansion of T cells. γδ T cells were present in the spleen of IL-7$^{-/-}$ K14-TSLP Tg animals (Figure 2B) in numbers corresponding to half that in WT controls (Figure 2C), indicating that TSLP was less efficient than IL-7 in maintaining the peripheral γδ T-cell pool.

In contrast to IL-7$^{-/-}$ mice, the absolute number of marginal zone (MZ) B cells in IL-7$^{-/-}$ K14-TSLP Tg animals was comparable with WT mice (Figure 2C). Follicular B- (FB) cell numbers were even 2.5-fold increased compared with controls (Figure 2C). It has been reported that TSLP promotes the accumulation of myeloid cells in the spleen.[25] In agreement with this, we found a 5- to 6-fold increase in the absolute number of Gr-1$^+$ CD11b$^+$ granulocytes (Figure S2). Altogether, these results indicate that TSLP promoted the accumulation of peripheral T, B, and myeloid cells.

### Systemic expression of TSLP in IL-7$^{-/-}$ K14-TSLP Tg animals

The analysis of IL-7$^{-/-}$ K14-TSLP Tg mice revealed that TSLP was able to support lymphopoiesis. We therefore investigated in which organs TSLP was expressed in Tg mice. Quantitative real-time PCR analysis revealed that, consistently with the K14 promoter specificity, TSLP mRNA was highly expressed in the skin (30-fold relative increase compared with WT) and thymus (8-fold) of Tg mice (Figure 3A). Interestingly, a 50-fold increase in TSLP transcripts was observed in the BM of Tg animals compared with WT controls. The amounts of TSLP transcripts in the spleen and mesenteric lymph nodes were not affected by the Tg expression. In the sera of IL-7$^{-/-}$ K14-TSLP Tg animals, an average concentration of 420 pg/mL TSLP was measured, while TSLP concentrations were less than 20 pg/mL in IL-7$^{-/-}$ and WT controls (Figure 3B). Therefore, the rescue of lymphopoiesis and of peripheral lymphocyte compartments might be the result of Tg-mediated increase of TSLP availability in primary lymphoid organs and in the serum.

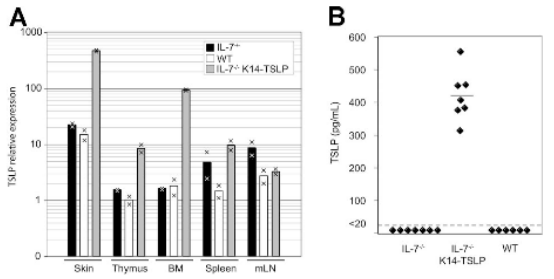


**Figure 3. TSLP is detectable in the serum of IL-7$^{-/-}$ K14-TSLP Tg mice.** (A) TSLP expression in skin, thymus, BM, et al. Each bar displays the mean of values obtained from cDNA from 2 mice. Results are representative of 3 independent experiments. (B) TSLP concentration in sera from IL-7$^{-/-}$, IL-7$^{-/-}$ K14-TSLP Tg, and WT mice was quantified by ELISA. Each symbol represents the result from an individual mouse. The mean of TSLP concentration is indicated.

### In B cells of adult IL-7$^{-/-}$ K14-TSLP Tg mice, the IgH locus contains additional N nucleotides

TSLP was previously shown to support fetal B lymphopoiesis.[20] The rescue of B-cell development in IL-7$^{-/-}$ K14-TSLP Tg mice could therefore be due to the effect of TSLP on fetal liver–derived progenitors. One hallmark of fetal lymphopoiesis is the absence of expression of terminal deoxynucleotidyl transferase (Tdt) in lymphocyte precursors,[38] leading to the generation of B cells that are completely devoid of N nucleotides at the junctions of their rearranged VDJ immunoglobulin heavy (IgH) chain genes.[39] To test whether the B cells generated in IL-7$^{-/-}$ K14-TSLP Tg mice originated from fetal or adult precursors, we examined whether rearranged IgH genes in B cells of 8-week-old IL-7$^{-/-}$ K14-TSLP Tg mice carried N region nucleotide additions. Table 1 shows that of 26 B-cell clone sequences from IL-7$^{-/-}$ K14-TSLP Tg adult spleen, all of them displayed N nucleotide additions. This result indicates that the B lymphopoiesis in adult IL-7$^{-/-}$ K14-TSLP mice was promoted by the action of TSLP on Tdt$^{+}$ cells and therefore, most likely on adult BM-derived progenitors.

### TSLP supports the differentiation of adult BM progenitor cells toward lymphoid lineages

To further assess whether the lymphopoiesis observed in IL-7$^{-/-}$ K14-TSLP Tg mice relied on the differentiation of adult precursors, we generated BM chimeras by adoptively transferring $10^7$ total BM cells from adult WT mice (CD45.1$^{+}$) into lethally irradiated IL-7$^{-/-}$ K14-TSLP Tg, IL-7$^{-/-}$, or WT recipients (CD45.2$^{+}$). Six weeks after transfer, donor chimerism was higher than 95% in all lymphoid organs of each mouse. Donor BM cell numbers were similar for each of the 3 experimental groups (Figure 4A). Donor-derived B-cell precursors were almost undetectable in the BM of IL-7$^{-/-}$ recipients, whereas all stages of B-cell development were present in both IL-7$^{-/-}$ K14-TSLP Tg mice and WT recipients (Figure 4B). Donor-derived thymocyte numbers were increased 270-fold in IL-7$^{-/-}$ K14-TSLP Tg compared with IL-7$^{-/-}$ recipients but remained 1.4-fold below numbers of WT controls (Figure 4C). Similar percentages of donor-derived DN, CD4$^{+}$ SP, CD8$^{+}$ SP, and DP were found in WT and IL-7$^{-/-}$ K14-TSLP mice (Figure 4D). The number of donor-derived splenocytes was substantially increased in IL-7$^{-/-}$ K14-TSLP Tg compared with IL-7$^{-/-}$ hosts (Figure 4E). Normal percentage of peripheral CD4$^{+}$ T cells, CD8$^{+}$ T cells, and CD19$^{+}$ B cells was found in reconstituted IL-7$^{-/-}$ K14-TSLP recipients. In agreement with our previous data, splenic B cells in IL-7$^{-/-}$ recipients were mainly composed of MZ B cells, while those found in IL-7$^{-/-}$ K14-TSLP Tg and WT recipients were mainly FB cells (Figure 4F).

**Table 1. Splenic B cells in IL-7$^{-/-}$ K14-TSLP Tg mice display N nucleotide additions at VDJ junctions**

| Clone | V | P | N1 | P | D | P | N2 | P | JH4 | D element |
|---|---|---|---|---|---|---|---|---|---|---|
| 3 | tgtaccgg | | — | | gattacg | | gggc | | attactatgctatggactactgg | SP2.2*01 |
| 4 | tgtgcaaga | | — | | tggg | | gg | | tactatgctatggactactgg | DQ52*01 |
| 5 | tgtgcaaga | tc | ctcg | | tatgattac | | gga | | atggactactgg | SP2.2*01 |
| 10 | tgtgcaaga | | aggg | | ttattactacgg | | c | | ttactatgctatggactactgg | FL16.1*01 |
| 12 | tgtgcaaga | | gg | gg | cctactatagtaac | | ccagg | | ggactactgg | SP2.x*01 |
| 14 | tgtgcaaga | | cgg | | agtaac | | — | | tacgatgctatggactactgg | SP2.x*01 |
| 16 | tgtgcaaga | | gagaagg | | attactacggtagtagct | | — | | ctatggactactgg | FL16.1*01 |
| 19 | tgtgcaaga | | aacccc | | ctacgg | | G | | actatgctatggactactgg | FL16.2*01 |
| 20 | tgtgc | | cct | | ctatga | | C | | tactatgctatggactactgg | SP2.2*01, SP2.9*01 |
| 23 | tgtgcgag | | ggacaca | | gggct | | — | | ctatgctatggactactgg | ST4 |
| 24 | tgtgcaaga | | tccc | | gta | | Agaagg | | atgctatggactactgg | FL16.1*01, SP2.x,.1,.5,.7,.8 |
| 25 | tgtgcaaga | | cg | | ggga | | G | | atgctatggactactgg | DQ52*01 |
| 28 | tgtgcgaga | | aatttcgcc | | tattactacggtagtag | | g | | actatgctatggactactgg | FL16.1*01 |
| 32 | tgtgcaaga | t | gggattttag | | cggta | | g | | ttactatgctatggactactgg | FL16.1*01 |
| 40 | tgtgcaaga | | gaga | | atgattacgac | g | gg | | tactatgctatggactactgg | SP2.2*01 |
| 50 | tgtgcaaga | | a | | aactggg | g | gcg | | actatgctatggactactgg | DQ52*01 |
| 51 | tgtgcaaga | | — | | attactacggtagtag | | ctc | | actatgctatggactactgg | FL16.1*01 |
| 56 | tgtgcaa | | tgacg | | attactacggtagtagc | | ggggg | | actatgctatggactactgg | FL16.1*01 |
| 57 | tgtgcaaga | | aggaatcg | | agttact | | tggg | | atgctatggactactgg | SP2.12*01 |
| 58 | tgtgcaaga | | gga | | tattactacggtagtagc | | ctctatggg | | tatgctatggactactgg | FL16.1*01 |
| 64 | tgtgc | | c | ga | tctactatggtaac | | cacggagggg | | tgctatggactactgg | SP2.1*01 |
| 65 | tgtgcaaga | | gcg | | tattactacggtag | | — | | tgctatggactactgg | FL16.1*01 |
| 67 | tgtgcaaga | tc | ggtgg | | gattacgac | | agaaggggtc | | ttactatgctatggactactgg | SP2.2*01 |
| 73 | tgtgcaaga | | atggaggg | | tac | | acttct | | ctatgctatggactactgg | All, except DQ52 |
| 75 | tgtgcaaga | | gaga | | atgattacgac | g | gg | | tactatgctatggactactgg | SP2.2*01 |
| 80 | tgtgcaaga | | tggac | | tactacggtagtagct | | gaaggaggg | | atggactactgg | FL16.1*01 |

Sequences of VHJ558-JH4 rearrangements from 26 individual PCR fragments derived from sorted B220$^{+}$CD19$^{+}$ splenic B cells of 8-week-old IL-7$^{-/-}$ K14-TSLP Tg mice are shown.

— indicates no N nucleotide addition.

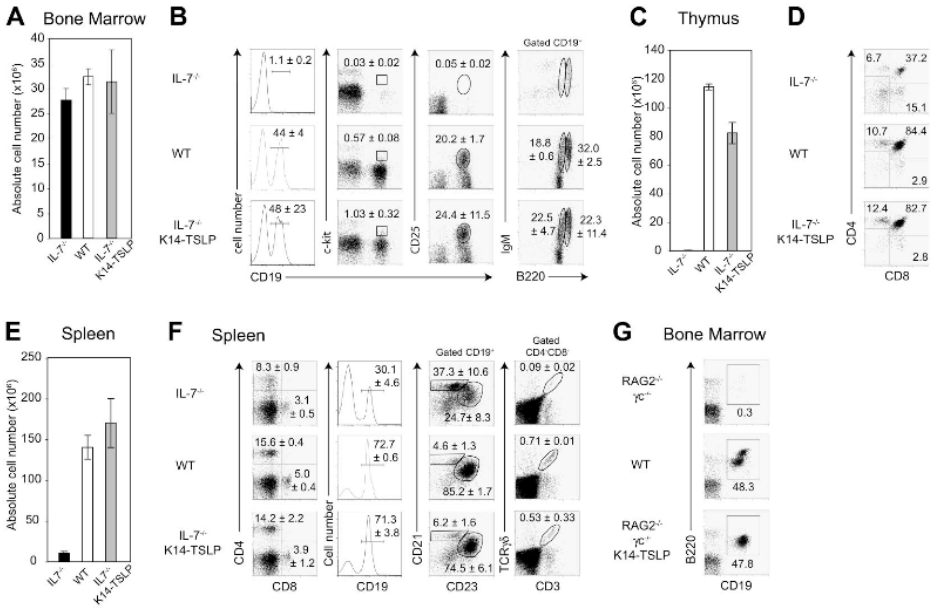


**Figure 4. TSLP sustains the differentiation of WT BM progenitors toward the B- and T-cell lineages in vivo.** (A) Absolute cell number per 2 femurs 6 weeks after reconstitution of lethally irradiated IL-7$^{-/-}$, WT, and IL-7$^{-/-}$ K14-TSLP Tg recipients with $10^7$ BM cells (CD45.1$^+$) is shown. (B) FACS profiles were gated on CD45.1$^+$ donor cells. Regions indicate the pro-B/pre-B-I (CD19$^+$c-kit$^+$), pre-B-II (CD19$^+$CD25$^+$), mature (CD19$^+$B220$^{high}$IgM$^+$), and immature (CD19$^+$B220$^+$IgM$^+$) B cells. Numbers are mean and standard deviation of percentage (n = 3). (C) Absolute CD45.1$^+$ thymocyte number and (D) percentages of donor-derived SP (CD4$^+$CD8$^-$, CD4$^-$CD8$^+$), DP (CD4$^+$CD8$^+$) thymocytes are shown. (E) Absolute CD45.1$^+$ splenocyte number. Histograms represent the mean and standard deviation from analyzing 3 animals. (F) Regions indicate splenic CD4$^+$, CD8$^+$, and γδ$^+$ T cells, and CD19$^+$ B cells containing follicular (CD23$^+$CD21$^+$) and MZ (CD21$^{high}$ CD23$^-$) B cells. Numbers are mean and standard deviation of percentage (n = 3). (G) RAG2$^{-/-}$ γc$^{-/-}$, WT, and RAG2$^{-/-}$ γc$^{-/-}$ K14-TSLP Tg mice were analyzed at 10 weeks of age. Regions indicate the committed (CD19$^+$B220$^+$) B cells in the BM. Representative FACS analyses of 1 of 3 mice.

Donor-derived cells were able to develop into TCRγδ$^+$ T cells in IL-7$^{-/-}$ K14-TSLP Tg mice. These results collectively show that TSLP could efficiently sustain the differentiation of adult BM progenitors toward both B- and T-cell lineages in vivo. Our data contrast in vitro studies showing that in adult BM only pre-BCR$^+$ progenitors are able to respond to TSLP.[40] RAG2$^{-/-}$ γ$_c^{-/-}$ mice are unresponsive to IL-7 and hence unable to generate pro-B cells. In contrast, TSLP Tg expression in adult RAG2$^{-/-}$γ$_c^{-/-}$ mice clearly promoted pro-B-cell generation in vivo (Figure 4G).

To test whether the K14-TSLP Tg had an additional effect on erythroid and myeloid compartments, we analyzed BM chimeras 6 months after reconstitution. Percentages and absolute numbers of donor-derived granulocytes (Gr-1$^+$ CD11b$^+$) and erythroblasts (Ter119$^+$ CD71$^+$) were similar in the BM of IL-7$^{-/-}$ K14-TSLP Tg and WT recipients (Figure 5A-B). In contrast, a significant increase in granulocyte and erythroblast numbers was found in the spleen of IL-7$^{-/-}$ K14-TSLP Tg mice (Figure 5C,D). Altogether, our results show that adult BM progenitors efficiently differentiated toward B and T lineages in response to TSLP and that TSLP promoted the accumulation of myeloid cells in the spleen.

### TSLP promotes B- and early T-cell development from EPLMs in vitro

The in vivo effect of TSLP prompted us to study whether TSLP could promote lineage commitment from a lymphoid/myeloid BM progenitor cell. EPLMs have been identified as B220$^+$ CD117$^+$ CD19$^-$ NK1.1$^-$ BM cells that can give rise to both myeloid and lymphoid lineages.[31] To test if TSLP promoted the development of B and T cells from EPLMs, we plated $5 \times 10^3$ FACS-sorted EPLMs on either OP9 or OP9 stromal cells expressing the Notch ligand Delta-like-1 (OP9-DL1) and added either TSLP or IL-7.

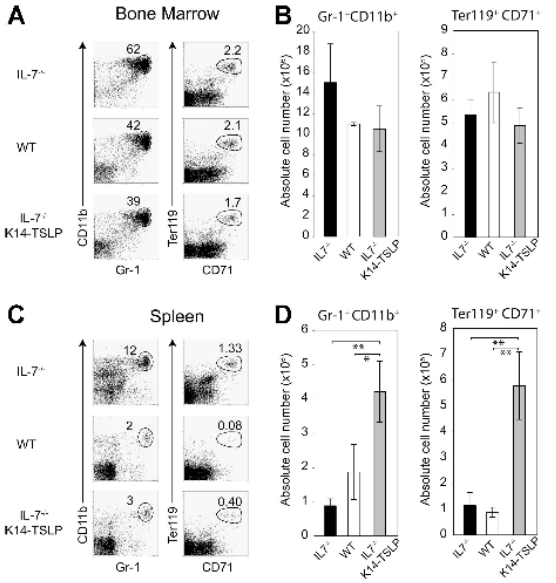


**Figure 5. TSLP Tg expression leads to the accumulation of granulocytes and erythroid precursors in the spleen.** Six months after reconstitution of lethally irradiated IL-7$^{-/-}$, IL-7$^{-/-}$ K14-TSLP, or WT recipient mice with $1 \times 10^7$ BM cells from CD45.1$^+$ mice, recipients were analyzed. All FACS profiles were gated on CD45.1$^+$ donor cells. (A) Granulocytes (Gr-1$^+$ CD11b$^+$) and erythroblasts (Ter119$^+$ CD71$^+$) in the BM are shown. (B) Absolute number of granulocytes and erythroblasts in BM. (C) Granulocytes (Gr-1$^+$ CD11b$^+$) and erythroblasts (Ter119$^+$ CD71$^+$) in the spleen are shown. (D) Absolute number of granulocytes and erythroblasts in spleen. *$P < .05$; **$P < .005$ (Student $t$ test). Histograms represent the mean and standard deviation from analyzing 3 animals.

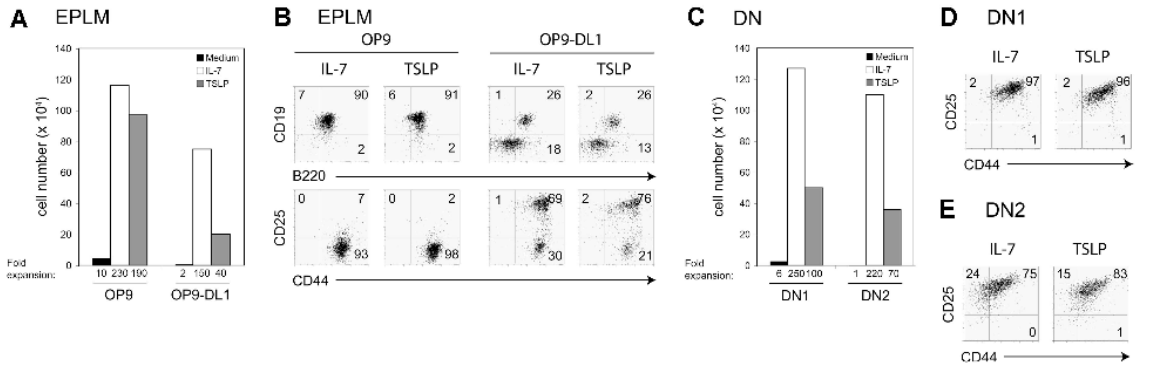


**Figure 6. TSLP promotes the expansion and the differentiation of EPLMs, and DN1 and DN2 cells in vitro.** (A) Absolute cell numbers harvested after a 7-day culture period of EPLMs (B220$^+$ CD19$^-$ NK1.1$^-$ CD117$^+$ CD93$^+$) on either OP9 or OP9-DL1 cells in the presence of TSLP or IL-7, or without additional cytokine. (B) The percentage of CD19$^+$ B220$^+$ B cells, DN1 (CD25$^-$ CD44$^+$), DN2 (CD25$^+$ CD44$^+$), and DN3 (CD25$^+$ CD44$^-$) T-cell precursors is shown. Data shown here are from 1 representative experiment of 2. (C) Sorted DN1 (CD25$^-$ CD44$^+$ CD117$^{high}$) or DN2 (CD25$^+$ CD44$^+$ CD117$^{high}$) cells were plated on OP9-DL1 in the presence of TSLP or IL-7, or without additional cytokine. Absolute cell numbers were calculated after 7 days of culture. Flow cytometric analysis of (D) DN1 or (E) DN2 cells cultured for 7 days on OP9-DL1 in the presence of TSLP or IL-7. Data shown here are from 1 representative experiment of 2.

EPLMs cultured on OP9 cells without additional cytokine did not substantially expand (Figure 6A). In contrast, cells cultured with IL-7 or TSLP underwent a 230- and a 190-fold expansion, respectively. The majority of EPLMs cultured with either TSLP or IL-7 had differentiated along the B-cell lineage, as 90% coexpressed CD19 and B220 (Figure 6B). When placed on OP9-DL1, EPLMs cultured with IL-7 or TSLP expanded 150- and 40-fold, respectively (Figure 6A). TSLP promoted the differentiation of EPLMs toward the T-cell lineage when cultured on OP9-DL1 cells (Figure 6B). Indeed, after 7 days of coculture, 76% showed a DN2 (CD25$^+$ CD44$^+$) phenotype, whereas 26% had entered the B lineage (CD19$^+$ B220$^+$). Comparably, cocultures supplemented with IL-7 gave rise to 69% DN2 cells and 26% B-committed cells. These results show that TSLP efficiently supports both B- and T-cell differentiation from adult lymphoid/myeloid BM progenitors.

#### DN1 and DN2 thymocytes are responsive to TSLP

We investigated which T-cell progenitors were directly responsive to TSLP. DN1 (Figure 6C,D) or DN2 (Figure 6C,E) cells were FACS sorted and cultured on OP9-DL1 stromal cells either alone or in the presence of TSLP or IL-7. Sorted DN1 cells cultured for 1 week with IL-7 underwent a 250-fold expansion, whereas cells cultured with TSLP expanded 100-fold (Figure 6C). Most of the cells had progressed to the DN2 stage after a culture period of 7 days (Figure 6D). DN2 cells grown in the presence of IL-7 or TSLP expanded 220- and 70-fold, respectively (Figure 6C). After 7 days in medium supplemented with TSLP, 15% of the originally sorted DN2 cells had a DN3 (CD25$^+$ CD44$^-$) phenotype, whereas 83% retained the DN2 phenotype (Figure 6E). Similar results were obtained from culturing DN2 cells with IL-7. Taken together, these results show that TSLP is able to promote the expansion and differentiation of adult DN1 and DN2 thymocytes in vitro.

#### IL-7$^{-/-}$ K14-TSLP Tg mice efficiently mount Ab responses to T-independent and T-dependent Ags

The functionality of B and T cells developing in IL-7$^{-/-}$ K14-TSLP Tg mice was assessed by immunization with the T-independent Ag NP-Ficoll or the T-dependent Ag NP-OVA. Ab titers in preimmune sera from IL-7$^{-/-}$ K14-TSLP Tg mice and IL-7$^{-/-}$ mice were higher compared with preimmune sera from WT mice (Figure 7A). Ten days after intravenous immunization with 100 μg NP-Ficoll, IL-7$^{-/-}$ mice failed to mount a significant anti–NP IgM response. In contrast, IL-7$^{-/-}$ K14-TSLP Tg animals mounted a clear anti–NP IgM response reflected by a 10-fold increase in specific Ab titer (Figure 7A), an increase similar to that seen in WT controls.

When immunized with 50 μg of the T-dependent Ag NP-OVA, IL-7$^{-/-}$ K14-TSLP Tg mice showed an increase in anti–NP IgG titers (Figure 7B). These results indicated that both B and T cells generated in IL-7$^{-/-}$ K14-TSLP Tg mice were functional and could effectively collaborate in mounting an Ab response to a T-dependent Ag. Further investigation will be required to understand the mechanisms underlying the high variation in anti–NP IgG titers observed in IL-7$^{-/-}$ K14-TSLP Tg immunized mice. Taken together, our results show that B and T cells generated in IL-7$^{-/-}$ K14-TSLP Tg mice were functional.

## Discussion

Mouse models for studying the function of TSLP have left several questions unanswered regarding its role in adult B- and T-cell differentiation and myeloid cell expansion. We show here a so-far-unappreciated capacity of TSLP to promote B- and T-cell

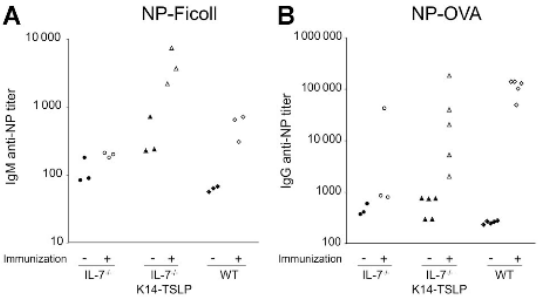


**Figure 7. NP-specific Ab titers in the sera of immunized mice.** Ten- to 12-week-old IL-7$^{-/-}$ K14-TSLP Tg, IL-7$^{-/-}$, and C57Bl/6 mice were immunized and sera were analyzed as indicated. Each symbol represents the result from an individual mouse. Filled symbols represent titers prior to immunization; empty symbols represent titers after immunization. (A) Titers of NP-specific IgM prior and 10 days after intravenous NP-Ficoll immunization. (B) Titers of NP-specific IgG titers prior and 15 days after intraperitoneal NP-OVA immunization.

development in adult mice. BM lymphoid/myeloid progenitors as well as DN thymocytes were responsive to TSLP. In the absence of IL-7, TSLP overexpression was able to restore central and peripheral lymphoid compartments and to amplify myeloid cells in the periphery.

TSLP mRNA is expressed at low levels in primary lymphoid organs[26] and TSLP concentrations are low ($< 20$ pg/mL) in the serum of WT and IL-7$^{-/-}$ mice[41,42] (Figure 3). These endogenous TSLP levels fail to overcome IL-7 deficiency. Here, we show that TSLP serum concentrations of 420 pg/mL were sufficient to compensate for the lack of IL-7. While even lower levels of TSLP affect B-cell development in a WT background,[24] the minimal concentration that is required locally or in the serum to sustain lymphopoiesis in absence of IL-7 remains to be determined.

TSLP overexpression restored all stages of B-cell differentiation in the BM of IL-7$^{-/-}$ mice (Figure 1B). Adoptively transferred adult WT BM progenitors replenished all B-cell compartments in BM and spleen of IL-7$^{-/-}$ K14-TSLP Tg recipient (Figure 4). Together with the finding that IgH junctions of B cells displayed N regions (Table 1), our data indicate that adult BM progenitors were responsive to TSLP. This is further supported by the fact that TSLP induced the differentiation of adult EPLMs toward CD19$^+$ B cells in vitro (Figure 6). In addition, pro-B cells developed in RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice in response to TSLP Tg expression (Figure 4G). These results contrast a previous study showing that in vitro, pre-BCR expression was required for adult B-cell precursors to be TSLP responsive.[40] The differences in our findings might be a result of different requirements for B-cell development in vitro and in vivo.

This study further shows that TSLP promotes the differentiation of adult BM progenitors toward T-cell lineage in vitro (Figure 6A,B) and that DN1 and DN2 cells are directly responsive to TSLP (Figure 6D-E). TSLP Tg expression restored DN1 and DN2 thymocyte compartments in IL-7$^{-/-}$ mice (Figure 1D) and normalized the organization of the thymus (Figure S1). Since DN thymocytes play a crucial role in cTEC maturation,[36,37] our results suggest that the TSLP-driven generation of DN1 and DN2 cells corrected cTEC differentiation in IL-7$^{-/-}$ mice. TSLP Tg expression also rescued the generation of γδ T cells. In IL-7$^{-/-}$ fetal thymus, Vγ3$^+$ T cells are generated but do not persist in the adult.[3] The presence of Vγ3$^+$ T cells in the skin of IL-7$^{-/-}$ K14-TSLP Tg mice shows that TSLP could replace IL-7 in maintaining the dendritic epidermal Vγ3$^+$ T cells.

The effect of TSLP on in vitro and in vivo thymocyte expansion was lower compared with IL-7. This could be due to a less stimulatory activity of TSLP on thymocytes. Indeed, TSLP has a weaker effect on α-CD3 stimulated SP thymocytes than IL-7.[12] Alternatively, it is possible that the frequency of TSLP-responding precursors might be lower than those of IL-7–responding precursors.

In the spleen, however, absolute T-cell numbers were restored and CD4$^+$ T cells were even significantly increased in IL-7$^{-/-}$ K14-TSLP mice compared with WT controls. A substantial percentage of CD4$^+$ and CD8$^+$ T cells in IL-7$^{-/-}$ K14-TSLP Tg mice showed a naive CD62L$^+$ CD44$^-$ phenotype, indicating that they were recent thymic emigrants. On the other hand, 44% of CD4$^+$ and 23% of CD8$^+$ T cells in IL-7$^{-/-}$ K14-TSLP mice showed a CD44$^{high}$ activated phenotype (Figure 2D), suggesting that TSLP-driven peripheral T-cell expansion could compensate for the relatively low thymic output. This result might explain the high variation of NP titer observed in T-dependent Ab response (Figure 7B), as activated T cells might be less efficient in their helper function.

The robust accumulation of FB cells in response to TSLP overexpression confirms previous results in K5-TSLP Tg mice[24] and is reminiscent of the effect of IL-7.[43] In addition, TSLP promoted the accumulation of myeloid precursors in the spleen. Whether this is a direct or indirect in vivo effect of TSLP remains to be clarified. High systemic concentration of TSLP in β-actin–TSLP Tg mice leads to myeloid hyperplasia in the spleen and, surprisingly, impairs lymphopoiesis.[25] In contrast, we find that TSLP can sustain T and B lymphopoiesis. This discrepancy might be a result of significantly lower amounts of TSLP Tg expressed in our mouse model. It will be important to elucidate the mechanism by which high amounts of TSLP inhibit lymphopoiesis.

Altogether, our data demonstrate that TSLP can promote adult B and T lymphopoiesis, restore peripheral lymphocyte compartments, and induce peripheral accumulation of myeloid cells. The compromised lymphopoiesis found in IL-7$^{-/-}$ mice might therefore not be due to the inability of adult hematopoietic cells to respond to TSLP but is rather a consequence of limited TSLP availability.

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## Authorship

Contribution: D.F. designed research; S.C. and A.G.R. participated in designing research; S.C. performed research; A.G.F., L.F., and A.G.R participated in performing research; A.G.F. contributed vital reagent; S.C., D.F., and A.G.R. analyzed and interpreted data; S.C. and D.F. wrote the paper.

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

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Part of the results presented here have been submitted for publication

## 5.1 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.

## 5.2 Results and Discussion

### 5.2.1 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 (Figure 1B, gate A), but that BAFF-R was highly expressed on B220$^{high}$ IgM$^+$ recirculating B cells (Figure 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 (Figure 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.

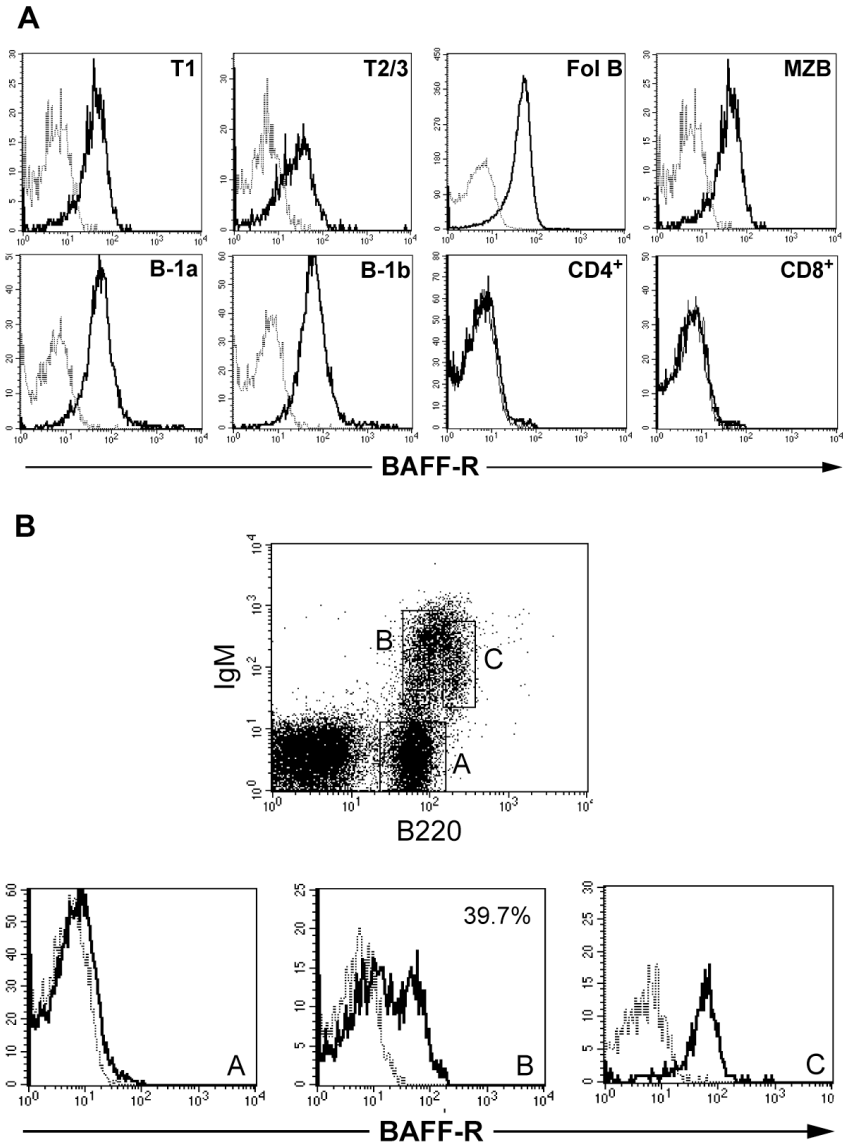


**Figure 1. BAFF-R expression on B and T cell subsets.**

**(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.

### 5.2.2 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 (Figure 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 (Figure 2B, right), whereas $CD19^{+}$ $CD93^{+}$ $IgM^{+}$ $CD23^{-}$ BM B cells are heterogeneous concerning BAFF-R expression (Figure 2B, left). Since it could only be assumed that within the $CD19^{+}$ $CD93^{+}$ $IgM^{+}$ $CD23^{-}$ B cells there is a $BAFF\text{-}R^{-}$ and a $BAFF\text{-}R^{+}$ fraction, we sorted and reanalyzed the putative two populations. We found, that two clearly definable fractions exist, a $BAFF\text{-}R^{-}$ and a $BAFF\text{-}R^{+}$ one (Figure 2C, left). Upon analysis of the surface IgM expression we found that the level was lower on the $BAFF\text{-}R^{-}$ fraction of $CD19^{+}$ $CD93^{+}$ $IgM^{+}$ $CD23^{-}$ B cells compared to the $BAFF\text{-}R^{+}$ fraction (Figure 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.

### 5.2.3 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 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

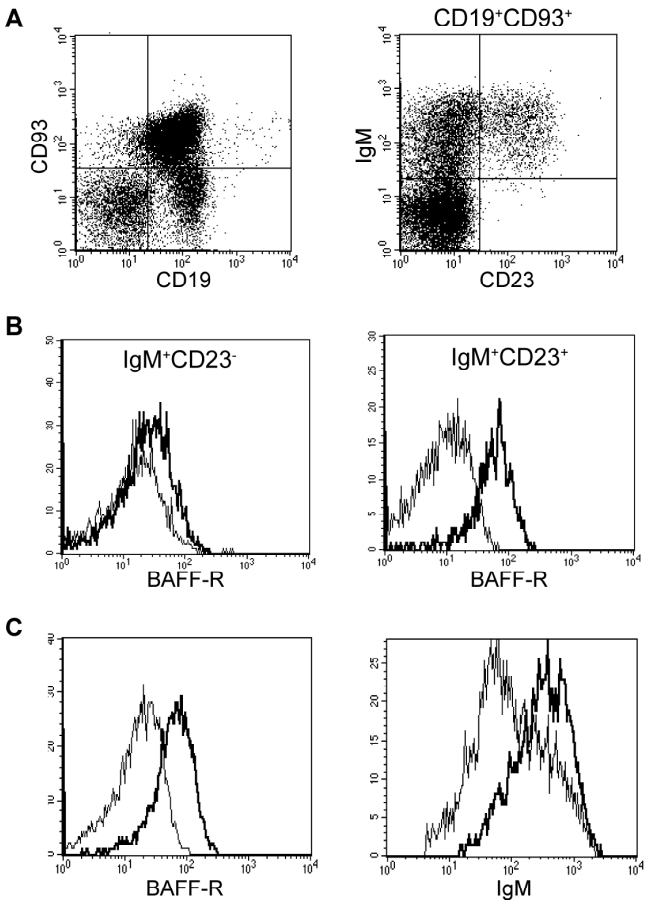


**Figure 2. Newly formed B cells from the bone marrow can be subdivided into a BAFF-R$^-$ and a BAFF-R$^+$ fraction.**

**(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.

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 (Figure 3, fraction B), which is in contrast to the finding in BM of WT mice (Figure 1B, fraction B). As expected and in accordance with the results obtained for WT mice (Figure 1B, fractions A and C), IgM$^-$ B220$^+$ B cells in these knock-in mice are negative for BAFF-R expression (Figure 3, fraction A), and all IgM$^+$ B220$^{high}$ recirculating B cells express BAFF-R (Figure 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.

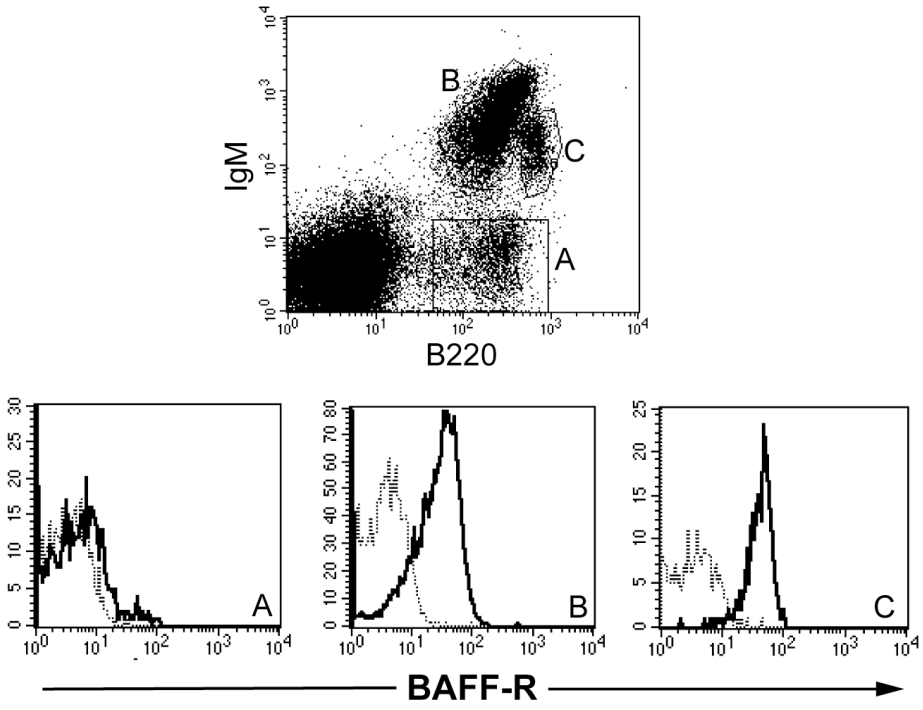


**Figure 3. BAFF-R is expressed homogenously on newly formed BM B, which do not undergo receptor editing.**

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.

### 5.2.4 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 RT-PCR. When cultured in the absence of

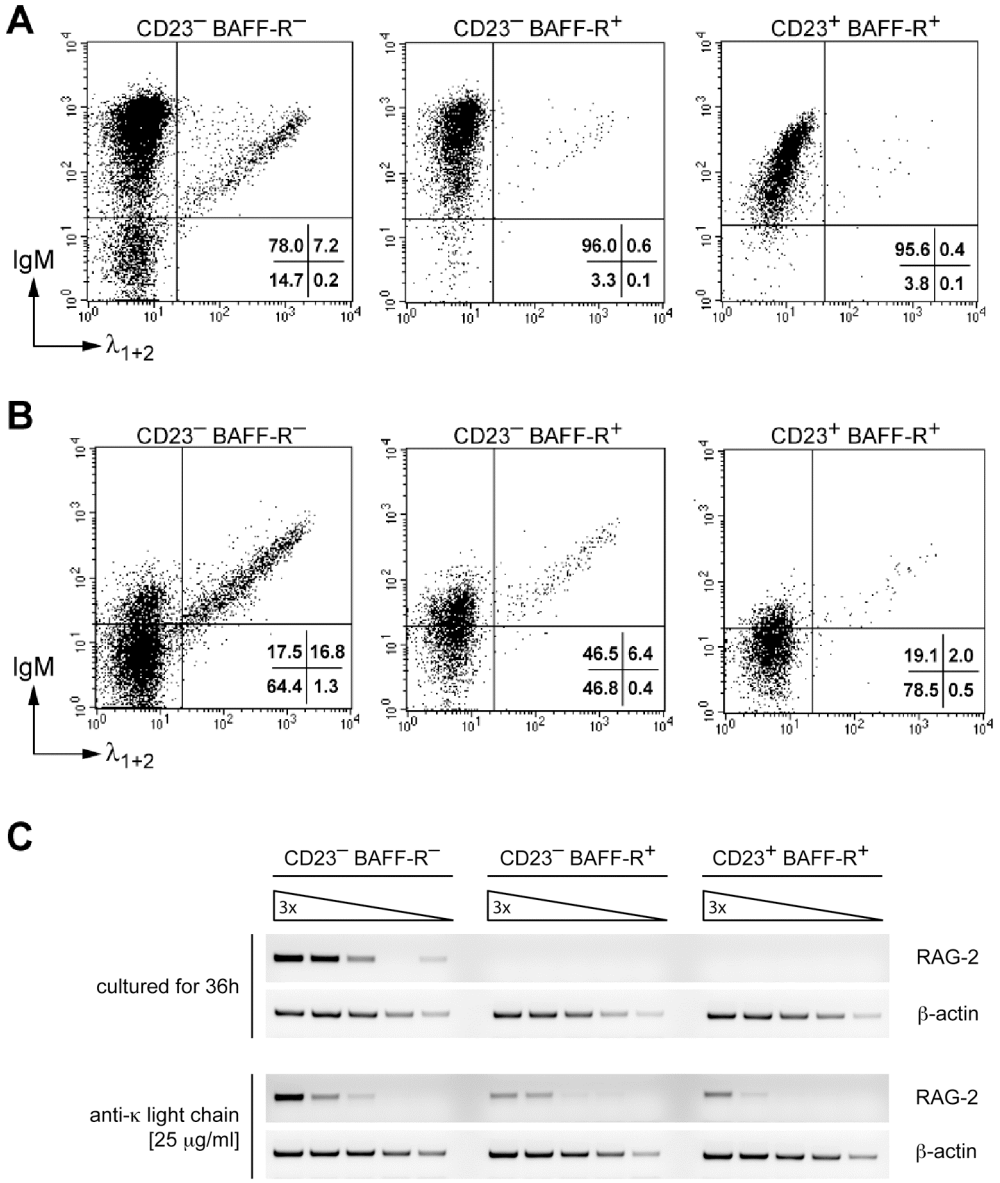


**Figure 4. RAG-2 is expressed only in BAFF-R$^-$ newly formed B cells from the BM, but can be induced in BAFF-R$^+$ newly formed B cells from the BM by culturing with anti-κ-LC antibody.**

**(A, 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 $\lambda_{1+2}$-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.

anti-κ-LC antibody, CD23$^-$ BAFF-R$^-$ B cells (Figure 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 (Figure 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 (Figure 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 (Figure 4B), then $CD23^-$ BAFF-$R^-$ BM B cells showed increased LC editing, which was apparent from the about 17% λ-$LC^+$ B cells (Figure 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 (Figure 4B, middle and right). Cells that were not able to edit the BCR from κ- to λ-LC showed reduced surface IgM expression levels (Figure 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 (Figure 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).

### 5.2.5 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, $IgM^+$ $CD93^+$ $BAFF\text{-}R^-$ $CD23^-$ and $IgM^+$ $CD93^+$ $BAFF\text{-}R^+$ $CD23^-$ as well as $IgM^+$ $CD93^+$ $BAFF\text{-}R^+$ $CD23^+$ 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 (Figure 5A, thick solid histogram).

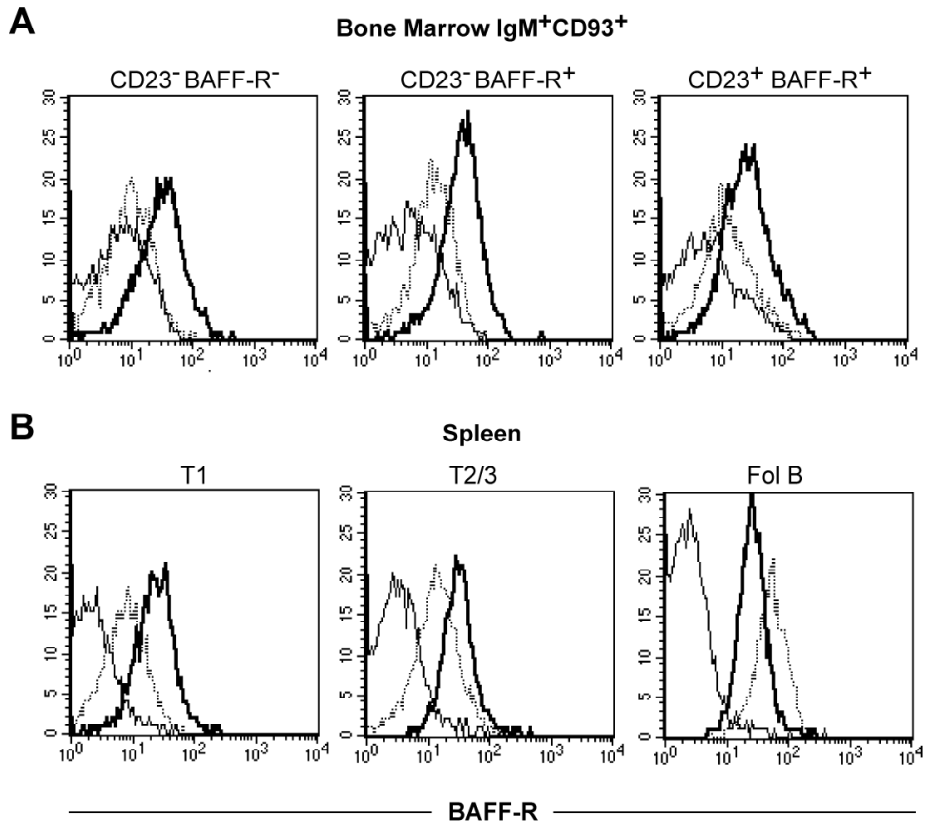


**Figure 5. Surface BAFF-R levels on B cell subsets change during culturing with anti-IgM.**

**(A)** BM B cells were sorted as $CD19^+$ $CD93^+$ $\kappa\text{-}LC^+$ $CD23^-$ $BAFF\text{-}R^-$ (left panel), $CD19^+$ $CD93^+$ $\kappa\text{-}LC^+$ $CD23^-$ $BAFF\text{-}R^+$ (middle) and $CD19^+$ $CD93^+$ $\kappa\text{-}LC^+$ $CD23^+$ $BAFF\text{-}R^+$ (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 $CD19^+$ $CD93^+$ $CD23^-$ $\kappa\text{-}LC^+$ (T1), $CD19^+$ $CD93^+$ $CD23^+$ $\kappa\text{-}LC^+$ (T2/3) or $CD19^+$ $CD93^-$ $CD23^+$ $\kappa\text{-}LC^+$ (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.

$IgM^+$ $CD93^+$ $CD23^-$ BAFF-$R^-$ B cells become 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 (Figure 5A, dotted histogram). Incubation of $CD23^-$ BAFF-$R^-$ cells in the presence of antibody (Figure 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 (Figure 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 (Figure 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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# 6 NEWB CELLS, A PUTATIVE NEW B CELL POPULATION

## 6.1 Introduction

Immature sIgM$^+$ B cells produced in the bone marrow migrate to the spleen, through the terminal branches of the splenic central arterioles, where they further differentiate into mature, immuno-competent B cells. In adult mice 2x10$^7$ immature B cells are produced daily of which 10-20% enter the spleen, but only 5-10% will enter the pool of mature B cells. Several distinct immature and mature B cell compartments have been characterized by their properties such as marker expression, functionality, turnover and life span. However, some of the defined compartments might yet still represent heterogeneous cell populations. The development of monoclonal antibodies (clones 493 and AA4.1) against the complement C1q-like receptor C1qRp (CD93) facilitated the analysis of immature (CD93$^+$) and mature (CD93$^-$) B cell compartments (1, 2).

The mature B cells in the spleen of a non-immunized mouse are represented predominantly by the relatively long-lived, circulating follicular B cells (FOB: CD19$^+$ CD93$^-$ CD21$^+$ CD23$^+$ IgM$^+$ IgD$^{hi}$) and to a lesser extent by the more sessile marginal zone B cells (MZB: CD19$^+$ CD93$^-$ CD21$^{hi}$ CD23$^{-/lo}$ IgM$^{hi}$ IgD$^{lo}$), which are exclusively located in the marginal zone of the spleen.

However, it is not clear yet what determines the selection of B cells into one or the other known mature B cell compartment. Factors, which have suggested involve the specificity of the B cell receptor, BCR-mediated signaling strength, B cell homeostasis, ontogeny and origin of precursors (3-5). Precursors of the FOB and the MZB cells are not well defined, as well as where in the FOB and MZB development their paths branch off (see General Introduction).

The mature B cell compartment (CD19$^+$ CD93$^-$) can be subdivided into CD23$^+$ cells comprising mainly the FOB cells and into CD23$^{-/lo}$ cells.

By looking on the second compartment, it became apparent that this population is heterogeneous (Figure 1). Within this population, which includes the CD21$^{hi}$ expressing MZB cells, a reasonable fraction of cells, however, expresses low levels of CD21. Compared to the MZB cells, which are relatively large in size and express high levels of IgM, but low levels of IgD, the cells of this fraction are also smaller in size and express intermediate levels of IgM and IgD (6). One could, therefore, speculate that this new B-cell subset represents yet another B-cell subpopulation.

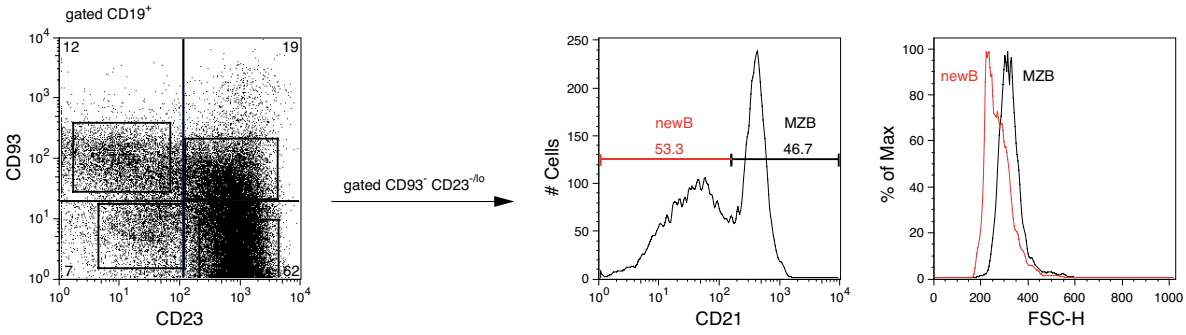


**Figure 1. Putative new B cell population in spleen.**

Splenocytes (C57BL/6) were stained for CD19, CD93, CD21 and CD23. Gating for $CD19^{+}$ $CD93^{-}$ cells reveals a $CD21^{hi}$ population comprising MZB cells and a $CD21^{-/lo}$ population, which is smaller in size (FSC) as the $CD21^{hi}$ cells.

In this study it is attempted to characterize this new B-cell subset and try to verify that it is a truly new, so far not defined, B-cell subpopulation. To that end, the cells were tested for a panel of additional markers and were also further characterized in various functional assays. The results obtained are discussed in the context of the well-described splenic immature and mature B cell compartments.

## 6.2 Results

### 6.2.1 Discovery of a putative new B cell population within the mature B cell compartment

When mature splenic B cells ($CD19^+$ $CD93^-$) are stained additionally for CD21 (complement receptor for C3d fragment) and CD23 (low affinity Ig-E receptor) surface markers, two major populations become visible (Figure 2). The majority (>80%) is $CD23^{hi}$ and comprises the FOB cells, whereas a minority (<10%) is $CD21^{hi}$ and comprises the MZB cells. The remaining, 1-4% double negative cells are the subject of this study. This putative new B cell population ($CD19^+$ $CD93^-$ $CD21^{-/lo}$ $CD23^{-/lo}$) was tested for the expression of a panel of other markers, which are differentially expressed in the characterized immature and mature B cell compartments of the spleen.

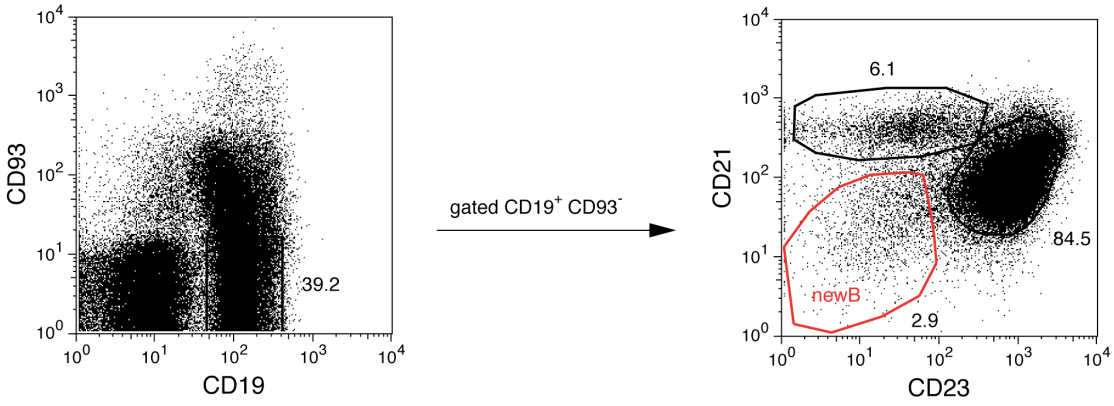


**Figure 2. Putative new B cell population ($CD19^+$ $CD93^-$ $CD21^{-/lo}$ $CD23^{-/lo}$) in the spleen of a C57BL/6 mouse.**

Splenocytes (C57BL/6) were stained for CD19, CD93, CD21 and CD23. Gating for $CD19^+$ $CD93^-$ cells reveals FOB ($CD21^+$ $CD23^{hi}$), MZB ($CD21^{hi}$ $CD23^{-/lo}$) cells and the putative new B cell population ($CD21^{-/lo}$ $CD23^{-/lo}$).

B1-a cells have been described as being $CD21^-$ $CD23^-$. Therefore these cells might fall into our newB cell gate. However, compared to the B2 B cells, B1-a cells are in addition $CD5^+$ (7).

Indeed, staining for CD5 revealed two subpopulations within this cell population. A subpopulation was $CD5^-$, while a second subpopulation was clearly $CD5^+$. The $CD5^-$ cells are smaller in size (forward scatter) than the $CD5^+$ cells and make up around 60% of the total $CD19^+$ $CD93^-$ $CD21^{-/lo}$ $CD23^{-/lo}$ cell population in the spleen of wild-type C57BL/6 mice (Table 2). This putative new B

cell population was named “newB” and defined as CD19$^{+}$ CD93$^{-}$ CD21$^{-/lo}$ CD23$^{-/lo}$ CD5$^{-}$ cells (Figure 3).

The CD5$^{+}$ subpopulation (40%) most probably comprises the B-1 cell population B-1a (7). Compared to the CD5$^{-}$ newB cells as well as the FOB and MZB cells, the CD5$^{+}$ subpopulation showed a dull staining for the B220 marker (data not shown).

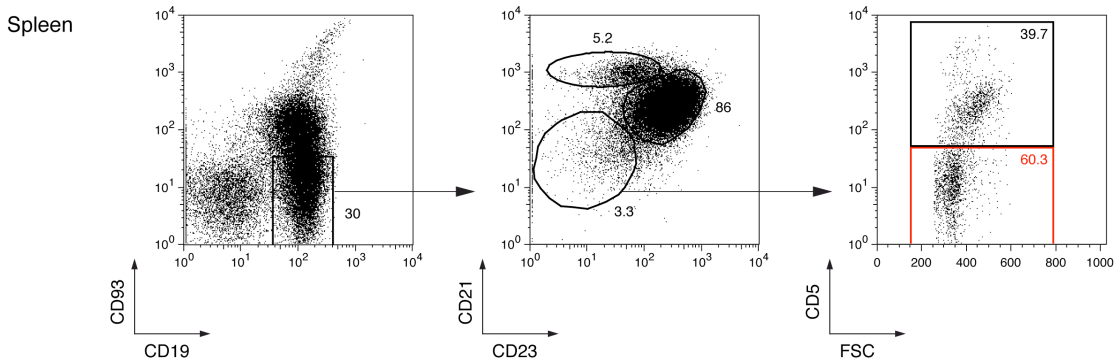


**Figure 3. New B cells are CD5$^{-}$.**

Mature B cell populations of non-immunized 8-wks-old C57BL/6 mice. Representative FACS profiles (n=5).

### 6.2.2 Surface markers expressed on newB cells compared to the FOB and MZB cell compartment

Two different approaches were used to further characterize the newB cell compartment phenotypically. Either the newB cells (CD19$^{+}$ CD93$^{-}$ CD21$^{-/lo}$ CD23$^{-/lo}$ CD5$^{-}$) were sorted in a 5-colour FACS sort and the sorted population re-stained for additional surface markers or splenocytes were depleted of CD5$^{+}$ cells, immature CD93$^{+}$ B cells as well as NK1.1$^{+}$ cells (C57BL/6 background only) using magnetic-bead separation (MACS). Then the MACS-purified mature B cells were stained for B220, CD21 and CD23 in combination with antibodies against additional surface markers. For comparison FOB and MZB cells were included in the analysis. The results are shown in figure 4 and are summarized in table 1.

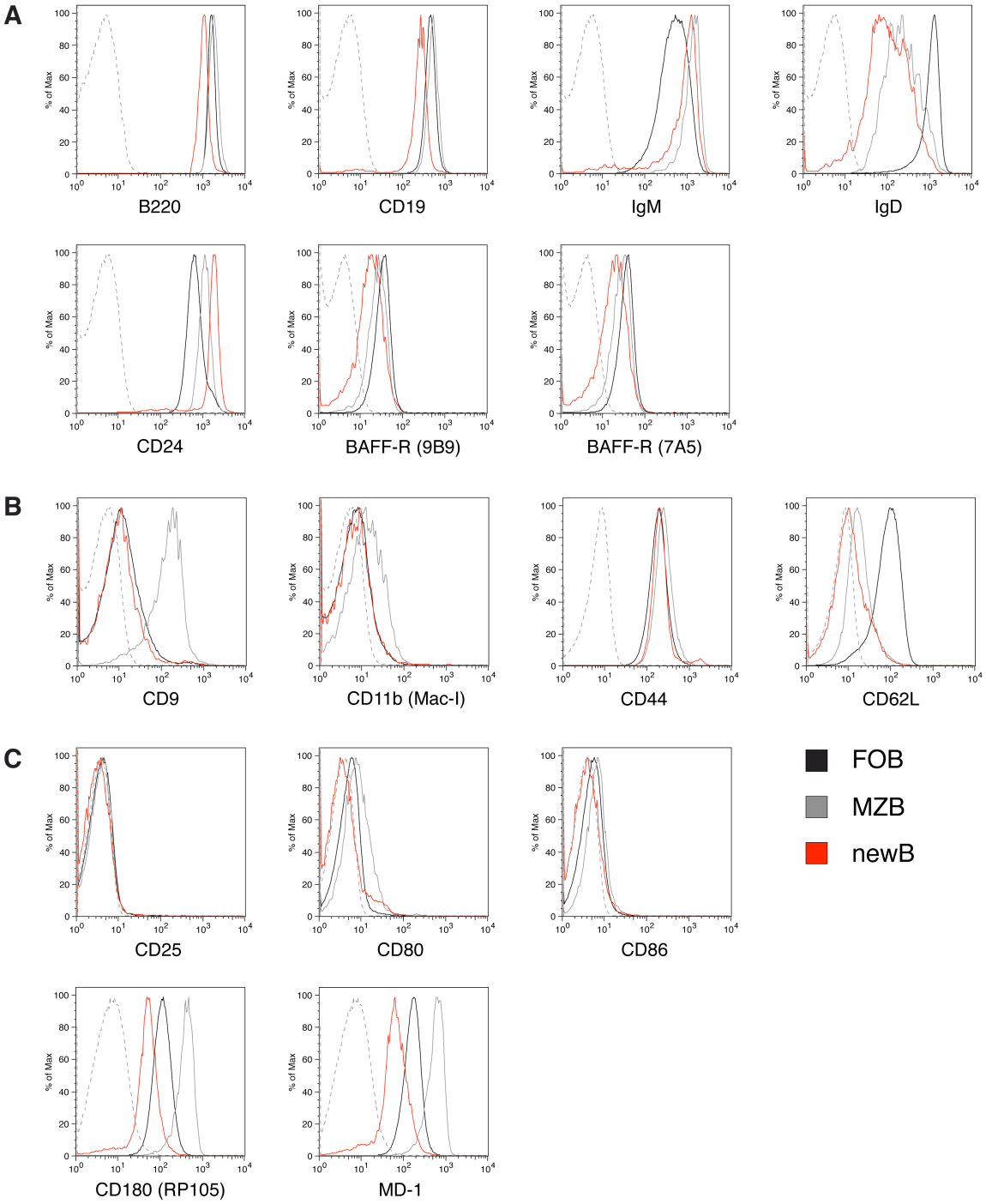


**Figure 4. Surface marker expression on newB cells compared to FOB and MZB cells.**

Splenocytes from C57BL/6 mice were depleted for $CD93^{+}$, $CD5^{+}$ and $NK1.1^{+}$ cells by MACS and stained with B220, CD21, CD23 and one of the indicated markers for FACS analysis. FOB (black: $B220^{+}$ $CD21^{+}$ $CD23^{hi}$); MZB (grey: $B220^{+}$ $CD21^{hi}$ $CD23^{-/lo}$); newB (red: $B220^{+}$ $CD21^{-/lo}$ $CD23^{-/lo}$); negative control (grey dashed line). Markers used were for **(A)** B cell and maturation; **(B)** Homing and adhesion; **(C)** Activation markers.

Thus, both IgM and IgD isotypes are differentially expressed on FOB and MZB cells. The newB cells demonstrate a level of IgM expression, which is as high as for MZB cells, but some cells show a lower IgM expression level. It cannot completely ruled out at that stage, that this low IgM-expressing cells yet represent another population or might even be in transit from or to the newB cell compartment. The IgD expression level is as low and varied like that seen in MZB cells. Compared to them, FOB cells express IgD more homogenously and at higher levels.

Both, CD19 and B220 are expressed at slightly lower levels on newB cells than on FOB and MZB cells. CD19 functions to mediate the amplification of Src family protein kinase activity in BCR signaling, and therefore its level is critical for B cell activation. Mice over-expressing CD19 on their B cells spontaneously develop autoimmunity (8).

The transitional 1 (T1) B cells are $IgD^{low}$ $CD21^{-}$ $CD23^{-}$ $CD93^{+}$ and express high levels of CD24 (9). In our FACS analysis newB cells show a higher expression level than MZB and FOB cells, suggesting that the newB cells might be of a less mature phenotype, although, by definition they belong to the mature B cell compartment, as they are $CD93^{-}$.

BAFF signaling through BAFF-R is known to be important for the survival of immature B cells and their further development into mature B cells. BAFF-R levels are regulated by BCR signaling and absence of BAFF-R largely prevents the development of B-2 B cells beyond the T1 stage (10, 11). Testing B cells for BAFF-R expression using two different anti-BAFF-R antibodies recently developed in our group (12) showed that newB cells do express BAFF-R, although slightly less than FOB and MZB cells. However, we could show that expression of BAFF-R can be detected as early as on a population of $CD19^{+}$ $CD93^{+}$ $CD23^{-}$ bone marrow cells (Rauch et. al., for submission).

Within the mature B cell compartment CD9 has been so far described as a unique marker for MZB cells, subsets of B-1 cells and plasma cells (13). While the MZB cells are $CD9^{+}$, both the newB and the FOB cells do not express CD9.

In contrast to B-1 cells, which are defined in part through their $CD11b^{+}$ phenotype (14), both newB and FOB cells are negative for CD11b, while MZB cells show a low expression.

NewB, FOB and MZB do express CD44 to the same extent. CD44 functions as a receptor for extracellular matrix components including hyaluronic acid, is implicated in lymphocyte homing and is elevated on activated B cells (15).

L-selectin CD62L mediates lymphocyte homing to high endothelial venules of peripheral lymphoid tissue and to activated endothelium at inflammatory sites (16). Interestingly, FOB cells express CD62L, while both MZB and the newB cells are $CD62L^{-}$. It is worth the notion that FOB cells, in contrast to the sessile MZB cells, are re-circulating cells. This may indicate that newB cells

represent a sessile B cell population much as MZB cells, alternatively, they do not fulfill other criteria to leave for the circulation.

| **Surface marker expression on newB cells in comparison to FOB and MZB cells** | | | |
|---|---|---|---|
| **(A)** B cell and B cell maturation | | | |
| | newB | FOB | MZB |
| CD19 | + | + | + |
| IgM | ++ | + | ++ |
| IgD | + | ++ | + |
| CD21 | -/+ | + | -/+ |
| CD23 | -/+ | + | -/+ |
| CD24 | ++ | + | + |
| CD93 | - | - | - |
| BAFF-R | + | + | + |
| **(B)** Homing, adhesion | | | |
| | newB | FOB | MZB |
| CD9 | - | - | + |
| CD11b (Mac-I) | - | - | -/+ |
| CD44 | + | + | + |
| CD62L | - | + | - |
| **(C)** Activation | | | |
| | newB | FOB | MZB |
| CD25 | - | - | - |
| CD80 | - | - | - |
| CD86 | - | - | - |
| CD180 (RP105) | + | + | ++ |
| MD-1 | + | + | ++ |

**Table 1. Overview: surface marker expression on newB cells compared to FOB and MZB cells.**
**(A)** B cell and B cell maturation; **(B)** Homing and adhesion; **(C)** Activation markers.

Tested surface markers known to be elevated on activated B cells, CD25, CD80 and CD86 are not higher expressed on newB cells as compared to the naive FOB or MZB cells, suggesting again, that newB cells are not in an activated state. Additionally, it was shown that memory B cell populations

have increased levels of CD80 and CD86 expression, which again, makes it unlikely that newB cells are related to memory B cells (17, 18).

The TLR proteins CD180 (RP105) and MD-1, known to influence signaling via the BCR in context of bacterial antigens such as LPS, are expressed at a lower level than in FOB, and especially lower than in MZB cells. The higher expression level of these proteins in MZB cells has been also described by others and may help these cells to more readily respond to bacterial antigens (19-21).

### 6.2.3 NewB cells can be found in lymph nodes

The major mature B cell population in lymph nodes is represented by the recirculating FOB cells. In contrast, MZB cells are absent in lymph nodes as they exclusively reside in the spleen. To investigate whether newB cells are present in lymph nodes, we performed 5-colour FACS analyses of cells isolated from axillary, inguinal and mesenteric lymph nodes.

Indeed, newB cells could be detected, although to relatively smaller numbers compared to the spleen (Figure 5 and Table 2). The $CD5^+$ cells could also be found in low numbers, which is not surprising, since B-1a cells have been described to be found in lymph nodes, as well (22, 23). Interestingly, the ratio between newB and $CD5^+$ B cells is similar in the spleen as in the lymph nodes (Table 5).

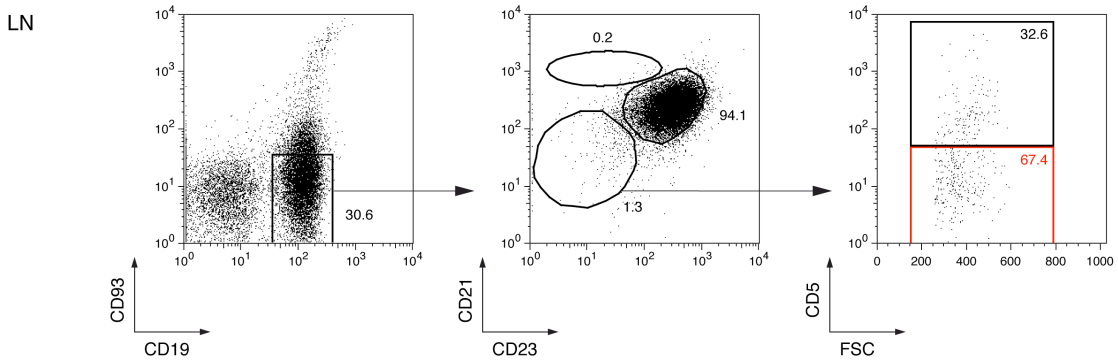


**Figure 4. NewB cells can be found in low numbers in LNs.**

Mature B cell populations of non-immunized 8-wks-old C57BL/6 mouse. (LNs: pooled axillary, inguinal and mesenteric). Representative FACS profiles (n=5).

| **NewB cell population size within the mature B cell compartment in spleen and lymph nodes** (% of total mature B cell compartment) | | |
|---|---|---|
| Population | Spleen | Lymph nodes |
| FOB | 84.2 ± 1.0 % | 92.8 ± 1.1 % |
| MZB | 6.2 ± 0.6 % | — |
| newB | 2.1 ± 0.1 % | 0.9 ± 0.3 % |
| $CD5^+$ B | 1.4 ± 0.2 % | 0.6 ± 0.2 % |
| **% of newB cells within the $CD19^+$ $CD93^-$ $CD21^{-/lo}$ $CD23^{-/lo}$ population** | | |
| newB | 60.2 ± 2.7 % | 66.1 ± 3.7 % |
| $CD5^+$ B | 39.8 ± 2.7 % | 33.9 ± 3.7 % |

**Table 2. NewB cell population size within the mature B cell compartment in spleen and lymph nodes.**

Cell suspensions from spleen and lymph nodes (pooled axillary, inguinal and mesenteric) of non-immunized 8-wks-old C57BL/6 mice (n=5) were stained with antibodies against CD19, CD93, CD21, CD23 and CD5 and analysed by 5-colour FACS.

### 6.2.4 Do newB cells have somatically hypermutated Ig chains?

Somatic hypermutation is an important aspect in generating antibody diversity in the maturation of an immune response. The process leads to higher affinity antibodies with the final goal to elicit a better response.

The λ locus is the smallest of the mouse Ig loci, and germline sequence differences combined with junctional imprecision in joining $V_\lambda$-$J_\lambda$ gene elements give only little diversity. Therefore the λ locus is a convenient target for the analysis of somatic hypermutations (24).

The analysis of the λ1 light chains in newB cells should give information if this putative new B cell population is involved in germinal center reactions and might be related to some form of memory B cells. It could be shown for germinal center B cells ($B220^+$ $PNA^{hi}$) that they contain readily hypermutated $V_\lambda1$-$J_\lambda1$ rearranged genes (25).

For the λ1 light chain analysis newB ($CD19^+$ $CD93^-$ $CD21^{-/lo}$ $CD23^{-/lo}$), FOB ($CD19^+$ $CD93^-$ $CD21^+$ $CD^+$) and immature transitional T1 ($CD19^+$ $CD93^-$ $CD21^-$ $CD23^-$) B cells were sorted from splenocytes of C57BL/6 mice. λ1 light chain-rearrangements were amplified from genomic DNA of sorted cells by a semi-nested PCR (26). The resulting PCR products were cloned generating λ1 light chain-libraries. Sequences obtained from each library were aligned and analysed for mutations in the CDR regions (27).

**Somatic hypermutations are absent in λ1 light chains of newB cells**

| | CDR1 | | | | | | | | | | | | | CDR2 | | | | | | | CDR3 | | | | | | | | Jλ1 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 24 | 25 | 26 | 27a | 27b | 27c | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 89 | 90 | 91 | 92 | 93 | 94 | 95 | | 96 | 97 | 98 | 99 | 100 |
| | S | S | T | G | A | V | T | T | S | N | Y | A | N | G | T | N | N | R | A | P | A | L | W | Y | S | N | H | | W | V | F | G | G |
| **Germline** | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | TTC C | TGG | GTG | TTC | GGT | GGA... |
| **newB** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | --- - | TGG | GTG | TTC | GGT | GGA... |
| 3 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | C-- | --- C | TGG | GTG | TTC | GGT | GGA... |
| 4 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | TT- - | --- | GTG | TTC | GGT | GGA... |
| 7 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | TT- - | --G | GTG | TTC | GGT | GGA... |
| 16 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | TTC - | -GG | GTG | TTC | GGT | GGA... |
| 22 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | TTC - | TGG | GTG | TTC | GGT | GGA... |
| 24 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | T-- - | -GG | GTG | TTC | GGT | GGA... |
| **FOB** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | C-- | --- C | TGG | GTG | TTC | GGT | GGA... |
| 2 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | T-- - | TGG | GTG | TTC | GGT | GGA... |
| 3 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | TTC - | -GG | GTG | TTC | GGT | GGA... |
| 7 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | --- - | TGG | GTG | TTC | GGT | GGA... |
| 8 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | TT- - | --- | GTG | TTC | GGT | GGA... |
| 10 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | TT- - | --G | **A**TG | TTC | CGG | TGG... |
| 14 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CT**G** | TGG | TAC | AGC | AAC | CA- | --- C | TGG | GTG | TTC | GGT | GGA... |
| **T1** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGC | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | TT- - | --G | GTG | TTC | GGT | GGA... |
| 2 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | T-- - | TGG | GTG | TTC | GGT | GGA... |
| 3 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | TTC - | --G | GTG | TTC | GGT | GGA... |
| 5 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | --- - | TGG | GTG | TTC | GGT | GGA... |
| 6 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | --- C | TGG | GTG | TTC | GGT | GGA... |
| 10 | TCA | AGT | ACT | GGG | GCT | GTT | ACA | ACT | AGT | AAC | TAT | GCC | AAC | GGT | ACC | AAC | AAC | CGA | GCT | CCA | GCT | CTA | TGG | TAC | AGC | AAC | CAT | TT- - | -GG | GTG | TTC | GGT | GGA... |

**Table 3. Somatic hypermutations are absent in λ1 light chains of newB cells.**

Nucleotide sequences (CDR1, CDR2, CDR3 and 5' of Jλ1) of λ1 light chain rearrangements with unique VJ-joining sequences isolated from newB, FOB and immature transitional T1 B cells are shown. A total of 40 newB-, 15 FOB- and 15 T1 B cell-derived $V_{\lambda 1}J_{\lambda 1}$-rearrangements were sequenced from splenocytes of C57BL/6 mice (n=3). Mutations in bold.

Germ-line sequence of λ1 is shown at top as a reference; codons numbered according to Kabat et al., 1991(27); CDR: complementarity-determining region.

We found only two mutations in the set of analysed unique λ1 light chain rearrangements in FOB cells, which are most probably due to PCR errors and none in the analysed newB and MZB cells (Table 3).

Thus no somatic hypermutations were detected in the set of λ1 light chains analysed in newB, FOB and transitional T1 cells. For germinal center B cells ($B220^+$ $PNA^{hi}$) it has been reported that 77% of all analysed clones showed >1 mutation per sequence, irrespective whether the $V_\lambda 1$-$J_\lambda 1$ junction was in- or out-of-frame (25). Thus, our finding is significant and demonstrates that the newB cells are not subjected to somatic hypermutation and most probably do not belong to germinal center B cells.

### 6.2.5 Response of newB cells to B cell stimuli

NewB cells were tested for their ability to respond to mitogenic stimulation with LPS and the oligodeoxynucleotid CpG, or to a BCR-dependent stimulus (α-IgM) in combination with BCR-independent stimuli (α-CD40 or IL-4) mimicking T cell-derived signals. The responses were compared with those of immature, transitional B cells and of mature B cell populations.

To get sufficient numbers of the rare newB cells a purification protocol was established where splenocytes were first depleted of $NK1.1^+$, $CD93^+$ and $CD5^+$ cells by magnetic bead separation (MACS). The remaining splenocytes were stained for B220, CD21 and CD23 expression. From this population, enriched for $CD93^-$ B cells, a preparation of newB cells ($B220^+$ $CD21^{-/lo}$ $CD23^{-/lo}$) could then be FACS-sorted in numbers sufficient for B cell stimulation assays ($1x10^5$ cells/well in a 96-well plate). The immature, transitional B, FOB and MZB cells were directly FACS-sorted by a 4-colour staining (CD19, CD93, CD21 and CD23).

Purified newB, transitional B and mature B cells were cultured *in vitro* in the presence of LPS, α-IgM + α-CD40 or α-IgM + IL-4 for 3 days. The cellular proliferation was quantified by the incorporation of [$^3$H]thymidine.

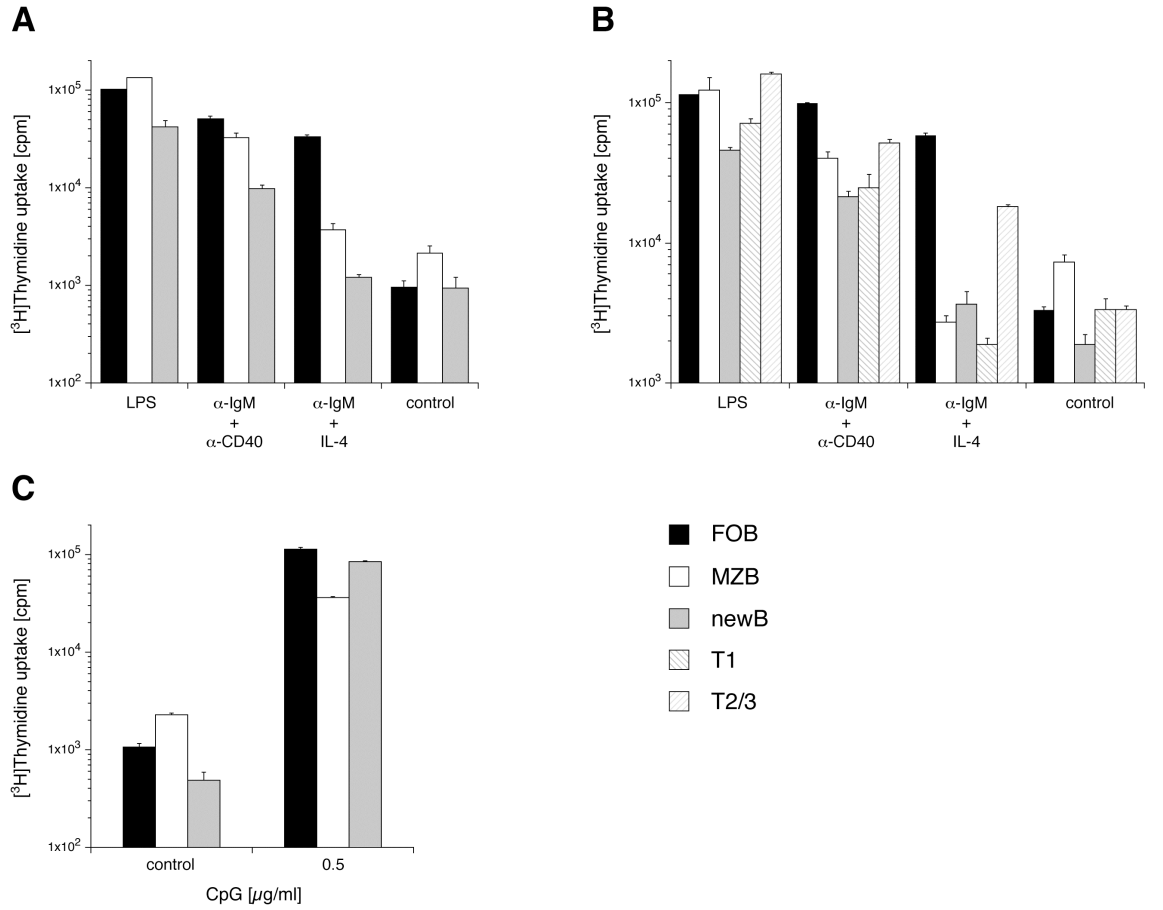


**Figure 6. NewB cells proliferate in response to LPS and to α-IgM + α-CD40 to a lesser extent.**

FACS-sorted immature and mature B cells, **(A)** newB, FOB and MZB cells, **(B)** newB, FOB, MZB, transitional B cells (T1 and T2/3), were stimulated *in vitro* with LPS [10 μg/ml], α-IgM [25 μg/ml] + α-CD40 [20 μg/ml] or α-IgM [25 μg/ml] + IL-4 [10% IL-4 sup.]. **(C)** NewB, FOB and MZB cells were stimulated *in vitro* with the oligodeoxynucleotid CpG (CpG1826 [0.5 μg/ml]). Cells were incubated for 72h. The last 9 hours dividing cells were labeled by adding [$^3$H]thymidine (1μCi/96-well). Control: unstimulated cells.

NewB cells did respond to LPS and to α-IgM + α-CD40, although they proliferated to a lesser extent than the mature FOB and MZB cells. However, a significant response to α-IgM + IL-4 could not be detected. Interestingly, the immature transitional T2/3 cells responded to all stimuli significantly higher than the newB cells (Figure 6A and B).

Motifs containing unmethylated CpG dinucleotides in bacterial DNA or in synthetic oligodeoxynucleotides (ODN) are known to activate B cells and promote their proliferation via TLR9 signalling (28, 29). NewB cells treated with CpG responded well, comparable to FOB cells (Figure 6C).

### 6.2.6 NewB cells in different mouse strains

Furthermore, the existence and numbers of newB cells in different strains were analysed. The analysed strains were either impaired in the output of immature B cells from the bone marrow (λ5 deficient) or in the formation of germinal center and memory B cells (CD40-deficient), or have an enlarged mature B cell compartment due to transgenes for survival factors (e.g. Bcl-2 or BAFF transgenic mice)(Figure 7).

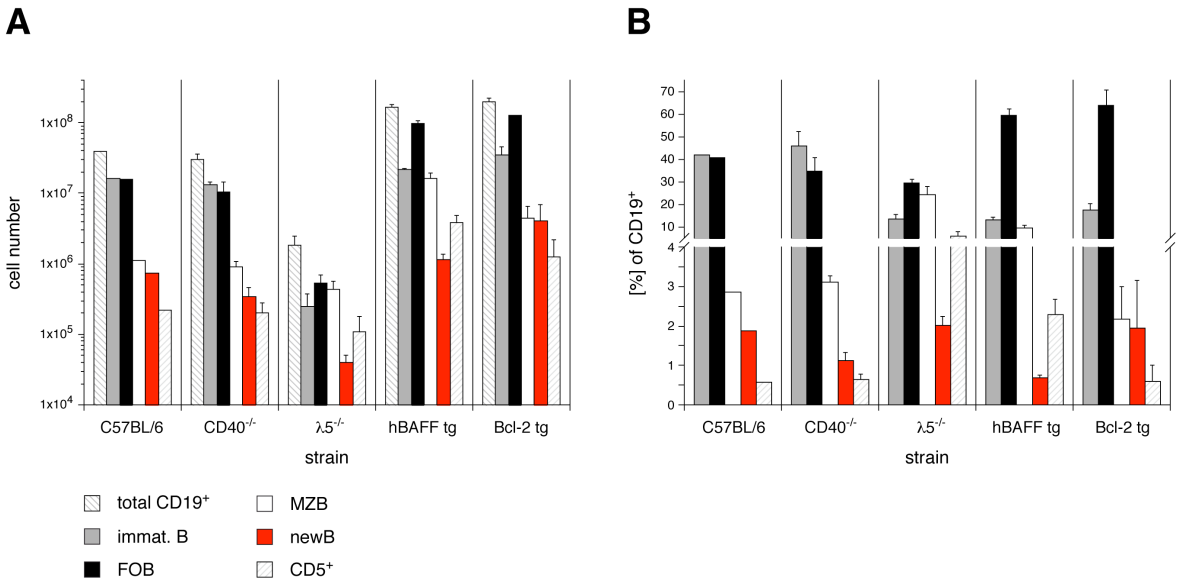


**Figure 7. NewB cells compared to other B cell populations in the spleen of wild-type, different k.o. and transgenic mouse strains.**

Splenocytes from C57BL/6, CD40$^{-/-}$, λ5$^{-/-}$, hBAFF tg (BALB/c) and Bcl-2 tg mice (n=2-4) were analysed for CD19, CD93, CD21, CD23 and CD5 by 5-colour FACS analysis.

NewB cells could be detected in spleens of λ5- and CD40-deficient, as well as in Bcl-2 and hBAFF transgenic mouse strains.

Absolute numbers of newB cells were reduced in λ5-deficient mice and must reflect the lower influx to the spleen of immature B cells from the bone marrow as there is an overall reduction of CD19$^+$ cells in these mice. However, the proportion of newB cells as compared to the total CD19$^+$ cells is similar as in wild-type C57BL/6 mice (~2%).

NewB cells in the mouse lacking CD40, which show impaired Ig class switching and lack germinal center formation (30) were found slightly reduced in absolute numbers and their proportion to total $CD19^+$ cells was less than in wild type mice.
In Bcl-2 tg mice the absolute number of newB cells is increased, as are all other B cell populations analysed. However, the proportion of the newB cells as compared to the total $CD19^+$ cells remains similar to the number found in wild-type C57BL/6 mice.
Interestingly, in hBAFF tg mice, where the number of FOB, MZB and $CD5^+$ B cells, and especially the proportion of MZB cells is increased, the total number of newB cells remains similar to what was found in wild-type C57BL/6 mice, while the proportion of newB cells as compared to the total $CD19^+$ cells is reduced.

### 6.2.7 Turnover of newB cells compared to other immature and mature B cell populations in the spleen

Finally, the turnover of newB cells was assed and compared to other immature and mature splenic B cells. 4-5 weeks old C57BL/6 mice were injected i.p. with the thymidine analogue BrdU and thereafter treated with BrdU in the drinking water for 18 days. The incorporation of BrdU into the DNA of splenic B cell populations was determined at the end of the BrdU-labeling period and after a chase period of 21 days (n=5 per group). Cells were isolated by FACS-sort with phenotypic markers (see above) to distinguish the newB cells, transitional T1 and T2/3, FOB, MZB and $CD5^+$ B cells. After the isolation $BrdU^+$ cells in each sorted population were detected with an intracellular anti-BrdU staining by FACS (Figure 8).
Transitional B cells showed an almost complete BrdU labeling after 18 days, being somewhat lower (88%) for the T2/3 cells, but in accordance with their sequential development. The nearly complete disappearance after the chase period indicates a high turnover of these cells and reflects the transitional state of these cells in development.
FOB and MZB cells were labeled to 40% and 58%, respectively after 18 days. Compared to the transitional B cells, these major mature splenic B cell populations remained to 27% and 45%, respectively, $BrdU^+$ after the chase period. This data is indicative of a slow turnover and most likely reflects the stage and function of these cells as being differentiated to relatively long-lived B cells and fulfill the function to monitor the organism for antigen.

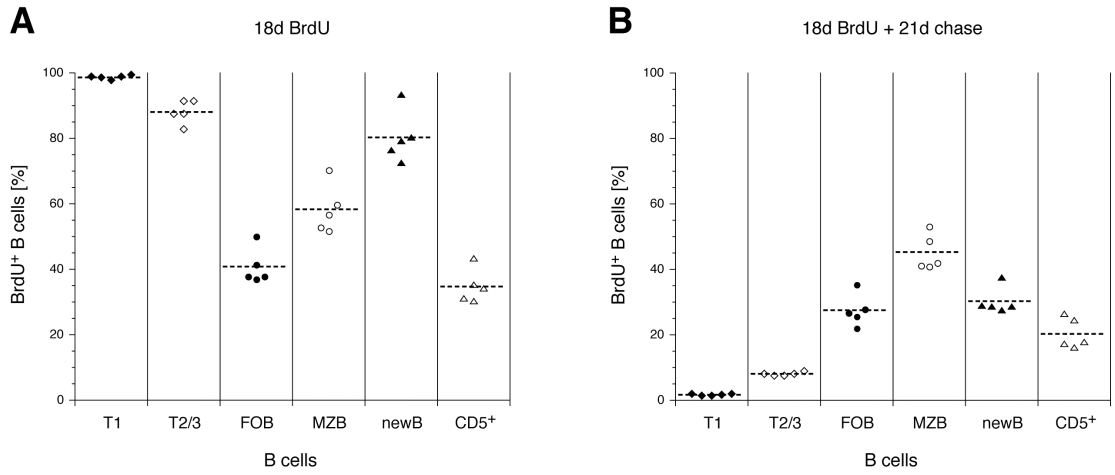


**Figure 8. Turnover of newB and other immature and mature splenic B cell populations.**

C57BL/6 mice (age 4-5 weeks) were treated with initially 1 mg BrdU i.p. and 1 mg/ml BrdU in drinking water for 18 days. Groups of mice (n=5) were analysed at end of BrdU-labeling and after a chase period of 21 days. Cells were isolated by FACS-sort with phenotypic markers to distinguish (◆) transitional T1, (◇) transitional T2/3, (●) FOB, (○) MZB, (▲) newB and (△) $CD5^+$ cells. $BrdU^+$ cells were detected with an intracellular anti-BrdU staining.

NewB cells were labeled significantly higher than the other mature ($CD19^+$ $CD93^-$) B cells showing $BrdU^+$ cell levels (80%) in-between the values obtained for T2/3 (88%) and MZB (57%) cells after the 18 days. Interestingly, after the 3 weeks chase period still 30% of the newB cells were $BrdU^+$.

The $CD5^+$ cells, in contrast showed the lowest labeling kinetics of all analysed B cell populations in the spleen.

## 6.3 Discussion

A putative new B cell population initially found in the spleen and to be of $CD19^{+}$ $CD93^{-}$ $CD21^{-/lo}$ $CD23^{-/lo}$ phenotype could be further defined and characterized (6). The initially described population could be shown to be heterogeneous for CD5 expression. The $CD5^{-/-}$ subpopulation of cells, now termed newB cells ($CD19^{+}$ $CD93^{-}$ $CD21^{-/lo}$ $CD23^{-/lo}$ $CD5^{-}$), are small in size, comparable to FOB cells, and represent 2% of $CD93^{-}$ (mature) B cells in spleens of wild-type C57BL/6 mice. In contrast, cells of the $CD5^{+}$ subpopulation (1%) are larger in size than the $CD5^{-}$ cells, have lower but more varied B220 expression levels (data not shown) and most probably comprise B-1a cells.

The analysis of surface markers for B cell maturation, homing, adhesion and activation suggest that newB cells have overall a less mature phenotype. While Ig expression levels on newB cells are comparable to levels in MZB cells with a low and varied IgD expression but high IgM expression, CD19 and B220 expressions are slightly lower. Additionally, BAFF-R expression, known to be regulated by BCR signaling (10, 11), is slightly reduced, while the expression of CD24, described to be higher on less mature B cells (9), is elevated. Additionally, the TLR proteins CD180 and MD-1, known to support signaling via the BCR and TLR4 in context of bacterial antigen, i.e. LPS (21) are expressed at lower levels. Compatible to these findings, activation markers such as CD25, CD44, CD80 and CD86 are not elevated on newB cells suggesting that newB cells do not represent activated B cells. Interestingly, CD62L, a L-selectin mediating lymphocyte homing to high endothelial venules of peripheral lymphoid tissue and to activated endothelium at inflammatory sites, is not expressed on newB and MZB cells, while FOB cells are $CD62L^{+}$. This may indicate that newB cells represent a sessile B cell population, much as MZB cells. Alternatively, given the less mature phenotype, they do not fulfill criteria for re-circulation and, as a consequence, do not express CD62L. In addition, newB cells are negative for the surface markers CD5, CD9 and CD11b, that are all used in defining B-1 cell subpopulations.

The findings of the analysis of λ1 light chain sequences demonstrated that newB cells are not subjected to somatic hypermutations and hence, most probably, do not belong to germinal center B cells and B cell memory.

In functional assays newB cells did respond with cell proliferation to LPS, CpG and α-IgM + α-CD40. However, the response to LPS and α-IgM + α-CD40 was significantly weaker than what was found for mature FOB and MZB cells, and even immature T2/3 cells showed a higher response. However, when stimulated with CpG, newB cells showed a response as strong as mature B cells. While CpG promote the proliferation of B cells via the TLR9 pathway, independently of the B cell receptor complex, signaling to LPS and α-IgM + α-CD40 in B cells does involve the

BCR complex. Therefore, it seems that triggering the BCR complex in newB cells leads only to an attenuated signal in these cells. In support of these findings were the results of an initial experiment measuring the $Ca^{2+}$-flux in newB, FOB and MZB cells shortly after triggering the cells with a polyclonal α-IgM antibody. While a $Ca^{2+}$-flux could be detected for MZB and FOB cells, it was not significantly detectable for newB cells (data not shown). It is therefore tempting to speculate that newB cells represent a population of anergic B cells, possibly as a consequence of carrying an autoreactive BCR. In order to test for this potential autoreactivity of cells of the newB cell population we produced hybridomas. Testing supernatants from individual newB cell-derived hybridomas in comparison to supernatants from FOB- and MZB-derived hybridomas though, did not show a higher frequency of auto-reactivity when tested on mouse kidney sections (data not shown). However these negative results are not finally conclusive, since the frequency of hybrids obtained only represent 0.1% of the B cells and also only less than hundred clones were tested from each subpopulation. Thus, it still remains possible that the new B cells represent an anergic B cell population.

The findings, that newB cells show a relatively high turn-over (in between what was shown for transitional B and MZB cells) would be supportive of a theory that newB cells are less mature and are precursors of mature B cells.

NewB cells were found in spleens of $\lambda5^{-/-}$ mouse in low numbers, however the proportion as compared to the total $CD19^{+}$ cells is similar as in wild-type C57BL/6 mice (~2%). It seems therefore, that the rate of influx of immature B cells from the bone marrow does influence the development of cells with the newB cell phenotype only proportionally to other B-2 B cell subsets. In contrast, it was demonstrated that B-1 B cells develop to normal numbers in these mice either through an independent fetal precursor or, alternatively, through self-replenishment in the peritoneum (31). While both, Bcl-2 and BAFF over-expression lead to increased absolute numbers of newB cells in spleens of transgenic animals, the proportion of newB cells compared to the total $CD19^{+}$ in Bcl-2 Tg animals did compare to normal C57BL/6 mice, whereas in BAFF Tg animals this proportion was reduced (~0.5%). However, the proportion of MZB cells is increased in these mice and it is highly speculative therefore, that newB cells might be involved in the development towards MZB cells.

It is not yet clear, where newB cells could be placed precisely in the pathway along B cell development. However, a more detailed fingerprint emerged from the results of phenotypic analysis and functional assays. These cells show a generally less mature phenotype with a lower responsiveness to B cell stimuli (anergy), a relatively high turnover, while showing no evidence of belonging to the B cell memory or the B-1 B cell lineage. The collected data therefore supports the

idea that newB cells could represent an additional intermediate cell population in the transition of immature to mature B cells, or alternatively, newB cells could represent cells, which for as yet, unknown reasons get selected out from the pool of mature, reactive B cells later, because they do not fulfill criteria to remain and therefore are rendered anergic and regain a more immature phenotype.

The latter concept could explain the somewhat conflicting finding that newB cells can also be found in lymph nodes. However, if these cells are selected out, it is difficult to understand, why these cells are not rapidly cleared from the circulation. Recently, our group observed a tremendous increase in the proportion of newB cells in spleens of very old mice. An observation, which indicates that newB cells are constantly acquired during adult B cell development and are therefore not of fetal origin.

Nevertheless, the insight gained so far through this study should help in designing additional experiments eventually solving the mystery of this newly defined B cell subset.

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# 7 DIFFERENTIAL RESPONSE OF MATURE SPLENIC B CELLS TO LPS IN C57BL/6 VERSUS BALB/C STRAIN OF MICE

## 7.1 Introduction

Gram-negative bacteria represent a major group of pathogens causing human diseases. The glycolipid lipopolysaccharide (LPS), also called endotoxin, is the principal bacterial component of the cell wall of all these pathogens and consists of a lipid A moiety, a core polysaccharide and an O-polysaccharide of variable length. Lipid A, which is responsible for the endotoxic effect, consists of a variable number of $C_{12-14}$ fatty acids linked to a phosphorylated N-acetylglucosamine dimer. The composition and number of acyl side chains lead to different lipid A structures and explain the activation of differential signaling pathways depending on the origin of the lipopolysaccharide. Microbial variants with long O-polysaccharide chains form smooth colonies, whereas variants lacking the O-polysaccharide chain form rough colonies. All of these variants of LPS have implications for the host immune responses to Gram-negative bacteria (1-3).
Recognition of LPS by various cell types of both the innate and the adaptive immune systems leads to pleiotropic cellular activation and responses with the ultimate goal to eliminate the invading pathogen. However, unregulated responses resulting in the release of an excessive amount of cytokines can lead to the pathological condition of septic shock and may eventually be fatal due to multiple organ failure.

### 7.1.1 Toll-like receptors

Although it is known already for many years how the adaptive immune system generates a broad and highly specific repertoire of antigen specific receptors, the faster responding innate immune system has been considered as being relatively unspecific, characterized by engulfment and digestion of microorganisms and foreign substances by macrophages and leukocytes. However, recent studies revealed that the innate immune system possess a higher degree of specificity. This became apparent with the discovery of a family of receptors, now known as the Toll-like receptors (TLRs), which recognize specific patterns of microbial components that are conserved among pathogens. Originally the Toll receptor was identified in *Drosophila* as an essential receptor for the

dorso-ventral patterning in embryos (4). Later, while analyzing Toll-mutant flies, it was discovered that they where highly susceptible to fungal infections. This study revealed, for the first time, that the immune system, particularly the innate immune system, could detect invading microorganisms using structures different from the B and T cell receptors of the adaptive immune system (5). Subsequently, mammalian homologues (TLRs) of the *Drosophila* Toll receptor where identified. They include at least 11 type I integral membrane glycoproteins and are members of a larger superfamily including the IL-1 receptor (6).

The cytoplasmic portion of TLRs has high similarity to that of the IL-1 receptor family and is therefore called Toll/IL-1 receptor (TIR) domain. The TIR domain binds to adaptor molecules involved in downstream signaling pathways. Unlike the Ig-like extracellular domain of IL-1 receptors, the extracellular domain of TLRs contains leucine-rich repeats (LRRs) and interacts specifically with microbial components.

After ligand binding and crosslinking of the TLRs, signaling originates from the cytoplasmic TIR domains. A TIR-domain containing adaptor protein, MyD88, which was first characterized to be utilized in IL-1R signaling has been shown to be also involved in TLR signaling (7, 8). It is now recognized that, at least, TLR signaling pathways consist of a MyD88-dependent pathway common to all TLRs and a MyD88-independent pathway that is especially involved in the TLR3- and TLR4 signaling pathways (9).

#### *7.1.1.1 MyD88-dependent pathway*

MyD88 associates with the TIR domain of TLRs or in the case of TLR2 and TLR4 via the adaptor protein TIRAP (10, 11). Upon receptor stimulation the homodimeric MyD88 recruits IL-1 receptor kinase-1 (IRAK1), which gets activated by phosphorylation through IRAK4, and then associates with tumor necrosis factor receptor (TNFR)-associated factor-6 (TRAF6). The IRAK1-TRAF6 complex is released from the receptor complex and forms a complex with TGF-β-activated kinase 1 (TAK1) and the TAK1-binding proteins TAB1 and TAB2. TAK1 activates NF-κB-inducing kinase (NIK), which leads to NF-κB activation as well as the JNK and p38 MAPK pathways, which activate AP-1 complexes. NF-κB and AP-1 enter the nucleus where they activate the transcription of inflammatory genes, particularly cytokines (Figure 1).

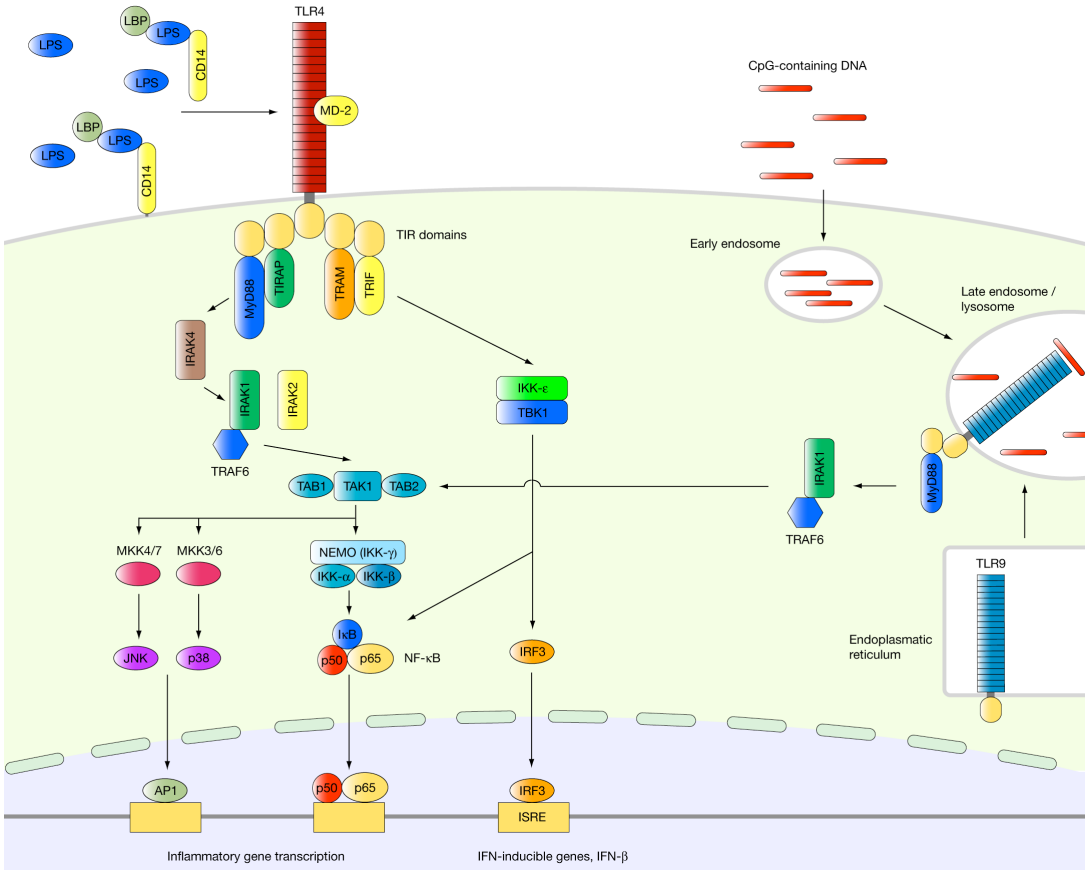


**Figure 1. TLR4 and TLR9 signaling pathways.**

TLR4 signaling in response to LPS includes the MyD88-dependent and MyD88-independent pathways. Intracellular TLR9 signaling is MyD88-dependent.

TIR (Toll/IL-1 receptor domain), LBP (LPS binding protein), IRAKs (IL-1 receptor kinases), IRF3 (Interferon regulatory factor 3), TAB1,2 (TAK1-binding proteins), TAK1 (TGF-β-activated kinase), TRAF6 (TNFR-associated factor 6), TRAM (TRIF-related adaptor molecule), TRIF (TIR-domain adapter inducing IFN-β), TRK1 (TANK-binding kinase 1)

### *7.1.1.2 MyD88-independent pathway*

Stimulation of TLR3 or TLR4 results in the induction of type I interferons (IFN-α/β) in a MyD88-independent manner. This pathway depends on the adaptor protein TIR domain-containing adapter inducing IFN-beta (TRIF) (12). In MyD88-independent signaling through TLR4 an additional adapter protein, TRAM, bridges TLR4 to TRIF in a similar fashion as TIRAP for MyD88-dependent signaling through TLR4 (13). Signaling through TRIF-TRAM leads to the activation of the non-canonical IκB kinases (IKKs), TANK-binding kinase 1 (TBK1) and IKKε/IKK*i*, as well as the IFN-inducible protein kinase PKR. The actions of these kinases result both in NF-κB

activation, enhancing the transcription of inflammatory cytokines, and the activation of STAT1 and IRF-3 and induction of both IFN-β and IFN-inducible genes (14-16) (Figure 1).

### 7.1.2 The role of TLR4 in response to lipopolysaccharides (LPS)

It has been shown, that after microbial infection, LPS is bound by LPS-binding circulating protein (LBP) in the plasma and can be recruited to the pattern recognition receptor CD14, which is present in the plasma as well as on the cell surface of mononuclear phagocytes (17, 18). However the GPI-anchored CD14 is missing a cytoplasmic signaling domain and other cells like B cells hardly express CD14 but respond to LPS. The identification of a membrane receptor involved in signaling to LPS was facilitated by the existence of LPS-resistant mouse strains [C3H/HeJ, C57BL/10ScCr and C57BL/10ScN]. By analyzing these strains it was found that allelic mutations of a single locus, then called LPS locus, were shown to abrogate the response to LPS (19-22). Later on, positional cloning identified the *tlr4* gene as the only candidate gene in the LPS locus (23). The generation of a TLR4 knockout mouse further confirmed the essential role of TLR4 in LPS responsiveness (24). Furthermore it could be shown that the TLR4 expression level determines the degree of LPS-susceptibility as by B cell mitogenicity, cytokine induction and lethal toxicity (25). However, transfection of cell lines with TLR4 did not enable these cell lines to respond to LPS, suggesting that yet another factor links TLR4 to LPS signaling. MD-2, a secreted protein, was then shown to associate with TLR4 and functions as an adaptor for LPS recognition thus defining the TLR4/MD-2 complex as the LPS signaling receptor. In addition, MD-2 functions as a chaperone guiding TLR4 through maturation to the plasma membrane as absence of MD-2 abolishes surface expression of TLR4 (26-28).

### 7.1.3 TLR4 signaling in B cells

The majority of work on TLR and TLR signaling in mammalian cells has been done in the context of dendritic cells and monocytes/macrophages. In recent years, studies have specifically examined the TLR expression in B cells. The majority of TLRs in human B cells upregulate their expression upon activation. Although, these studies mainly focused on human B cells, the results presented were presumed to extend to rodent B cells. However, there is evidence that species specific differences exist as naïve B cells from mice are known to express TLR4 and undergo proliferation

and plasmacytoid differentiation *in vitro* in response to LPS, while human B cells seem to lack significant TLR4 expression in either naïve or memory B cells (29-32). In the search for monoclonal antibodies with radioprotective activity with the initial goal to identify receptors on B cells, which control apoptosis, a 105kDa molecule, referred to as RP105 (CD180), has been found. Originally it was found that RP105 expression is restricted to mature B cells, but later been shown to be also expressed in monocytic and dendritic cells. A structural analysis revealed that RP105 belongs to the family of Toll-like receptors. But unlike other TLR family members, RP105 does not contain a cytoplasmic TIR domain (33, 34). For efficient cell surface expression RP105 needs the coexpression of the MD-2 homologous protein MD-1 (35). The stimulation of mature B cells with an anti-RP105 Ab induced their proliferation and blastogenesis. While B cells of $TLR4^{-/-}$ or $MD\text{-}2^{-/-}$ mice do not respond to LPS, $RP105^{-/-}$ or $MD\text{-}1^{-/-}$ mice show reduced responses to LPS. Furthermore in a NF-κB reporter assay expressing TLR4 and RP105/MD-1 together an increased response to LPS was measured compared to cell lines expressing TLR4/MD-2 alone suggesting that these receptors act synergistically in B cells. In contrast to the situation in B cells, RP105 seems to have an inhibitory role in TLR4 signaling in monocytic and dendritic cells (DCs) and macrophages and DCs from $RP105^{-/-}$ or $MD\text{-}1^{-/-}$ mice do not show a reduced response to LPS indicating that there are differences in signaling between B cells and macrophages or DCs in the response to LPS (33, 36-38). It could be shown that RP105 activates similar pathways to B cell receptor signaling such as the Lyn/CD19/Vav complex, the PI 3-kinase, Btk, and other molecules of the B cell receptor (BCR) signalosome. LPS binding to RP105 induces Lyn activation and phosphorylation of CD19, which in turn augments the Lyn kinase activation. The PI 3-kinase and Btk are activated independently of CD19 and regulate intracellular calcium flux in response to RP105 signaling. As the cytoplasmic domain of RP105 is only 11 aa, it has been suggested that RP105 acts by recruiting signaling components into rafts and thereby facilitating signaling to LPS. It could be shown that RP105 induces the CD19 translocation into lipid rafts together with the constitutively lipid raft-located Lyn (39, 40). Moreover, as RP105 activates similar pathways to BCR signaling that might directly link innate and adaptive immunity in B cells. Nevertheless, the precise mechanism how RP105 and TLR4 might cooperatively regulate responses to LPS in B cells is still unclear (Figure 2).

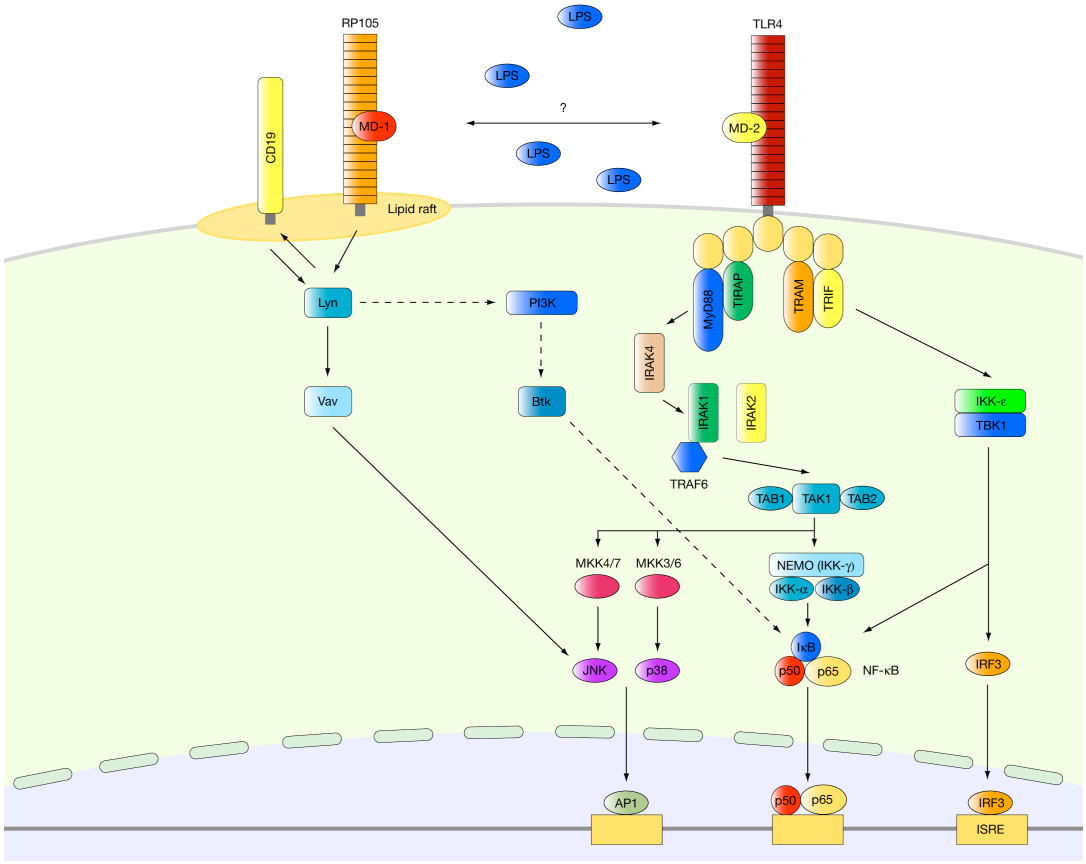


**Figure 2. TLR4 signaling in B cells.**

TLR4 signaling to LPS in B cells involves RP105 and integrates components of the BCR-signalosome (Modified, adapted from (40)).

### 7.1.4 TLR9 mediates signaling in response to CpG

Unlike TLR4, members of the TLR9 subfamily (TLR7, 8, 9) sense pathogen-derived RNA and DNA motifs generated during intracellular infections. TLR9 is expressed intracellularly in the endosomal-lysosomal compartment and detects hypomethylated CpG motifs. Upon non-specific cellular uptake of CpG-ODN into the acidified endosomal compartment, either through internalization or generated due to an intracellular infection, TLR9 directly binds sequence specifically and initiates signaling in a MyD88-dependent fashion (Figure 1) (32).

### 7.1.5 Response of mature B cells to the mitogens LPS and CpG in mice of different genetic backgrounds

It has been shown that frequencies of mitogen-reactive B cells in mice of different genetic background differ. Limiting dilution experiments showed that 1 out of 6 splenocytes of a C57BL/6 mouse responded to LPS, while in the spleen of BALB/c mice only 1 in 20 cells responded (41). However, at this time the knowledge about the different B cell compartments in the spleen was still very limited. In addition, the pattern recognition receptors (PRRs), including the TLRs were not yet identified.

We investigated the responsiveness of the major splenic mature B cell populations comprising the follicular B (FOB: $CD19^{+}$ $CD93^{-}$ $CD21^{+}$ $CD23^{+}$) and marginal zone B (MZB: $CD19^{+}$ $CD93^{-}$ $CD21^{hi}$ $CD23^{-/lo}$) cells from mice of different genetic backgrounds to LPS and CpG *in vitro*. Furthermore, we tried a genetic approach in order to help understanding the differences in responsiveness of mature B cells in mice of different genetic backgrounds, as well as between FOB and MZB cells.

## 7.2 Results

### 7.2.1 Reactivity of follicular B cells stimulated *in vitro* with LPS and CpG

Splenic mature B cells of FOB ($CD19^+$ $CD93^-$ $CD21^+$ $CD23^+$) phenotype were isolated from spleens of young adult mice of either C57BL/6 or BALB/c strain by FACS sorting. The cells were stimulated *in vitro* either with the TLR4 ligand LPS or the TLR9 ligand CpG. To avoid signaling through other TLRs, like TLR2 as a consequence of lipoprotein-contaminants, purified, phenol-extracted LPS was used (42). TLR9 detects microbial DNA with hypomethylated CpG motifs. Unlike TLR4, which is expressed on the cell surface, TLR9 is expressed in the endoplasmic reticulum and signals from an endosomal compartment upon CpG DNA internalization (43).
The reactivity of the cells to these stimuli was measured after 3 days of culture by means of their [$^3$H]thymidine uptake into DNA of proliferating cells.

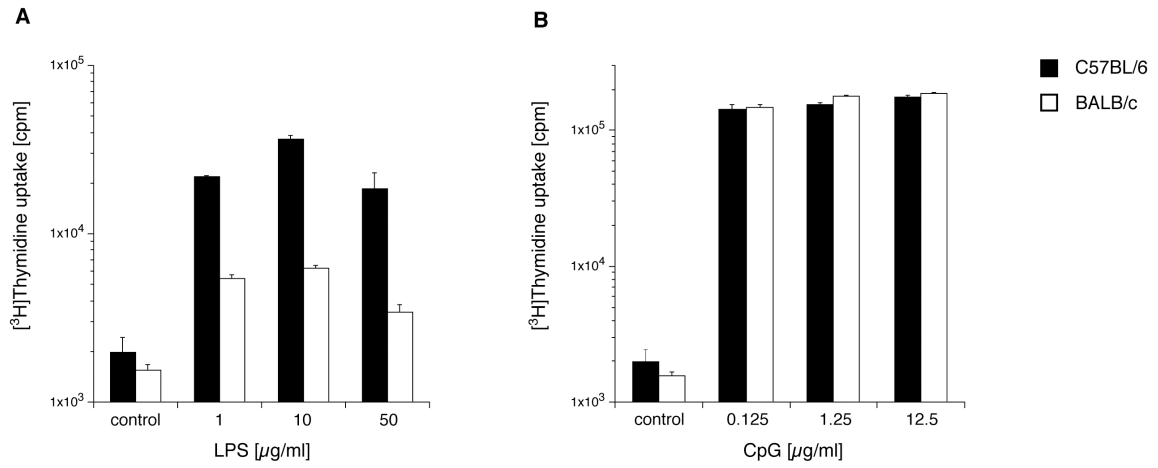


**Figure 3. Stimulation of sorted FOB cells (C57BL/6 and BALB/c strains) with lipopolysaccharide (LPS) or CpG**

**(A)** LPS [0-50µg/ml] or **(B)** CpG (CpG1826 [0-12.5 µg/ml]) for 72h. Measurement of [$^3$H]thymidine uptake for the last 9h of culture. Control: unstimulated cells.

After *in vitro* LPS induced activation, FOB cells of C57BL/6 mice show a significantly higher level of [$^3$H]thymidine uptake than those of the BALB/c mouse strain at all concentrations of LPS tested.
However, when FOB cells were stimulated with CpG, the proliferative response was more vigorous than with LPS, but both strains responded equally strong. Thus, the difference observed between

the two strains in their response of FOB cells to LPS might involve components of the TLR4 pathway, which are not shared with the TLR9 signaling pathway.

### 7.2.2 Expression levels of TLR4/MD-2 and RP105/MD-1 in C57BL/6 strain compared to BALB/c strain

B cells have a non-redundant requirement for the expression of the LPS receptors TLR4 and RP105, which both function as recognition and signal transducing receptors. TLR4 is ubiquitously expressed in leucocytes, but compared to macrophages and dendritic cells, B cells express relatively little TLR4. In contrast, the expression of RP105 (CD180) is more limited to mature B cells (34, 44), which includes FOB cells. In a first attempt to test whether the differences in LPS reactivity seen in FOB cells of the two mouse strains is simply a result of different expression levels for the receptors TLR4 and RP105 together with their adaptors MD-2 and MD-1, respectively, the level of transcripts of these components were measured in sorted FOB cells using semi-quantitative RT-PCR. In addition the level of TLR9 transcripts was also determined.

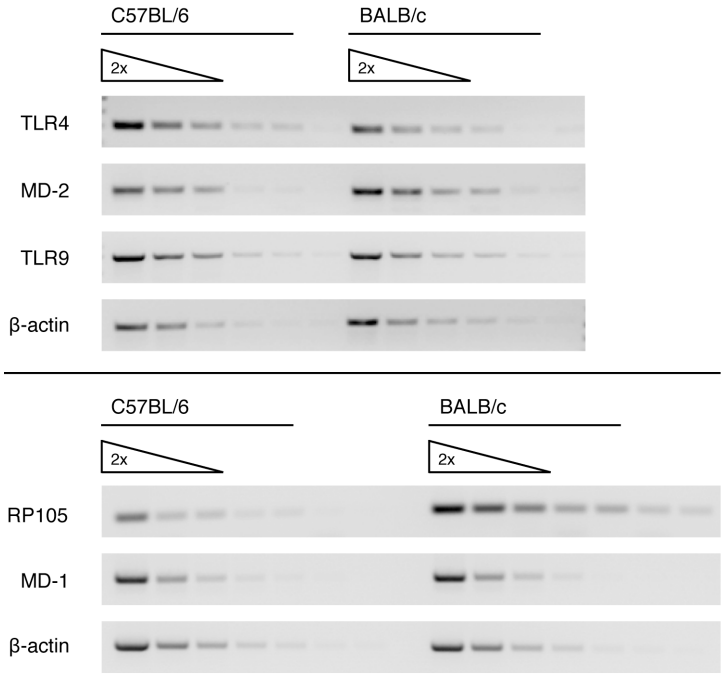


**Figure 4. Transcript levels of LPS and CpG receptor components in FOB cells**

Semi-quantitative RT-PCR analyses of the transcript levels for the LPS receptor components TLR4, MD-2, RP105, MD-1 and the CpG receptor TLR9 in sorted FOB cells. As internal standard, the transcript level of β-actin was included.

Some differences in transcript levels were detected for TLR4 and RP105. While TLR4 showed a higher level in FOB cells of the C57BL/6 strain, RP105 showed a higher level in FOB cells of the low responder strain BALB/c. However, both adaptor molecules, MD-2 for TLR4 and MD-1 for RP-105 were detected with similar levels in both strains. Given the fact that complexes of these molecules are expressed in 1:1 ratios for the functional receptors with their adaptors it is, however, not clear how that determines the final LPS stimulation (Figure 4).
As expected from the results obtained by stimulating the cells with CpG, no difference in the transcript level for TLR9 could be detected for the two mouse strains (Figure 4, upper part).

Additionaly, the surface expression of TLR4 / MD-2, RP105 and MD-1 on purified mature B cells from C57BL/6 and BALB/c mice was determined by FACS. Splenocytes were depleted for $CD5^{+}$ cells, such as B1-a B cells and T cells, and immature transitional $CD93^{+}$ B cells by MACS. The remaining cells were stained for B220, CD21 and CD23 in combination with antibodies specific either for TLR4/MD-2, RP105 or MD-1. This allowed for distinguishing the FOB ($B220^{hi}$ $CD21^{+}$ $CD23^{+}$) and the MZB cells ($B220^{hi}$ $CD21^{hi}$ $CD23^{-/lo}$).
TLR4/MD-2 was detected only weakly on the surface of FOB and MZB cells by FACS. RP105 and MD-1 are expressed on both FOB and MZB cells from C57BL/6 and BALB/c mice. However, there is only a very small shift seen between the two strains, which can hardly explain the difference seen in LPS responsiveness of the FOB cells. Interestingly, the surface expression levels of RP105 and MD-1 as calculated from the mean fluorescence intensities (MFI) are 3.1-3.5 times higher on MZB compared to FOB cells for both strains, an observation, which has also been described, at least for RP105, by others (45-47) (Figure 5).

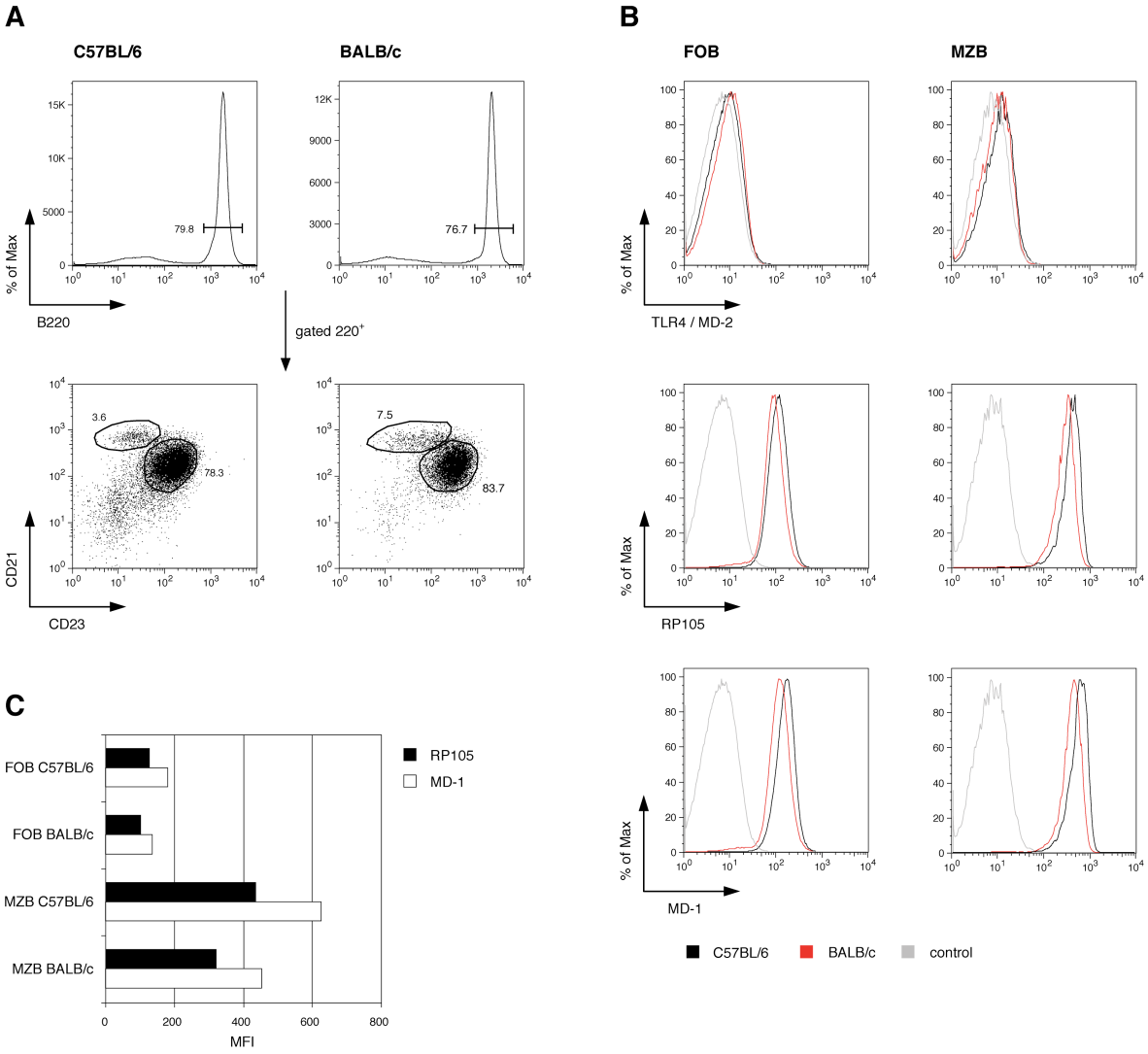


**Figure 5. Surface expression of TLR4/MD-2 and RP105/MD-1 in FOB and MZB cells of C57BL/6 and BALB/c mice.**

Splenocytes from adult C57BL/6 and BALB/c mice (pools of 3 spleens/strain) were MACS-depleted for $CD5^+$ and $CD93^+$ cells. Remaining cells were stained for B220, CD21 and CD23 in combination either for TLR4/MD-2, RP105 or MD-1. **(A)** Mature B cell populations after MACS-depletion. **(B)** TLR4/MD-2, RP105 and MD-1 surface expression on FOB ($B220^{hi}$ $CD21^+$ $CD23^+$) and MZB ($B220^{hi}$ $CD21^{hi}$ $CD23^{-/lo}$) cells of C57BL/6 and BALB/c mice. **(C)** Mean fluorescence intensities (MFI) for RP105 and MD-1 surface expression.

### 7.2.3 Kinetics of *in vitro* LPS-stimulated FOB cells: C57BL/6 versus BALB/c strain

Sorted FOB cells from C57BL/6 and BALB/c strains were loaded with CFSE (Carboxyfluorescein succinimidyl ester) and cultured *in vitro* in the presence of LPS [5µg/ml] for 1 week. Every 0.5-1 d sample of cells were analysed for the content of CFSE and viability (PI) by FACS.

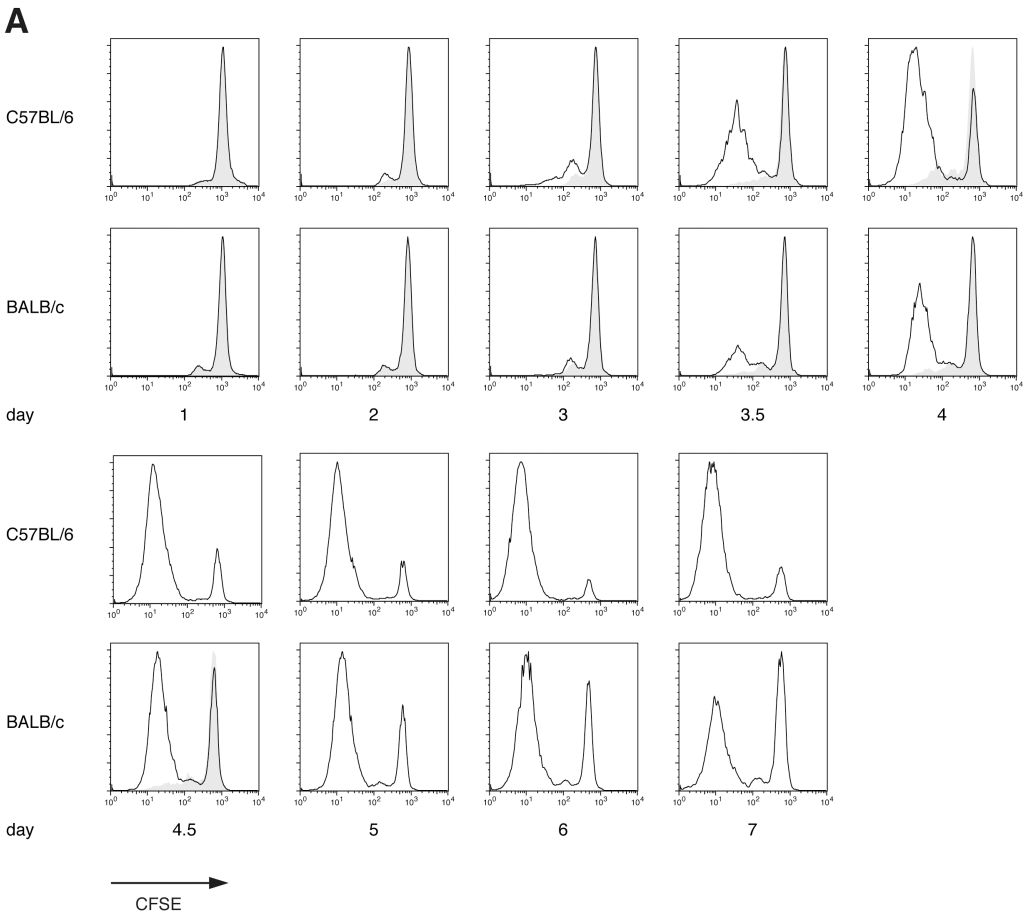


**Figure 6. Kinetics of LPS-stimulated FOB cells *in vitro*: C57BL/6 versus BALB/c strain**

For panel B and description see next page.

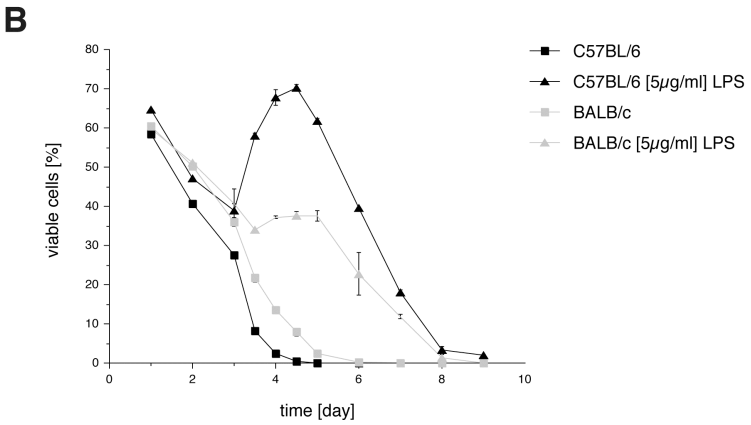


**Figure 6. Kinetics of LPS-stimulated FOB cells *in vitro*: C57BL/6 versus BALB/c strain**

Sorted FOB cells from C57BL/6 and BALB/c strain (from pooled splenocytes of 3 mice per strain) were loaded with CFSE and stimulated in culture ($1x10^5$ cells/96-well) with [5µg/ml] lipoprotein-free LPS and analysed (gated for life cells) at intervals of 0.5-1 d. **(A)** The decrease in CFSE levels (filled grey: unstimulated cells) and **(B)** the percentage of viable cells (PI) was monitored over time.

FOB cells of C57BL/6 strain stimulated with LPS responded quicker (approx. 0.5 d) than of the BALB/c strain. They also responded more vigorously and show 6-7 divisions by day 4, while cells of the BALB/c strain proliferated one generation less (Figure 6).

## 7.2.4 Response of MZB cells from C57BL/6 and BALB/c mice to LPS

As FOB cells show a differential reactivity to LPS in the two, tested mouse strains the reactivity of the less abundant marginal zone B (MZB) cells was also tested. MZB cells ($CD19^+$ $CD93^-$ $CD21^{hi}$ $CD23^{-/lo}$) were isolated from spleens of young adult mice by FACS. The cells were stimulated *in vitro* with lipoprotein-free LPS and the reactivity was measured as [$^3$H]thymidine uptake into DNA of proliferating cells.

Interestingly, MZB cells of both mouse strains show a similar strong proliferative response to LPS (Figure 7). Compared to FOB cells the response is stronger and starts earlier as has been seen by the visual appearance of blasts in the cultures within 24 h. This has been also confirmed by others (data not shown; (45, 48, 49)) and could be explained, at least in part, by the higher expression levels of RP105 and MD-1 on MZB cells (Figure 5B and C). Furthermore, it has been recently shown that MZB cells possess an enhanced secretory apparatus including a more abundant rough endoplasmatic reticulum, which enables MZB to react rapidly to bacterial components like LPS (45).

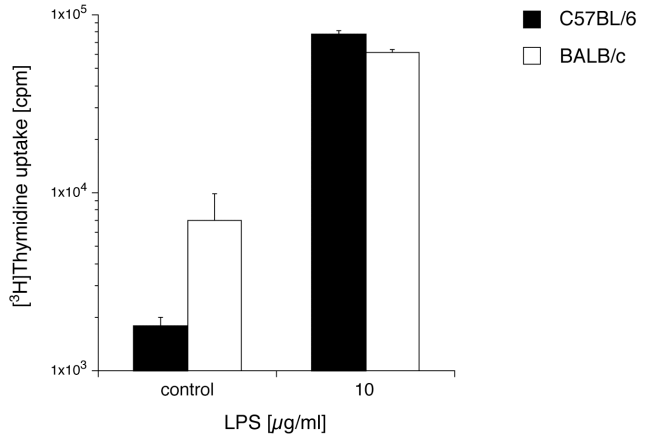


**Figure 7. Stimulation of sorted MZB cells (C57Bl/6 and BALB/c strains) with lipopolysaccharide (LPS)**
Sorted cells were cultured in the absence (control) or presence of LPS [10µg/ml] for 72h. [$^3$H]thymidine uptake during the last 9h of culture was recorded.

However, this raises the question if some of the components involved in signaling through the TLR4 pathway are differentially expressed.

### 7.2.5 Genetic approach: responsiveness of FOB cells from intercrosses and backcrosses

A genetic approach was set up with the ultimate goal to identify the locus responsible for the differential response of FOB cells to LPS in the two mouse strains. With the data so far known from others and obtained through our *in vitro* proliferation assays one could have expected to find a component involved in the TLR4 signaling pathway. By generating F1 (BALB/c x C57BL/6) hybrid animals and backcrossing them on either the C57BL/6 or BALB/c strain and considering the Mendelian law of genetics we expected to receive low and high responder mice in terms of the proliferative response of their FOB cells to LPS. With the help of existing microsatellite markers (polymorphism) for the C57BL/6 and BALB/c strain it would then be possible to identify the responsible locus.

### *7.2.5.1 Response of FOB cells from F1 (BALB/c x C57BL/6) animals to LPS*

FOB cells from C57BL/6, BALB/c and from F1 (BALB/c x C57BL/6) hybrid young adult (6-8 weeks) mice were stimulated with LPS for three days *in vitro*. The proliferative response of the FOB cells isolated from F1 (BALB/c x C57BL/6) hybrid mice was exactly in between (50%) the values obtained for C57BL/6 (100%) and BALB/c animals (26%) (Figure 8). This might indicate that components necessary for the LPS induced proliferative response are limiting in the FOB cells of the BALB/c strain and that is only partly restored by one allele from the high responder strain C57BL/6. However, new F1 animals, where the mother is C57BL/6 could be tested in addition to test for a possible effect of maternal imprinting.

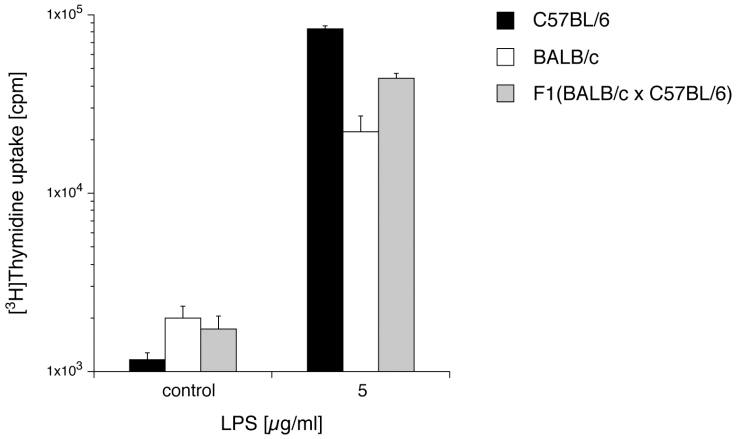


**Figure 8. Response of FOB cells to LPS from F1 (BALB/c x C57BL/6) animals**

Sorted cells were cultured in the absence (control) or presence of LPS [5µg/ml| for 72h. [$^3$H]thymidine uptake during the last 9h of culture was recorded.

### *7.2.5.2 Response of FOB cells from F2 BALB/c x (BALB/c x C57BL/6) backcrosses to LPS*

FOB cells sorted individually from F2 BALB/c x (BALB/c x C57BL/6) offsprings were stimulated for three days *in vitro* with LPS. As a high responder control (100%) sorted FOB cells from a C57BL/6 mouse were used. The results obtained show a more complex picture with responses over a wide range (9-56%) but mostly staying below half of the proliferative response of FOB cells from the C57BL/6 high responder strain (Figure 9A). This is contrary to what one expects for a one-locus event responsible for the difference in responses observed earlier in the F1 animals, where

now in the F2 generation one would expect segregation into low and intermediate responders. However, keeping in mind that multiple loci might be involved, that could explain the observed results. Therefore a backcross of the F1 animals onto the high responder C57BL/6 strain should at least show an overall higher response of the FOB cells to LPS in these mice considering a locus dosage effect.

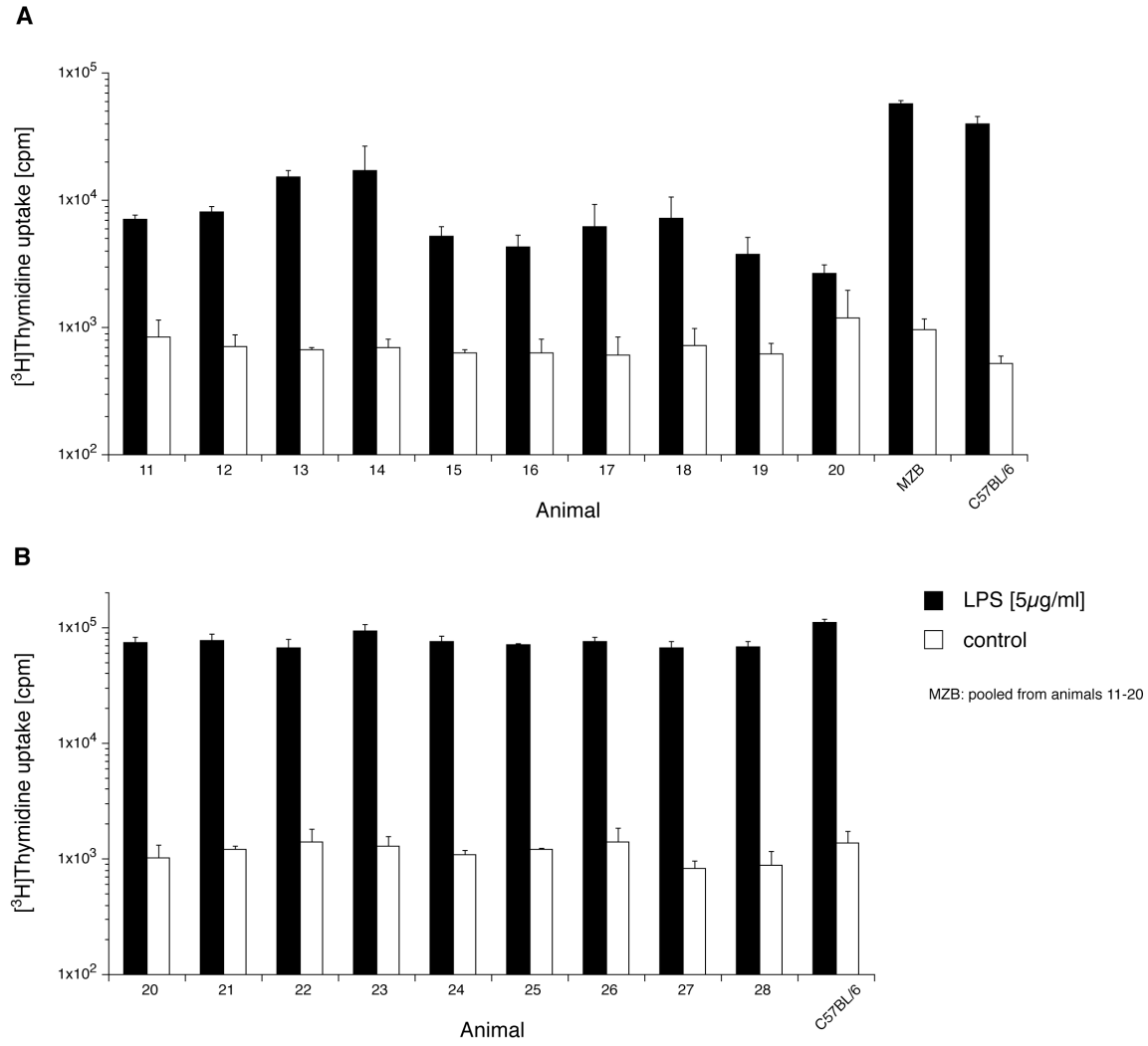


**Figure 9. LPS response of FOB cells from F2 animals**

**(A)** F2 BALB/c x (BALB/c x C57BL/6); MZB: pooled from animals 11-20.
**(B)** F2 C57BL/6 x (BALB/c x C57BL/6).
Sorted cells were stimulated with LPS [5µg/ml] for 72h. [$^3$H]thymidine uptake was determined during the last 9h of the culture. Control: unstimulated cells.

#### *7.2.5.3 Response of FOB cells from F2 C57BL/6 x (BALB/c x C57BL/6) backcrosses to LPS*

When FOB cells were sorted from individual spleens of the other F2 breeding, C57BL/6 x (BALB/c x C57BL/6), and stimulated with LPS, FOB cells from all individual F2 animals showed indeed overall higher proliferative responses (59-84%) (Figure 9B). Therefore, the results indicate that multiple genes might control the response of FOB to LPS in C57BL/6 and BALB/c strains in a differential fashion. Alternatively, a single gene for the component limiting LPS responses in BALB/c might be subject to maternal imprinting when the mother is a high responder. However, for both of these backcrosses, more animals have to be tested including controls of F1 FOB cells.

### 7.2.6 Influence of the MyD88-dependent pathway

TLR signaling pathways consist of a MyD88-dependent pathway common to all TLRs and a MyD88-independent pathway involved in the TLR3- and TLR4 signaling pathways (9). The current view is that LPS responsiveness of B cells is mainly mediated through the MyD88-dependent pathway. To address the role of the MyD88-dependent pathway in the different outcome of LPS-stimulated FOB cells in the two different mouse strains and to exclude that the effect observed is not due to a contribution of the MyD88-independent pathway, we analysed mature B cells of MyD88-deficient animals of either C57BL/6 or BALB/c background.

FOB cells sorted from C57BL/6, BALB/c, C57BL/6 MyD88$^{-/-}$ and BALB/c MyD88$^{-/-}$ young adult (6-8 weeks) mice were stimulated with different LPS concentrations [1-25µg/ml] during three days *in vitro.* The proliferative response of the cells was again determined by [$^{3}$H]thymidine uptake into the DNA.

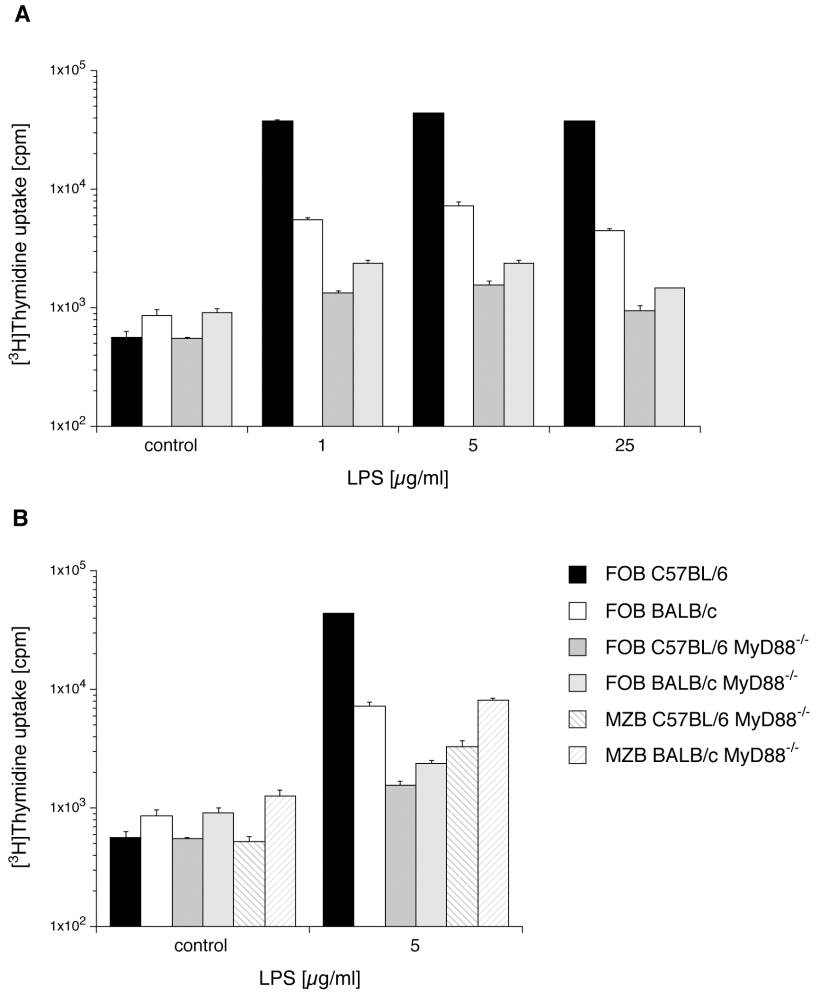


**Figure 10. Proliferation of FOB cells from normal or MyD88-deficient mice of C57BL/6 and BALB/c background.**

Sorted cells were stimulated with LPS **(A):** [0-25 µg/ml **(A)**] for 72h or **(B):** [5µg/ml **(B)**] including MZB cells of MyD88$^{-/-}$ mice for 72h. [$^3$H]thymidine uptake was determined during the last 9h of the culture. Control: unstimulated cells.

As expected, FOB cells from MyD88$^{-/-}$ animals of both backgrounds showed a reduced proliferative response to LPS. However, in contrast to wild-type BALB/c mice, FOB cells from MyD88$^{-/-}$ animals with BALB/c background showed even a slightly higher proliferative response than from MyD88$^{-/-}$ animals of C57BL/6 background (Figure 10A). In addition, MZB cells from MyD88$^{-/-}$ mice of BALB/c background still showed a proliferation as strong as normal FOB cells of BALB/c strain (Figure 10B).

Based on these results one might assume that the difference seen in LPS reactivity between FOB cells of C57BL/6 and BALB/c strains must either involve components within the MyD88-

dependent pathway or involves components, which can modulate the MyD88-dependent signaling pathway.

### 7.2.7 Is differential responsiveness to LPS due to the haplotype of the MHC class II locus ?

Recently, Rodo et al. (50) investigated also the differential response of mature B cells to LPS in C57BL/6 and BALB/c mice. They claim to have located the MHC class II locus to be responsible and therefore that the *d* haplotype of the BALB/c strain leads to a lower responsiveness of mature B cells to LPS in this strain. However, in their study total $B220^+$ B cells were used without distinguishing the FOB and MZB cell compartments, which show different reactivity to LPS and are actually represented in slightly different ratios in C57BL/6 and BALB/c mice. Nevertheless, to test their results, we analysed the response to LPS and CpG stimulation of FOB and MZB cells from BALB/b mice. BALB/b differ from BALB/c mice only in the MHC, where the *b* haplotype is present, which they share with the high responder C57BL/6 strain. Therefore, according to the claim of Rodo et al. one would expect a similar response for FOB cells of BALB/b and C57BL/6 mice. Sorted FOB and MZB cells from C57BL/6, BALB/c and BALB/b mice were stimulated with LPS for three days *in vitro* and the proliferative response was measured by [$^3$H]thymidine uptake into DNA of proliferating cells.

Interestingly, FOB cells from the BALB/b strain showed a low response similar to FOB cells from the BALB/c strain (Figure 11A). This is in contrast to what one could have expected for the *b* haplotype, if the MHC haplotype is responsible for the outcome of LPS-stimulated FOB cells. LPS-stimulated MZB cells from the three strains proliferated to a very similar extent (Figure 11B). Likewise, FOB cells stimulated with CpG responded equally strong in all three strains tested (Figure 11C).

In addition, the level of CD69 surface expression on FOB cells was addressed in the absence or presence of LPS on *in vitro* cultured FOB cells from C57BL/6, BALB/c and BALB/b mice. The expression of the common early lymphocyte activation marker CD69, a c-type lectin, is augmented shortly after the activation of lymphocytes (51). For this, sorted FOB C57BL/6, BALB/c and BALB/b mice were cultured in the absence or presence of LPS. After 24h of culture the expression of CD69 was only elevated on LPS-stimulated FOB sorted from C57BL/6 mice (Figure 11D).

We suggest therefore, that the difference seen between C57BL/6 and BALB/c derived LPS-stimulated FOB cells is unlikely to be principally determined by the MHC class II haplotype of these strains.

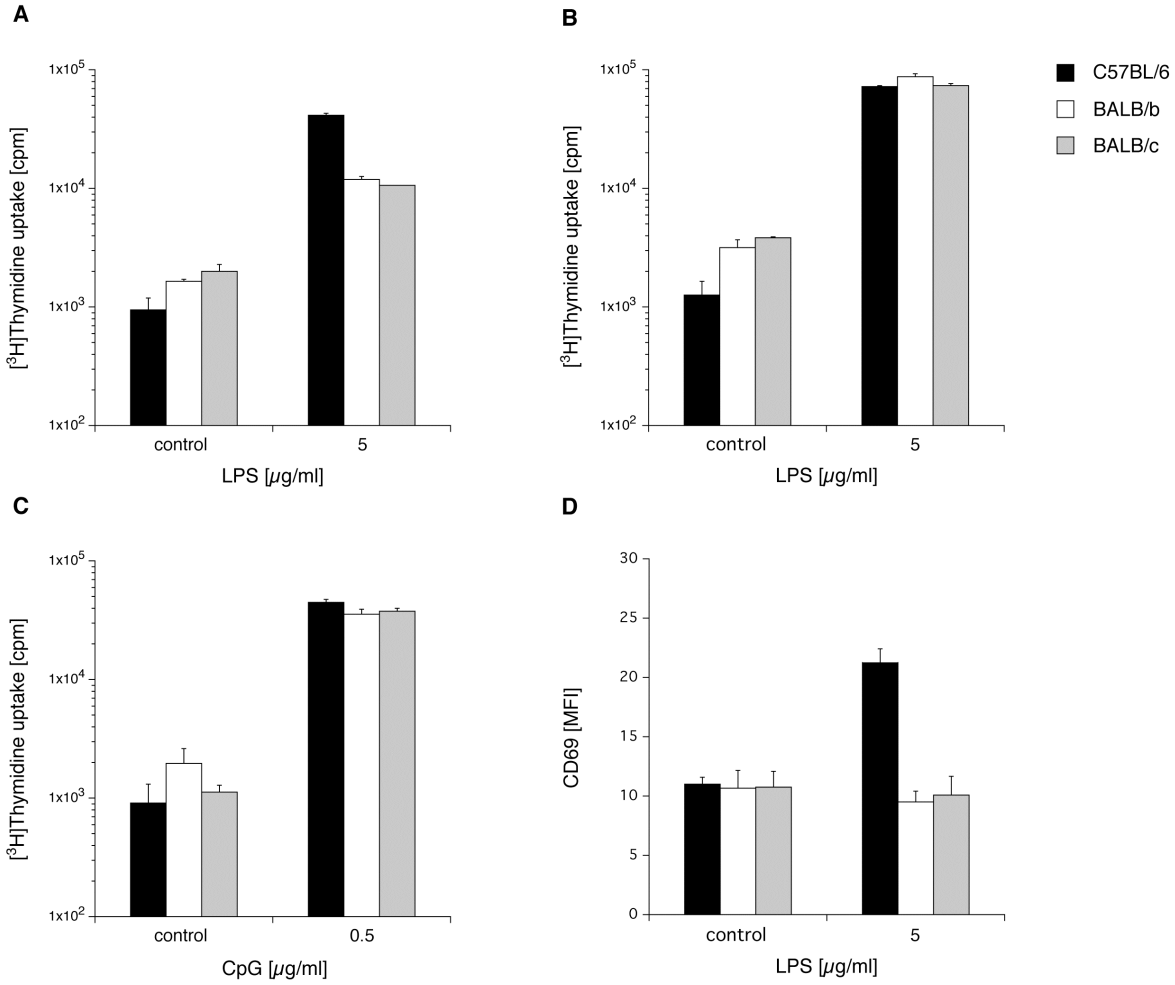


**Figure 11. Proliferation and CD69 surface expression of sorted FOB and MZB cells (C57BL/6 versus BALB/c and BALB/b strains) in response to LPS and CpG stimulation**

Sorted FOB **(A)** or MZB **(B)** cells were stimulated with LPS [5µg/ml] for 72h. In addition, FOB cells were also stimulated with CpG [0.5µg/ml] **(C)**. Measurement of [$^3$H]thymidine uptake during the last 9h of the experiment.
**(D)** Surface expression of CD69 (MFI) on unstimulated and LPS-stimulated FOB cells ([5µg/ml] for 24h *in vitro*).
**(A-D)** FOB and MZB cells sorted from pooled splenocytes (spleens: n=3 for each strain); cultures: 1x10$^5$ cells/96-well (n=3). Control: unstimulated cells.

## 7.3 Discussion

A variety of mitogenes, many of them are bacterial components, stimulate murine B lymphocytes polyclonally to proliferate and to differentiate into Ig-secreting plasma cells.

It was known that frequencies of LPS-reactive B cells from C57BL/6 and BALB/c mice differ showing a higher frequency in C57BL/6 mice (41). However, at this time little was known about the different B cell compartments within the spleen and many receptors for mitogenes, members of the Toll-like receptor family (TLRs), were not yet identified.

Here, distinguishing the two most abundant, mature B cell types present in the spleen, follicular B (FOB) and marginal zone B (MZB) cells, we could show that actually the FOB cells of C57BL/6 respond stronger to LPS than FOB cells of the BALB/c strain.

The unique location of MZB in close proximity to the marginal sinus in the spleen makes them ideally suited to respond to blood-borne antigens, like gram-negative, LPS -containing bacteria. In line with that, MZB cells adapted themselves to respond earlier and stronger to LPS than the more abundant recirculating FOB cells. MZB cells allocate an enhanced secretory apparatus with a more abundant rough endoplasmatic reticulum than FOB cells (45, 49). Additionally, MZB cells show a 3-4 times higher expression of the TLR proteins RP105 and MD-1 compared to FOB cells. Both proteins were shown to support the response of B cells to LPS. In this comparative study, MZB cells of both mouse strains showed a stronger response to LPS than the FOB cells. However, in contrast to the observation in FOB cells, MZB cells of both tested strains responded equally strong.

Having observed the stronger response of FOB cells from C57BL/6 mice an obvious question was, as to what extent expression levels of the LPS receptor molecules might vary between FOB cells of C57BL/6 and BALB/c strains. However, TLR4 / MD-2 expression levels could not be measured to be significantly different between the two strains. Additionally, while expression levels for RP105 and MD-1 were found to be 3-4 higher on MZB cells compared to FOB cells within both strains, the differences between the strains for both cell types were minimal.

Given the complexity of the TLR4 signaling pathway we tried a genetic approach. By generating F1 hybrid animals and backcrossing those onto either parental strain, we expected to obtain low and high responder animals and aimed at a genetic screening approach with the help of microsatellite markers (polymorphism) to possibly identify a responsible locus. We found that FOB cells from F1 animals responded with values exactly half of what was found for the parental strains. FOB cells of F2 BALB/c x (BALB/c x C57BL/6) backcross animals, however, showed responses below half of the response of the high responder FOB cells from C57BL/6 mice, while FOB cells from C57BL/6 x (BALB/c x C57BL/6) animals showed an overall higher response than

FOB cells from F1 animals. However, results from either backcrosses did not segregate into low and intermediate responders or intermediate and high responders, respectively.

Therefore, the results indicate that multiple genes control the response of FOB cells to LPS in C57BL/6 and BALB/c strains in a differential fashion. Given the complexity of the expression network with induction and repression of expression in a temporal cascade upon engagement of the LPS-receptor components, the results from the genetic approach are not completely surprising. To get more insight, many more animals need to be tested. An alternative approach to get more insight could involve the collection and analysis of temporal oligonucleotide microarray data during the response of FOB cells to LPS for the two strains.

However, alternatively, still, a single gene for the component limiting LPS responses in BALB/c might be subject to maternal imprinting when the mother is a high responder. This could be tested by breeding F1 animals for which the mother would be of the C57BL/6 strain.

While all TLRs rely on a MyD88-dependent pathway, signaling through TLR3 and TLR4 additionally involves a MyD88-independent pathway (9). Analysing the response to LPS of FOB cells from MyD88-deficient animals of both C57BL/6 and BALB/c backgrounds showed, that in absence of MyD88, the response of FOB cells of C57BL/6 background is strongly reduced, being even slightly weaker than for FOB cells of BALB/c background. This suggests that the stronger response of wild-type FOB cells of C57BL/6 either involves components of the MyD88-dependent pathway, or alternatively, components that modulate the MyD88-dependent pathway.

In contrast to stimulation with LPS, treatment with CpG led to a similar response of FOB cells of both strains and therefore supports the idea that the differential outcome to LPS is independent of components shared with the TLR9 pathway.

Investigations of another group claimed to have located the MHC class II locus to be responsible for the different strength of responses of mature B cells from C57BL/6 and BALB/c mice to LPS stimulation (50). They have identified the *d* haplotype of the BALB/c strain to be responsible for the lower response of mature B cells in that strain. However, the results presented were based on the analysis of total splenic $B220^+$ cells. To test their results we included the analysis of FOB and MZB cells from BALB/b mice, which differ from BALB/c mice in that they share the *b* haplotype with the C57BL/6 strain. In contrast to what was found by Rodo et al, FOB cells from the BALB/b *b* haplotype strain showed a similar low response as FOB cells from the BALB/c *d* haplotype strain. Additionally, MZB cells of all three tested mouse strains showed similar responses to LPS and CpG. We conclude, therefore, that the MHC class II haplotype is not a major contributor to the different strength of responses seen for FOB cells of the tested strains.

This work identified the FOB cells of C57BL/6 and BALB/c to respond with different strength and is dependent on the MyD88-dependent signaling pathway. It remains intriguing, why this effect could not be seen in MZB cells. Either the modulating components are additionally differentially regulated between FOB and MZB cells, or alternatively, the observed effect does not become apparent in MZB cells as these cells already respond stronger than FOB cells. However, given the abundance of FOB cells in the mouse, this effect must have a different overall impact in the response of the adaptive immune system upon challenges with LPS containing bacteria in the two tested mouse strains.

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# Abbreviations

| | |
|---|---|
| Ab | Antibody |
| ADCC | Antibody-dependent cellular cytotoxicity |
| Ag | Antigen |
| APC | Antigen presenting cell |
| BAFF | B cell activating factor of the TNF family |
| BAFF-R | BAFF receptor |
| Bcl-2 | B cell leukemia 2 (oncogene) |
| BCR | B cell receptor |
| BM | Bone marrow |
| bp | Base pair |
| BrdU | 5-bromo-2'-deoxyuridine |
| BSAP | B-cell-specific activator protein |
| C region | Constant region |
| CD | Cluster of differentiation |
| cDNA | Complementary DNA |
| CFSE | Carboxyfluorescein succinimidyl ester |
| CLP | Common lymphoid progenitor |
| CpG ODN | Oligodeoxynucleotides containing unmethylated CpG motifs |
| D region | Diversity region |
| DC | Dendritic cell |
| E2A | Transcription factor E2A |
| EBF | Early B cell factor |
| FACS | Fluorescence-activated cell sorter |
| FOB | Follicular B cell |
| HMG | High mobility group protein |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IgH | Immunoglobulin Heavy-chain |
| IgL | Immunoglobulni Light-chain |
| IKK | IκB kinase |

| | |
|---|---|
| IL | Interleukin |
| IRAK | IL-1 receptor kinase |
| ITAM | Immune tyrosine activation motif |
| ITAM | Immune tyrosine activation motif |
| J region | Junctional region |
| LBP | LPS-binding protein |
| LN | Lymphnode |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| MHC | Major histocompatibility complex |
| MZ | Marginal zone |
| MZB | Marginal zone B cell |
| MZM | Marginal zone macrophage |
| NF-κB | Nuclear Factor kappa B |
| NHEJ | Nonhomologous end joining |
| PALS | Periarteriolar lymphoid sheath |
| PAX | Paired box transcription factors |
| PC | Phosphorylcholine |
| PCR | Polymerase chain reaction |
| PI | Propidium iodide |
| PKR | Protein kinase R |
| Pre-B | Pre-B cell |
| Pre-BCR | Pre-B cell receptor |
| Pre-TCR | Pre-T cell receptor |
| PtC | Phosphatidyl choline |
| RAG | Recombination activating gene |
| RSS | Recombination signal sequences |
| RT | Room temperature |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SL chain | Surrogate light chain |
| T1/2/3 | Transitional 1/2/3 cells |
| TAB | TAK-binding protein |

| | |
|---|---|
| TAK | TGF-β-activated kinase |
| TBK | TANK-binding kinase |
| TCR | T cell receptor |
| TdT | Terminal deoxynucleotidyl transferase |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TRAF-6 | TNF receptor-associated factor 6 |
| TRAM | TRIF-related adaptor molecule |
| TRIF | TIR domain-containing adapter inducing IFN-beta |
| TSLP | Thymic stromal lymphopoietin |
| TSLP-R | Thymic stromal lymphopoietin receptor |
| V region | Variable region |
| WT | Wild type |

# Curriculum vitae

**Personal data**

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| Name | Lukas Andreas Flück |
| Date of birth | 20.02.1971 |
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**Education**

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| 1978 - 1987 | Primary and Secondary School, Aesch/BL |
| 1987 - 1991 | Grammar school, Muenchenstein/BL<br>Degree: Matur (Typus C) |
| 10/1992 – 5/2002 | Federal Institute of Technology Zurich (ETHZ): Diploma in biochemistry<br>Title of diploma thesis: 'Putative role of RNP1- and RNP2-sequence elements for the antiviral function of mouse Mx-1 protein' |
| 6/2002 – 12/2006 | University of Basel: PhD student in immunology<br>Supervisor: Prof. Dr. Antonius G. Rolink<br>Thesis title: 'Regulation of murine B cell development and function' |
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**Working experience**

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| since January 2007 | Scientist, Head of Bioinformatics, 4-Antibody AG, Basel |

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| 1988 - 1992 | Administrator, Office for Radiation Safety,<br>Ciba-Geigy AG, Basel |
| 1992-1996 | IT support, software development and documentation,<br>Roche AG, Basel |
| 1999 - 2000 | Research assistant at the Institute for Medical Virology,<br>University of Zurich |
| 2000 - 2002 | Web application developer, CIN Technologies, Zurich |
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**Further training**

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| 3/2003 | Introductory course in laboratory animal science,<br>Institute for Laboratory Animal Science, University of Zurich |
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**Publications**

Chappaz, S., Flueck, L., Farr, A. G., Rolink, A. G. and Finke, D. (2007) Increased TSLP availability restores T- and B-cell compartments in adult IL-7 deficient mice. Blood 110 (12), 3862-70

Rauch, M., Flueck, L. and Rolink, A. G. (2009) Differential BAFF-R expression enables the discrimination between receptor editing and non-receptor editing immature bone marrow B cells. In prepraration.

**Lectures**

I have attended lectures of the following professors during the time of my PhD studies:

- Prof. Antonius G. Rolink
- Prof. Fritz Melchers
- Prof. Jan Andersson
- Prof. Daniela Finke
- Prof. Ed Palmer
- Prof. Gennaro De Libero
- Prof. Jean Pieters

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