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„Comparative characterization of mice overexpressing BCL2-like proteins in the hematopoietic system"

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Zusammenfassung

Apoptose ist eine Form des programmierten Zelltodes und spielt z.B. eine essentielle Rolle in der Entwicklung und der Gewebshomöostase. Nicht-funktionelle Apoptose kann zur Entstehung von Krebs und anderen Krankheiten führen. Einer der Signalwege der Apoptose wird durch die Bcl-2 Proteinfamilie reguliert, zu dieser Familie gehören Bcl-2, Mcl-1, Bcl-xL und Bfl-1 (bzw. A1 in Mäusen). Diese wirken anti-apoptotisch und besitzen dieselben evolutionär konservierten Domänen. An ihrer Rolle im Immunsystem wurde schon ausgiebig geforscht, da die Entwicklung und Aufrechterhaltung der Immunzellen zum großen Teil durch Apoptose garantiert wird. Zusätzlichen stellen die anti-apoptotischen Proteine ein interessantes Ziel in der Therapie von verschiedenen Erkrankungen dar. Da verschiedene Krebsarten, welche aus Zellen aus dem hämatopoietischen System entstanden sind, diese Proteine vermehrt exprimieren. Für Lymphome sind schon Therapien in Anwendung, die gezielt Bcl-2 und Bcl-xL inhibieren. Deren Spezifität im Immunsystem ist dennoch noch nicht vollkommen geklärt.

Unser Interesse lag darin festzustellen, ob es eine Hierarche zwischen den Proteinen gibt. Dazu haben wir die Auswirkungen der Proteine auf die Entwicklung des Immunsystems bzw. die Resistenz von Immunzellen verglichen, wenn diese im blutbildenden System verstärkt exprimiert werden. Dies sollte zu einem besseren Verständnis dieser Proteine und ihrer Wirkungsweise in normalen Geweben führen. Literaturrecherche ließ uns vermuten, dass eine Erhöhung der Bcl-xL bzw. Bfl-1 Expression zu einer Veränderung in der Zusammensetzung verschiedener Immunorgane führen würde. In unseren Experimenten konnten wir nichts dergleichen feststellen. Jedoch sahen wir die schon beschriebenen Veränderungen bei Mcl-1 und Bcl-2 Expressionserhöhung. In sogenannten „survival assays“ haben wir den Überlebensvorteil von Thymozyten, Pre-B- und reifen B- und T-Zellen, welche die anti-apoptotischen Proteine erhöht exprimieren, *in vitro* getestet. Bcl-xL, Mcl-1 und Bcl-2 transgene Zellen zeigten einen Überlebensvorteil. *Vav-Bfl-1* Zellen zeigten nur dann einen Schutz, wenn Bcl-2 und Bcl-xL durch bestimmte Inhibitoren gehemmt waren. Erwähnenswert ist, dass *Vav-Bcl-2* Mäuse höhere transgene Proteinexpression aufweisen, als *Vav-Bcl-xL* und *Vav-Mcl-1* Mäuse. Wir beobachteten, dass Mäuse, die Bcl-xL vermehrt bildeten, eine höhere Immunantwort aufwiesen, wenn sie immunisiert wurden. Das war sichtbar an der Menge gebildeter Keimzentrums- B-Zellen. Interessanterweise haben Forscher festgestellt, dass dies nicht der Fall ist, wenn die Erhöhung von Bcl-xL nur in der B-Zell Linie geschieht. Unsere Ergebnisse deuten an, dass dieser Effekt T-Zell abhängig ist.

Insgesamt weisen unsere Ergebnisse darauf hin, dass Bcl-xL eine sehr wichtige Rolle in der Immunzellentwicklung und –überleben spielt, jedoch möglicherweise eine geringere Rolle als Bcl-2 und Mcl-1. Viele Fragen bleiben trotzdem offen und es wäre weiterhin von Wichtigkeit die Rolle von Bcl-xL und Bfl-1 gegenüber den restlichen Familienmitgliedern zu erforschen.

Summary

Apoptosis is a form of programmed cell death; it plays a crucial role in development and its deregulation is often involved in cancer and other disorders. The mitochondrial pathway of apoptosis is tightly regulated by members of the BCL-2 family, subdivided into proteins promoting or inhibiting cell death. Bcl-2, Mcl-1, Bcl-xL and Bfl-1 (respectively A1 in mice) belong to the anti-apoptotic family members. They have been found to share the same evolutionary conserved domains, but still are not redundant respectively, only to limited extends. Their roles in the immune system have been well studied, since the development and homeostasis of immune cells are warranted to a major part through apoptosis. Numerous cancers derived from these compartments and autoimmune diseases display a deregulation of these proteins – making these proteins an interesting target for therapy. Although Bcl-2 and Bcl-xL are already targeted in lymphoma therapy, their specificity in the immune system is still not fully understood.

Therefore, we investigated if there is some kind of hierarchy between the pro-survival proteins by analyzing the effect of different overexpressed BCL-2 family proteins on the immune system side by side – contributing to a better understanding of these effectors in normal tissue and to the ongoing work of investigating the role of these proteins in healthy cells.

We could not find major deviations in immune cell distribution upon Bcl-xL and Bfl-1 elevation in the hematopoietic system *in vivo*, neither in immature nor in mature lymphocytes. This was surprising, regarding the described importance of these proteins in different immune cell stages. However, we were able to confirm the already described phenotypes of Mcl-1 and Bcl-2 overexpression. When testing the survival benefit of thymocytes, pre-B and mature B and T cells, which expressed the transgene in *in vitro* apoptosis assays, we were able to see a clear advantage when Bcl-xL was overexpressed and also were able to confirm the described survival advantage upon Mcl-1 and Bcl-2 elevation. We were not able to see a protection of *Vav-Bfl-1* cells, when they were challenged with common apoptotic stimuli like γ-irradiation, non-selective protein kinase inhibitors and glucocorticoids. Only upon specific inhibition of Bcl-2 and Bcl-xL by using BH3-mimetics, Bfl-1 overexpression led to a survival benefit. Noteworthy, higher protein expression was found of Bcl-2 in tg mice compared to other tg mice. Bcl-xL overexpression was also found to lead to an elevated immune response in mice, when compared to normal mice. Interestingly, it was reported before that when Bcl-xL is elevated in the B cell compartment alone there is no effect. Our findings might suggest that this effect is T cell dependent.

Altogether, our results indicate an important role of Bcl-xL in immune cell development and survival, but it might have a less important role than Bcl-2 and Mcl-1. Its importance compared to the other proteins and the role of Bfl-1 still needs to be investigated further.

Contents

Introduction

Apoptosis

Cells in multicellular organisms are able to undergo a complex and tightly regulated form of cell death called apoptosis. It plays a crucial role in development and tissue homeostasis. Unlike necrosis, a different form for cells to die, the cells undergoing apoptosis die in a controlled manner, without causing harm to surrounding cells or tissue[1]. Due to these characteristics apoptosis also accounts as a form of cell suicide process referred to as programmed cell death and is needed for normal development. In contrast to other forms of programmed cell death, apoptosis is undoubtedly the best investigated and understood one[2]. In human, deregulation of apoptosis can have far-reaching consequences. It is known to be an important step during tumor cell development and therefore resisting cell death accounts for one of the "hallmarks of cancer"[3]. Furthermore, dysfunctional apoptosis may also lead to autoimmune diseases and degenerative disorders[4].

A cell undergoing apoptosis exhibits characteristic morphological changes due to biochemical events taking place within the cell. The cytoskeleton collapses, the nuclear envelope dissembles and the chromosomal DNA breaks up into fragments. Consequently, the cell starts to shrink and condense and finally forms membrane-enclosed apoptotic bodies. On the outside of these bodies phosphatidylserine (PS), a negatively charged phospholipid, informs surrounding phagocytic cells to remove these apoptotic bodies to prevent inflammation. Usually, PS is located on the inside of the cell, but upon activation of the apoptotic program it is actively flipped to the outside[5].

The apoptotic program can be initiated through two distinct pathways, the "extrinsic" and the "intrinsic" pathway[6]. Both rely on specific cysteine-aspartate proteases, called caspases. Caspases are separated into two classes, initiator caspases like caspase-8, -9 and -10 and effector caspases like caspase-3, -6 and -7. They are present within the cell as inactive precursors and are only active when proteolytically cleaved[7]. Upon apoptotic stimuli the initiator caspases are processed and therefore activated. Once activated through autoproteolytical cleavage, initiator caspases can in turn cleave and activate effector caspases, which have many substrates including nuclear lamins, thereby leading to the destruction of the cell[8]. When extracellular signals drive cells into apoptosis the so-called extrinsic pathway is activated via transmembrane death receptors. These death receptors are composed of an extracellular ligand-binding domain, a single transmembrane domain and an intracellular death domain[9]. The extracellular domain is bound by their respective ligands of the tumor necrosis factor family (e.g. Fas/TNFα), leading to an activation of the intracellular death domain, which in turn recruits and activates initiator caspases-8 and -10. The whole complex then is called death inducing

signaling complex (DISC). Intracellular stresses like DNA damage, growth-factor deprivation and viral infection initiate the intrinsic pathway of apoptosis. The intrinsic pathway is also known as the "mitochondrial" or "BCL-2-regulated" pathway since it is tightly regulated by members of the BCL-2 protein family. Members of the family can cause mitochondrial outer membrane permeabilization (MOMP), which leads to the release of mitochondrial proteins into the cytosol[10]. One of these proteins, cytochrome c, binds to and initiates oligomerization of the pro-caspase-activating factor-1 (APAF-1) to form a so-called apoptosome. The APAF-1 proteins in the apoptosome complex can recruit and initiator caspase-9 proteins that then autoprocess and subsequently activate the apoptotic effector caspase-cascade and finally lead to programmed cell death[11].

MOMP and the BCL-2 protein family

MOMP is considered as the point of no return in apoptosis, once started, there is no way back, and it is regulated by BCL-2 family proteins. All BCL-2 protein family members share particular structural similarities, known as BCL-2 homology domains (BH-domains) (Fig. 1). Depending on the number of BH-domains present they are subdivided into three classes, which then either promote or inhibit apoptosis[12]. The anti-apoptotic sub-class comprises the proteins Bcl-2, Bcl-xL, Mcl-1, Bcl-w and A1/Bfl-1. They share four highly conserved BH-domains (designated BH1-4). Multidomain pro-apoptotic proteins include BAX, BAK and BOK that also possess four such domains. BAX and BAK are the effectors of MOMP while the role of BOK is not completely understood[13]. BAK and BAK oligomerize in the outer mitochondrial membrane (OMM) and thereby form pores enabling cytochrome c release into the cytosol. The other group of pro-apoptotic proteins covers Bmf, Hrk, Bik, Bid, Bim, Bad, Noxa and Puma. These proteins share only the BH3 domain with the remaining BCL-2 family and are therefore termed BH3-only proteins[14].

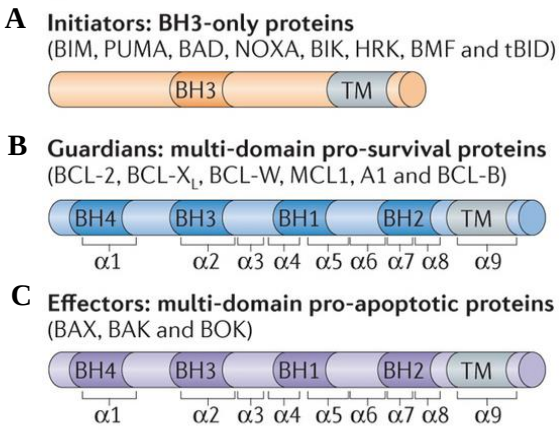

Figure 1. BCL-2 family members sub-divided by regions of sequence (BcCL2-homology regions: BH1-BH4) and structural homology into 3 classes: (A) pro-apoptotic BH3-only proteins, (B) multi-domain pro-survival proteins (BCL2-like proteins) and (C) multi-domain pro-apoptotic proteins. BH1/BH2/BH3/BH4, BCL2 homology-1/2/3/4 domains; TM, transmembrane domain. Figure adopted from Czabotar[15].

BCL2-proteins interact and regulate each other via their BH-domains[16]. Not all members of a sub-class can interact with all proteins of another sub-class and certain binding preferences have been defined (Fig. 2). Interestingly, anti-apoptotic proteins Bcl-2, Bcl-xL, Bxl-w, Mcl-1 and A1/Bfl-1 show different binding preferences to BH3-only proteins, as well to BAK and BAK[17]. This already indicates that the pro-survival proteins might not be fully redundant in their activities.

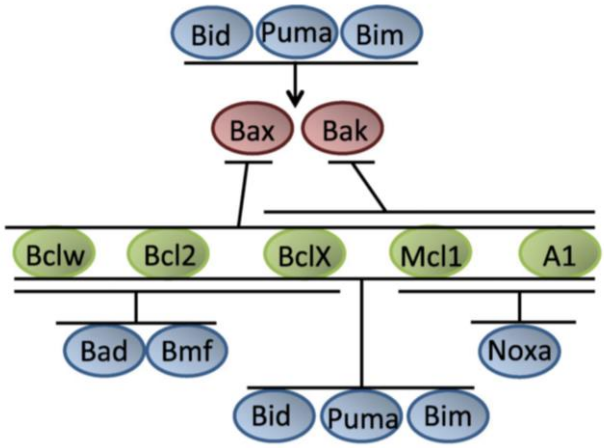

Figure 2. Relations between BCL-2 family members. Different members of the BCL-2 family, categorized into three classes of proteins that control mitochondrial cell death by complex protein–protein interactions, can interact with each other. These interactions are based on different affinities between individual family members and are influenced by various post-translational protein modifications. Blue proteins: BH3-only proteins; green proteins: anti-apoptotic BCL2-like proteins; red proteins: pro-apoptotic multidomain proteins. Picture adopted from Sochalska et.al, 2015[17].

Two major models exist trying to explain how BAX/BAK-dependent MOMP is controlled by pro- and anti-apoptotic BCL-2 family proteins. The "direct activation model" suggested, that pro apoptotic BH3-only proteins are subdivided into two classes, "sensitizers" and "direct activators", where the latter can directly trigger BAX/BAK-dependent MOMP formation[18]. However, the alternative "neutralizing model" proposed that pro-survival BCL2-like proteins sequester and inhibit BAX and BAK, until they are themselves sequestered through inhibition by BH3-only proteins[19]. Taking both theories, which found both plentiful support, into account also a "unified model" was proposed, but is not discussed in detail here[20].

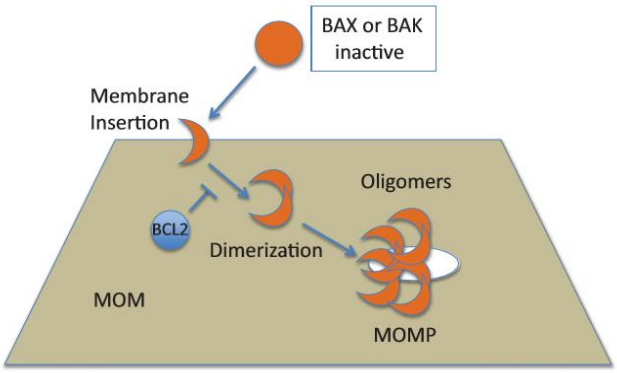

Figure 3. Pro-apoptotic BAX and BAK can spontaneously accumulate in the outer mitochondrial membrane, homo-oligomerize and trigger MOMP, when no pro-survival BCL2-like counterparts are present. MOMP activation is independent from any known BH3-only protein and p53 or Rb. Figure adopted from García Sáez AJ and Villunger A, 2016[21].

Using CRISPR/Cas9 technology researchers recently generated cells completely missing BH3-only proteins (and p53/Rb)[21]. Interestingly, MOMP formation and further caspase activation was still possible upon BCL2-like protein neutralization, thereby implicating that there is no need of direct BH3-only protein activation. Strikingly, even after deleting all BCL2-like proteins (Bcl-2 allKO), both BAK and BAX were able to spontaneously associate with the OMM and form pores. The only essential features for BAX/BAK activation therefore are implicated to reside in their respective helix 9 (C-terminal end) and the OMM[22]. To avoid this spontaneous homo-oligomerization of BAX and BAK in healthy non-apoptotic cells binding by pro-survival BCL-2 proteins appears essential (Fig. 3). BH3-only proteins may shift the balance towards MOMP by only neutralizing the anti-apoptotic BCL-2 proteins sequestering BAK and BAK upon apoptotic stimuli[21].

Apoptosis in immune cell development

Among other essential tasks, apoptosis plays a crucial role in normal lymphocyte development and function. At each developmental stage in B and T cell maturation those cells have to undergo selection processes. Many immature B and T cells are sorted out because they fail to undergo productive rearrangement of their immunoglobulin or respectively T cell receptor genes and therefore do not receive essential signals for survival during positive selection. Positive selection rescues maturing T cells from apoptosis and enables further maturation and differentiation[23]. On the other hand, when lymphocytes bind with their antigen-receptors to self-antigens with high affinity they undergo apoptosis in response to the signaling generated by high-affinity binding, also called negative selection[24,25]. This prevents the development of autoreactive B and T cells. More than 90% of B and T cells undergo apoptosis during their development in the bone marrow or the thymus[26]. Not only during development, but also after a successful immune reaction the immune system strongly relies on

apoptosis. During an infection lymphocytes expand through clonal proliferation to be able to fight pathogens. After the infection is cleared nearly all clones, apart from some that develop into memory cells, die by programmed cell death to ensure homeostatic conditions[27]. Consequently, deregulation or defects in the cell death program can cause either immunodeficiency, when positive selection is impaired, or autoimmunity and/or lymphoma, when negative selection is disturbed[28].

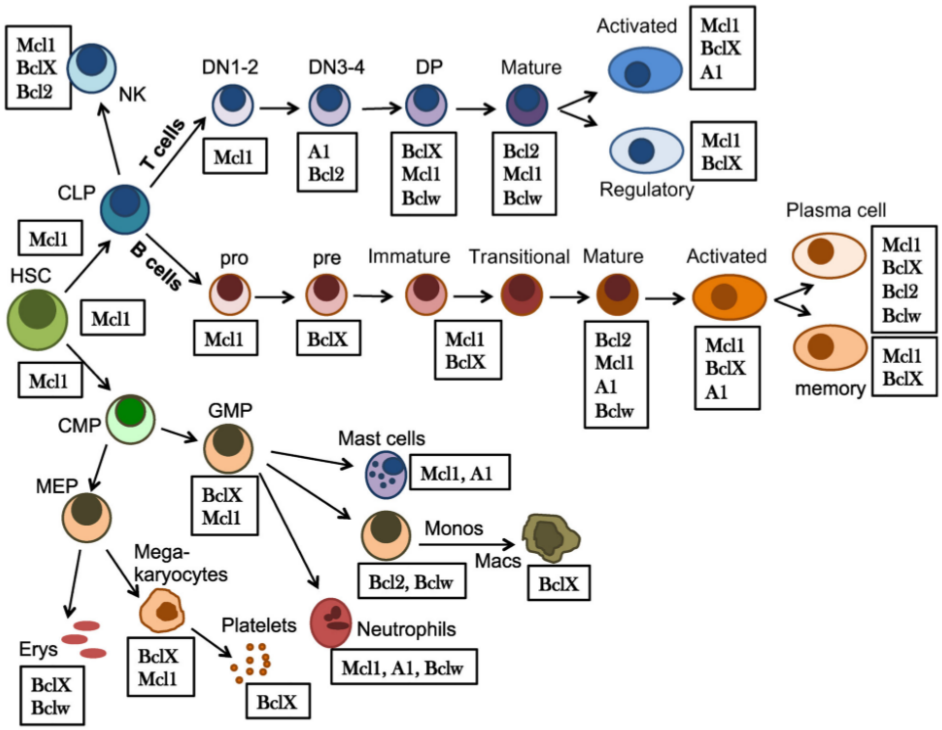

Figure 4. The expression of anti-apoptotic BCL-2 proteins is dynamically regulated during the development of the hematopoietic system. At a given stage in development usually more than one protein is expressed. Therefore, loss of function of one protein is often compensated by the presence of another. Still, some losses in function are fatal – for example loss of Mcl-1 can kill cells, despite the fact that Bcl-2 and Bcl-xL are present. In the adapted figure the proteins are ranked according to the severity of the reported knockout phenotypes[17]. Red boxes highlight the immune cell subsets we chose for further experiments because of the suspected importance of Bcl-xL in it, green boxes highlight the ones where A1/Bfl-1 might is of importance.

Since apoptosis plays a crucial role in immune cell development and homeostasis also its key-factors, like the BCL-2 family proteins, themselves have major impacts. Knock-out studies reported that different family members have various functions[29].

Deletion of BAX leads to a mildly increased number of thymocytes and B cells in mice[30], whereas BAK-deficient mice develop normally[31]. Double knock-out of BAX and BAK causes a perinatal death in the majority of the mice because of multiple developmental defects[31], indicating redundancy between BAX and BAK. BH3-only protein Bim depletion lead to increased numbers of lymphoid and myeloid cells[32] and inhibition of apoptosis of autoreactive thymocytes[33]. *Bid*$^{-/-}$ mice can develop a myeloproliferative disorder on a mixed genetic background[34].

As my thesis mainly focuses on pro-survival BCL2-like proteins, I am going to describe the findings on them in more detail. In gain- and loss-of function studies pro-survival proteins showed highly selective roles in apoptotic signaling during lymphocyte development. Blastocyst-stage and early embryonic lethality of mice lacking Mcl-1 or Bcl-xL, respectively, and severely reduced life span of Bcl-2 deficient mice indicates an important role of these proteins, but challenged the progress of investigating their function.[17]. Consequences of Bcl-w loss appear to be limited to male infertility. In mice, the gene harboring *A1* is present in three functional copies (*A1-a, -b* and *-c*) and one pseudogene (*A1-d*). Therefore, loss-of-function studies were very difficult, leaving many questions about the physiological role of the protein largely undefined[35].

Further studies on Bcl-2 implicated critical roles for it in mature lymphocyte subsets, but a surprisingly restricted role in normal development[36]. Mcl-1 on the other hand seemed to play the most crucial role of all anti-apoptotic proteins during development, since its deletion is lethal in early embryogenesis[37]. Furthermore, multiple studies demonstrated that it is important in the development of the hematopoietic system and neurons[38,39]. Addressing the role of Bcl-xL in lymphocyte development multiple lines of transgenic mice were generated (*SV 40-Eμ-Bcl-xL; Lck-Bcl-X*)[40–42]. Chimeric mice, generated by transplantation of *Bcl-xL*$^{-/-}$ embryonic stem cells into C57BL/6 mice, showed a reduction in pre-B and immature B cell numbers in the bone marrow, a significant loss of B and T cells in the spleen as well as a shifted T cell ratio in the thymus[43]. Conditional deletion of Bcl-xL in double-positive thymocytes ($CD4^+$ $CD8^+$ thymocytes) reduces cell number only to a minor part, although Bcl-xL is expressed prominently in this compartment[44]. Redundancy with Mcl-1, also strongly expressed in this compartment, is thought to explain this phenomenon[38]. Furthermore, Bcl-xL was reported to be strongly induced upon T cell activation, but controversially deletion of the protein only shows minimal effects on T cell response[45].

Regarding studies on A1, knock-out of *A1-a,* lead to enhanced spontaneous neutrophil apoptosis[46]. *In vivo* expression of shRNAs resulted in a constitutive knockdown (KD) of all functional A1 mRNAs. This KD led to slightly delayed thymic development, impaired myeloid and B cell homeostasis *in vivo*[47,39]. Taken together, these results suggest an involvement of A1 in lymphoid and myeloid cells. Using progenitor lines committed to the neutrophil lineage researchers found that A1/Bfl-1 regulates the survival of neutrophils and their homeostasis[39]. These progenitor cell lines were established by conditional estrogen-regulated activation of the transcription factor Hoxb8, which prevented cells from differentiation into neutrophils as long as it was active. However, the role of A1/Bfl-1 in leukocyte development and homeostasis is still less clear than its subfamily members and needs to be further investigated.

Anti-apoptotic BCL-2 family members and cancer

Sustaining resistance to cell death is known as one of the hallmarks of cancer[3]. Due to their central function in the apoptotic machinery BCL2-like proteins are often deregulated in cancer.

B-cell lymphoma protein-2 (Bcl-2) was discovered in B cell follicular lymphoma, where its transcription was excessively upregulated by a chromosomal translocation[48]. Later, it was described that its overexpression inhibits cell death – it was the first regulatory protein found to be involved in apoptosis[49]. A genome-wide screen also identified Bcl-xL and Mcl-1 being highly amplified in various cancer cells that depend on the expression of these genes for their survival[50]. Pro-survival BCL-2 family members are often overexpressed in and therefore contribute to cancer. Their undue expression does not only contribute to tumorigenesis but is also involved in resistance to chemotherapeutic drugs and failure to anti-cancer treatments.

In order to study the effects of Bcl-2 overexpression in the hematopoietic system a *Vav-Bcl-2* mouse model was used. These mice displayed splenomegaly[51], an overabundance of enlarged germinal centers and greatly elevated numbers of cycling B cells. Many mice older than 10 months developed follicular lymphoma[52]. *Vav-Mcl-1* mice showed dramatically accelerated Myc-driven lymphomagenesis, enhanced drug resistance and aging mice developed spontaneous lymphomas with a stem/progenitor cell phenotype or pre-B cell tumor[53]. Exogenous Bcl-xL expression in the T cell compartment resulted in protection from a variety of different apoptotic stimuli and altered thymocyte maturation, but did not cause malignant diseases in mice[40]. Murine A1-a expressed under the control of the *Eμ* enhancer, leading to an overexpression in the B and T cell compartment, triggered disturbance of B cell development in mice and extended survival of early B cells and thymocytes[54]. So far there were no transgenic mouse models exploring the consequences of Bcl-xL and A1/Bfl-1 overexpression under control of the pan-hematopoietic Vav gene promoter.

Based on their importance in controlling cell death in different malignant diseases BCL2-like family proteins became an interesting target in anti-cancer research and drugs that inhibited these proteins came into focus. Via the 3D structure of the proteins, compounds were developed that mimic the function of BH3-only proteins (BH3-mimetics)[55], which should bind and inhibit the anti-apoptotic function of BCL-2-like proteins in cancer cells, leading to cell death. ABT-737, which is able to bind Bcl-2, Bcl-xL and Bcl-w, was the first compound of its kind and found to successfully kill cancer cells *in vitro* and *in vivo.* Its successor ABT-263, known as Navitoclax, is already clinically tested in combination therapy. Another compound, ABT-199 (Venetoclax), was developed, which only binds to and inhibits Bcl-2 has been recently approved by the Food and Drug Administration (FDA) for the treatment of certain cases of chronic lymphocytic leukemia (CLL). It is better tolerated than Navitoclax, because it does not target Bcl-xL, which reduces platelet lifespan when inhibited. BH3-mimetics combined with conventional chemotherapeutics are also into trial[56].

Aim

Although a lot is already known about them, some questions still are unclear concerning anti-apoptotic BCL-2 family proteins. Some studies implied redundancy of the BCL2-like proteins, others pointed out differences in cell death protection, depending on cell type or stimulus. Given their crucial role in development, the immune system and cancer it is of great interest if there is some kind of hierarchy or redundancy between them, which would be important for biomedical research and drug-design.

Focusing on the immune system and immune cell development we chose to concentrate on Bcl-xL and A1/Bfl-1 protein. The investigated specific cell subsets for the ongoing experiments were chosen relying on the described dynamic expression pattern of BCL-2 family proteins in hematopoiesis[17]. With newly generated *Vav-Bcl-xL* and *Vav-Bfl-1* and already existing *Vav-Mcl-1* and *Vav-Bcl-2* mouse models we planned on comparing the impact of overexpression of these proteins on immune cell development and maintenance in a side by side comparison. I intended to gain deeper insight on their function at different stages of immune cell development and thereby contribute to improve strategies to develop cures for varying diseases of the immune system.

Material and Methods

Mice

Two transgenic mouse models on C57BL/6 genetic background were generated. The aim was to obtain mice overexpressing anti-apoptotic Bcl-xL, respectively, Bfl-1, the human homolog of A1, in the hematopoietic system. Therefore, pronuclear injection of plasmids encoding the desired gene of interest into oocytes of C57BL/6 mice was performed by Prof. Thomas Rülicke at the Univ. of Vet. Medicine in Vienna. Using this method, plasmids integrate randomly but stably into the mouse genome. Expression of *Bcl-xL* gene and *Bfl-1* gene are driven by the *Vav*-promoter, which is only active in the hematopoietic system, to ensure expression solely there. Both genes were fused to a FLAG sequence (with sequence motif DYKDDDDK) at the N-terminus. This tag allows the detection of the transgenic proteins by anti-FLAG antibodies without recognizing endogenous protein. Additionally, it enables the comparison of the amount of protein overexpressed between the different transgenic mouse lines. The *Vav-Bfl-1*gene construct is shown as an example in Fig. 5.

Already existing and kindly provided by researchers form the Walter and Eliza Hall Institute of Medical Research (WEHI) in Melbourne, AUS, were *Vav-Bcl-2* mice and *Vav-FLAG-Mcl-1* mice, also on a C57BL/6 genetic background. These lines are already described[51,53] and served as controls.

Offspring from two individual founders, *Vav-Bcl-xL* line #A and *Vav-Bcl-xL* line #B, showed the strongest and most stable expression of the Bcl-xL transgene and were therefore chosen for further experiments. *Vav-FLAG-Bfl-1* line #1 and line #3 were selected.

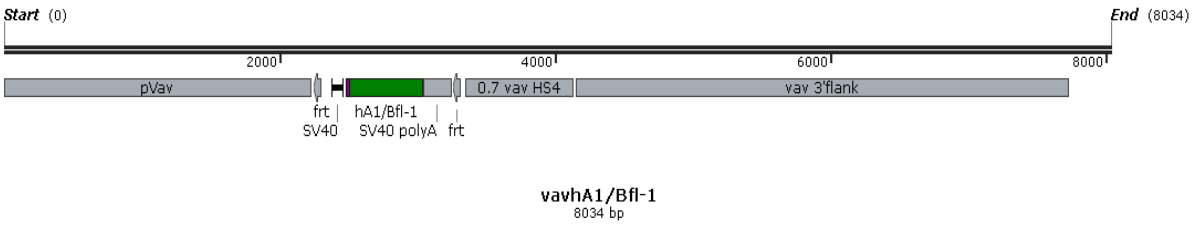

Figure 5. Construct of the *Vav-Bfl-1* sequence which integrated randomly into the mouse genome.

Cell isolation

Lymph nodes, bone marrow, thymus and spleen were isolated from mice and single cell suspension was made using a cell strainer and a syringe plunge. The washing buffer used for the cells was KDS/BSS buffer solution (149nM NaCl, 3.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 0.8 mM K2HPO4 and 7.4 mM HEPES in dH2O) supplemented with 10% fetal calf serum

(FCS, PAA FBS Gold #A15-151), 2 nM L-Glutamine (PAA, #M11-004), 1% Penicillin plus Streptomycin (10.000 U/mL Penicillin and 10 mg/mL Streptomycin in 0.9% NaCl) (PAA, P11-010) and 50 µg/mL Gentamicin (GIBCO #15750-037).

Splenocytes were incubated with Red Blood Cell Removal Buffer at room temperature (145.6 mM NH4Cl, 0.127 mM EDTA, 23.8 mM NaHCO3 in nanopure water) to assure the removal of erythrocytes. The reaction was stopped after 2 minutes adding x ml of FCS.

Cell sorting

BD FacsAriaTMIII Cell sorter (BD Biosciences) was used for cell sorting.

Single cell suspensions were prepared from either spleen or bone marrow and stained with fluorochrome-labelled antibodies recognizing specific cell surface antigens. The cells were stained for 20 min on ice. Clumps of cells were avoided by filtering the cells through a glass Pasteur pipette with a plug of cotton wool. After washing cells with KDS/BSS and resuspension in complete RPMI 1640, they were ready for sorting procedure.

$B220^{-}Gr\text{-}1^{+}$ granulocytes, $B220^{+}Gr\text{-}1^{-}IgM^{-}CD25^{-}ckit^{+}IgD^{-}$pro B cells, and $B220^{+}IgM^{-}CD25^{+}ckit^{-}IgD^{-}$ pre B cells were sorted from bone marrow. The spleen was sorted for $B220^{+}TCR\beta^{-}$ B cells and $B220^{-}TCR\beta^{+}$ T cells.

4′,6-Diamidin-2-phenylindol (DAPI – 12.5µg/mL) was used as marker to exclude dead cells.

Negative selection cell sorting

Cells isolated from organs were negatively selected following MagniSortTM protocol (ebiosciences affymetrix). Thereby cell solutions were incubated with biotinylated antibodies to enable binding to streptavidin coated magnetic beads. Cells coupled to beads were excluded from solution by exposing cell solution to a magnetic field. The remaining cells in the suspension were then negative for specific markers. Granulocytes were obtained by excluding $B220^{+}$, $CD19^{+}$, $Terr119^{+}$, $CD3^{+}$, and $NK1.1^{+}$ cells from the bone marrow. B cells were purified by clearing $CD3^{+}$, $Gr\text{-}1^{+}$, $CD11b^{+}$, $Terr119^{+}$, $NK1.1^{+}$ and T cells clearing $B220^{+}$, $CD19^{+}$, $Terr119^{+}$, -$CD11b^{+}$, $Gr\text{-}1^{+}$, and $NK1.1^{+}$ cells from the suspension. After negative selection the purity of the desired cell type was verified using flow cytometry and cells were additionally discriminated for viability of cells (Annexin V-/7AAD-). Purified granulocytes, B and T cells were then used for further experiments (e.g. apoptosis assay).

Gene expression and translation

To ensure that the newly established transgenic mice also express and translate the desired protein western blot analysis was performed on cell lysates derived from Bcl-xL and Bfl-1 transgenic mice.

Protein isolation

Cells were isolated from different organs (Thymus, Spleen, Lymph Nodes and Bone Marrow) and immediately frozen at -80°C. The cells were thawed on ice and resuspended in 20 µL lysis buffer (Tris-HCl 50 nM pH 7.4, NaCl 150 mM, NP-40 0.5%, NaF 50 nM, Na3VO4 1 mM, PMSF 1mM, DNAseI 0.03 mg/mL). Following an incubation time of 30-60 min the cell suspension was centrifuged at 15000 g for 15 min at 4°C. The supernatant was transferred into a new reaction tube. Lysates were kept on ice or stored at -20°C (-80 °C for long term storage).

Bradford assay

For protein concentration analysis the lysates were diluted (1:5) and 1µL of the dilution was mixed with 99 µL PBS and 900 µL Bradford Reagent (Biorad – diluted 1/5 in A.D). Bovine serum albumin (BSA) with f.c. of 0 µg, 2 µg, 4 µg, 6 µg, 8 µg and 10 µg diluted in 1 mL Bradford Reagent was used as a standard for photometric analysis (595 nm).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were denaturized using a 5x SDS sample buffer (250 mM TRIS pH 6.8, 50% glycerol, 500 mM β-mercaptoethanol, 0.5% brom phenole blue) and incubated at 95°C for 5 min. Denaturized proteins are stable for storage at -20°C.

20-80 µg (depending on available amount) denaturized proteins were loaded on 13% acrylamid gels, peqGOLD Protein Marker V (Peqlab Cat. No.: 27-2210) was used as a marker. A SDS running buffer (1 M Tris, 1 M Tricin, 1% SDS) was used and proteins were separated at a constant electric current of 16mA (per gel) for about 2h.

Immunoblotting

The transfer of the proteins from the PAGE to a nitrocellulose membrane (GE healthcare) was achieved by using an X Cell sure lock wet transfer system (Invitrogen) and Western-Transfer Buffer (3.03 g TRIS, 14.4 g glycin, 200 ml ethanol, apply to 1000 ml with a.d.) for 75 min at current of 100 V on ice. Ponceau-staining (0.3% Ponceau-red, 3% trichloroacetic acid) was performed to confirm successful transfer and was washed off with PBS-Tween20 (PBS-T; PBS containing 0.1% Tween20).

The nitrocellulose membrane was blocked in 5% skimmed milk (in PBS-T) for 1 h at room temperature and incubated with desired primary antibody overnight at 4 °C. The membrane was washed with PBS-T 5x for 5 min, to remove primary antibody not bound to specific proteins, before incubating the membrane with required secondary antibody (in PBS-T) for 1 h at room temperature. After incubation, the membrane was washed 4x for 5 min with PBS-T and 1x for 5 min with PBS, then was incubated for 3 min with ECL (Pierce) and transferred to a cassette used for film exposure.

For detection X-ray films (GE healthcare/AGFA) were used. The exposure time (10 sec – 90 min) varied depending on used antibodies. Used primary antibodies were rb α Bcl-xL (polyclonal, Cell Signaling), ms α FLAG (Sigma, clone M2), rb α Mcl-1 (Rockland, polyclonal), rb α Bcl2A1 (Bfl-1) (J. Borst (NKI), polyclonal), rb α VAV1 (Cell Signaling, polyclonal) and ms α Bcl-2- (BD Pharmigen, clone Bcl-2-100).

Flow cytometry

Single cell solutions were incubated with different antibodies, allowing distinction between cell subsets and analyzing distribution of these subsets.

Following fluorochrome-labeled monoclonal antibodies used for extracellular staining: rat α mouse Ly-6G (RB6-8C5), rat α mouse/human CD11b (M1/70), rat α mouse/human CD45R (RA3-6B2), rat α mouse IgD (11-26c.2a), rat α mouse IgM (RMM-1), hamster α mouse TCRβ (H57-597), rat α mouse CD8 (53-6.7), rat α mouse CD25 (3C7), rat α mouse Ter119 (TER-119), rat α mouse CD317 (927), rat α mouse CD117 (2B8), rat α mouse CD45 (30-F11), Annexin-V FITC and Streptavidin APC from BioLegend; rat α mouse CD44 (IM-7), rat α mouse CD62L (MEL-14) and rat α mouse CD138 (281-2) from BD Biosciences; rat α mouse CD4 (RM4-5), Annexin-V Alexa647, rat α mouse CD23 (B3B4), Annexin-V eFluor450, rat α mouse CD71 (R17217), Streptavidin PerCP-Cyanine 5.5, rat α mouse CD3 (145-2C11) and rat α mouse γδTCR (eBioGL3) from eBiosciences.

The flow cytometry analyzer LSRFortessa (BD Biosciences) was used for performing flow cytometry measurements, running on FacsDiva Software (BD Biosciences). For analysis of the flow cytometry data FlowJo Software was used.

Extracellular staining

Depending on the cell subset we wanted to investigate different fluorochrome-labelled antibodies (Tab. 1) were chosen and diluted in plain sterile PBS or 1:1 1x PBS:BD Brilliant Violet (BD Biosciences). 3 x 10^6 cells were washed in 1x phosphate buffered saline (PBS, 6.5 nM Na2HPO4, 1.5 mM KH2PO4, 2,5 mM KCl, 140 mM NaCl, pH 7.25) and resuspended in 30 µL antibody dilution. Antibody incubation time was 20 min on ice, then the cells were washed with 2 mL 1x PBS for direct analysis by flow cytometry, incubated with 2^{nd} antibody or resuspended in a fluorochrome-labelled Annexin V antibody solution.

Cell culture & Survival assays

Isolated primary cells were cultured in Roswell Park Memorial Institute 1640 medium (PAA RPMI1640 #E15-039), containing 10% FCS, 2 nM L-Glutamine, 1% Penicillin plus Streptomycin (10.000 U/mL Penicillin and 10 mg/mL Streptomycin in 0.9% NaCl), 5 x 10-5 M 2-mercaptoethanol,

50 µg/mL Gentamicin, 100µM nonessential amino acids (Gibco #1091607) and 1 mM sodium pyruvate (Gibco #1046485) in a flat-bottom 96 well plate. Culture conditions were 5% CO2 at 37°C.

To observe survival benefits the cells were challenged with different cell death inducing stimuli, including incubation with corticosterone at 312.5 and 625 nM, staurosporine (STS) at 50 and 100nm, ABT-199, WEHI539 and ABT-737 at 0,5 µM, and single irradiation with 1.25 Gray and 5 Gray. After 0h, 4h, 8h, 12h, 20h, 30h, 48h and 72h thymocytes, B cells and T cells were harvested and FACS analyzed for viability, respectively, pro B cells and granulocytes after 0h, 8h, 18h, 30h and 48h. Therefore, cells were transferred into a FACS tube, washed with 2 mL PBS, centrifuged for 5min and 1500rpm at 4°C. After removal of supernatant the cells were stained with 1:1000 7-AAD and 1:400 Annexin V (Pacific blue), which bind to cell death receptors on the cell surface. Viability analysis was performed using flow cytometry. Only cells being double negative for both cell death markers 7-AAD and Annexin V were considered alive and used for further calculations.

Used cell-death inducers

Staurosporine: As an alkaloid isolated from Streptomyces staurosporesa, Staurosporine works as a non-selective protein kinase inhibitor by preventing ATP to bind to kinases. This is achieved by the strong affinity to the ATP-binding site, therefore STS serves as an ATP competitor. For research purposes it is used to induce apoptosis. It can lead to apoptosis both caspase-dependent and caspase-independent[57].

γ- irradiation: Electromagnetic radiation of an extremely high frequency that can cause biological damage. Exposing cells to gamma rays leads to single or double-strand brakes of the DNA caused by the ionization of the atoms that make up the DNA chain. The amount of irradiation correlates with the amount of DNA damage. Depending on the dose the DNA repair machinery repairs the damage or - when it's overloaded - severe mutations and chromosome aberrations pile up and finally lead to cell death[58].

Corticosterone: Corticosterone is a 21-carbon steroid hormone of the corticosteroid type. It belongs to the class of glucocorticoids (GC), which are produced by the adrenal cortex. GCs bind to the glucocorticoid receptor (GR) and are therefore part of the feedback mechanism of the immune system. They are known to reduce aspects of the immune function, like reduction of inflammation. Due to this function, they are used in medicine to treat disease caused by an over reactive immune system (allergies, asthma, autoimmune diseases)[59]. In mice corticosterone is the main GC, known to be important in T cell selection and proliferation in the thymus. GCs are known as an agent able to regulate DP thymocyte number and also to induce apoptosis of them[60].

FasL (CD95-ligand): FasL is a ligand of Fas, which is a so-called death receptor playing an important role in apoptosis. When Fas is bound, a conformational change in the intracellular domain of the

receptor happens, which then allows the binding of specific proteins and finally leading to the activation of the extrinsic pathway of apoptosis. FasL induced apoptosis in primary lymphocytes is independent of Bcl-2 family proteins[61].

ABT inhibitors (BH3-mimetics):

- ABT-199 (GDC-0199) serves as a novel and specific inhibitor of (Bcl-2). It is >4800-fold more selective versus Bcl-xL and Bcl-w, but has no activity to Mcl-1. When treated with ABT-199, Bcl-2 dependent cells die of apoptosis, since their anti-apoptotic mitochondrial pathway is suppressed. This is not the case for Bcl-xL dependent cells[62].
- WEHI-539 was designed as a Bcl-xL inhibitor with high affinity and selectivity. It interacts with the binding groove of Bcl-xL and effectively kills cells by inhibiting the pro-survival activity of Bcl-xL. WEHI-539 cannot kill cells lacking the cell death mediator BAK, because BAK is regulated by BCL-XL and MCL-1[63].
- ABT-737 functions as novel and potent inhibitor of Bcl-xL, Bcl-2 and Bcl-w. There was no inhibition observed against Mcl-1, Bcl-B or Bfl-1. ABT-737 could also induce the disruption of the Bcl-2/BAX complex and BAK-dependent but BIM-independent activation of the intrinsic apoptotic pathway[64].

Immunization of mice

Sheep blood (Biozol – CL2581-100D) was washed with PBS by transferring 4mL sheep blood into a 50mL reaction tube and adding 46mL PBS. The mixture was centrifuged at 1500rpm for 5 min. After carefully discarding the supernatant, remaining red blood cells (SRBC) were resuspended in 4mL PBS. 200µL/mouse was injected *i.v.* into mice narcotized by inhalation of Isoflurane. After 7 days, serum, spleen, inguinal and mesenteric lymph nodes were isolated from mice and a single cell suspension was made using a cell strainer and a syringe plunge. The washing buffer used for the cells was KDS/BSS buffer supplemented with 10% fetal calf serum, 2 nM L-Glutamine, 1% Penicillin plus Streptomycin and 50 µg/mL Gentamicin. $3x10^6$ cells were used for cell characterization analysis. Cells were stained with antibodies specific for germinal center B cells and plasma cells. Germinal center staining: IgG1, Fas, CD38, B220, IgD, IgM and CD19; Plasma cell staining: CD138, TACI, B220, IgM and CD19 (see Tab.1).

Statistical analysis

Statistical comparisons were made using GraphPad Prism 6. Estimation of statistical differences between groups was carried out using the unpaired Student t-test or two-way ANOVA analysis. P-values of <0.05 were considered to indicate statistically significant differences.

Results

Establishing transgene expression profiles in whole organs and isolated leukocyte subsets

Transgenic *Vav-FLAG-Bcl-xL* overexpression

To verify that the exogenous transgene is not only inserted into the genome but also transcribed and expressed, we performed western blot analysis for Bcl-xL on spleen, thymus, lymph nodes and bone marrow derived from *Vav-Bcl-xL* transgenic mice generated from two independent transgene-positive founders. Transgene expression was confirmed for all hematopoietic organs (Fig. 1). The faster migrating (lower) band in the blots represents endogenous Bcl-xL, the upper band exogenous Bcl-xL, which is linked to a FLAG-tag sequence at the N-terminus and therefore runs slower than the endogenous protein. A difference in exogenous protein expression was found between the organs which was also independent from the transgene-promoter used; as is visible in comparison to the signal generated using a Vav-specific antibody (Fig. 1). Notably, differences in expression were also found between the two independently generated transgenic Bcl-xL mouse lines analyzed.

Using an antibody targeting the FLAG sequence of the transgenic protein (Flag M2), the expression of FLAG-Mcl-1 and FLAG-Bcl-xL was found comparable. *Vav-Bcl-xL* line A expresses nearly the same amount of transgene than the *Vav-FLAG-Mcl-1* transgenic mouse line, whereas *Vav-Bcl-xL* line #B was found to overexpress less protein than line #A or the Vav-Mcl-1 mouse line in some of the cell lines analyzed (Fig. 1). Differences in transgene expression between different founders are not uncommon, as the model used here relies on random transgene insertion into the host genome that can affect expression levels[65].

To define which cell types of the hematopoietic system actually express the transgene, protein expression was also analyzed in selected FACS-sorted cell subsets, including pre B cells and granulocytes from bone marrow, thymocytes (representing T cells in primary lymphatic organs), splenic T- and B cells and T- and B cells from lymph nodes (Fig. 7). A variation of expression between the two lines was again observed in primary lymphoid organs and in particular in the B cell linage in the periphery; *Vav-Bcl-xL* tg line #A showing a significantly stronger signal than line #B. Both lines were found to express equal amounts of the transgene in T cells (Fig. 7).

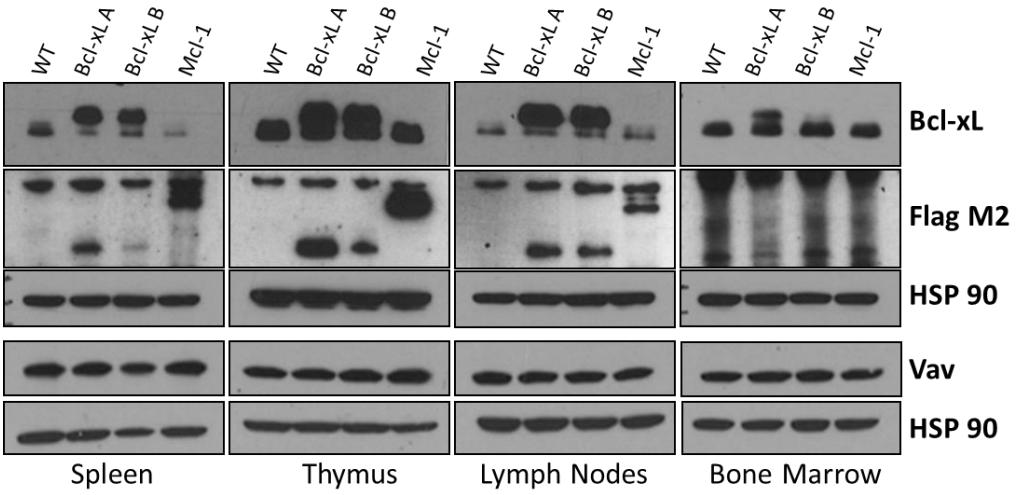

Figure 6: Western blot analysis was performed on protein lysates from whole organs (spleen, thymus, lymph nodes and bone marrow) on a 13% polyacrylamide gel. Bcl-xL antibody recognizes both exogenous (upper band) and endogenous (lower band) Bcl-xL; Flag M2 antibody binds to introduced FLAG-protein construct; HSP 90 antibody serving as control for protein expression; Vav antibody recognizing Vav-protein, which is only present in the hematopoietic system (and therefore representing the Vav-promoter activity).

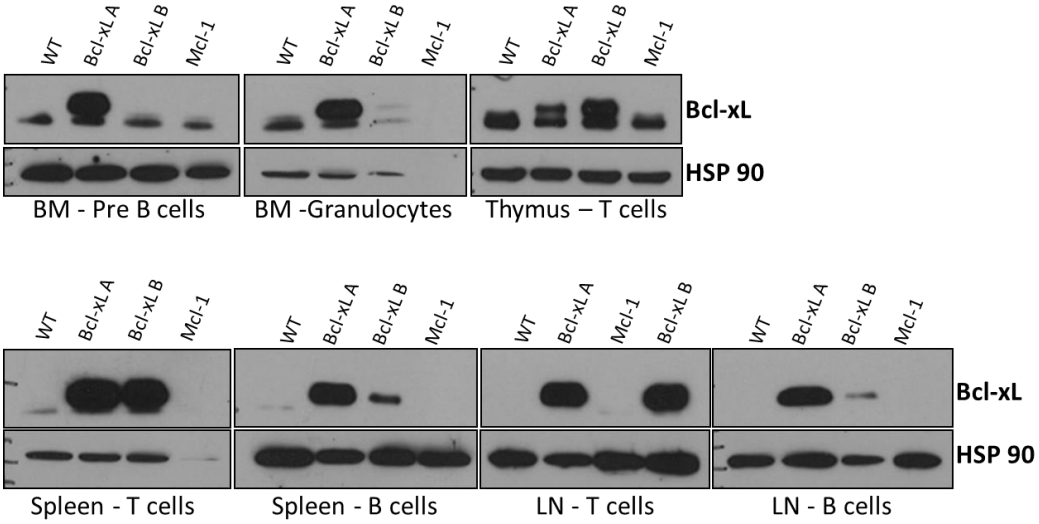

Figure 7: Western blot on proteins from sorted cellular subsets (Pre B cells, Granulocytes, B and T cells) on a 13% polyacrylamide gel.

Transgenic *Vav-FLAG-Bfl-1* overexpression

In the lymphatic tissues, such as lymph nodes, spleen and thymus Bfl-1 expression was evaluated. From the two established mouse lines from transgene-positive founders, which were chosen for experiments, mouse line #3 was found more potent in transgene expression than line #1. Due to sensitivity problems with the available Bfl-1 antibody we could not confirm expression of the transgene within isolated cell subsets (including T cells, B cells, pre B cells and granulocytes). A FLAG-signal could also not be detected, making a quantitative comparison of transgene expression with transgenic mice overexpressing Mcl-1 or Bcl-xL impossible.

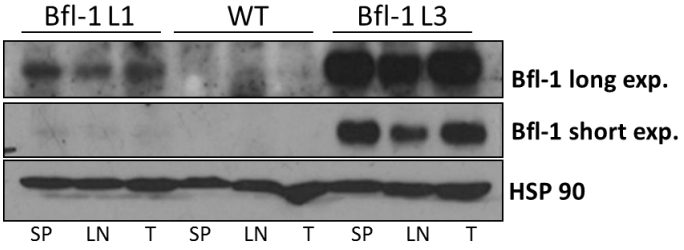

Figure 8: Western blot on proteins from whole organs (spleen, thymus and lymph nodes) on a 13% polyacrylamide gel. Bfl-1 antiserum detecting transgenic Bfl-1 expression; HSP 90 serving as control for protein expression.

Impact of pro-survival protein overexpression on lymphocyte subset distribution

After protein expression of both transgenes (*Vav-Bcl-xL* and *Vav-Bfl-1*) was confirmed, we went on to investigate their impact on the development of different immune cell subsets. Therefore, FACS analysis of primary and secondary hematopoietic organs was performed. We used transgene-negative littermate mice as a control for normal lymphocyte development and compared the hematopoietic system form *Vav-Bcl-xL* and *Vav-Bfl-1* tg mice to that found in *Vav-Bcl-2* and *Vav-Mcl-1* animals, since their influence on lymphocyte distribution has already been described.

Since each anti-apoptotic BCL-2 like protein is of importance at different developmental stages of the immune cell subsets, we chose to focus first on the subsets where Bcl-xL and A1/Bfl-1 are reported to have an important function during development, such as in T cells, B cells and granulocytes.

Impact of Bcl-xL overexpression on immune cell distribution

Bcl-xL was specified to be a crucial player in double positive (DP) thymocytes, pre B cells and in activated B- and T- cells[17]. Hence, it was of interest whether an overexpression of this protein would influence the composition of these compartments.

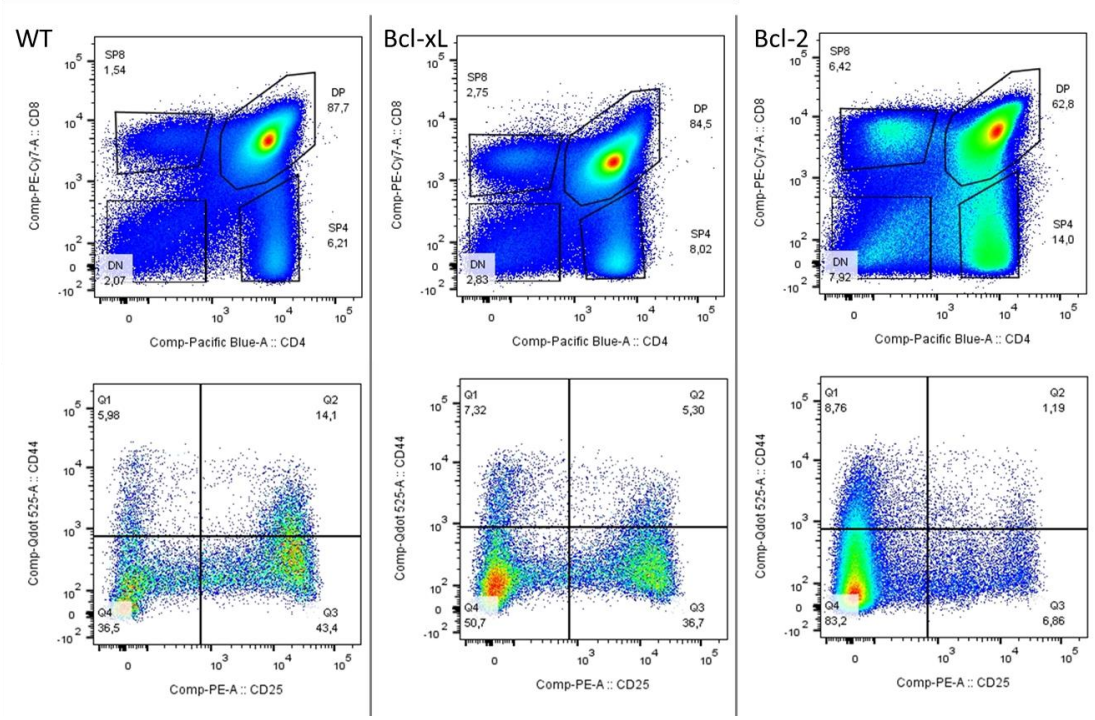

Figure 9 Representative FACS blot from thymus. WT, Bcl-xL and Bcl-2 transgenic mice are compared. Upper row demonstrates thymic subset composition based on the cell surface markers, CD8 vs. CD4, used to discriminate between DN, SP4, SP8 and DP thymocytes. Lower panel compares early thymocytes within the DN cell subset, based on the cell surface markers, CD44 vs. CD25, defining clockwise double-negative (DN)1, DN2, DN3 and DN4 developmental stages. Immune cells were analyzed by flow cytometry.

Depending on expressed CD8 and/or CD4 cell surface markers thymic composition is divided into double-negative, DN ($CD4^-CD8^-$), single positive, SP4 ($CD4^+CD8^-$), single positive, SP8 ($CD4^-CD8^+$) and double-positive DP ($CD4^+CD8^+$) cells. Under steady state conditions no abnormal distribution within these subsets could be found in *Vav-Bcl-xL A, Vav-Bcl-xL B, Vav-Bfl-1 L1, Vav-Bfl-1 L and Vav-Mcl-1 mice when* compared to the WT controls (Fig. 10). Bcl-2 overexpression led, as already described[51], to a significant decrease in DP thymocytes and an significant elevation of the double negative (DN), single positive (SP) for CD8 (SP8) and SP4 compartments (Fig. 10 A). The $CD4^-CD8^-$ double-negative (DN) stage, or committed T-cell precursor stage, can be subdivided further into four sequential stages of differentiation: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$)[66,67]. In this compartment Mcl-1 and Bcl-xL overexpression caused an accumulation of DN4 cells (Fig. 10 B), accompanied by a consequent decrease of DN3 cells.

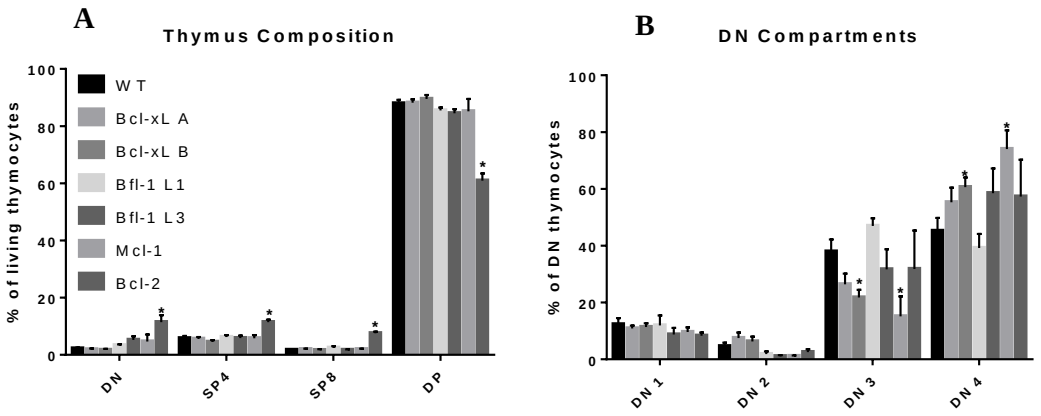

Figure 10 Distribution of thymocyte subsets in vivo under steady state conditions. Thymocytes were isolated from WT, *Vav-Bcl-xL A, Vav-Bcl-xL B, Vav-Bfl-1 L1, Vav-Bfl-1 L3, Vav-Mcl-1* and *Vav-Bcl-2* mice. Developmental stages DN, SP4 SP8 and DP (A) and DN compartment of the thymus were analyzed by flow cytometry. Bars represent means ± s.e.m. *P< 0,05 (Two-way ANOVA); n = 4-10.

No significant changes could be observed for the distribution of lymphocytes in the spleen between *Vav-Bcl-xL #A, Vav-Bcl-xL #B, Vav-Bfl-1 #L1, Vav-Bfl-1 #L3, Vav-Mcl-1* and *Vav-Bcl-2* and WT mice. The ratio between lymphocytes and myeloid cells ($CD11^+Gr1^+$) was in the normal range for all genotypes (Fig. 11 A). It is noteworthy, that spleen cellularity was massively increased upon *Vav-Bcl-2*, but also *Vav-Mcl-1* overexpression (Fig. 11). This phenomenon was already described[53,68] and also observed here. Importantly, our *Vav-Bcl-xL* and *Vav-Bfl-1* tg lines did not show this increase in cellularity.

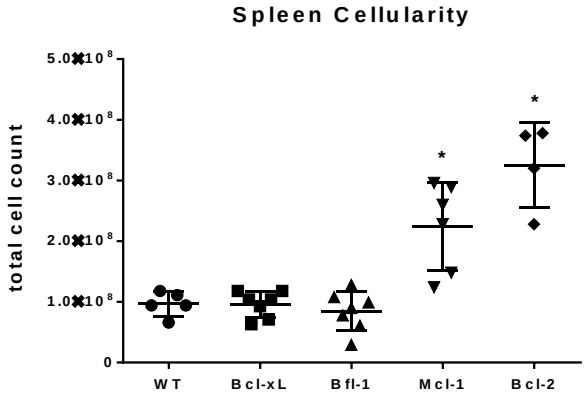

Figure 11 Bar-graph representing total cell count of spleens taken from WT and transgenic mice (*Vav-Bcl-xL, Vav-Bfl-1, Vav-Mcl-1, Vav Bcl-2*). Summary of mean splenic cellularity per genotype ± s.e.m. *P< 0,05 (unpaired Student t-test); n = 4-10.

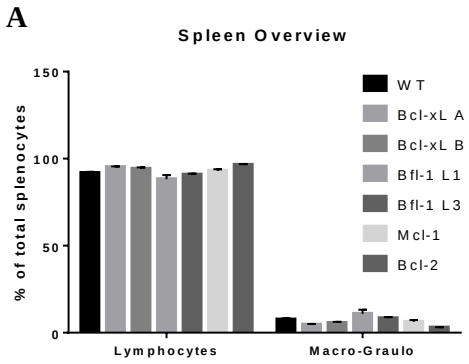

Figure 12 Distribution of leukocyte subsets in the spleen *in vivo* under steady state conditions. Splenocytes were isolated from WT, *Vav-Bcl-xl A, Vav-Bcl-xL B, Vav-Bfl-1 L1, Vav-Bfl-1 L3, Vav-Mcl-1* and *Vav-Bcl-2* mice. Immune cell composition was analyzed by flow cytometry. Bars represent means ± s.e.m. *P< 0,05 (Two-way ANOVA); n = 4-10.

Within the lymphocyte subset a slight increase in the T cell compartment ($TCR\beta^+B220^-$) was found for *Vav-Bcl-xL* transgenic mice (Fig. 14 B). Also Bfl-1 L3 and Mcl-1 overexpression showed a tendency of an elevation in T cells, but did not proof to be stable between different experiments. Further investigation of the T cell subset (Fig. 13) in the spleen could not confirm significant elevation of T cells in Vav-Bcl-xL mice (WT 36,25% and Bcl-xL #B 43,84% T cells) (Fig. 14 A). Furthermore, the ratio between helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) appeared to be normal (Fig. 14 B). Only *Vav-Bcl-2* transgenic mice showed a slight shift within the activation status of helper and cytotoxic T cells from the naïve T cell population ($CD62L^+CD44^-$), towards an increase in both central ($CD62L^+CD44^+$) and effector memory T cells ($CD62L^-CD44^+$) (Fig. 14 C-D).

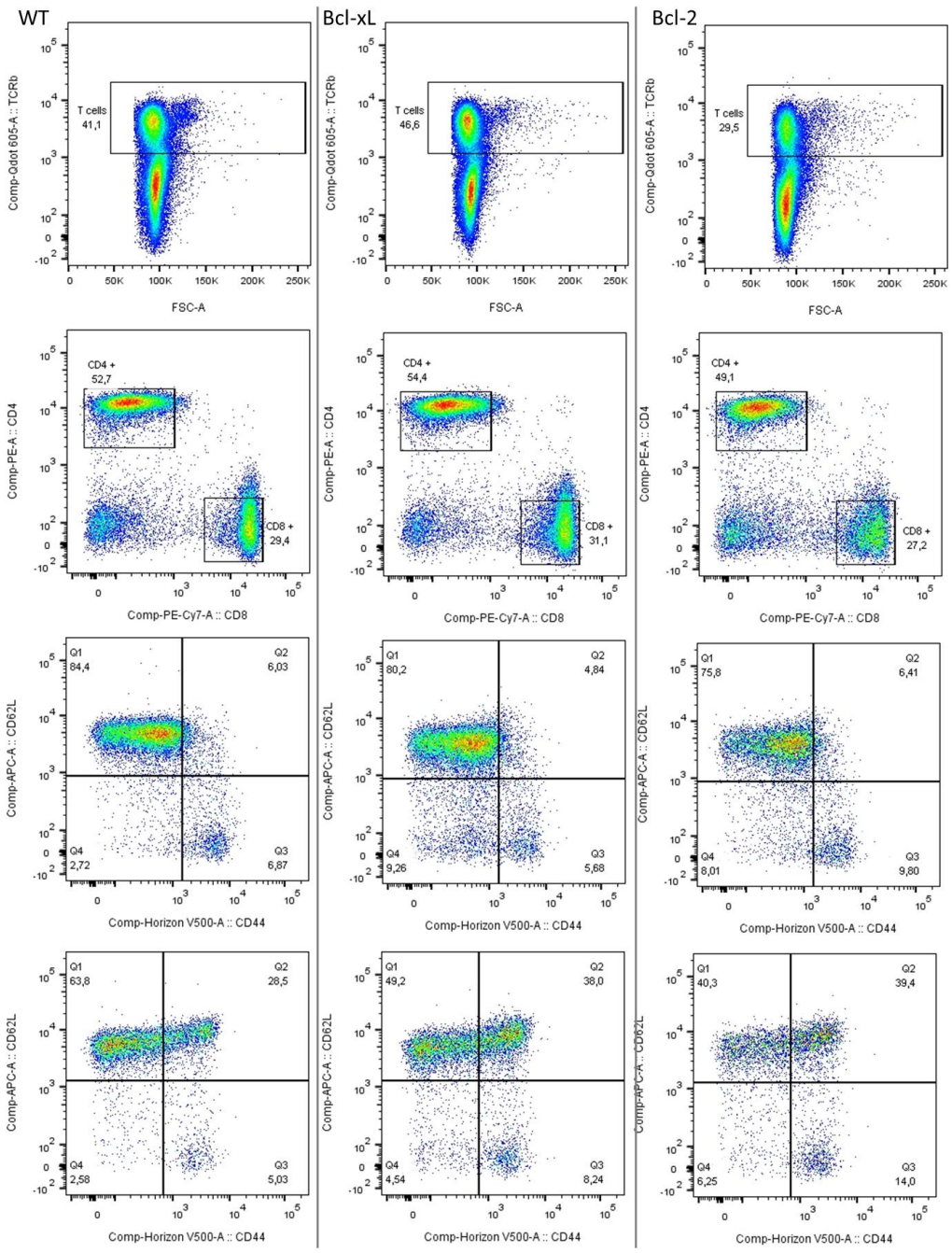

Figure 13 Representative FACS blots of splenic T cells. WT, *Vav-Bcl-xL* and *Vav-Bcl-2* genotypes are compared. The first row shows TCRβ$^+$ cells, assumed to be T cells, present in the spleen. In the 2nd row the cell surface markers CD4 and CD8 are used to discriminate between T cells expressing either the one or the other receptor. Depending on the expression of CD62L and CD44 CD8$^+$ respectively CD4$^+$ cells are subdivided into naïve (CD62L$^+$CD44$^-$), central memory (CD62L$^+$CD44$^+$) and effector memory (CD62L$^-$CD44$^+$) cells, shown in 3rd row (CD4$^+$ cells), respectively. 4th row shows CD8$^+$ cells based on CD62L and CD44 expression. Immune cells were analyzed by flow cytometry.

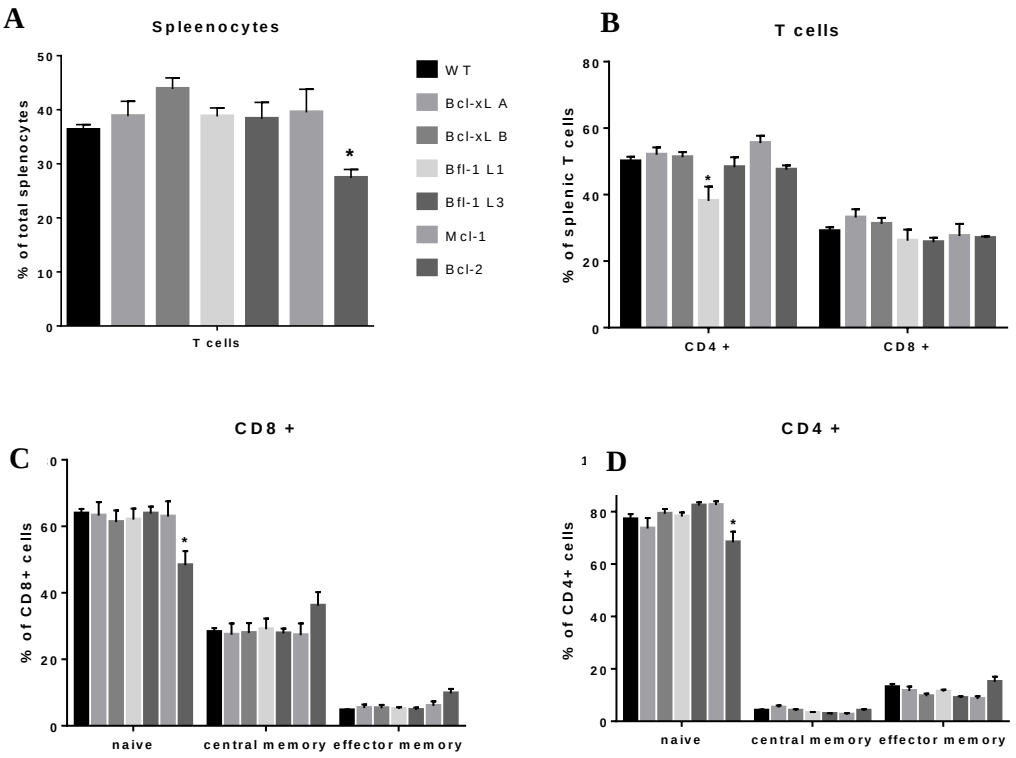

Figure 14 Distribution of splenic T cell compartments under steady state conditions. Splenocytes were isolated from WT, *Vav-Bcl-xl A, Vav-Bcl-xL B, Vav-Bfl-1 L1, Vav-Bfl-1 L3, Vav-Mcl-1* and *Vav-Bcl-2* mice. Immune cell composition was analyzed by flow cytometry. Bars represent means ± s.e.m. *P< 0,05 (unpaired student t test; Two-way ANOVA); n = 4-10.

The $B220^+$ B cell compartment of the spleen was not influenced by the overexpression of Bcl-xL, Bfl-1 or Mcl-1 (Fig. 16 A-E). *Vav-Bcl-2* transgenic mice seem to loose IgM and IgD receptors due to accumulation of class-switched B cells as well as plasma cells, therefore having a relative decrease in the percentage of mature/naive, memory, marginal zone and T1 B cells (Fig. 16 C and E).

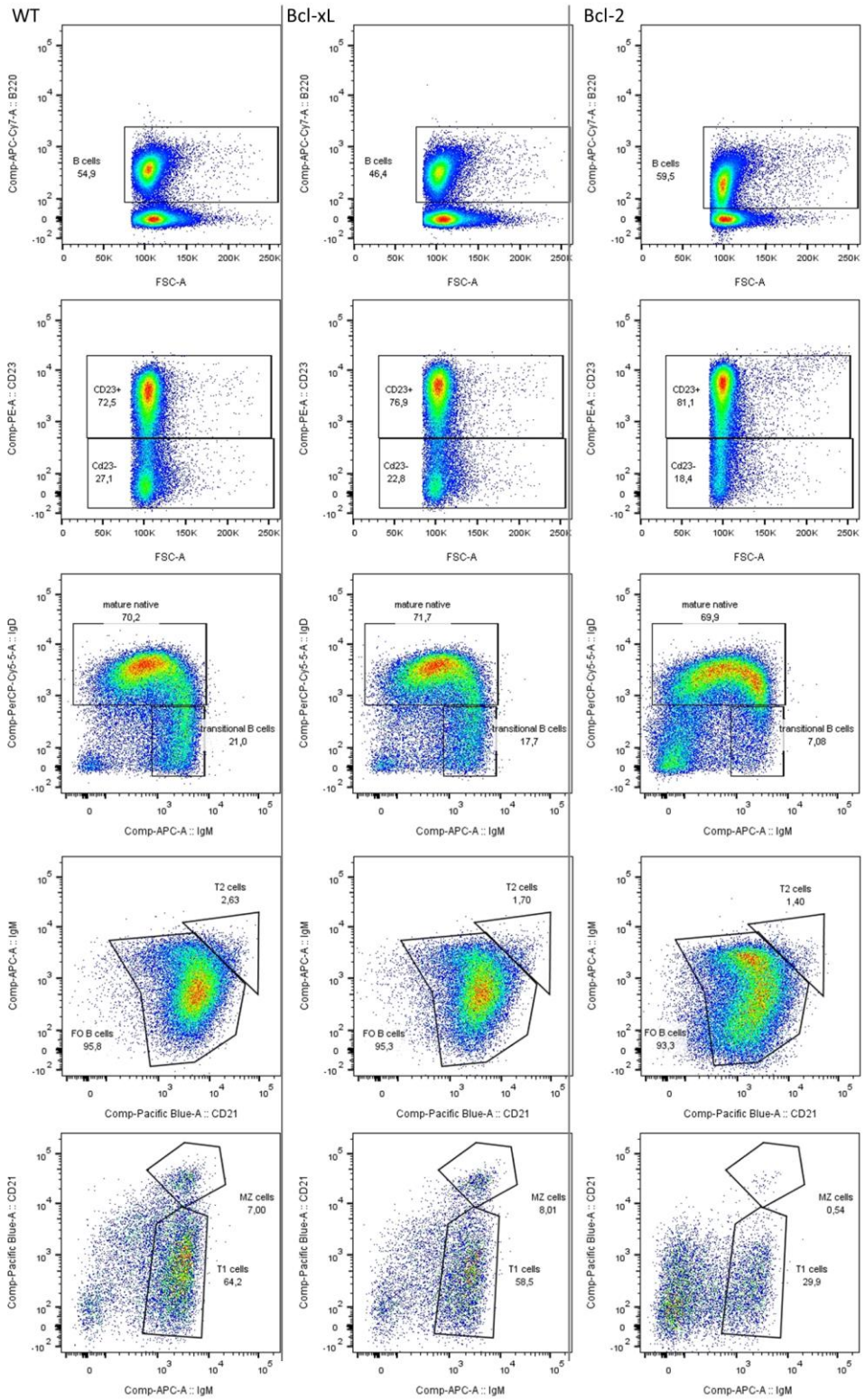

Figure 15 Representative FACS blot – splenic B cells. WT, *Vav-Bcl-xL* and *Vav-Bcl-2* genotypes are compared. The first row shows B220^{+} cells, representing B cells, present in the spleen. In the 2nd row the cell surface marker CD23 discriminates between B cells being positive or negative for it. In the middle row IgM and IgD surface markers enable to differentiate between mature and memory B cells. Within the CD23^{+} cell compartment, T2 cells and follicular B cells are classified using IgM and CD21 markers (4th row). The same markers are used in CD23^{-} cell to differentiate between marginal zone (MZ) and T1 B cells. Immune cells were analyzed by flow cytometry.

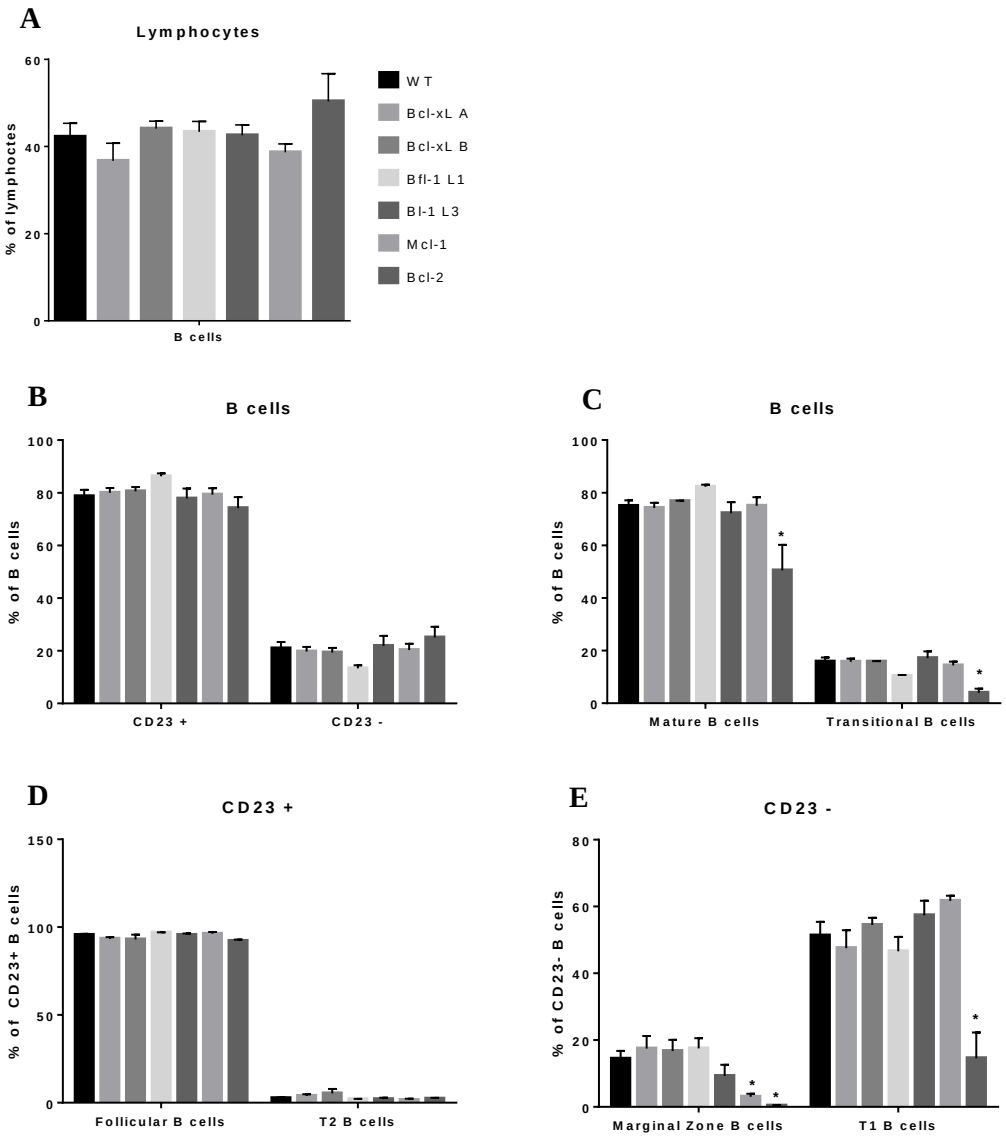

Figure 16 Distribution of splenic B cell compartments under steady state conditions. Splenocytes were isolated from WT, *Vav-Bcl-xl A, Vav-Bcl-xL B, Vav-Bfl-1 L1, Vav-Bfl-1 L3, Vav-Mcl-1* and *Vav-Bcl-2* mice. Immune cell composition was analyzed by flow cytometry. Bars represent means ± s.e.m. *P< 0,05 (unpaired student t test; Two-way ANOVA); n = 4-10.

When analyzing the subsets of T cells in lymph nodes, I could not observe any significant aberrations upon pro-survival protein overexpression (Fig. 17 A-D). Although, the B and T cell compartment in the lymph nodes showed to vary a little (upon *Vav-Bcl-xl #A*, *Vav-Mcl-1* and *Vav-Bcl-2* a slight decrease in total B cells was noted), this change did not proof to be significant.

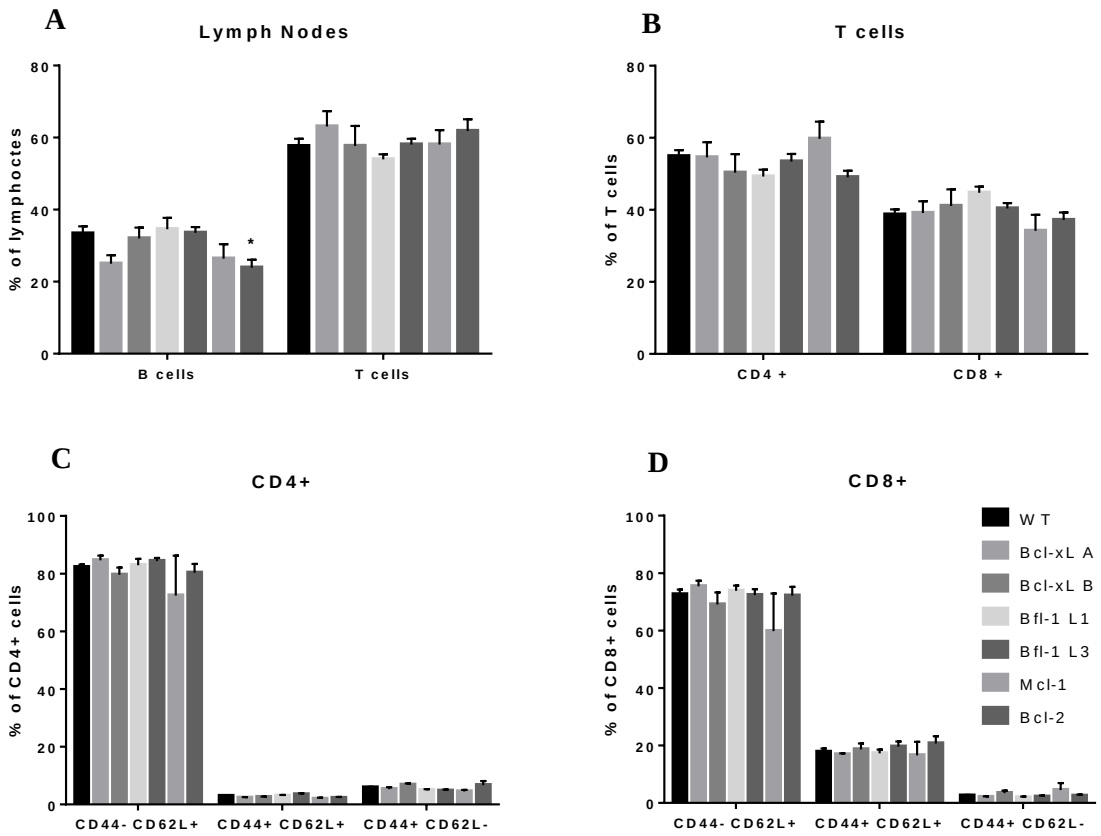

Figure 17 Distribution of cell subsets in inguinal lymph nodes under steady state conditions. Lymph nodes were isolated from WT, *Vav-Bcl-xl A, Vav-Bcl-xL B, Vav-Bfl-1 L1, Vav-Bfl-1 L3, Vav-Mcl-1* and *Vav-Bcl-2* mice. Immune cell composition was analyzed by flow cytometry. Bars represent means ± s.e.m. *P< 0,05 (Two-way ANOVA); n = 4-10.

Characterization of the bone marrow (Fig. 18 and Fig. 20) showed relatively wide variations in cellularity. *Vav-Mcl-1* and *Vav-Bcl-2* tg mice showed an accumulation of recirculating B cells. Bcl-2 overexpressing mice showed an elevation of helper T cells within the T cell compartment (Fig. 19 C).

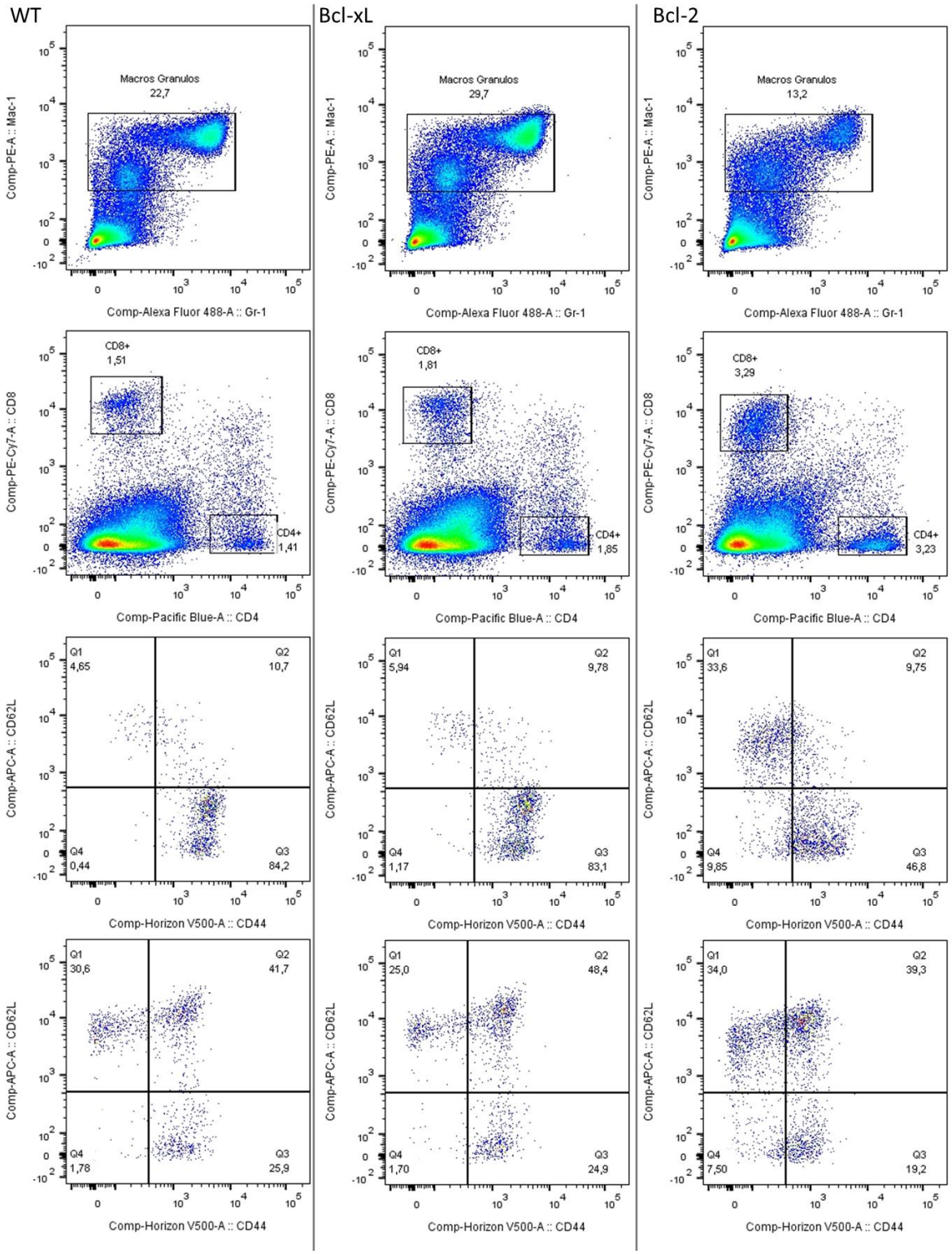

Figure 18 Representative FACS blot from cells from bone marrow. WT, *Vav-Bcl-xL* and *Vav-Bcl-2* genotypes are compared. The first row represents the FACS gating strategy showing macrophages and granulocytes (markers Gr-1 and CD11b). In the 2nd row the cell surface markers CD4 and CD8 are used to discriminate between T cells expressing either the one or the other receptor. Depending on the expression of CD62L and CD44 $CD8^+$ respectively $CD4^+$ cells are subdivided into $CD62L^+$ $CD44^-$, $CD62L^+$ $CD44^+$ and $CD62L^-$ $CD44^+$ cells, shown in 3rd row ($CD4^+$ cells) respectively, 4th row ($CD8^+$ cells). Immune cells were analyzed by flow cytometry.

Also regarding the B cell compartment of the bone marrow, only *Vav-Mcl-1* and *Vav-Bcl-2* tg mice showed an impact on the immune cell composition (Fig. 21). Upon *Vav-Bcl-2* overexpression the cell number of immature B cells was significantly elevated and an accumulation of recirculating B cells was observed in bone marrow from *Vav-Mcl-1* or *Vav-Bcl-2* mice.

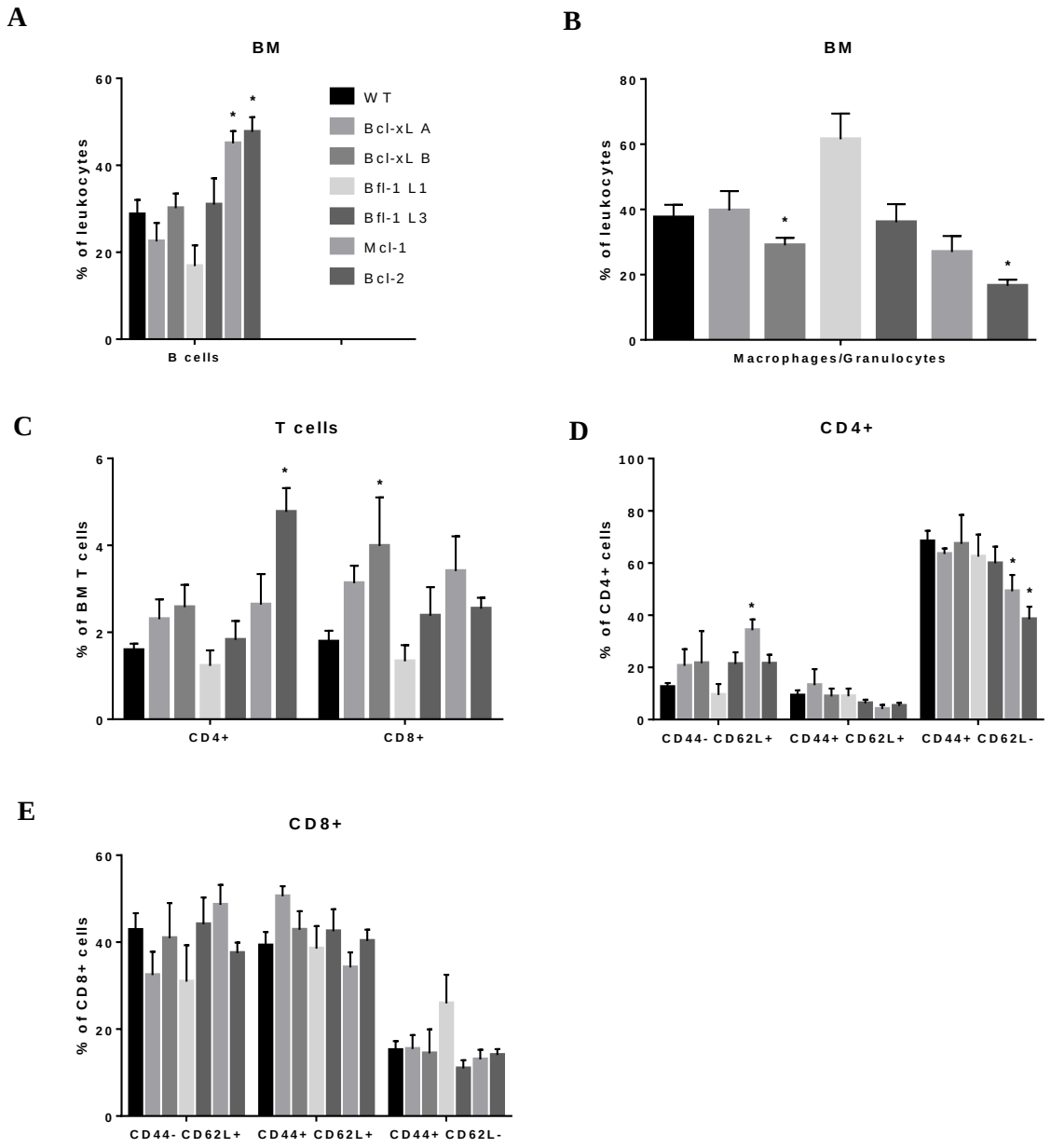

Figure 19 Distribution of T cell subsets in bone marrow under steady state conditions. Bone marrow was isolated from WT, *Vav-Bcl-xl A*, *Vav-Bcl-xL B*, *Vav-Bfl-1 L1*, *Vav-Bfl-1 L3*, *Vav-Mcl-1* and *Vav-Bcl-2* mice. Immune cell composition was analyzed by flow cytometry. Bars represent means ± s.e.m. *P< 0,05 (unpaired student t test; Two-way ANOVA); n = 4-10.

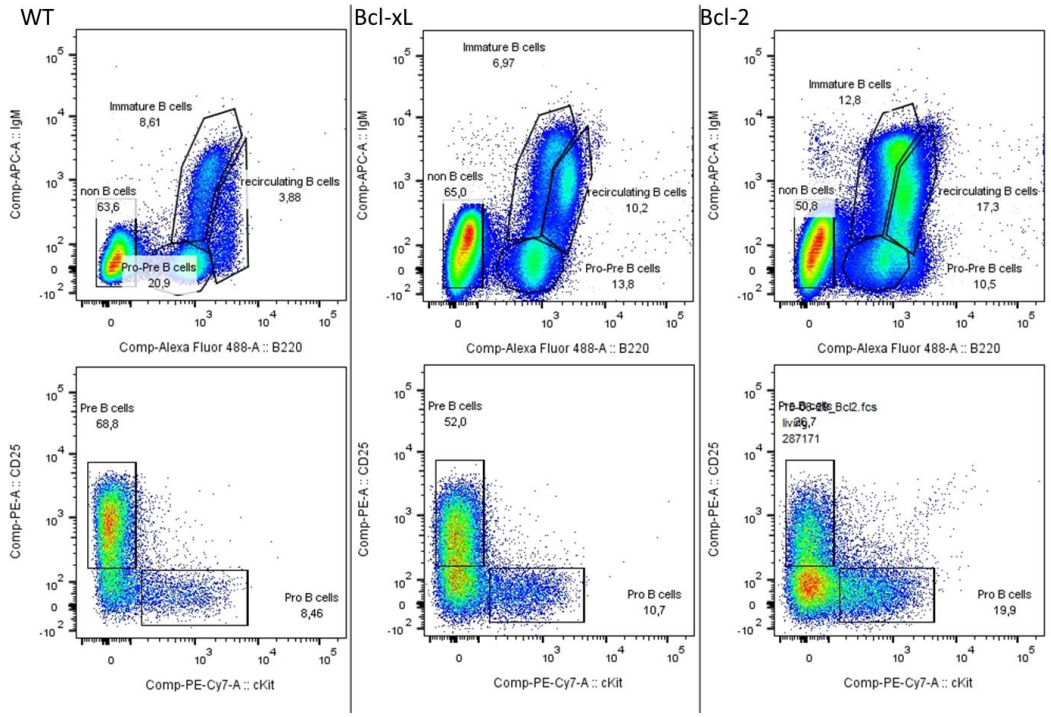

Figure 20 Representative FACS blot from B cells in bone marrow. WT, *Vav-Bcl-xL* and *Vav-Bcl-2* genotypes are compared. The first row represents the FACS strategy using IgM and B220 cell surface markers to discriminate between immature B cells, recirculating B cells and pro-pre B cells. Depending on the expression of CD25 and ckit pro-B, respectively pre-B cells, are differentiated from pro-pre B cells (2nd row). Immune cells were analyzed by flow cytometry.

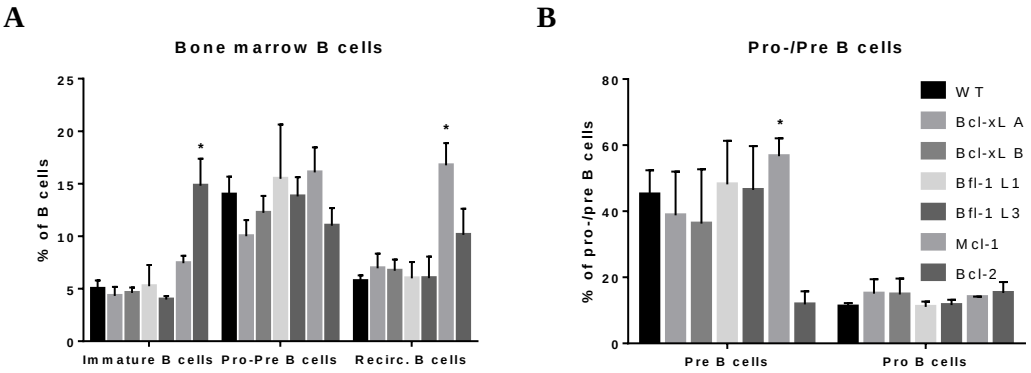

Figure 21 Distribution of B cell subsets in bone marrow under steady state conditions. Bone marrow was isolated from WT, Vav-Bcl-xl A, Vav-Bcl-xL B, Vav-Bfl-1 L1, Vav-Bfl-1 L3, Vav-Mcl-1 and Vav-Bcl-2 mice. Immune cell composition was analyzed by flow cytometry. Bars represent means ± s.e.m. *P< 0,05 (Two-way ANOVA); n = 4-10.

Evaluation of the anti-apoptotic potential of transgenic Bcl-xL and Bfl-1 protein

In different studies it was shown that the overexpression of pro-survival BCL-2 proteins in different cell types enhances their resistance against a broad range of death stimuli when compared to WT cells. Therefore, specific survival assays were set up to compare the protective effect of the different members of the anti-apoptotic BCL-2 proteins side by side.

Bcl-2 and Mcl-1 overexpression were already described to impose a survival benefit to lymphocytes upon cell death induction. My aim was to compare, weather Bcl-xL and Bfl-1 overexpression also display comparable effects. I tried to identify a hierarchy of potency between these proteins and evaluate if they are fully redundant in their function. As the Vav-gene promoter driving the transgenic expression is only active in the hematopoietic system, only cells isolated from primary or secondary lymphatics were chosen for these experiments.

Freshly isolated thymocytes from WT, *Vav-Bcl-xL* A, *Vav-Bcl-xL* B, *Vav-Bfl-1*, *Vav-Mcl-1* and *Vav-Bcl-2* tg mice were put into culture and treated with 100 nM staurosporine, 312.5 nM corticosterone, irradiated with 1,25 and 5 Gy, respectively, and survival was compared to untreated thymocytes over time. Cells were harvested after 4, 8, 12, 20, 30 or 48h, stained with Annexin V and 7-AAD and analyzed by flow cytometry. Cells were only considered alive when double-negative for both markers. Considering differences in spontaneous apoptosis (sA) between the genotypes, I referred to treatment-induced cell death (iA) using the formula: (iA-sA)/(100-sA)% for induced apoptosis.

Comparing protection of immature T cells overexpressing distinct anti-apoptotic Bcl-2 proteins

In former studies it was already reported that Bcl-2 overexpression *in vitro* protects cells from apoptosis[69]. In our experiment we were also able to show a prominent difference in survival for thymocytes isolated from *Vav-Bcl-2* transgenic mice compared to WT thymocytes. 50% of WT cells were AV^+7AAD^+ within 10h, whereas *Vav-Bcl-2* cells never reached 50% apoptotic rate within the 48h experiment. Thymocytes from the *Vav-Bcl-xL* lines #A and #B as well as the *Vav-Mcl-1* line showed moderate protection from spontaneous and drug-induced apoptosis, whereas Bfl-1 overexpression failed to protect from any of the stimuli used (Fig. 11 A-D).

Throughout the different treatments the same pattern of protection was observed. Bcl-2 overexpressing cells hardly seem to react to death stimuli and less than 30% of the cells were found to undergo specific apoptosis, even after 48h of stimulation. In contrast, *Vav-Bcl-xL* and *Vav-Mcl-1* transgenic cells resist cell death within the first 30h significantly, when compared to WT but ultimately died within 48h of incubation with staurosporine or corticosterone. Interestingly, DNA damage caused by irradiation was tolerated significantly better by *Vav-Bcl-xL* and *Vav-Mcl-1* overexpressing thymocytes

than WT thymocytes. About 70% of those transgenic cells were still alive after 48h. Notably, *Vav-Bfl-1* tg thymocytes did not show any protection from spontaneous or induced apoptosis when compared to WT cells.

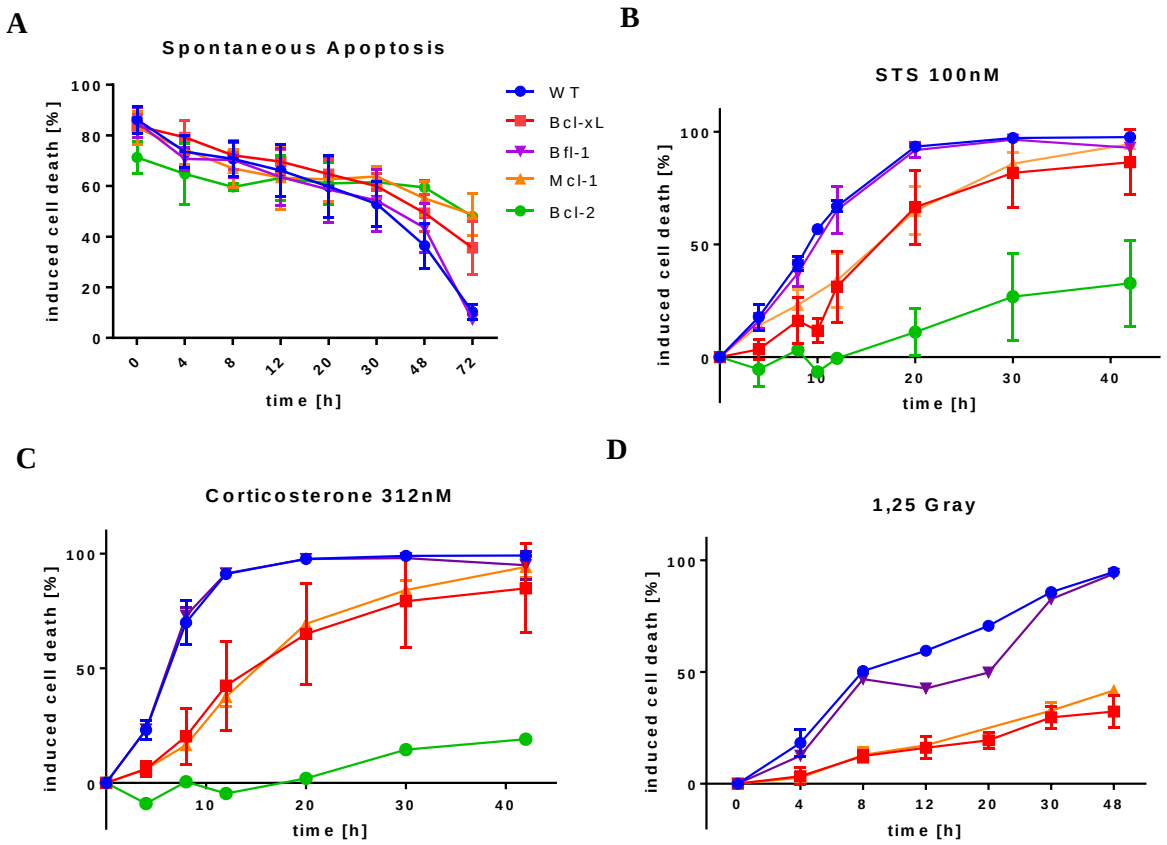

Figure 22 Thymocyte apoptosis upon different stimuli (A – spontaneous apoptosis in culture, B – 100nM Staurosporine, C – 312nM Corticosterone and D – radiation with 1,25 Gy). Thymocytes were isolated from WT, *Vav-Bcl-xl A*, *Vav-Bcl-xL B*, *Vav-Bfl-1 L1*, *Vav-Bfl-1 L3*, *Vav-Mcl-1* and *Vav-Bcl-2* mice. *Vav-Bcl-xL* and *Vav-Bfl-1* data of both lines was pooled. Survival of cells was analyzed by flow cytometry (7-AAD- Annexin V-). n = 2-6 mean ± SD (except for Bcl-2 treated with corticosterone and irradiated Bfl-1 – there we only had one animal each).

Evaluation of the anti-apoptotic potential of exogenous Bcl-xL and Bfl-1 in immature and mature B cells

To analyze the effects of transgene expression in developing B cells, we sorted the bone marrow for c-Kit^{+}CD25^{-} cells. We could not observe any protection of pre B cells overexpressing Bcl-xL respectively Bfl-1, neither in spontaneous apoptosis, nor when treated with staurosporine or irradiation. In contrast, Mcl-1 overexpression was significantly protecting pre B cells from apoptosis. No experiments were done for Bcl-2 overexpression in pre B cells, but a protective effect in *Vav-Bcl-2* mice was reported before[52].

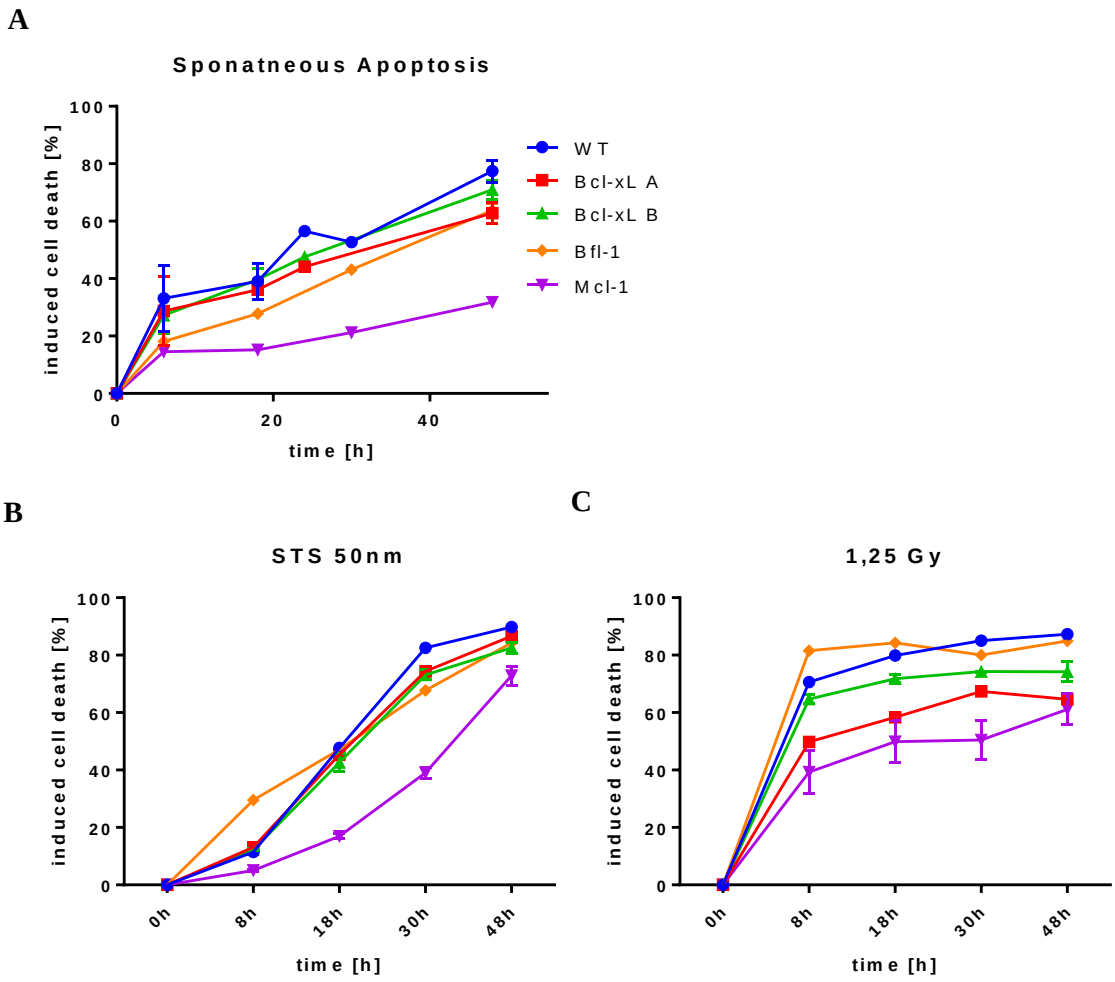

Figure 23 Pre B- cell apoptosis upon different stimuli (A – spontaneous apoptosis in culture, B – 50nM Staurosporine, C –and radiation with 1,25 Gy). Pre B cells were isolated from WT, *Vav-Bcl-xl A, Vav-Bcl-xL B, Vav-Bfl-1 L1, Vav-Bfl-1 L3, Vav-Mcl-1* and *Vav-Bcl-2* mice. *Vav-Bfl-1* data was pooled. Survival of cells was analyzed by flow cytometry (7-AAD- Annexin V-). n = 1-2 mean

Protection of mature T cells overexpressing anti-apoptotic proteins

After being able to show a survival benefit in thymocytes expressing Bcl-xL we went on to repeat the assay using mature T cells isolated from the periphery. Therefore, we sorted TCRß$^{+}$B220^{-} cells from the spleen and lymph nodes and challenged these cells also with 100nm staurosporine or irradiation (1,25 Gy). Transgenic T cells derived from *Vav-Bcl-2*, *Vav-Bcl-xL line A* or *line B* as well as *Vav-Mcl-1* showed prolonged survival under normal culture conditions compared to WT cells (spontaneous apoptosis fig.12 A). Bfl-1 overexpression, however, did not enhance the life span of these cells in culture. Upon stimulation with staurosporine or irradiation, cells overexpressing Bcl-2 seemed not to be driven into cell death at all, but also cells from the *Vav-Bcl-xL B* line displayed a strong survival benefit. Overexpression of Mcl-1 or Bcl-xL in line A provided minor protection, whereas Bfl-1 overexpression did not protect peripheral T cells from cell death (Fig. 12 B-C).

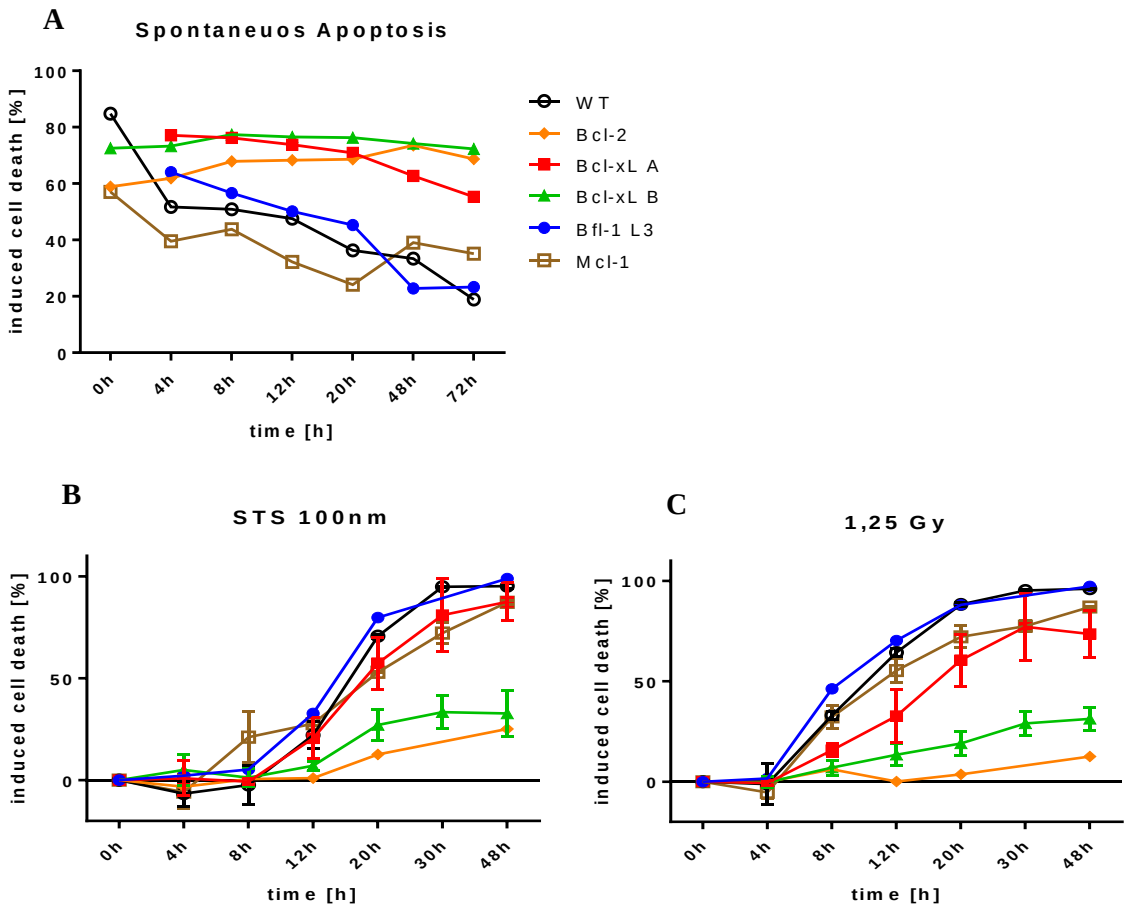

Figure 24 T- cell apoptosis upon different stimuli (A – spontaneous apoptosis in culture, B – 100nM Staurosporine, C –and radiation with 1,25 Gy). T cells were isolated from WT, *Vav-Bcl-xl A, Vav-Bcl-xL B, Vav-Bfl-1 L1, Vav-Bfl-1 L3, Vav-Mcl-1* and *Vav-Bcl-2* mice. *Vav-Bfl-1* data was pooled. Survival of cells was analyzed by flow cytometry (7-AAD-Annexin V-). n = 2-4 mean ± SD (except for Bcl-2 and Bfl-1 treated with STS and irradiation – there we only had one animal each).

Protection of peripheral B cells overexpressing anti-apoptotic proteins

Peripheral B cells from the spleen and the lymph nodes were sorted by selecting $B220^{+}$ $TCR\beta^{-}$ cells and challenged with 50 nM staurosporine or irradiation (1,25 Gy). The survival of the cells was compared to cells under normal culture conditions. *Vav-Bcl-2* overexpression in peripheral B cells showed the most prominent protection from drug-induced cell death but also from spontaneous apoptosis. Both *Vav-Bcl-xL* mouse lines differed in intensity of protection. Whereas line #B displayed a moderate effect in protection, line #A had only a slight benefit. *Vav-Bfl-1* and *Vav-Mcl-1* overexpression in peripheral B cells did not protect B cells from induced cell death.

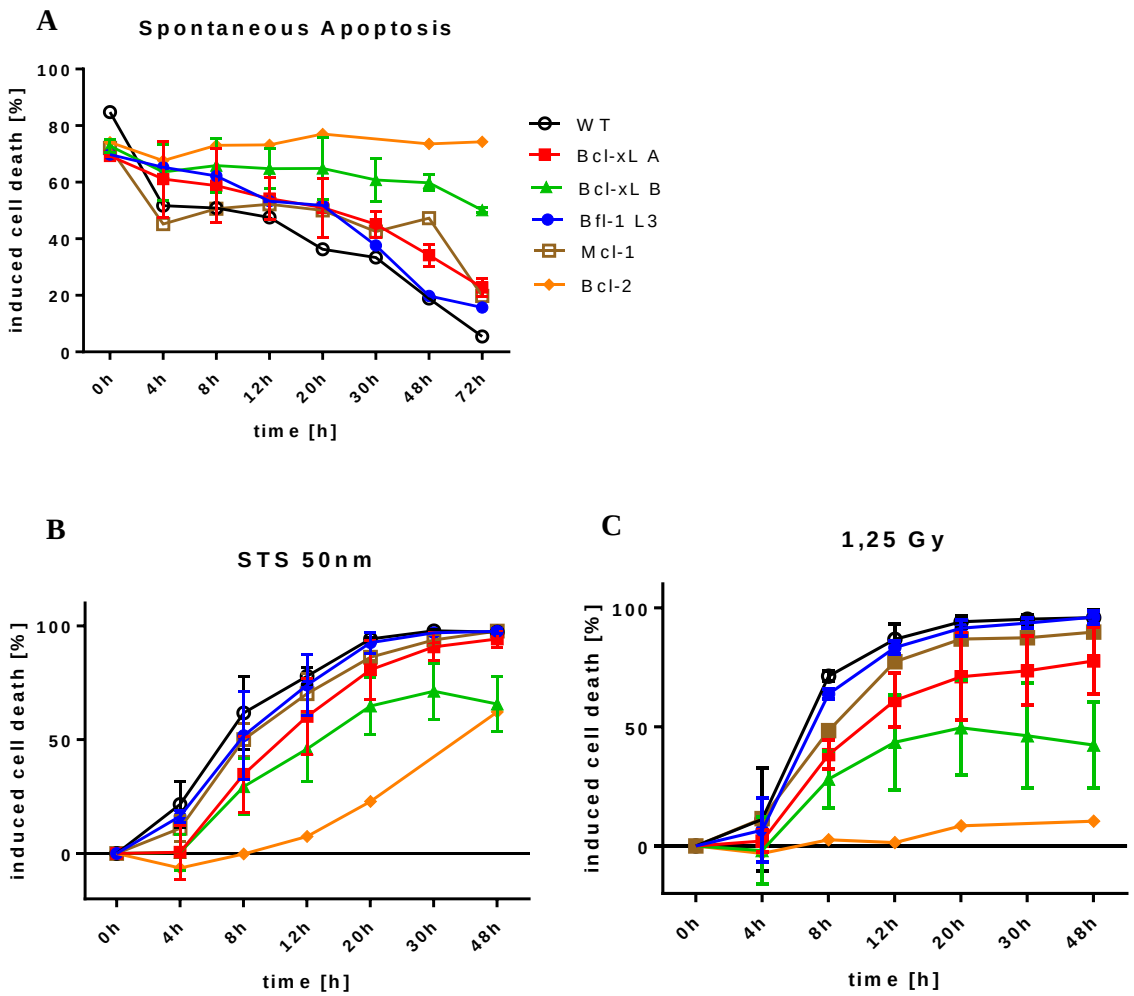

Figure 25 B- cell apoptosis upon different stimuli (A – spontaneous apoptosis in culture, B – 50nM Staurosporine, C – and radiation with 1,25 Gy). B cells were isolated from WT, *Vav-Bcl-xl A*, *Vav-Bcl-xL B*, *Vav-Bfl-1 L1*, *Vav-Bfl-1 L3*, *Vav-Mcl-1* and *Vav-Bcl-2* mice. *Vav-Bfl-1* data was pooled. Survival of cells was analyzed by flow cytometry (7-AAD-Annexin V-). n = 2-4 mean ± SD (except for Bcl-2 and Bfl-1 treated with STS and irradiation – there we only had one animal each).

Assessing BCL2-dependence in transgenic thymocytes using BH3-mimetics

The overexpression of *Vav-Bfl-1* had no effect on survival of cells or development of the immune system. Therefore, we questioned its functionality. BH3-mimetics are a group of small molecule inhibitors that target specific BCL-2 family proteins and inhibit their anti-apoptotic function. We used ABT-199 to inhibit Bcl-2, WEHI-539 to inhibit Bcl-xL and ABT-737 to inhibit both, Bcl-2 as well as Bcl-xL. None of the inhibitors target A1/Bfl-1[56]. Thymocytes were chosen for the experimental set up.

Vav-Bfl-1 derived thymocytes did not show a survival benefit when treated with ABT-199 or WEHI-539 (Fig.15 A and B). However, when treated with ABT-737, which inhibits both Bcl-2 and Bcl-xL a survival benefit was observed (Fig.15 C). This indicates that the *Bfl-1* transgene is expressed in its functional structure and is able to protect thymocytes from apoptosis when Bcl-2 and Bcl-xL protein

are blocked. As predicted, cells overexpressing Bcl-2 were susceptible to ABT199 or ABT-737, but protected from WEI539 that only targets Bcl-xL. Cells expressing Mcl-1 were least responsive to these compounds, as predicted based on their affinities.

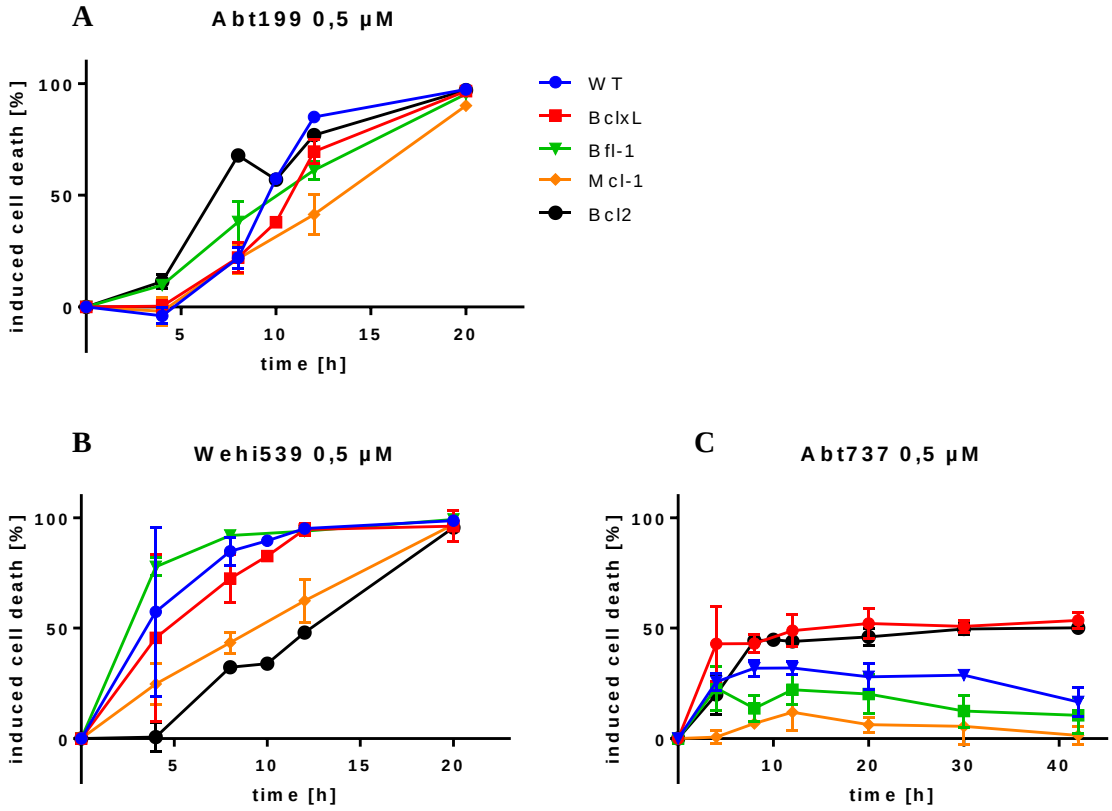

Figure 26 Thymocyte apoptosis upon different stimuli (A – ABT-199 0,5µM, B – WEHI-539 0,5µM, C – ABT-737 0,5µM). Thymocytes were isolated from WT, *Vav-Bcl-xl A, Vav-Bcl-xL B, Vav-Bfl-1 L1, Vav-Bfl-1 L3, Vav-Mcl-1* and *Vav-Bcl-2* mice. *Vav-Bcl-xL* and *Vav-Bfl-1* data of each 2 lines was pooled. Survival of cells was analyzed by flow cytometry (7-AAD- Annexin V-). n = 2-4 ± SD

Evaluation of transgene expression: *Vav-Bcl-2* to *Vav-Bcl-xL* mice

Due to the FLAG sequence linked to the transgenic *Vav-Bcl-xL* respectively *Vav-Mcl-1* sequence, the expressed amounts of these transgenes can be directly compared by FLAG immunoblotting. However, *Vav-Bcl-2* mice do not have a FLAG tag attached to their transgenic Bcl-2. As Bcl-2 overexpression showed the strongest effect throughout all experiments, we wondered whether Bcl-2 is more important than the other anti-apoptotic proteins or if there is simply higher transgene expression. Using two HEK239T cell lines transiently transfected with and therefore expressing HA-tagged Bcl-2 or HA-tagged Bcl-xL protein it was possible to compare the relative protein amounts. The HA signal of HEK239T cells expressing HA-Bcl-xL was about three times stronger than that of cells expressing HA-Bcl-2 as the HA signal of the 1/10 HA-Bcl2 was about as strong as the one of the 1/100 HA-Bcl-

xl (Fig.29 red boxes). In other words, one needs ten times the amount of HA-Bcl2 expressing HEK293T cells to get equal amounts of HA-Bcl2 in comparison to HA-Bcl-xl. Looking at the antibody signals of Bcl-xL and Bcl-2 (Fig. 29 blue boxes) it was clear that the thymus lysate from the *Vav-Bcl-2* mouse showed by far a stronger signal than the highest concentration of HEK239T cells expressing Bcl-2 (Fig.29 1st row). On the other hand, the signal of thymus lysates from *Vav-Bcl-xL* mice was as bright as the concentration 1/30 to 1/100 dilution of HEK239T cells expressing Bcl-xL (Fig. 29 2nd row). Although, the ratio between *HA-Bcl2* and *Vav-Bcl-2* would be a bit less striking if the HA-Bcl2 signal would be even ten times stronger, the transgene expression in *vav-Bcl2* mice is still distinctly stronger. Therefore, *Vav-Bcl-2* mice express considerably more transgenic protein than *Vav-Bcl-xL* mice.

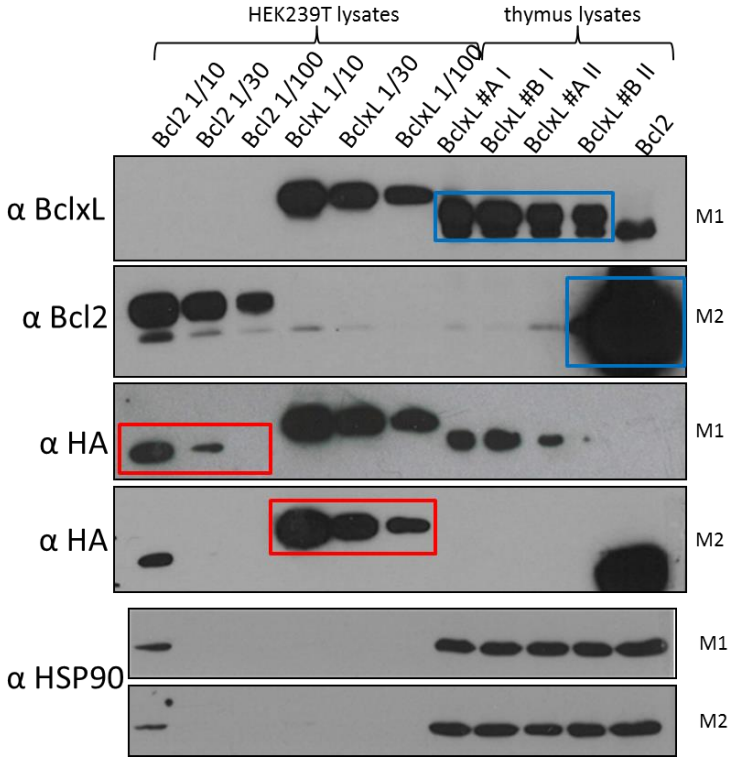

Figure 27 Western blot analysis was performed on protein lysates from whole V*av-Bcl-2* and *Vav-Bcl-xL* mouse thymus and HEK239T cell lysates expressing Bcl-2 respectively Bcl-xL on a 13% polyacrylamide gel. Bcl-2 antibody binds plasmid expressed HA-Bcl-2 as well as endogenous and exogenous Bcl-2 in thymus lysates, as well as Bcl-xL antibody (exogenous HA-Bcl-xL (upper band) and endogenous Bcl-xL (lower band)). HA antibody binds to exogenous HA-protein construct. HSP90 antibody served as control for protein expression. M1 –Membrane 1; M2 – Membrane 2.

Enhanced immune response in Bcl-xL overexpressing mice

Since Bcl-xL has been described to be an important player in activated T and B cell survival[70], it was of interest to investigate the impact of its overexpression upon antigen-driven T cell dependent B cell activation.

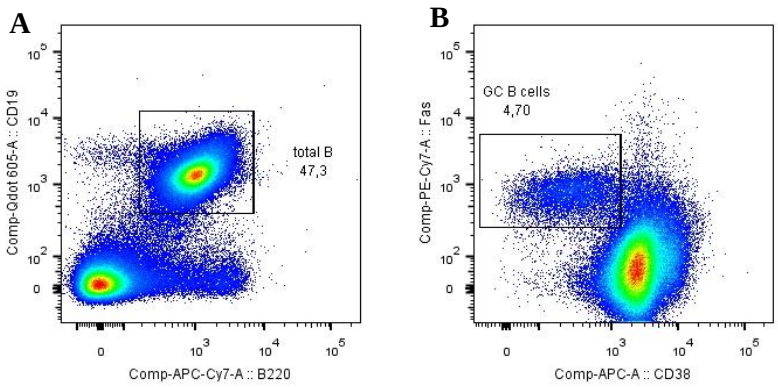

Figure 28 Gating strategy for total B cells in spleen, germinal center (GC) B cells and cell subtypes within GC B cells. (A) B220$^+$ and CD19$^+$ cells represent B cells. (B) Within the B cell compartment, GC B cells are found Fas$^+$ CD38^{low}. Splenic cells were analyzed by flow cytometry.

WT and *Vav-Bcl-xL* #A and #B mice were immunized with *i.v.* injection of sheep red blood cells (SRBC), causing an immune response in mice. 7 days after immunization mice were sacrificed and analyzed for germinal center (GC) reaction.

B220 and CD19 expression was used in flow cytometry to identify B cells in the spleen. Within the B cell compartment (B220$^+$CD19$^+$ cells), GC B cells were identified by expressing Fas/CD95, but low CD38 expression (Fig. 27). Mice overexpressing anti-apoptotic Bcl-xL protein showed enhanced in GC formation, meaning that due to the overexpression of Bcl-xL the GC cells show prolonged survival. In WT mice about 2,5% of total B cells were GC B cells, whereas in *Vav-Bcl-xL* #A mice about 6,6% and in *Vav-Bcl-xL* #B mice about 4,4% represented this specific cell type. In the non-immunized mouse only 0,6% of B cells were GC B cells. In inguinal and mesenteric lymph nodes also an elevated immune response was observed upon Bcl-xL overexpression. Again mouse line #A showed a stronger effect (Fig. 28 A) than line #B. Western blot analysis of the spleens was performed to evaluate Bcl-xL transgene expression. Line #A exhibited higher Bcl-xL transgene expression that line #B (Fig. 28 B).

Due to limited time, this experiment could not be repeated for other genotypes.

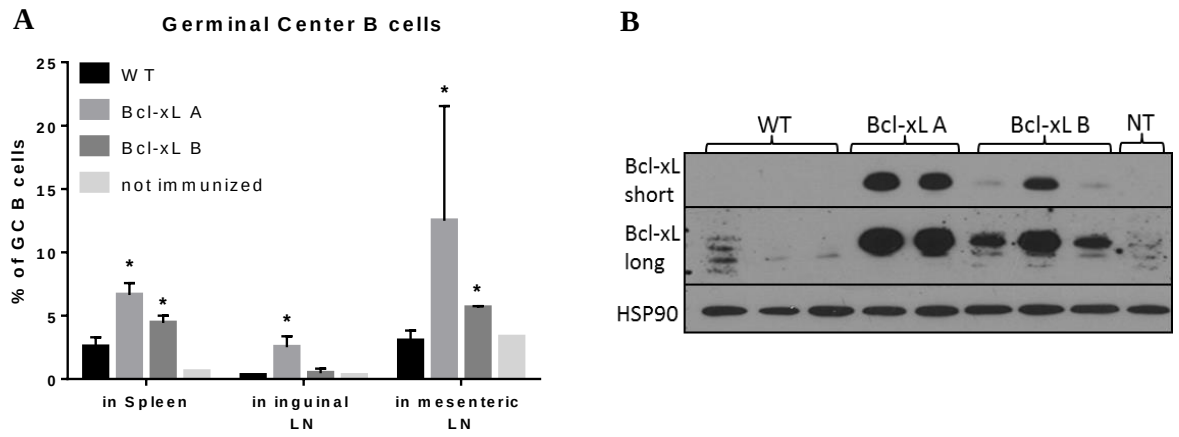

Figure 29 (A) Germinal center B cells within the total B cell compartment of the spleen (cell surface markers $B220^{+}CD19^{+}Fas^{+}CD38l^{ow}$) upon immunization with SRBC. GC formation was observed between WT, *Vav-Bcl-xL #A*, *Vav-Bcl-xL #B* and one non-immunized mouse. (B) Western blot analysis on whole spleen lysates of immunized mice and one non-immunized mouse on a 13% polyacrylamide gel. Bcl-xL antibody recognizes both exogenous (upper band) and endogenous (lower band) Bcl-xL. Bcl-xL "short" respectively "long" indicating the length of exposure. Bars represent means ± SD *P< 0,05 (unpaired Student t test); n = 2-3.

Discussion

The anti-apoptotic proteins Bcl-2, Bcl-xL, Mcl-1, Bcl-w and A1/Bfl-1 were already studied by different research groups. Yet many questions are left unanswered. Conditional and constitutive knockout mouse models helped to clarify the importance of individual proteins in immune cell development, homeostasis and survival. Interestingly, their importance does not always correlate with their differential expression levels during immune cell development [17]. Some studies also suggest that all these proteins are equal in their function and it is simply a matter of expression level and stability if they can protect cells against a certain type of stress or damage[71]. However, overexpression studies within the whole hematopoietic system of mice were only done independently for Bcl-2 and Mcl-1[51,72].

During my master thesis, I compared mouse models overexpressing Bcl-2, Mcl-1, Bcl-xL and Bfl-1 under control of the Vav-promoter to gain deeper insight on their function in the immune system, how immune cells behave when Bcl-xL and Bfl-1 expression is enforced and if they are protected from apoptosis to the same extent as cells from *Vav-Bcl-2* or *Vav-Mcl-1* tg mice. My aim was to find out whether there is some kind of hierarchy or cell type specificity between the pro-survival proteins when present in excess or if they are redundant, and to contributing to the ongoing work of investigating the role of these proteins in healthy cells. Considering the recent approval of a BH3-mimetic drug by the FDA my work is contributing to a better understanding of the Bcl-2 family in normal tissue.

The expression of the transgenes needed to be confirmed before we started a comparative cell survival analysis. Performing western blot analysis on whole tissue lysates of organs of the immune system and on sorted cell subsets (e.g. B cells, T cells, pre B cells and granulocytes), we could confirm expression, but it did not show to be entirely congruent in both established *Vav-Bcl-xL* mouse lines. Across different experiments, we could observe that the strength of transgene expression often varied, not only between litters, but sometimes also between littermates, independent from age and gender. Interestingly, we could also see variation in transgene expression between different organs that might be related to changes in tissue specific promoter activity or because of random transgene insertion into the genome at a location that may differ in accessibility between organs or cell types. There was no such phenomenon reported from groups who established *Vav-Bcl-2* or *Vav-Mcl-1* mice[51,72]. A possible explanation would be that the transgenic constructs inserted into regions of DNA that vary in their epigenetic status. Such obstacles could be circumvented by targeted single-copy insertion of transgenes in a predefined gene locus, such as ROSA 26 for example[73].

Bfl-1 expression analysis was challenged because of very weak reactivity of the Bfl-1 antibody when used to probe whole organ lysates of spleen, lymph nodes and thymus or the overall rather poor

expression of the Bfl-1 transgene. Unfortunately, we could not confirm expression in isolated B cells, T cells, pre B cells or granulocytes due to poor quality of the antibody. An alternative explanation might be that Bfl-1 is known to be very unstable and may be rapidly turned over. Lang et al. already reported difficulties with commercially available antibodies targeting human Bfl-1, because they found that these antibodies do not detect endogenous protein. They managed to generate an A1-specific antibody, recognizing mouse protein, but this antibody unfortunately does not recognize endogenous levels of human Bfl-1[74]. We confirmed mRNA expression, but the Bfl-1 mRNA expression data may not correlate well with the Bfl-1 protein levels, since A1 and Bfl-1 protein is regulated by ubiquitin-dependent proteasomal degradation[74]. Given the limited amount of protein we isolated from B cells, T cells, pre B cells and granulocytes and the very weak antibody signal we obtained from whole organ lysates, we assumed that protein expression in these immune cell subsets of our *Vav-Bfl-1* mice was present but overall very low. This assumption was supported by the findings made in different mouse lines that showed mRNA levels correlating with protein expression. Although, Bfl-1 was supposedly tagged with a FLAG sequence at its N terminus, we could also not obtain a detectable signal using anti-FLAG antibodies. This suggested that the protein was highly labile or that translation might preferentially start at an internal ATG, excluding translation of the FLAG sequence.

In order to identify the role of BCL2-like proteins in immune cell development we investigated immune cell distribution within different organs.

It was reported that Bcl-xL and Mcl-1 play an important role in DP thymocytes[38]. Despite this knowledge we did not expect to observe an impact of Bcl-xL overexpression on the thymus composition, since DP thymocytes make up around 90% of thymocytes[75] – a fact that does not leave much space for further accumulation of these cells, even when important effectors push towards this stage. Our results on Bcl-2 overexpression in thymocytes confirmed what Ogilvy et al. already described in 1999[51]; namely altered T cell development in the thymus evident by decreased intermediate stage (DP) and elevation of cells at the very immature (DN) and most mature stages (SP4 and SP8). A1/Bfl-1 expression was reported to be increased upon pre-TCR signaling[76]. However, our data did not show aberrations of the composition of the thymus in steady state in the DN subset, when Bfl-1 was overexpressed.

Splenomegaly in mice upon Bcl-2 and Mcl-1 protein expression was first described by Ogilvy et al. respectively Campbell et al.[51,72]. This is due to accumulation of B and T cells that are protected by the overexpression of the anti-apoptotic proteins leading to increased numbers of lymphoid follicles, and expanded germinal centers. Given the assumed survival benefit of B and T cells also upon Bcl-xL[44] and Bfl-1 overexpression we expected to obtain a similar phenotype for our *Vav-Bcl-xL* and *Vav-Bfl-1* mice. Interestingly, the cellularity of the spleen in these mice was normal. Probably the expression of

these transgenes is not sufficient to prolong survival of peripheral B and T cells or - in the case of Bfl-1 – might be due to rather poor expression of the transgene. Regarding the distribution of lymphocytes and myeloid cells (e.g. macrophages and granulocytes) in the spleen, none of the genotypes caused aberrations. This matches the findings Ogilvy et.al made in Vav-Bcl-2 mice.

The high induction of A1/ Bfl-1 upon T cell activation[77] made us wonder if the phenotype of mature T cells (i.e. naïve vs. memory-like) in the spleen would change upon its overexpression. Our data did not support this hypothesis. Only Bcl-2 overexpression led to a reduction of naïve CD4 and CD8 T cells and thereby to an accumulation of $CD8^+$ central and effector memory T cells and $CD4^+$ effector memory T cells. This again confirmed already described phenotypes of Bcl-2 overexpressing mice[36]. Also, in the B cell compartment only the overexpression of Bcl-2 caused a major phenotype, which is reflected in the accumulation of total B cells.

Surprisingly, when we further investigated the different B cell compartments in the spleen of *Vav-Bcl-2* tg mice, we observed a reduction in mature B cells, as well as in transitional B cells. They also lost nearly all B cells of the marginal zone[78] and T1 B cells. I did not expect the loss of mature B cells, since Nakayama et al. reported the importance of Bcl-2 in the lymphoid system[79]. In lymph nodes, no changes upon anti-apoptotic protein overexpression were detected. From literature we know that Bcl-xL depletion in chimeric mice did not affect the lymph nodes[44]. In Mcl-1 overexpressing mice we found a reduction of marginal zone B cells.

Consistent with the first observations in *Vav-Bcl-2* mice, we found an increase in B cells within the bone marrow of the mice[51]. Further investigating this compartment, we found that this was due to an accumulation of immature B cells. Also in *Vav-Mcl-1* mice we were able to confirm the already described phenotype[72] namely an increase in recirculating B cells. Within the bone marrow we could not find changes in immune cell composition for *Vav-Bcl-xL* and *Vav-Bfl-1* mice. Since Motoyama et al. reported cell death of immature hematopoietic cells in Bcl-xL deficient mice[43] – we expected an elevation of these cells upon overexpression. However, this hypothesis could not be confirmed. Also enforced Bfl-1 expression left the bone marrow compartments unchanged.

It has already been shown that enforced Bcl-xL expression protects thymocytes from glucocorticoids and γ-irradiation induced apoptosis. This was studied using Bcl-xL expression under the *lck* promoter by Chao et al. *Vav-Bcl-2* and *Vav-Mcl-1* mice were described to enhance survival capacity of all lymphoid cells[53]. Therefore, we were keen to compare the impact of mice overexpressing Bcl-xL and Bfl-1 also throughout the hematopoietic linage.

Thymocytes from *Vav-Bcl-2*, *Vav-Mcl-1* and *Vav-Bcl-xL* mice displayed a benefit in survival compared to WT and *Vav-Bfl-1* mice when challenged *in vitro* with different insults. Upon staurosporine and corticosterone stimulation thymocytes from *Vav-Bcl-2* mice were most resistant, but

also Bcl-xL and Mcl-1 elevation lead to a significant protection of thymocytes. Protein expression analysis of the FLAG-tag that is fused to both transgenes showed similar amounts of expressed Bcl-xL and Mcl-1 exogenous protein, explaining the same degree of protection. The strongest apoptotic protection in *Vav-Mcl-1* and *Vav-Bcl-xL* thymocytes was observed when cells were γ-irradiated, which causes DNA damage (Please note that Vav-Bcl-2 tg thymocytes were not included in irradiation experiments).

Mature B and T cells, as well as immature B cells from the bone marrow, did not show to be protected in the same extend as thymocytes upon anti-apoptotic protein overexpression in our experiments. Mature T cells from *Vav-Bcl-2* and *Vav-Bcl-xL* #B mice were strongly protected from staurosporine and irradiation, whereas the other transgenic cells only had a small (*Vav-Bcl-xL*#A and *Vav-Mcl-1*) or no survival benefit (*Vav-Bfl-1*). This was especially interesting, since *Vav-BclxL* tg thymocytes from both lines had only moderate benefits when compared to those with Bcl-2 overexpression. The observed differences in T cell survival between the two *Vav-Bcl-xL* tg mouse lines can be explained by the stronger expression of the transgene in T cells from Vav-Bcl-xL #B mice (not visible in Fig. 7 – but throughout the time of analyses we found differences in expression within the T cell compartment of the two mouse lines). Immature and mature B cells were highly protected when Bcl-2 was overexpressed, which aligned with findings reported in the literature[72]. Interestingly, Bcl-xL seemed to show more protection in mature B cells and Mcl-1 was more potent in protecting pre B cells from induced apoptosis.

Although Chuang et al. reported a perturbation of B cell development in mice overexpressing A1-a (Eμ-A1 mice) and also extended survival of thymocytes and early B cells[54], we could not detect any effect in our *Vav-Bfl-1* mice. Therefore, we tested the functionality of the transgene.

Throughout all cell death assays and immune cell composition analysis, we started to wonder if this is because Bcl-2 itself is much more potent than its relatives, or because *Vav-Bcl-2* mice simply express more transgenic protein than the other strains of mice. We aimed to compare the protein amounts of the expressed transgene in total thymus lysates from *Vav-Bcl-2* and *Vav-Bcl-xL* mice (see Fig. 29). Using a comparative method, we were able to show that by far more *Bcl-2* protein is expressed in *Vav-Bcl2*-transgenic mice than Bcl-xL in the *Vav-Bcl-xL* strain. Therefore, we propose that the higher protein expression causes the stronger protection in cell survival and immune cell composition changes. However, it is still possible that the overexpressed proteins are neutralized by increased expression of BH3 only proteins and the higher expression of Vav-Bcl-2 is not relevant. This would imply that there is a hierarchy between the anti-apoptotic proteins. These questions still remain to be addressed in more detail.

Also the amount of Bfl-1 protein compared to the other transgene could not be addressed in *Vav-Bfl-1* mice. The overall low levels may not suffice to protect lymphocytes from the apoptosis-inducers used.

Using ABT inhibitors as apoptotic insults, we challenged thymocytes derived from transgenic mice. ABT inhibitors are novel BH3-mimetics, which are able to bind and inhibit individual or multiple BCL2-like family proteins[56]. Cells overexpressing a pro-survival Bcl-2 family member, which is targeted by an ABT inhibitor, die even faster than normal cells when treated with this inhibitor that is neutralizing their survival advantage. Strikingly, upon ABT737 treatment, which targets Bcl-2, Bcl-xL and Bcl-w, Bfl-1 overexpressing thymocytes showed a clear protection. This finally confirmed Bfl-1 functionality. However, the question if there was too little Bfl-1 expression in transgenic mice to offer survival benefit effects in response to other insults or if the protein is only active when most of its family relatives are inhibited remains unanswered.

It was shown, that once B cells are mature, they no longer depend on Bcl-xL but on Bcl-2 and Mcl-1[51]. Conditional removal of Bcl-xL in germinal centers only had minimal impact on GC or memory B cell formation; compared to the importance Mcl-1 has in this cell subset[80]. However, it is still interesting to see that the survival benefit of *Vav-Bcl-xL* tg T and B cells we observed *in vitro* had also an influence on lymphocyte function *in vivo*, particularly as we did not note a significant increase in B cell number. Our data showed that both *Vav-Bcl-xL* lines displayed an elevation of germinal center formation upon immunization, being significantly stronger in *Vav-Bcl-xL* #A mice. This effect was also correlating with a higher transgene expression in the immunized *Vav-Bcl-xL* #A mice (Fig. 28 B). Taken together, our data suggests a major role of Bcl-xL in GC formation. Interestingly, Takahashi et al. showed that Bcl-xL overexpression does not lead to more GC B cells in their mouse models[81], which only expressed the transgene in B cells[82]. This indicates that the effect we have seen might well be T cell dependent. It would be of interest to repeat this experiment with Vav-Bfl-1 transgenic mice.

Overall, my analyses suggest that expression level is the key for the protection of different lymphocyte subsets from apoptotic cell death. This is based largely on the direct comparison of cells expressing comparable levels of Bcl-xL or Mcl-1. Bfl-1 may either be a poor cell death inhibitor or the expression levels achieved were simply not sufficient to provide protection to other apoptosis inducers, next the ABT737. Bcl-2 overexpression, at first sight, seems to be most potent in protecting lymphocytes from stress-induced apoptosis, but based on our semi-quantitative comparison of protein levels, *Vav-Bcl2* transgenic mice appear to express the highest transgene levels, correlating with superior protection. In essence this supports the idea that expression levels dictate biological response that relative differences in binding affinities for cell death inducing Bcl-2 proteins can be overcome by increasing expression of their inhibitors.

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List of abbreviations

7-AAD: 7-amonoactomycin D

ANOVA: analysis of variance

APAF-1: apoptotic protease activating factor 1

BAK: BCL2-antagonist/killer 1

BAX: Bcl2-associated X protein

BH-domain: Bcl-2 homology domain

Bcl-2: B-cell leukemia/lymphoma 2

Bcl-w: BCL2-like protein 2

Bcl-xL: BCL2-like 1 isoform 1

Bfl-1 (A1): BCL2-related protein A1 (murine form)

d: days

caspase: cysteine aspartate protease

CLL: chronic lymphocytic leukemia

DISC: death inducing signaling complex

DNA: Deoxyribonucleotid acid

FACS: fluorescent activated cell sorting

FAS: TNF receptor superfamily, member 6

FCS: fetal calf serum

FDA: Food and Drug Administration

FITC: Fluorescein isothiocyanate

GC: germinal center

GC: Glucocorticoid

h: hours

i.v.: intra venous

Ig: Immunoglobulin

KD: knock-down

KO: knockout

Mcl-1: Induced myeloid leukemia cell differentiation protein

min: minutes

MOMP: Mitochondrial outer membrane permeabilization

ms: mouse

n: number of performed experiments

PE: phycoerithrin

PS: phosphatidylserine

rb: rabbit

tg: transgene/transgenic

WT: Wild-type