Conditional Expression of Pim1 and c-Myc in Murine B Lineage Cells and their Functional Consequences

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1 Abstract

This thesis was aimed at identifying proto-oncogenes that contribute to cell cycle entry, proliferation and survival of mouse B-lymphocytes at different stages of their development, i.e. in preB-I, preB-II, immature B-cells, mature resting and mature activated B-cells.

A central notion of tumour development of the B-lymphocyte cell lineages is that the activation of a single proto-oncogene, or the loss of a single tumour suppressor gene, is not sufficient to transform cells to full malignancy. One such example with which this cooperation of oncogenes has been studied is Eμ-*myc* induced lymphomagenesis. Transgenic Eμ-*myc* mice express the *c-myc* gene under the control of the Ig_H-chain gene enhancer in B-lineage cells. B-cell lymphomas are generated in these mice within several weeks. Retroviral insertional mutagenesis has identified *pim1* as cooperating oncogene which accelerates lymphomagenesis *in vivo*.

Hence, I have chosen *myc* and *pim1* to introduce them into B-cell progenitors by retroviral transduction, to test their effects alone or together on preB-I, preB-II, immature and mature B-cells *in vitro* and, upon transplantation into recipient mice, *in vivo*. As target cells for retroviral transduction, mouse fetal liver-derived preB-I-cells were chosen, since they proliferate long term *in vitro* on stromal cells in the presence of IL7.

I used inducible forms of these genes to turn on and off their expression in the transfected cells at different times for different time periods. After initial tests with the inducible estrogen-receptor system, the TetON inducible expression system was chosen which allows the expression of inducible transgenes upon addition of doxycycline.

In the here presented work show that overexpression of Myc alone in pre-BI cells *in vitro* enhances cell cycle entry without impairing apoptotis of pre-BI cells induced by the removal of IL-7 from the culture. Pim1 overexpression alone did not show any effect. However, in cooperation with Myc, Pim1 led to growth-factor-independent long term *in vitro* proliferation of pre-BI cells deprived of IL-7. This induction of proliferation could be reversed when doxycycline was removed again from the culture. During long term proliferation of these cells, differentiation to pre-BII and immature B-cells was slowed down but not completely blocked *in vitro*. The differentiated pre-BII and IgM^+ immature B-cells also were induced to growth factor-independent proliferation by Pim1 plus Myc.

Transplantation of Myc-overexpressing pre-BI cells into sublethally irradiated Rag KO mice did not increase the numbers of B cells developing in the transplanted mice compared with mice transplanted with doxycycline-non-induced pre-BI cells. On the other hand, transplantation of pre-BI cells overexpressing Pim1 and Myc together expanded the immature IgM^- (pre-B) cell compartment 100 fold and the immature IgM^+ B-cell compartment 6 fold in the spleen within 1 month. Upon removal of doxycycline from the drinking water, the expanded numbers of B-cells reverted to almost normal levels, i.e. to the levels of B cell numbers of doxycycline-uninduced mice. Hence, overexpression of the proto-oncogenes Pim1 and Myc is able to induce growth-factor-independent proliferation of pre-B cells and immature IgM^+ cells, *in vitro* and *in vivo*.

By contrast, mature B cells overexpressing Myc and Pim1 together, isolated from the spleens of pre-BI cell-transplanted mice, were not induced to cytokine-independent proliferation *in vitro*. Even the stimulation by LPS, IL-5, IL-4 + αCD40, and anti-Ig *in vitro* did not induce prolonged proliferation beyond the normal stimulation observed with non-transgenic, or with transgenic B cells in the absence of doxycycline. Likewise, *in vivo* antigenic stimulation with KLH of mice transplanted with Myc and Pim1-overexpressing pre-BI cells in the presence or absence of co-transplanted T-cells did not result in expanded, KLH-specific, class-switched antibody responses. Hence, while pre-BI, pre-BII and immature

B cells expand cytokine-independently *in vitro* and *in vivo* when Pim1 and Myc are overexpressed together, mature B cells generated from these transplanted pre-B cells *in vivo* do not manifest deregulated proliferation or survival *in vitro* or *in vivo* upon T-cell-independent or T-cell-dependent stimulation.

Abbreviations

AP	alkaline phosphatase
BCR	B-cell receptor
BM	bone marrow
EtOH	ethanol
FACS	fluorescence activated cell separation
FCS	fetal calf serum
FL	fetal liver
Flt3L	Fms-related tyrosine kinase 3 ligand
h	hours
IL	interleukin
IRES	internal ribosome entry site
KLH	keyhole limpet hemocyanin
KO	knockout
LPS	lipopolysaccharide
MACS	magnetic activated cell separation
MoMulV	Moloney murine leukemia virus
MPI-IB	Max-Planck Institute for Infection Biology
Myc, *myc*	C-Myc, V-Myc myelocytomatosis viral oncogene homolog, *gene*
MZ, MZB	marginal zone B cells
ON	over night
PC	peritoneal cavity
PI	propidium iodide
Pim1, *pim1*	proviral integration site 1 protein, *gene*
RT	room temperature
RT-PCR	real time PCR
SIN	self inactivating (retroviral vector)
T_H	T helper cell
TD	T-dependent (antigen)
TdT	terminal desoxyribonucleotidyl transferase
TI	T-independent (antigen)
TLR	toll-like receptor
TSLP	thymic stromal lymphopoietin

2 Introduction

2.1 B-Cells: Development and Function

B-cells and T-cells constitute their adaptive part of the vertebrate immune system. While T-cells confer cellular immunity, B-cells are responsible for humoral immunity by secreting antibodies which circulate in the body fluids to capture and neutralise foreign antigens.

2.1.1 B-Cell Development in the Mouse

The first wave of B-cells in the murine embryo is generated from primitive pluripotent hematopoietic stem cells (pHSCs) first aggregating at day 7.5-8 p.c. (*post coitum*) in the AGM (aorta-gonad-mesonephros), which lies between the notochord and the somatic mesoderm and contains the dorsal aorta, the genital ridges and mesonephros [106]. These pHSC-progenitors then migrate to the fetal liver around day 11 p.c.. Around birth, i.e. at day 18 p.c., B cell development reaches a maximum in fetal liver, and is initiated in the bone marrow, where B-cell development takes place during the rest of life from resident pHSCs.

Bone marrow-derived B cells differ from fetal liver-derived ones in several aspects [72]. Only bone marrow-derived antibodies have insertions of random bases (N regions) at the joints of the rearranged segments, since the enzyme TdT (terminal desoxyribonucleotidyl transferase) is expressed and active in the bone marrow, but not in the fetal liver [80].

Another difference between bone marrow and fetal liver preB-cells is the absolute requirement of bone marrow B-cell precursors for IL-7, whereas fetal liver preB-cells can develop IL-7-dependently, but do not rely on this cytokine only. Hence, in IL-7 deficient mice, B-cell development is only observed during fetal and perinatal life [15]. These IL-7 deficient mice only have B1- and marginal zone B-cells and show enlarged IgM and IgG-serum levels if T-cells are present. They grow in the absence of IL-7 if supplemented with IL-3 or thymic stromal lymphopoietin (TSLP) [113]. Hence, in *in vitro* culture, fetal liver pre-BI cells can be grown in the absence of IL-7 if supplemented with IL-3 or thymic stromal lymphopoietin (TSLP) [113].

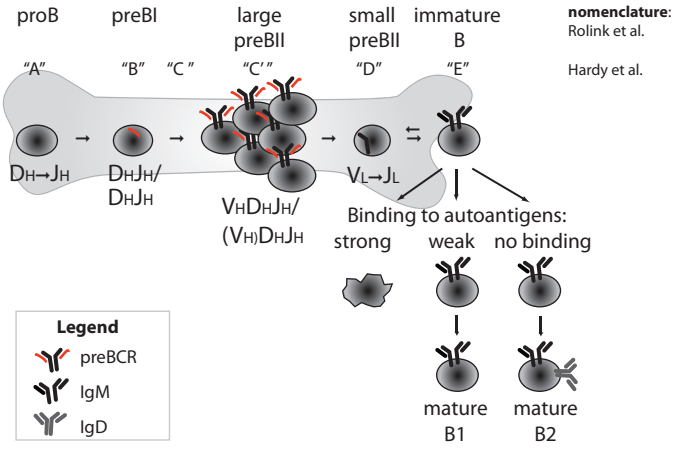

Figure 2.1 – Outline of the development of B lymphocytes in the bone marrow. Shown are two nomenclatures, the "Basel" separation system developed by Rolink et al., and the ABC nomenclature developed by Hardy et al., see the text for further details. Proliferation of preB-cells can mainly be observed in large pre-BII cells and to a lesser extent in pre-BI cells. All other immature stages do not divide. $D_H \rightarrow J_H$, etc: rearrangement of the designated segments is ongoing, $D_H J_H$, etc: rearrangement of the designated segments (V, D, and J) is finished. $_H$: heavy chain, $_L$: light chain.

Development of B cells can be tracked by their state of rearrangements of the V, D and J segments of the B-cell receptor, and by FACS analysis, since different stages of B cells express characterising surface receptors. Several different systems have been established to identify the different B cell maturation stages. Herein, the surface marker separation system developed by Rolink et al. [111] [113] is used. In figure 2.1, this system is compared to the Hardy ABC-system [49].

The earliest event which shows that a cell is committed to the B lineage is the transcription of the non-rearranged germline μ heavy chain gene locus, as well as the transcription of the V_{preB} and $\lambda 5$ genes, which are parts of the pre-B cell receptor [91]. These cells are $B220^+$ but still do not express the pan-B cell marker CD19 and are called pre/pro-B cells. Rearrangement of D_H segments to J_H segments of the Ig gene loci is already detectable at this stage.

The next distinguishable stage on the way to a mature B-cell has almost completely rearranged all alleles of the heavy chain to $D_H J_H$, and expresses c-kit and CD19 [90]. This stage is referred to as pre-BI stage.

V_H to DJ_H rearrangements mark the transition from pre-BI cells to large pre-BII cells. pre-BII cells are $CD19^+$c-$kit^-CD25^+CD43^+$. As soon as a productive VDJ_H-rearranged allele is made and the μ-heavy chain is deposited on the cell surface together with the surrogate light chain (μH + $V_{preB} + \lambda 5$, = preBCR, preB-cell receptor), allelic exclusion inhibits further rearrangement of the other allele. The deposition of the preBCR on the cell surface induces these cells to enter the cell cycle and divide around two to five times. Cells deficient for the preBCR do not proliferate [73]. As soon as the preBCR is formed, expression of the V_{preB} and $\lambda 5$ proteins stops [42] and subsequently, proliferation of pre-BII cells ceases.

The cells now become small (since they are resting) and start to rearrange the light chain gene segments V_L to J_L by activating the rearrangement machinery again and opening the light-chain gene loci for these rearrangements. They are now named small pre-BII. In contrast to humans, mice do not reactivate TdT anymore for rearranging the light chain. Hence, mouse VJ_L joints do not have N regions. Rearrangements of the κ and λ light chain loci are independent of each other, but the κ locus becomes first accessible, and the rate of rearrangement at the κ_L-chain locus is 5 times higher than at the λ_L locus. Only one allele of the κ locus is rearranged at a time [99].

When a functional light chain is produced which can pair with the heavy chain, the immature B-cell is tested for reactivity with self-antigens. If the produced B-cell receptor (BCR) can strongly bind to self-antigen, the respective immature B-cells can be rescued by "editing" their receptor by secondary light-chain rearrangements [110]. If receptor editing is not possible or if the secondary rearrangement does not change the autoreactivity, the cells acquire an unresponsive, anergic state or are clonally deleted [53]. Immature B-cells expressing a B-cell receptor which binds self-antigen with low affinity are thought to be positively selected and to enter the B1- and MZ (marginal zone) compartment. Immature B-cells carrying a BCR receptor which does not bind autoantigen enter mainly the B2 compartment.

2.1.2 B-cell Subsets and their Characterisation

B-cells can be divided into at least 2 lineages: B1 cells, and conventional (B2) B-cells [3].

B1 cells are IgM^{hi} IgD^{lo} $CD11b^+$ and comprise the majority of B-cells in the peritoneal cavity. The B1 compartment is further divided into B1-a and B1-b cells according to their expression of CD5. B1-a cells express CD5, whereas B1-b cells are CD5-negative. The B1-a cells are thought to be one main source of "natural antibodies" that are produced also in the absence of antigenic stimulations, e.g. infections. These natural antibodies recognise high molecular weight polymeric antigens and are thought to be important during the early response to encapsulated extracellular bacteria [44]. In contrast, B1-b cells produce

antibodies only after exposure to antigens. Activation of B1-b cells can be T-helper cell (T_H) independent and can generate long lasting IgM memory [4]. Until recently there was a debate if B1- and B2 cells are derived from the same ancestor, or if there are different progenitors for each lineage. It has recently been shown that B1 B-cells have their own progenitor among the $CD45R^{lo-neg}CD19^{+}$ population which is highly abundant in fetal bone marrow and less abundant in postnatal bone marrow [96]. Therefore, it is not very surprising that fetal liver pre-BI cells repopulate preferentially the B1 cell compartment, whereas bone marrow pre-BI cells more efficiently generate the B2 cell compartment [55], [50].

The B2 cell compartment is further subdivided into follicular B-cells and marginal zone B-cells (MZB). In rodents, the latter are mainly found in the marginal zone of the spleen which surrounds the B-cell follicles. MZB-cells are sessile [43] and express high levels of surface immunoglobulin M (sIgM) and CD21 (complement receptor 2) and low levels of sIgD. MZB-cells can respond more rapidly following exposure to antigen [86] and, together with the B1-cells, are largely responsible for rapid early T_H-cell-independent B-cell responses.

The follicular B-cells are $IgM^{+}IgD^{+}CD23^{+}CD21^{lo}$ and are found in the follicles of spleen and lymph nodes, but also circulate in the blood. They can react to T_H-cell dependent protein antigens, and organize themselves in germinal centers where they develop to somatically hypermutated, Ig-class switched long lived memory and plasma cells harbouring high affinity antibodies to the immunising antigen.

2.1.3 Activation of B Cells

B-cells can be activated either with or without the help of T-cells (TD, T-dependent or TI, T-independent), depending on the nature of the antigen and the receptors involved. T-independent antigens fall into two classes, which activate B-cells by two different mechanisms.

TI-1 antigens can activate B-cells independently of the BCR. This polyclonal activation can occur e.g. via the toll-like receptors (TLRs) present on all B-cells which recognise bacterial cell wall components, bacterial DNA, flagella and other bacterial components (figure 2.2). TI-1 antigens need to be present at high concentrations in order to polyclonally activate B-cells. An example of a TI-1 antigen often used in research is lipopolysaccharide (LPS).

TI-2 antigens on the other hand are highly repetitive molecules such as bacterial polysaccharides which can crosslink several BCRs and hereby activate the B-cells [10]. A typical example of TI-2 antigens used in research is anti-IgM coupled to dextran or sepharose beads.

Protein antigens without repetitive antigenic structures can only activate naive B-cells with the help of activated T helper cells (T_H). If a naive B-cell recognises a protein antigen with its BCRs, it internalises the antigen, processes it by proteolytic degradation, and presents the peptide fragments via MHC-II complexes on the cell surface. This preactivated B-cell is preferentially trapped in T-cell rich zones of secondary lymphoid tissues such as spleen and lymph nodes. T_H cells which have a T-cell receptor (TCR) specific for the MHCII-antigen complexes on the B-cell are subsequently activated by the B-cell. Thereafter, the T-cell expresses CD40-L [103] [104] and releases cytokines such as IL-2 or IL-4, which in turn further activate the B-cell via CD40 and corresponding cytokine receptors (figure 2.2). The TNF-family member CD40 plays a central role in T-cell dependent B-cell activation and germinal center formation [74] [39]. The interaction of activated T-cells with B-cells via CD40 can be mimicked with CD40-specific antibodies [115]. Finally, B-cells upregulate the expression of B7-1 and B7-2, which interact with CD28 or CTLA-4 on helper T-cells which either enhance (CD28) or inhibit (CTLA-4) the T-B collaboration.

Upon T-cell dependent activation of B-cells, there are several possible pathways a B-cell can take

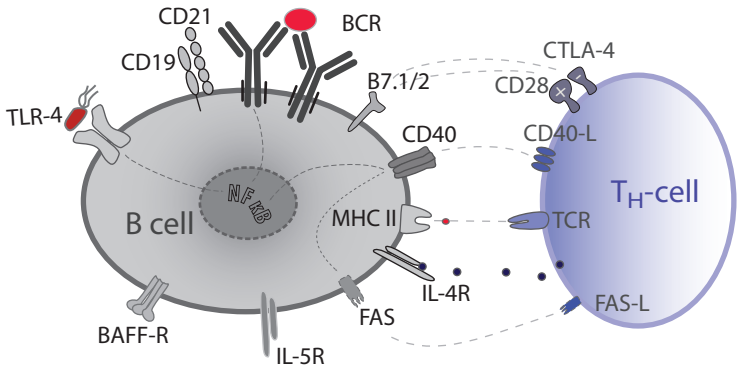

Figure 2.2 – B-cells can be activated and influenced via a set of different surface receptors. The main receptor is the B-cell receptor, which is surrounded by the coreceptor signalling complex consisting of Igα/β, CD19, CD81, and the complement receptor CD21. CD21 can bind the complement fragments C3d and C3g of antigen-complement clusters and leads to enhanced activation of B-cells [11]. Toll like receptors (TLR) recognise bacterial components and activate B-cells BCR-independently. Different cytokine receptors specific for IL-2, IL-4, IL-5, IL6, etc can influence the reactions of activated B-cells. Signalling via BaffR leads to enhanced survival, whereas signalling via FAS leads to apoptosis of activated B-cells. FAS is upregulated after B-cell activation in the germinal center. CD40 recognises CD40-ligand on activated T-cells. Signalling via CD40 costimulates and activates B-cells which were preactivated by signalling via BCR. B-cells in turn can activate T-cells by presenting antigen fragments on their MHC II molecules. Many different signalling pathways activate the NFκB transcription factors which are involved in activation and survival of B-cells.

(figure 2.3). First, activated B-cells can move to the border of the T-cell zone and the medulla in the spleen or to the medullary cords in the lymph nodes. Then they proliferate and subsequently differentiate into short lived plasma cells which secrete mainly IgM (figure 2.3).

Second, T-cell-activated B-cells can migrate to follicles in the B-cell zone and form a germinal center. In the germinal center, proliferating B-cells form the dark zone. These cells downregulate their sIg molecules and divide every 6-8 hours (centroblasts), whereas in the light zone of a germinal center, there is a mixture of mainly non-proliferating B-cells (centrocytes) deriving from the centroblast, T-cells and follicular dendritic cells (FDCs). The dark zone is thought to be the place where the B-cells begin to acquire somatic hypermutations in the variable regions of their antibody genes, while the light zone is the place where these mutated cells are selected for BCRs with enhanced affinities to the original antigen by interactions with FDCs which present a nondegraded, native form of the protein antigen to the B-cells, thereby allowing selection of hypermutated B-cells with specific BCRs for 3-dimensional antigen determinants on the protein antigen. Hypermutated B-cells with higher affinity to the antigen are positively selected and switch their antibody heavy chains to different isotypes. The mainly produced isotypes during a GC reaction are dependent on the cytokines which are present [125]. The cytokine milieu itself is dependent on the reactions of the cells of innate immunity. Here also, CD40 signalling is important for the survival and Ig isotype switching of germinal center B-cells [76] [82].

Finally, hypermutated, Ig-class-switched B-cells either become long lived plasma cells and migrate to niches in the bone marrow, or they differentiate into long-lived memory cells which are quickly reactivated upon re-encounter with the same antigen.

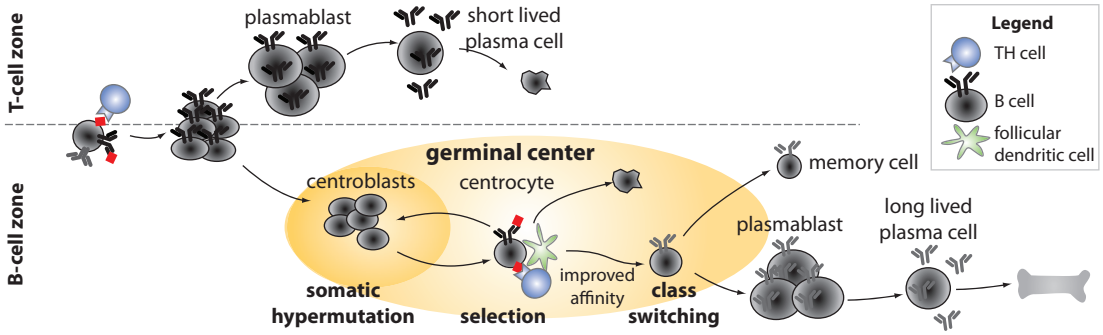

Figure 2.3 – T-cell dependent activation of B-cells in the spleen after antigen exposure.

2.1.4 Growth Factors and Stimulants for B-Cells

For the different maturation stages and subsets of B-cells, many growth factors and survival factors are known.

The main growth factor for preB-cells is interleukin 7 (IL-7). In IL-7 KO mice, B-cell development in the bone marrow is blocked precisely at the transition between pro-B-cells and pre-B-cells [137]. Vice versa, overexpression of IL-7 under the control of the MHCII promoter results in the expansion of the early immature B-cell compartment [37]. IL-3 has been shown to be able to replace IL-7 in the cultivation of fetal preB-cell lines, but in contrast to IL-7, it also induces differentiation of hematopoietic precursors to myeloid cell fates in mixed bone marrow cultures [139].

Some of the main growth factors for mature B-cells are IL-2, the T_H2-cytokines IL-3, IL-4, IL-5, and IL6. IL-2 is known to promote proliferation and differentiation of stimulated B-cells [140]. IL-3 can enhance growth of normal human B-cells and of human follicular B-cell lymphoma cell lines [20]. Human tonsillar B-cells activated with *Staphylococcus aureus* have a higher proliferation rate if IL-3 is present. IL-3 acts synergistically with IL-2, which promotes proliferation and differentiation of stimulated B-cells [140]. IL-3 also cooperates with IL6 in inducing differentiation and Ig secretion in human B-cells and -lines [128].

IL-4 prevents the death of naive B lymphocytes through the up-regulation of antiapoptotic proteins [30]. Furthermore, in B-cells activated by CD40- or BCR-engagement, IL-4 (together with IL-21) enhanced proliferation of purified mouse B-cells. On the other hand, IL-4 and IL-21 inhibited the proliferative response to LPS or CpG DNA [64]. IL-4 also induces AID-expression and therefore plays a role during class switching [71].

IL-5 promotes B1 cell growth and differentiation. In consequence, IL-5R$\alpha^{-/-}$mice have less than 30% of peritoneal B1 cells compared to wild type littermates. In contrast, cell numbers in spleen and lymph nodes are unaltered in these mice, as well as the B2 compartment sizes [56] [129]. In addition, IL-5 is needed for responses of B1 B-cells to stimulation with IL-4 and αCD40. In contrast, B2 cells mainly rely on IL-4 in combination with CD40-stimulation [66] [34].

IL6 is a known growth factor for plasmacytomas and hybridomas, and can induce maturation to plasma cells [78]. Furthermore, it has been shown to allow survival of murine bone marrow-derived plasma cells *in vitro* [16].

2.1.5 *In vitro* Culture of B-Cells

In vitro cultivation of B-cells is a helpful tool to examine their properties and functions. So far, the only murine B-cell stage which can easily be cultured by continuous proliferation *in vitro* is the pre-BI-cell stage. Fetal liver pre-BI cells can be grown for several months if supplemented with bone marrow stromal cells and the cytokine IL-7 [112]. The pre-BI cells keep their phenotype of surface markers and proliferate long-term, i.e. for hundreds of divisions. In contrast to the fetal liver pre-BI cells, bone marrow pre-BI cells cultured in the presence of stromal feeder layer cells and IL-7 only grow for approximately 30 days before they die (personal communication, F. Melchers).

Other differentiation stages of B-cells can only be kept *in vitro* for some days. Mature naive B-cells for example can be stimulated to proliferate for short periods of time *in vitro* with αIgM molecules coupled to sepharose beads in the presence of cytokines, or with αCD40 antibody and IL-4 which simulate the activation signals of T-cells [115]. B-cells with a memory phenotype can be generated *in vitro* out of human GC cells by the addition of αCD40, IL-2 and IL-10, whereas plasma cells can be generated in the presence of IL-2 and IL-10 alone [5]. None of them proliferate long-term.

Long-term cultivation, i.e. proliferation, of specific antibody secreting cells is only possible if activated/proliferating B-cells are fused to hybrid myeloma (hybridoma) cell lines, a technique developed by G. Köhler and C. Milstein in 1975 [70].

In the herein presented work, the cooperating oncogenes *Pim1* and *Myc* were integrated into B-lineage cells with the aim to generate long-term proliferating naive B-cells, memory B-cells, or plasma cells.

2.2 Myc and Pim1

Around 20 years ago, a number of experiments was conducted to find new genes involved in tumorigenesis. *Myc* and *Pim1* loci were found to be involved very often in retroviral insertion of the Moloney Murine Leukemia Virus (MoMuLV). Moreover, the 2 proto-oncogenes were shown to cooperate in the generation of T-cell lymphomas [2] [133] [134].

2.2.1 Structure of Myc

The carboxyterminal end of the Myc protein contains a basic-helix-loop-helix-leucine zipper (b-HLH-LZ) domain (figure 2.4). This region is required for dimerization with other members of the b-HLH-LZ family. In the case of Myc, it is Max, another b-HLH-LZ family member [13]. The Myc/Max heterodimers can then bind DNA by recognizing the so called E-Box sequence CACGTG [12]. Dimerization of the Myc-partner Max with the HLH-LZ family member Mad and family members thereof results in transcriptional repression which antagonizes the trancriptional and transforming activities of Myc [7].

Figure 2.4 – Structure of Myc. bHLH: basic helix-loop-helix; zip: leucine zipper; NLS: nuclear localisation signal; MB I-III: conserved Myc Boxes; TAD: transactivation domain; P: one of the phosphorylation sites.

Myc contains 3 so called Myc homology boxes (MB I-III), which are highly conserved within the Myc family members and also within Myc proteins of different species. All of them lie in the N-terminal part of the protein. The first box, Myc homology box I or MB I, contains 2 phosphorylation sites (Thr and

Ser), which are important for the stability of Myc. MB II is important for the transforming activity of Myc, but also seems to be involved in transcriptional repression of genes [79], [107]. Both Myc boxes carry also independent signals for protein degradation [38]. The Myc boxes therefore also play an important role in regulating the levels of the Myc protein. MB III plays a role in transcriptional repression of Myc and has recently been found to negatively regulate the pro-apoptotic function of Myc [54].

Myc exists in two major protein forms, which are generated by differential translational initiation. The short form, Myc2, is initiated at a canonical ATG in exon 2, whereas the long form, Myc1, is derived from an alternative non-AUG codon near the 3' end of exon 1 [47]. In humans and mice, this is a CTG codon 15 codons upstream of the ATG codon. There are no apparent differences between these two proteins in subcellular localization, stability, or post-translational modifications [46]. In growing cells, Myc1 protein has been shown to be only 10-15% of the level of Myc2 protein levels [48]. Upon methionine deprivation as occurring during nutrient deprivation, Myc1 synthesis is increased 5-10x, whereas synthesis of the AUG-initiated Myc2 is minimally affected [48]. The increase in Myc1-expression is not observable in cells subjected to stress factors (hypertonic treatment, heat shock, serum deprivation, growth inhibition by the lack of essential amino acids other than methionine). The small N-terminal tail of the larger Myc1 seems to offer an additional DNA-binding domain, which can specifically activate transcription of the C/EBP sequences within the EFII enhancer element of the Rous sarcoma virus LTR. Overexpression of murine Myc1 protein in COS cells by replacing the alternative start codon to ATG results in reduced proliferation of cells compared to overexpression of the short Myc form by destroying the alternative start codon [45].

2.2.2 Regulation of Myc

Myc is highly regulated at transcriptional, post-transcriptional, and post-translational levels [124] [138] [114] [121]. In general, Myc is expressed in proliferating cells and is downregulated in quiescent and differentiated cells. After serum- or mitogen stimulation of quiescent cells, Myc levels peak within several hours and subsequently drop to a basal level which is dependent on the presence of growth factors [136].

2.2.3 Function of Myc

A null Myc mutation causes lethality before day 10.5 of gestation in homozygotes and reduces fertility in heterozygous female mice. [28]

Myc has a wide range of activities in cells coupled to proliferation. During B lymphocyte development, Myc enhances protein synthesis and cell size [62]. This increase in cell size occurs independently of cell cycle phase.

Myc can provoke proliferation, but at the same time induces apoptosis. For example, Myc induces suppression of bcl-X_L and bcl-2 in hematopoietic cells [33]. This suppression works indirectly, since *de novo* protein synthesis is required. Vice versa, coexpression of the anti-apoptotic BCL2 protein accelerates Myc induced lymphomagenesis [127]. In addition to block survival proteins such as BCL2, Myc activates the ARF-Mdm2-p53 tumour suppressor pathway, which is frequently disabled in human cancers [123]. Inhibition of p53 prevents Myc-induced apoptosis [63]. Taken together, these lines of evidence propose that the most important mechanism of cooperation with Myc in tumour formation is suppression of apoptosis. However, even ARFnull Eμ-*Myc* mice whose B-cells show decreased apoptotic rates, only develop clonal tumours [33]. Hence, it is likely that additional transformation events are necessary to develop tumours. In normal hematopoietic cells, apoptosis induced by Myc can be suppressed by cytokines, but if Myc is expressed at high levels, it overrides the protective effects of these survival factors [101].

2.2.4 Myc and Cancer

Myc by itself is most often not sufficient to transform cells. Eμ-*Myc* mice develop B-cell tumours with a mean latency of 12-16 weeks of age, suggesting that deregulation of at least one more proto-oncogene is necessary for full transformation [134]. Before the development of tumours, B-cell progenitors appear relatively normal, but the early B-cell compartment is expanded and shows increased apoptosis [75]. Burkitt's lymphoma (BL) is associated with translocations and, hence, deregulation, of the *Myc* gene almost by definition. In BL, Myc overexpression leads to a centroblast-like phenotype of the B-cells including upregulation of bcl-6 and expression of the human GC marker CD77 [118]. Deregulated Myc cooperates with BCL-Xl to cause plasma cell neoplasms in mice [19].

2.2.5 Myc and B-Cell Development

Transcription of *Myc* is upregulated in immature B-lymphocytes upon IL-7 signalling [98], and in mature B lymphocytes by growth factors such as LPS. Within 1-3 hours of stimulation, *Myc* RNA levels increase 10-40 fold and decline thereafter and during subsequent proliferative cell cycles of activated B-cells to very low levels [68].

In human tonsils, Myc is predominantly expressed in IgD^-, $CD38^+$, $CD77^-$ centroblasts [87]. Myc expression is also regulated by CD40 signalling in B-cells [117]. Myc transcription is repressed by BLIMP-1 during final maturation to plasma cells [83]. This repression is necessary for terminal differentiation, but not sufficient [81].

2.2.6 The Pim Kinase Family

The Pim family comprises 3 Ser/Thr kinases, Pim1, Pim2 and Pim3, with high degrees of sequence- and structural similarities which are well conserved in vertebrates [93]. Pim1 and Pim2 are both known targets of proviral integration which is associated with a rapid development of T-cell lymphomas [133] [25].

2.2.7 Structure and Function of Pim1

The *Pim1* gene comprises around 5 kb and contains six exons. It encodes two related proteins of 34 kD and 44 kD due to the use of alternative initiation sites at AUG and CUG [116]. The catalytic domain lies in the central part of the protein starting encompassing the amino acids 38-290 of totally 313 amino acids in mouse and human. The lysine at position 67 is crucial for kinase activity, and replacement of lysine 67 by methionine results in a kinase dead *Pim1* mutant. Pim1 is highly expressed in thymus, spleen, bone marrow and fetal liver, and also in some non-hematopoietic tissues such as prostate and hippocampus [31]. Pim1 protein is found in both cytoplasm and nucleus. Pim1 is also regulated at the post-transcriptional level. Its 5'UTR (untranslated region) contains a GC rich region which inhibits translation. Eukaryotic translation initiation factor 4E (eIF-4E) can relieve this translational inhibition [61]. The half life of Pim1 protein in human leukemic cells is around 1.7 hours [122].

2.2.8 Pim1 in B-Cells

Expression of Pim1 is associated with the survival and proliferation of hematopoietic cells. Pim1 has been found to co-localise with the pro-apoptotic protein BAD resulting in a phosphorylation of BAD on serine 112, which is a gatekeeper site for BAD inactivation. This suggests a direct role of Pim1 in preventing cell death, since the inactivation of Bad can enhance BCL-2 activity and thereby promotes cell survival [1].

In addition, Pim1 binds and phosphorylates the phosphatase Cdc25A, which is a positive G1-specific cell cycle regulator [95]. Furthermore, Pim1 also phosphorylates Cdc25-associated Kinase 1 (C-TAK1) and thereby inhibits its activity, which suggests a activating role of Pim1 at the G2/M transition of the cell cycle [8].

Mice deficient for all three Pim kinases are viable and fertile, but have a reduced body size throughout life. Furthermore, the *in vitro* response of distinct hematopoietic cell populations to growth factors such as IL-3 or IL-7 is impaired, e.g. proliferation of peripheral T lymphocytes upon stimulation and IL-7-mediated proliferation of late pre-B-cells [93]. LPS- and CD40-signalling in B-cells upregulate the expression of the Pim1 kinase via the NFκB pathway and increase kinase activity [141].

Pim1 expression is induced by a variety of cytokines, growth factors and mitogens including IL-2, IL-3, IL-6 and IL-7 [27] [9].

2.2.9 Pim and Tumours - Pim-p my Myc!

Pim1 by itself is a weak oncogene, but as indicated above cooperates with Myc to cause preB-cell lymphomas [134] and T-cell lymphomas. Zippo et al. have recently shown that Pim1 interacts with the Myc BoxII domain and mediates phosphorylation of histone H3 at serine 10 of Myc target genes, which contributes to the activation of a subset of Myc target genes [143]. Moreover, grafting Pim1 onto the N-terminus of Myc bypasses the requirement for Myc-BoxII in cellular transformation. Though, binding of Pim1 cannot be the only function of MycBoxII, since among others, TRRAP (transformation/transcription domain-associated protein) also binds to a different part of the MycBoxII. This protein is a core component of several histone acetylase complexes [89]. So, Pim1-dependent phosphorylation of Myc-target genes is possibly the second mechanism in Myc-induced transformation together with its role in inhibition of Myc induced apoptosis. Pim1 and Pim2 are also required for efficient transformation of pre-B-cells by the V-ABL oncogene [18]. Eμ-*Pim1*-transgenic mice often develop T-cell tumours with a mean latency period of 7-8 weeks [133]. These tumours often have deregulated Myc expression patterns.

2.3 Thesis Objectives

This project was aimed at examining the effect of the proto-oncogenes *Pim1* and *Myc* to cell cycle entry, proliferation, and sustained survival in murine B-lymphocytes at different stages of B-cell maturation; i.e. in preB-cells, immature IgM$^+$ cells, mature naive B-cells, and activated B-cells such as germinal center B-cells and plasma cell precursors.

3 Materials

3.1 Machines

Cell culture	
CO_2 incubator for cell culture	Binder CB210
Laminar flow hood	Haereus
H_2O-distillery	Destamat Bi-Distiller Bi 18E, GCS
N_2 tank	Arpege 170, Air Liquide
Inverse microscopes	Nicon Eclipse TS100, Leica DMIL with Leica DFL 300 FX camera
Gamma irradiator	Gamma Cell 40, MDS Nordion
FACS analyser	LSRII, BD Dickinson, 405 nm violet laser (filters: A: 630LP, 655/8; B: 595LP, 605/12; C: 575LP, 585/15; D: 545LP, 560/20; E: 475LP, 525/50; F: 450/50; G,H: empty); 355 nm UV laser (filters: A: 505LP, 530/30; B: 450/50, C: empty), 633 nm red laser (filters: A: 755 LP, 780/60; B: 710LP, 730/45; C: 660/20) and 488 nm blue laser (filters: A: 755LP, 780/60; B: 685LP, 695/40; C: 655LP, 660/20; D: 600LP, 610/20; E: 550LP, 575/26; F: 505LP, 530/30; G: SSC, H: empty)
MACS multistand	Miltenyi
Mini-MACS magnet	Miltenyi
Midi-MACS magnet	Miltenyi
Electrophoresis & Visualisation	
Electrophoresis chamber (SDS page)	Mini-Protean 3 Electrophoresis Module, Biorad
Blotter for SDS page gels	Trans-Blot SD Semi Dry transfer cell, Biorad
Power supply for gel electrophoresis	Power Pac Basic, Biorad
Gel analyzer (Agarose Gels)	Gel Doc 2000, Biorad
Luminescent image analyzer	LAS 3000, Fujifilm
PCR, molecular Biology	
Real time PCR machine	7900 HT sequence detection system, ABI Prism
Thermal cycler	DNA Engine PTC 200, Biorad
Tube heating block	Thermomixer comfort, Eppendorf
DNA-electroporator	Gene Pulser X-cell total system, Biorad
Bio-Photometer	Eppendorf
Tabletop centrifuges	Eppendorf 5417R, Eppendorf 5810R

3.2 Software

FACS acquisition & analysis	Diva 6.1, BD Biosciences
FACS analysis	Flow Jo, Tree Star, Inc.
Design of illustrations	Adobe Illustrator CS3
Processing of photos	Adobe Photoshop CS3
Real-time PCR acquisition	SDS v2.0, ABI Prism
Scientific graphics	Prism 5.0, Graphpad software

3.3 Kits

Gel extraction kit	Qiagen
Midiprep kit endo-free for extraction of plasmid DNA	Qiagen
Miniprep kit for extraction of plasmid DNA	Qiagen, Zymo Research
Superscript III first strand synthesis system	Invitrogen
Topo-TA cloning kit	Invitrogen
QuantiTect SYBR Green RT-PCR Kit	Qiagen

3.4 Labware

product	**description**	**manufacturer**
cell culture flasks 25 cm^2 filter cap (T25)	Nunclon surface	Nunc
cell culture flasks 75 cm^2 filter cap (T75)		TPP
cell culture plates 15 cm diam.	Nunclon surface	Nunc
cell culture plates	6 - 96-well	Corning
cell culture plates	6-well, Cell-Bind surface (for transfection)	Corning
cell culture pipettes	pyrogen free	Corning and Sarstedt
cell strainer	40 μ, nylon	BD Falcon
conical tube 0.5-2 ml		Eppendorf
conical tube 15 & 50 ml	Corning	
dialysis tubing	(20/30), wall thickness 0.05 mm	Visking
Electroporation cuvettes		Biorad
bottletop filters 250 - 1000 ml	Express-Plus Steritop	Millipore
MACS LD columns	for cell depletion	Miltenyi
MACS MS columns + plungers	for cell enrichment	Miltenyi
Needles 30G (0.3x13mm)	Microlance 3	BD
Needles 18G (1.2x40mm)	Sterican hypodermic needle, BC/SB	B. Braun Melsungen AG
Needles 26G (0.45x12mm)	Sterican disposable insulin needle, BL/LB	B. Braun, Melsungen AG
Nitrocellulose membrane	Nybond ECL 0.2 μ	Amersham Biosciences
PCR 8-well stripe + caps	Biozym	
Polypropylene tubes 5 ml	round bottom, for FACS analysis	BD Falcon
PVDF membrane	f. Western blot, Immobilon 0.45 μ	Millipore
plastic eppendorf tube crushers		Eppendorf
Pre-separation filters	for MACS, 30 μm	Miltenyi Biotech
Syringe 1 ml	Injekt-F, tuberculin, with plastic stopper	B.Braun, Melsungen AG
Syringe 1 ml	Omnifix, tuberculin, with rubber stopper	B. Braun, Melsungen AG
96-well plates for ELISA	Immuno-Maxisorp U-bottom	Nunc
96-well plates for RT-PCR	Biozym	
250 ml-1 l storage bottle	Stericup	Millipore
250 ml-1 l storage bottle		Corning

3.5 Antibodies for FACS Analysis

molecule	dye	company	clone name	isotype
CD4	PE	eBioscience	Gk1.5	Rat IgG2b
CD5	FITC	eBioscience	53-7.3	Rat IgG2a
CD8a	FITC	eBioscience	53-6.7	Rat IgG2a
CD19	PerCP-Cy5.5	eBioscience	eBio1D3	Rat IgG2a
CD21	FITC	eBioscience	eBio8D9	Rat IgG2a
CD23	PE	eBioscience	B3B4	Rat IgG2a
CD25	PeCy7	eBioscience	eBio3C7	Rat IgG2b
CD43	FITC	eBioscience	eBioR2/60	Rat IgM
CD93	PE	eBioscience	aa4.1 [88]	Rat IgG2b
CD95	PE	BD Pharmingen	Jo2	Armenian hamster IgG2
CD138	PE	BD Pharmingen	281-2	Rat IgG2a
c-kit	APC	eBioscience	ACK 2	
c-kit	APC	in-house	ACK4	
GL-7	FITC	BD Pharmingen	GL-7	
IgD	PE	eBioscience	11-26	Rat IgG2a
IgM	APC	eBioscience	II41	Rat IgG2a
IgM	FITC	in-house	M41 (C3 specific) [77]	Rat IgG1
kappa	APC	in-house	187-1	

3.6 Antibodies for ELISA

molecule	dye	company	clone name	isotype
goat anti-mouse IgM	- and AP	AbD Serotec	polycl.	
goat anti-mouse total IgG	- and AP	Southern Biotech	polycl.	
goat anti-mouse IgG2b	AP	Southern Biotech	polycl.	
goat anti-mouse IgG2c	AP	Southern Biotech	polycl.	
goat anti-mouse IgG3	AP	Southern Biotech	polycl.	
goat anti-mouse IgA	- and AP	Southern Biotech	polycl.	
mouse IgA (Standard)	-	Southern Biotech	S107	
mouse IgG (Standard)	-	Southern Biotech	polycl.	
mouse IgG (Standard)	-	homemade	UCHT-1	IgG1
mouse gM (Standard)	-	Chemicon Int.	polycl.	
mouse anti-KLH (TNP-KLH)	-	BD Pharmingen	A112-3	IgG3, κ

3.7 Antibodies for Western Blotting

molecule	dye	company	clone/isotype	immunogen, expected size
mouse anti-c-Myc	-	Santa Cruz	9E10, IgG1	AA408-439 of hc-Myc, 64, 67kD
rabbit anti-mouse Hdac1	-	eBioscience	polycl., IgG	hHDAC1, c-terminal part, 60kD
goat-anti Pim-1 (m,h,rat)	-	Santa Cruz	polycl., IgG	hPim1, interal part, 33kD
rabbit anti-goat IgG (H&L)	HRP	Pierce	polycl.	
goat anti-mouse IgG (H&L)	HRP	Pierce	polycl.	
goat anti-rabbit IgG (H&L)	HRP	Pierce	polycl.	

3.8 DNA Primers

m068	Linker for pSuperRetroPuro-fo, contains the restriction endonuclease sites EcoRI, HpaI, AvrII, XhoI, NruI, AflIII, HindII, ClaI and MluI	cgacgcgttaacatcgattcgcgaattctcgagcctagg
m074	Linker for pSuperRetroPuro-re	tcgacctaggctcgagaattcgcgaatcgatgttaacgcgt
m069	pgk promoter + PuroR MluI-fo	cgcacgcgtaattctaccgggtaggggagg
m070	pgk promoter + PuroR MluI-re	cgcacgcgttcgtgcgctcctttcggtcg
m073	Sequencing primer for linker	catcgattcgcgaattctcgagc
m095	histidinol resistance gene-re (BamHI)	cgggatcctcatgcttgctccttgaggg
m099	*pim1* BglII-fo	gaagatctatgctcctgtccaagatcaactccctg
m100	*pim1* HindIII-re	gaccaagctttatttgcccttctacttgctggatcc
m101	myc HindIII-re	gagcgaagctttatttccttacgcacaagagttccgtag
m102	myc, BamHI-fo	cgggatccatgcccctcaacgttagcttcaccaac
m122	*egfp*, BamHI+NcoI-fo	ctgggatccatggtgagcaagggcgagga
m123	*egfp*, HindIII-re	gaagcttgcttacttgtacagctcgtccatgc
m136	pgk promoter-fo (XhoI)	cactcgaggcgttcgcaattctaccgggtagg
m137	pgk promoter-re (EcoRI)	gagaattctagcttgggctgcaggtcgaaag
m147	histidinol resistance gene-fo	catcatgagcttcaataccctgattgac
m167	RT-PCR primer *myc*-fo	atgcccctcaacgttagcttc
m168	RT-PCR primer *myc*-re	cgcaacataggatggagagca
m169	RT-PCR primer *pim1*-fo	ctggagtcgcagtaccagg
m170	RT-PCR primer *pim1*-re	cagttctccccaatcggaaatc
m179	RT-PCR primer *pim1*neu-B-fo	gagaacatcttaatcgacctgagc
m180	RT-PCR primer *pim1*neu-B-re	ggtagcgatggtagcgaatc
m181	RT-PCR primer *gapdh*-fo	catgttccagtatgactccactc
m182	RT-PCR primer *gapdh*	gtagactccacgacatactcagc

fo=forward/5', re=reverse/3' primer

3.9 Enzymes

Restriction endonucleases	New England Biolabs (NEB), Fermentas
Recombinant Taq polymerase	Fermentas
RNAse-A, DNAse free	Qiagen
Platinum Taq polymerase	Fermentas
CIAP (calf intestinal phosphatase)	Roche
T4-DNA ligase	NEB

3.10 Tissue Culture Media and Additives

Media	
D-MEM + Glutamax I + 4.5g/lt glucose - pyruvate	Gibco/Invitrogen

IMDM powder with L-Glutamine w/o NaHCO3	Gibco/Invitrogen 42200-030
MEM alpha powder	Gibco/Invitrogen
Opti-MEM	Gibco/Invitrogen
PBS w/o Ca, Mg	PAA Laboratories
RPMI-1640 medium + L-glutamine	Gibco/Invitrogen
Additives	
L-glutamine 200mM (100x)	Gibco/Invitrogen
Insulin from bovine pancreas	Sigma-Aldrich I5500
MEM non-essential amino acids 100x, 100 ml	Gibco/Invitrogen
2-mercapto-ethanol	Fluka/Sigma-Aldrich
Primatone	RL-Quest International 5X59057 www.sheffield-products.com
5% Trypsin-EDTA	Gibco/Invitrogen
Antibiotics	
Kanamycin-sulfate solution 100x, 100ml	Gibco/Invitrogen
Penicillin-Streptomycin solution 100x, 100 ml	Gibco/Invitrogen
L(-) Histidinol dihydrochloride 99%	Sigma Aldrich
Hygromycin	Roche, Mannheim; Carl Roth GmbH
Puromycin	Calbiochem, Beeston
Interleukins	
BAFF, murine, recombinant	R&D Systems
Interleukin-21, murine, recombinant	R&D Systems
TPO	Peprotech, stocks of 10 μg/ml in PBS

3.11 Chemicals

Ampicillin sodium salt	Sigma-Aldrich
APS	Carl Roth GmbH
CFSE / 5(6)-Carboxyfluorescein diacetate, N-succinimidyl ester	Alexis Biochemicals
Chloroform / NH_4Cl	Carl Roth GmbH
CNBr-activated sepharose 4B, lyophilized, average bead size 90 μm	GE Healthcare
DAPI	Carl Roth GmbH
Diethanolamine	Merck
DMSO Hybri Max	Sigma-Aldrich
Doxycycline hyclate	Sigma-Aldrich
EDTA disodium salt $\cdot$ 2 H_2O	Carl Roth GmbH
Ethidium bromide solution 0.5%	Carl Roth GmbH
Ethanol	Carl Roth GmbH
Glycerol	Sigma
Glycine electrophoresis grade	Carl Roth GmbH
HCl	Merck
HEPES 1M	Sigma-Aldrich
H_2O, RNAse free, DNAse free	Gibco/Invitrogen
Isopropanol	Acros Organics

KCl	Carl Roth GmbH
$KHCO_3$	Carl Roth GmbH
KLH, highly soluble, low endotoxin	Alexis Biochemicals
Methanol	Carl Roth GmbH
$MgCl_2 \cdot 6\ H_2O$	Carl Roth GmbH
Na-acetate	Carl Roth GmbH
NaCl	Fluka/Sigma-Aldrich
$NaHCO_3$	Fluka/Sigma-Aldrich
NaOH	Fluka/Sigma-Aldrich
Propidium iodide HPLC	Sigma-Aldrich
RedSafe nucleic acid staining solution 20'000x	INtRON Biotech
SDS solution ultra	Fluka/Sigma-Aldrich
Temed	Carl Roth GmbH
Tris Pufferan	Carl Roth GmbH
Tween20	Sigma-Aldrich

3.12 Molecular- and Cell-Biology Reagents

Bradford solution	Biorad
BSA, fatty acid free, low endotoxin, for cell culture	Sigma
BSA fraction V, for western blotting, ELISA, etc.	Serva
BSA Standard 2 mg/ml for bradford measurements	Biorad
DNA ladder (Gene Ruler 1kb plus)	Fermentas
dNTP set 4x100 μM	Fermentas
dnpp tablets for 20 ml solution per tablet	Sigma
Lipofectamine	Invitrogen
MACS α-mouse CD19 beads	Miltenyi
Ripa buffer	Sigma
Protease inhibitor cocktail 40x, contains 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin, and sodium EDTA	Sigma, P2714
Protein marker for western blot (Precision Plus Dual Color)	Biorad
Protogel	Biorad
Skinman Soft disinfectant	Ecolab Germany
TiterMax gold adjuvant	Sigma-Aldrich
Trizol	Invitrogen
Western Lightning chemiluminescence reagent plus	Perkin Elmer

3.13 Buffers and Solutions

Designation	**Recipe, Storage**
Ampicillin stock solution	100 mg/ml in water, -20°C
APS stock solution	10% APS in water, 4°C
Doxycycline stock solution	1 mg/ml doxycycline hyclate in H_2O, sterile filtered. -20°C.
Erythrocyte lysis buffer	0.01M $KHCO_3$, 0.155M NH_4Cl, 0.1 mM EDTA, sterile filtered, RT

FACS Buffer	2% heat-inactivated FCS in PBS, 4°C
Laemmli buffer	250 mM Tris-Cl pH 6.8, 40% v/v glycerol, 5% p/v SDS, 0.005% bromophenol blue, 10% β-mercapto ethanol, -20°C
LB-Medium	done by the media kitchen staff of the MPI-IB (10g Bacto-tryptone,5g yeast extract, 10g NaCl per liter, pH 7.5, autoclaved. Storage at 4°C)
PBS	done by the media kitchen staff of the MPI-IB, RT
TBS	6.05 g Tris, 8.76 g NaCl, distilled water ad 1000 ml, RT

3.14 Plasmid Vectors

Designation	**manufacturer**
pTre-Tight	BD Biosciences
pSuperRetroPuro w/o Stuffer	OligoEngine
pCR4-TOPO	Invitrogen
pim1-CS41	in pSP65 vector (for *in vitro* transcription, contains murine pim-1, generates messages for 34 & 44 kD, kind gift of Anton Berns, Netherlands Cancer Institute
pBJ4-cmyc	chimera of human and murine c-myc-2 in pBJ4ω, kind gift of Ulf Rapp
pSRL5P Vpmut IRES nsEGFP	contains the IRES sequence and a non-sticky variant of *egfp*, kindly provided by Ozan Guezelbey

3.15 Bacteria

Top10 electrocompetent *E. coli*	*F- mcrA* Δ *(mrr-hsdRMS-mcrBC)* Φ*80lacZ*Δ*M15*Δ*lacX74 recA1 araD139* Δ*(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG* λ -

3.16 Cell Lines

name	**description**
Fetal liver preBI-cell lines	
Fld18Ber3	fetal liver preBI cell line, made in Berlin at the MPI for Infection Biology by Szandor Simmons in our Lab from a fetal liver of a C57Bl/6 mouse embryo at day 18 of gestation
Fld18Ber3-rtta1 pim/myc	fetal liver preBI cell pool carrying the rtTA-TetON vector and the TetON-myc & TetON-*pim1* vectors (non-clonal)
Fld18Ber3-rtta1 myc	fetal liver preBI cell pool carrying the rtTA-TetON vector and the TetON-myc vector (non-clonal)
Fld18Ber3-rtta1 *pim1*	fetal liver preBI cell pool carrying the rtTA-TetON vector and the TetON-*pim1* vector (non-clonal)
Makn	fetal liver preBI cell line, made in Berlin at the MPI for Infection Biology by Marko Knoll

Makn rtTA myc	Makn cell line carrying the rtTA-TetON vector and the TetON-myc vector
Stromal cell lines	
OP9	stromal cells established from the bone marrow of newborn B6C3F1 op/op mouse calvaria (which lacks M-CSF)
ST2	a stromal cell line established from murine fetal liver [51]
Packaging cell lines	
Phoenix-eco	ecotropic retroviral packaging cell line based on the Hek-293 cell line
Plat-E	ecotropic retrovirus packaging cell line named Platinum-E (Plat-E) based on the 293T cell line. This cell line contains the MoMulv gag-pol and env coding sequences under the control of the strong EF1-alpha promoter. The promoter also drives resistance genes for puromycin and blasticidin, which are attached to the gag-pol and env coding regions by an IRES (internal ribosomal entry site) [97]
Cytokine producers	
CHO-SCF	SCF producer cell line [144], kind gift of T. Feyerabend, University of Ulm
J-558L-IL-7	IL-7 producer cell line [139]
SP2/0-Flt3L	Flt3L producer cell line, kind gift of P. Vieira, Institute Pasteur [29]
X-63-IL-2	IL-2 producer cell line [67]
X-63-IL-3	IL-3 producer cell line [67]
X63-IL-4	IL-4 producer cell line [67]
X-63-IL-5	IL-5 producer cell line [67]
X-63-IL-6	IL-6 producer cell line [67]
B cell lines	
Daudi	human Burkitt's Lymphoma cell line

3.17 Mouse Strains

strain	**Ly5 allele**	**remarks**
C57Bl/6*J*	Ly5.2	
C57Bl/6*J* rag1^{-}/$-$	Ly5.2	
Jax C57Bl/6*N* rag2^{-}/$-$	Ly5.2	Taconic
C57Bl/6*J* rag1^{-}/$-$ Ly5.1	Ly5.1	Jax, bad breeders, low viability

Mice were bred under specific pathogen free conditions in IVC (individually ventilated) cages in the animal facilities of the Max Planck Institute in Marienfelde and at the MPI for Infection Biology. For transplantation experiments, mice aged from 6 to 10 weeks were used. Experimental setups done in this work were approved by the LAGeSo (Landesamt für Gesundheit und Soziales), Berlin, approval number G0099/08, "Beeinflussung der Blutzelldifferenzierung der Maus durch Regulatoren und Modulatoren der Gen-Expression und der Signaltransduktion in Vorläuferzellen des hämatopoietischen Zellsystems; Zusatz:

Immunisierung der mit prä-B- und T-Zellen transplantierten Mäuse mit dem Protein-Antigen Keyhole Limpet Hemocyanin (=KLH) zur Messung der Funktionsfähigkeit der adaptiven Immunreaktion der in den Empfängermäusen rekonstituierten gereiften B- und T- Zellen".

4 Methods

4.1 Molecular Biology

4.1.1 Cultivation of *E. coli*

Bacteria were cultivated in LB medium containing the appropriate antibiotic for selection of plasmids, usually 100 μg/ml ampicillin. Bacteria carrying high-copy plasmids were grown for 12-16 hours in 2 ml LB-medium for minipreps and 100 ml LB medium for midipreps.

4.1.2 Preparation of Electrocompetent Bacteria

Top10 bacteria picked with a stick from frozen stocks were grown over night in a 37°C shaker in a volume of 100 ml of LB broth without antibiotics. The next day, 25 ml of the resulting broth was resuspended per 500 ml of LB broth without antibotics in 2 liter Erlenmeyer flasks and shaken for 2-4 hours until the OD_{600} reached 0.6-0.8. The bacteria were cooled down on ice for 20 minutes and then centrifuged at 2600 g in a tabletop centrifuge in 400 ml flasks for 20 minutes at 4°C. From this step on, bacteria were kept on ice, and handling was done in a cold room (7°C). All solutions in contact with the bacteria were sterile filtered and cooled to 4°C on ice before use. All quantities mentioned are calculated for 1 liter of starting culture and have to be adjusted accordingly. Pelleted cells were resuspended slowly in 600 ml ice-cold 0.1 M HEPES. Then, the cells were centrifuged for 15 minutes at 2600 g at 4°C, and the resulting pellet was resuspended in 400 ml 0.1 M HEPES. Once again, the cell suspension was centrifuged at 2600 g at 4°C, and the pellet was resuspended in 20 ml 0.1 M HEPES supplemented with 10% glycerol. The solution was pelleted a last time and resuspended in 3 ml 0.1 M HEPES supplemented with 10% glycerol and portioned at 200 μl per precooled 0.2 ml conical tubes. The cells were shock-frosted in dry ice and kept at -80°C.

4.1.3 Electroporation of Bacteria

40 μl of electrocompetent Top10 bacteria were thawed on ice and put into ice-cold 1 mm electroporation cuvettes. 1 μl of ligation product or maximally 100 ng of plasmid DNA was added and incubated on ice for 5 minutes before electroporation. Bacteria were electroporated with 1800 V, 25 $\mu Farad$, and 200 Ω in a 1 mm cuvette. After electroporation, bacteria were resuspended in 600 μl of antibiotic-free LB-medium and incubated for 30 minutes in a 37°C shaker to allow expression of the resistance gene, and subsequently plated onto agar plates containing the appropriate antibiotic for selection of plasmid-containing bacteria. Plates were incubated for 12-16 hours at 37°C.

4.1.4 Restriction Endonuclease Digests

Digests were done in a total volume of 30 μl using 2 μg of DNA and 5U of each enzyme and the recommended buffer from New England Biolabs. Digests were usually incubated over night at the recommended temperature.

4.1.5 DNA Gel Electrophoresis

Digested DNA was separated at 60V in 0.8-1.5% electrophoresis grade agarose dissolved in TBS. Bands were visualized using ethidium bromide or RedSafe in a gel imager.

4.1.6 Gel Extraction of DNA Fragments

DNA fragments were extracted from agarose gels using the Qiagen Gel Extraction Kit using the standard protocol, and eluted in 35 μl water.

4.1.7 Primer Design for PCR and RT-PCR

Primers were designed according to the following rules: 18-25 bases long, GC content of 45-60%, no intra-primer homology beyond 3 base pairs, no intra- and inter-primer complementary sequences beyond 2 base pairs, no AT or GC stretches beyond 3 base pairs, primers should end with G or C. Primer pairs should have approximately the same melting temperature +/- 0.5 °C.

4.1.8 PCR with Taq Polymerase

For colony PCR and detection of DNA, Taq polymerase was used. In totally 30 μl, 100 ng of purified DNA or 500-1000 ng of crude DNA, or 4 μl of bacterial suspension generated from a picked colony resuspended in 50 μl water were set up in 1x KCl-PCR buffer (Fermentas) supplemented with 200 μM dNTPs, 1.5 mM $MgSO_4$, 10 μM of each primer and 2U of enzyme in 0.2 ml tubes.

For GC-rich templates, up to 5% DMSO was added to the reaction to inhibit formation of secondary structures. The annealing temperature was lowered by 0.5-1°C when DMSO was added.

Cycling conditions: 94°C 2 min, 30x (94°C 20 sec, 49°C - 57°C 30 sec, 72°C (60 bases/kb + 10 sec)) 72°C 5 min, 10°C storage temperature

4.1.9 PCR with PFU Polymerase

For cloning, 100 ng of purified plasmid DNA or 500-1000 ng of crude DNA (genomic DNA) or 1 μl of c-DNA were set up in 1x PFU-PCR buffer w/o $MgSO_4$ from Fermentas, 150 μM dNTPs, 1.5 mM $MgSO_4$, 10 μM of each primer and 2U of enzyme in 0.2 ml tubes.

Cycling conditions: 95°C 1 min 30 sec, 30x(94°C 30 sec, 49°C - 57°C 35 sec, 72°C (1kb/min)) 72°C 5 min, 10°C storage temperature

4.1.10 Preparation of messenger RNA

Cells were counted, and $1x10^6$ to $1x10^7$ cells were pelleted by centrifugation (260 g, 5 minutes at 4°C), and resuspended in 30 μl PBS in 1.5 ml conical tubes until no clumps remained. Then, 1 ml Trizol was added and the cells were lysed by repetititve pipetting. After 5 minutes of incubation at RT, 0.2 ml chloroform per ml Trizol were added, and the tubes were shaken by hand for 15 seconds. The tubes were incubated on ice for 3 minutes and then centrifuged at 12'000 g for 10 minutes at 4°C. the colourless upper aqueous phase was transferred to a fresh tube, and RNA was precipitated by adding 0.5 ml isopropanol per ml Trizol. After 10 minutes of incubation at RT, the tubes were centrifuged for 10 minutes at 12'000 g at 4°C. RNA was washed once with 1 ml ice-cold 70 % EtOH, dried for 5 minutes at RT, redissolved in RNAse-free water, and heated to 55°C for 2 minutes. RNA was stored at -80°C.

4.1.11 Preparation of c-DNA

c-DNA was prepared from whole RNA generated by the Trizol method using the SuperscriptIII Kit from Invitrogen as proposed by the manufacturer.

4.1.12 Real Time PCR

RT-PCR was performed using the QuantiTect SYBR Green RT-PCR Kit (Qiagen). Reactions were set up in 96-well PCR plates covered with translucent plastic foil. Per reaction, 10-50 ng total RNA extracted from cells (see section 4.1.10), 0.8x SYBR-green PCR master mix (still providing consistent results), 0.25 μl RT-mix and 400 nM of each primer were used in a total volume of 25 μl. Primers were chosen to amplify a target of 80-150 base pairs. As a standard, *gapdh* mRNA levels were measured for each cell line and condition. Primers were used at a concentration of 2 μM. Before use, standard curves with each primer set were done to assess their usability. Samples were recorded in a 7900HT Real-time PCR machine (ABI Prism) using SDS 2.0 software using the following cycling parameters: 50°C 30 min, 95°C 15 min, 45x(94°C 20 sec, 60°C 40 sec, 72°C 40 sec), 95°C 15 sec, 60°C 15 sec, 95°C 15 sec.

Relative overexpression of genes was assessed using the ΔC_T method.

4.1.13 Oligo Linker Annealing

Oligos for the linker were designed to generate sticky ends when annealed, which were compatible to restriction-endonuclease cutting sites in the vector. Oligos were purified by HPLC or comparable methods to ensure that only full-length oligos were annealed.

2 μl of 100 μM forward oligo and 2 μl of 100 μM reverse oligo dissolved in H2O were mixed with 3 μl of 10x T4-DNA Ligase buffer from NEB in a total reaction volume of 30 μl. The reaction was heated to 65°C in a waterbath and cooled down slowly to RT before use for ligation. For ligation, 4 different ratios of linker versus backbone were set up. Dilutions of 1:10, 1:100 and 1:1000 of the linker were set up, and 1 μl of undiluted annealed linker and each of the 3 dilutions was used in a ligation reaction together with 1 μl of 35 μl linearized dephosphorylated vector (sections 4.1.4 and 4.1.6).

4.1.14 Addition of Adenine-Overhangs to blunt-ended PCR Products for TOPO-TA Cloning

PCR products were cleaned by gel extraction as usual (see 4.1.6), and 5 μl of PCR product were incubated with 1 μl of PCR buffer I (10x, 500 mM KCl, 100 mM Tris HCl pH 8.3, 15 mM $MgCl_2$), 1U of Taq (not HotStart), 0.8 μl dNTPs from a 2.5mM stock in a volume of totally 10 μl for 10-15 minutes at 70°C. Then the product was frozen to kill Taq polymerase. Subsequently, 2-4 μl of the product were used for TOPO-TA cloning.

4.1.15 Dephosphorylation of DNA Fragments

Linearized DNA fragments were dephosphorylated using Calf Intestinal Alkaline Phosphatase. DNA from originally 1-2 μg digested DNA was set up in a 40 μl reaction volume in 1x CIAP buffer supplemented with 4U of enzyme. The reaction was incubated for 1h at 30°C. After incubation, the DNA was purified using the Qiagen PCR Gelextraction Kit (PCR-purification protocol) and eluted in 20 μl water.

4.1.16 DNA Ligation

Ligations were done in a volume of 40 μl using 4U of T-4 DNA Ligase in the recommended buffer with 1/30 of 2 μg of digested and gel-extracted backbone and 3/30 of 2 μg digested and gel-extracted insert DNA. Ligations were incubated overnight at 16°C, frozen, and then transferred into electrocompetent Top10 bacteria.

4.1.17 Topo-TA Cloning

Topo-TA cloning was done as proposed in the manufacturer's protocol. In short, 4 μl of purified PCR product were mixed with 1:4 diluted salt from the kit and 1 μl of Topo-TA-enzyme mix, incubated for 15 minutes at RT, frozen, and then shocked into electrocompetent bacteria.

4.1.18 SDS Page

5x10^6 to 1x10^7 cells were pelleted by centrifugation at 260 g for 5 minutes at 4°C, resuspended in 5 μl PBS to minimize clumping, and lysed with 150 μl Ripa buffer (Sigma) supplemented with protease inhibitors from a 40x stock solution (Sigma) for 30 minutes on ice. Lysis was completed by crushing the cells using plastic eppendorf crushers and another round of incubation at 4°C for 30 minutes. The extract was centrifuged at 10'000 g for 10 minutes and the supernatant (= protein extract) was transferred to a clean conical tube. Protein concentration was assessed by the Bradford method. Standard BSA (Biorad) was set up at different concentrations ranging from 1500 μg/ml down to 100 μg/ml in H_2O. 5 μg of each dilution were pipetted into 245 μl of Bradford solution, incubated for 5 minutes, and measured in an conical Bio-Photometer using the preset Bradford program. Samples were diluted 1:5 in water, and 5 μl of each sample was pipetted into 245 μl of Bradford solution, incubated for 5 minutes, and measured in the Bio-photometer. The samples were diluted in 4x Laemmli buffer, heated up to 95°C, centrifuged at 10'000 g, and cooled down. Acrylamide solution for the separating gel was prepared freshly from stock solutions using 11% acrylamide (Protogel 30), 0.1% SDS, 0.03-0.05% APS, and 0.1% Temed in 0.37 M Tris base pH 8.8. Acrylamide solution for the stacking gel was prepared using 3.9% acrylamide (Protogel 30), 0.1% SDS, 0.05% APS and 0.1% Temed in 0.124 M Tris base pH 6.8.

Acrylamide gels were poured using the Protean-3 minigel module with combs with a thickness of 0.75 mm. The seperatation gel was covered with water during polymerization to ensure an even edge. After polymerization of the stacking gel, the combs were removed, and the gel was placed into the Protean3 gel chamber and the chamber was filled according to the Protean3-protocol with running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) prepared from a 5x stock.

20 to 40 μg of protein were loaded onto the gel. One lane was reserved for 5-10 μl of the protein marker (Precision plus, Biorad). The gels were run at10 mA (around 70 V) until the migrating front was just in front of the resolving gel. Then, the voltage was increased to 200 V for 1-1.5h until the migrating front was leaking out of the gel. Unused sections of the gel and the blue migrating band were cut away with a sharp scalpel, the left upper edge of the gel was removed to mark the orientation, and the gel was submerged in Bjerrum Schafer-Nielsen transfer buffer (48mM Tris, pH 9.2, 39mM Glycine, 20% Methanol, designed for semi-dry transfer) for 5 minutes for equilibration. A piece of PVDF or nitrocellulose membrane with a size slightly larger than the gel and 2 extra thick blotting papers with the size of the gel were prepared as described by the manufacturer (PVDF membranes were pretreated with 100% Methanol for 15 seconds and then submerged in H_2O, nitrocellulose membranes were submerged in H_2O) and equilibrated in transfer buffer for 5 minutes.

A stack of wet filter paper, the membrane, gel and another filter paper were piled onto the anode of the blotter, and remaining air bubbles were removed. 1 ml of transfer buffer was poured onto the stack, and spilled liquid was removed with a paper towel. The cathode was placed onto the stack, and the proteins were blotted for 30 minutes at 10-15 V for minigels as proposed by the manufacturer. After blotting, correct migration of the protein ladder was ensured, the membrane was marked at the upper left corner, and the membrane was washed 1x in TBS-T, and then incubated in blocking buffer (5% milk powder in TBS-T) for 1 h at RT. Then the membrane was probed with the first antibody diluted in TBS-T 1:1000-1:20000 according to the manufacturer for 2 h at RT up to ON at 4°C. After 2x washing in

TBST, the membrane was probed with the secondary HRP-labelled antibody diluted in TBST for 1h at RT. After another round of washing in TBST and finally TBS, the membrane was developed with 1-2 ml of detection agent (Western Lightning) per minigel size and viewed with a Luminescent Image analyser (LAS3000).

4.1.19 ELISA

Maxisorp ELISA 96-well plates were coated with 2 μg/ml unlabelled antibody in PBS overnight at 7°C or for 2 hours at RT. For coating of keyhole limpet hemocyanin (KLH), 2 μg/ml PBS were coated ON in the fridge at 7°C. Then the plates were blocked for 30 minutes at RT with 200 μl blocking buffer (0.005 % Tween-20, 2.5% BSA fraction V, PBS) and rinsed 2x with tap water (the wells were completely filled wit tap water and subsequently the plate was dumped and clapped onto paper towels to drain remaining liquid).

Sera were set up at 3-4 different dilutions in standard ELISA buffer (0.25% BSA fraction V, 0.05% Tween-20, PBS) depending on the desired target (total IgM, IgG: 1:500-1:20'000, specific IGs: starting from 1:100). In parallel, Standard antibodies were diluted in 7 serial 1:3 dilutions starting from 300 ng/ml (linear range of detection). If no standard antibody was available, standard C57Bl/6 serum from one big pooled frozen and aliquoted batch was used.

50 μl of serum dilutions and standards were pipetted into each well and the plates were incubated at RT for 1-3 hours. Then the plates were rinsed 4x with tap water, and clapped onto a paper towel to dry. Alkaline phosphatase-labelled antibody was diluted to 1 μg/ml standard ELISA buffer and 50 μl thereof were pipetted into each well and incubated for 1h at RT. Plates were washed again 3x with tap water as described above. 50 μl of developing solution (l tablet of dnpp in 20 ml 1M diethanolamine, pH 9.8) were pipetted quickly in each well, and after 15, 30, 45, and 60 minutes, plates were read at 405 nm in a plate reader.

For data analysis, standard curves were set up using scatter plots for the amount of antibody versus extinction at 405 nm, and the linear range of detection was determined. Only extinction measurements of the samples in the linear range were taken.

4.2 Cell Biology

4.2.1 Coupling of anti-IgM Antibodies to CNBr-Activated Sepharose

Figure 4.1 – modified 50 ml polypropylene tube.

Sepharose beads loaded with anti-mouse IgM antibodies can be used to induce murine B cell proliferation [108]. 5 mg anti-IgM antibodies (clone M41 [77]) were dialyzed over night in coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl, pH9, sterile) using 20/30-dialysis tubing from Visking with a wall thickness of 0.05 mm. Concentration of antibodies was measured by assessing the optical density at 280 nm. The resulting OD was multiplied with 1.4 and the dilution factor to get the concentration of antibody. Final concentration was 1.7 mg/ml. 300 mg of lyophilized CNBr-sepharose were swollen in 3 ml 1 mM HCl for 15 minutes at room temperature. Then, the beads were transferred with a sterile spatula into a 30 μm filter unit on a modified conical 50 ml polypropylene tube, see figure 4.1. This tube had been prepared by punching a hole (around 6 mm diameter) into the upper wall and inserting a conical hose-adapter taken from a Millipore steritop filtration unit. The hose-adapter was sealed with glue. By using this modified 50 ml conical tube, liquids residing in the filter unit could be sucked into the 50 ml conical

tube using vacuum. The beads were washed with totally 200 μl 1mM HCl and dried by applying vacuum. Then, the beads were washed with 5 ml sterile coupling buffer. After that, the beads were transferred quickly into 4 ml coupling buffer containing 1.7 mg/ml anti-IgM antibodies prepared before in a 15 ml polypropylene tube. The tube was rotated gently at RT for 2 hours. Then, the beads were spun at 200 g for 30 seconds, the supernatant was collected in a fresh 15 ml polypropylene tube, and the beads were resuspended in coupling buffer and transferred again onto the filter in the modified 50 ml conical tube and were washed 3x with coupling buffer. Then, the beads were transferred into 5 ml of coupling buffer containing 10% FCS and 100 μ l 100 mM ethanolamine and incubated for 2 h at RT to block residual binding sites. The beads were then washed 4x alternating between 5 ml low pH buffer (0.1M sodium acetate, pH 4, sterile) and 5 ml coupling buffer in the filter unit. Then, the beads were transferred into 1 ml sterile PBS and stored at 4°C.

4.2.2 FACS Staining

The cells were washed in PBS 2% FCS (FACS buffer), centrifuged at 260 g, and resuspended in FACS Buffer. 2x10^5 to 1x10^6 cells were stained at 4°C for 40 minutes in FACS buffer supplemented with the titrated amount of antibodies and Fcγ-receptorII blocking antibody. The cells were then washed 2x with FACS Buffer and resuspended in FACS buffer containing DAPI at 100 ng/ml for exclusion of dead cells.

4.2.3 FACS Sorting of B-Cells and B-Cell Precursors

The cells were washed in sterile PBS 2% FCS (FACS buffer), centrifuged at 260 g, and resuspended in FACS Buffer. Cell numbers to be stained were calculated according to the amount of sorted cells needed and the frequency of the cells to be sorted in the initial sample. Cell loss of sorted cells was estimated to be around 50%. The antibodies used for sorting were diluted in FACS buffer and filtrated with 2 μm conical tube filters. For cell sorting, neither dead cell exclusion nor FcγreceptorII blocking antibody was used to preserve the cells. 100 μl of staining solution was used per 2x10^6 cells, and the cells were stained for 40 minutes at 4°C in the dark. Cells were then washed in 4°C cold FACS buffer and filtrated with MACS preseparation filters (Miltenyi) and subsequently sorted using an Aria or DiVa cell sorter into tubes containing 500 μl of medium. The tubes were pretreated by flushing the inner tube surface with 500 μl of heat-inactivated FCS to minimize binding of cells to the plastic surface. After sorting, cells were washed, counted, and seeded into fresh medium containing Gentamycin and the appropriate growth factors.

4.2.4 Cell Cycle Analysis by PI Staining

Cells were washed in PBS and resuspended at 1x10^6/100 μl in ice-cold PBS in a 2 ml conical tube. Samples were then put into a Eppendorf Thermomixer at 800 rpm set to 10°C. 900 μl ice-cold 70% Ethanol were added dropwise per 100μl of shaking cell suspension to minimize clumping, and the cells were then fixed for at least 24 hours to maximum 4 days at -20°C. After fixation, cells were resuspended in PBS for further sorage or directly used for staining. For the staining of DNA, a staining solution of 25 μg/ml PI, 0.05% Triton-X, 100μg/ml RNAse A in PBS was made from stock solutions of 5 mg/ml PI in H_2O, 10 % Triton-X in H_2O and 100 mg/ml DNAse free RNAse A (Qiagen). The fixed cells were washed once in PBS and then stained with 1 ml of staining solution per 1x10^6 cells at 37°C for half an hour. Cells were then washed 1x in PBS and subjected to FACS analysis. Doublets were excluded from the analysis by plotting forward scatter-area against -width as well as side scatter-area against -width and gating of the corresponding singlet populations. PI was recorded in linear mode, and DNA-histograms

were analysed manually or using the FlowJo Cell Cycle tool (using the Watson algorithm, see FlowJo software guide).

4.2.5 CFSE Staining of B Cells

CFSE (5-/6-carboxyfluorescein diacetate succinimidyl ester, CFDA-SE) can easily penetrate cell membranes due to its lipophilic nature and thus is readily taken up by cells. Once inside the cells, cellular esterases can remove the two acetate groups. The resulting carbocyfluorescein succinimidyl ester (CFSE), in contrast to its precursor CFDA-SE, is highly fluorescent, and has a reduced lipophilicity. This results in reduced emigration of the dye, which enhances the chance that CFSE is coupled covalently to amino grous of intracellular molecules through its highly reactive succinimidyl side chain. Once covalently bound to intracellular macromolecules, the dye resides in the cell and is transmitted to daughter cells, so that they then have around half of the fluorenscence than the parent cell. This allows to see single divisions of different cell populations.

A 5 mM stock solution was prepared by dissolving 1 mg CFSE in 359 μl DMSO (anhydrous). Aliquots of stock solutions were quickly frozen at -20°C in the dark. Cells were washed in PBS + 0.1% BSA prewarmed to 37°C, resuspended at 5x10^6/ml, and filtrated at 30 μl with MACS preseparation filters (only single cell suspensions are labelled uniformely). A freshly thawed or freshly prepared stock solution of CFSE was diluted 1:200 in PBS. 110 μl of the 50 μM stock solution were added to 1 ml of cells (2.5 μM end concentration), and mixed rapidly by vortexing at low speed. The cells were then incubated for 6-8 minutes in a 37°C waterbath. Thereafter, 10 vol of icecold cultivation medium (SF-IMDM + 2% FCS) were added to quench the CFSE, and the cells were incubated for 5 minutes on ice. Then, cells were washed twice with 10 ml medium, centrifuged at 260 g for 4 minutes at 4°C, and resupended in fresh medium.

In vitro cell cultures were set up under appropriate conditions to study division. After 3 to 4 days, cells were harvested in the dark, stained for surface markers, and analysed by FACS analysis. An unstained sample was also acquired to determine autofluorescence of cells. CFSE staining was measured in logarithmic mode in the FITC channel, and analysis was done using FlowJo software (Proliferation Platform tool).

4.3 Cell Culture

4.3.1 Cell Culture Media

For preparation of cell cultivation media and stock solutions for cell cultivation, only water doubly-distilled with the destamat quartz-distillery was used.

SF-IMDM Medium 1 can of IMDM powder w glutamine w/o $NaHCO_3$ (for 10 liters) was dissolved in 5 liters of H_2O in a 10 lt narrow-necked volumetric flask. Then, 30.24 g of $NaHCO_3$ were added and dissolved.100 ml of 10x Kanamycin solution was added together with 10 ml insulin sock solution (see section 4.3.2) while shaking. After that, 10 ml of 10 mM 2-mercapto-ethanol were prediluted in 30 ml H_2O, and then added and mixed. Finally, 30 ml of Primatone stock solution (10% w/v dissolved in water and filtered) was added to the medium, and water was added to approximately 10 liters. The pH was set to pH 7.0 +/- 0.05 using 10M NaOH, and the volume was adjusted to exaxtly 10 liters with H_2O after removing the magnetic stirring bar.

SF-MEM-Alpha Medium SF-MEM-alpha medium was prepared as described above for SF-IMDM medium, but with the following modifications:

1 can of alpha-MEM powder instead of 1 can of IMDM powder, 22 g of $NaHCO_3$ instead of 30.24 g, no insulin added.

Medium was stored in the fridge in the dark for up to 3 months.

4.3.2 Preparation of Insulin Stock Solution 5 mg/ml

1 g insulin from bovine pancreas was put into a glass cylinder containing approximately 180 ml ultrapure H_2O in little portions under constant stirring. As soon as the suspension was homogenous, NaOH (at first 10N, then 1M) was added dropwise (1-3 drops) until the insulin dissolved (approximately pH 11). Then the volume was adjusted to 200 ml with H_2O, and pH was adusted to pH 7.5 with HCl 32% (2-3 drops). Aliquots of 10 ml in 15 ml polystyrene tubes were stored at -20°C.

Final working concentration was 5 μg/ml.

4.3.3 Cultivation Conditions of Cell Lines

name	medium and cultivation
CHO-SCF	SF-IMDM + 2% FCS + 200μM L-Glutamine + 100μM pyruvate, 10% CO_2
Daudi	RPMI1640 + 10% FCS, 5% CO_2
Fld18Ber3	SF-IMDM + 2% FCS + 1% IL-7 supernatant, 10% CO_2
J-558L-IL-7	SF-IMDM + 2% FCS, 10% CO_2
Makn	SF-IMDM + 2% FCS + 1% IL-7 supernatant, 10% CO_2
OP9	SF-α-MEM + 2% FCS, 10% CO_2
ST2	SF-IMDM + 2% FCS, 10% CO_2
Phoenix Eco	RPMI-1640 + 10% FCS + 200 μM L-Glutamine, 5% CO_2
SP2/0-Flt3L	Opti-MEM + 10% FCS + 50μM beta-mercapto-ethanol
Plat-E	D-MEM + 10% FCS + 1 μg/ml puromycin + 10μg/ml blasticidin, 10% CO_2
X-63-IL-2	SF-IMDM + 2% FCS, 10% CO_2
X-63-IL-3	SF-IMDM + 2% FCS, 10% CO_2
X-63-IL-4	SF-IMDM + 2% FCS, 10% CO_2
X-63-IL-5	SF-IMDM + 2% FCS, 10% CO_2
X-63-IL-6	SF-IMDM + 2% FCS, 10% CO_2

4.3.4 Preparation of Cytokine Supernatants and Stock Solutions

The different cell lines were expanded in their appropriate medium, and the culture volume was increased stepwise up to 200 ml per 225 cm^2 dish. When the pH indicator in the culture medium turned from red to orange (lower pH) and approximately 10% of the cells where dead, the cellular debris and cells where centrifuged at 260 g for 5 minutes, and the supernatant was sterile filtered and frozen in aliquots.

Stock solutions of TPO were prepared in PBS containing 2% BSA at 10 μg/ml, were sterile filtered using 0.2 μm filters and were used at a concentration of 10 ng/ml. Stock solutions of IL-21 were set up in PBS + 0.5% BSA at 100 μg/ml. Stock solutions of BAFF were set up in PBS + 0.5% BSA at 10 μg/ml.

average yield of cytokines in the supernatants

IL-3	approx. 160 ng/ml	determined by ELISA
IL-7	approx. 300 ng/ml	determined by comparing growth curves of cells of the 5/7 cell line in recombinant mouse IL-7 and IL-7 supernatant at different concentrations of IL-7
Flt3L	approx. 500 ng/ml	determined by comparing growth curves of bone marrow cells. Total bone marrow cells grown for 4-7 days in SCF and Flt3L were cultivated with SCF and variing cocentrations of Flt3L to be tested at a concentration of $4x10^5$/20 ml in 96-well plates. As a standard, recombinant Flt3L was used.
SCF	approx. 10 ng/ml	determined using an SCF-dependent cell line, titrated against recombinant SCF

4.3.5 Cryopreservation of Cells

cells where freshly harvested, centrifuged at 260 g in a tabletop centrifuge, and resuspended in 1 ml freezing medium at a concentration of $1x10^6$ to $5x10^6$ cell per ml and transferred to 1.8 ml cryopreservation tubes. The cells where subsequently frozen for at least 24h at -80°C in styrofoam boxes before being transferred to liquid nitrogen.

4.3.6 Reactivation of Cryopreserved Cells

Frozen cells where thawed at 37°C until only a small rest of frozen material was left in the tube, then the cells were resuspended in 1 ml medium and transferred into a T75 flask containing feeder layers if necessary and 25 ml prewarmed medium supplemented with the adequate cytokines. On the next day, the medium was exchanged.

4.3.7 Counting of Living Cells by Trypan Blue Exclusion

Suspensions of cells were diluted 1:10 for high densities of cells or 1:2 for low densities of cells in Trypan Blue and counted using a Neubauer chamber. bright living cells of 2 to 4 4x4-squares were counted under a microscope and averaged. In spleen and BM suspensions, erythrocytes were excluded from counting by size.

4.3.8 Cultivation of PreB-I Cells

PreB-I cells were grown in 75 cm^2 tissue culture flasks with vented caps on an irradiated OP9 feeder layer in 25 ml SF-IMDM medium containing 2% FCS and 1% IL-7 supernatant in incubators containing 10% CO_2 at 37°C. The flasks were preseeded with OP9 feeder layer cells 0 to maximum 3 days before passage of preB-I cells at a cell number which ensured a density of $1x10^6$ cells per 75cm^2 flask at the day of sub-passage of the preB-I cells. The doubling time of OP9 feeder layer was expected to be 1 day. PreB-I cells were subpassaged every third to maximal fourth day onto new feeder layer cells, at the latest when they were 90% confluent. At the time of subpassage, preB-I cells were detached from the feeder layer by tapping on the rim of the flask for 5-7 times, then the supernatant was harvested in a 50 ml polypropylene tube, centrifuged at 260 g for 5 minutes at 4°C, and the cell pellet was resuspended in 5 ml medium, counted, and $5x10^5$ cells were subpassaged into a new 75 cm^2 flask containing the above mentioned medium and feeders.

4.3.9 Cultivation of Hematopoietic Progenitors from the Bone Marrow

Hematopoietic progenitors were cultivated on OP-9 feeder layer cells prepared as described in section 4.3.8 in SF-IMDM medium containing 2% pretested FCS. For cultivaton of very early mulitpotent progenitors, IL-3 (3%), IL-6 (3%), SCF (3-6%) and recombinant TPO (10 ng/ml) were used. For B cell specific precursors, Flt-3L (2%), SCF (3-6%) and IL-7 (1-1.5%) were used. Cells were seeded at 1x10^6 per T75 flask and cultivated for 3-4 days before subpassaging. Harvest and handling was done as described for preB-I cells.

4.3.10 Trypsinization of Adherent Cell Lines

The medium of maximally 90% confluent cells was removed, and 10 ml trypsin solution per 180 cm^2 dish (accordingly less for smaller dishes) were added. The plates were incubated for 5 minutes at 37°C. Then the cells were flushed from the plates with a pipette and transferred to a 50 ml conical tube. 5 ml medium per 10 ml trypsin was added to stop the reaction, and the tubes were centrifuged for 5 minutes at 260 g at 4°C. The supernatant was aspirated, and the cells were resuspended in 5 ml of fresh medium.

4.3.11 Cultivation of Adherent Cell Lines

Adherent cell lines were trypsinized as explained in section 4.3.10, and seeded onto new plates according the following table:

OP9 and ST-2	2x10^5 per 15 cm round dish
Phoenix-eco	1x10^6 cells per 15 cm round dish
Plat-E	1x10^6 cells per 15 cm round dish

4.3.12 Transient Transfection of Phoenix-Eco & Plat-E Cells

Cells were harvested and counted one day before transfection. Cells were seeded in Cellbind 6-well plates at 5x10^5 cells per well for Phoenix-eco cells and at 6e5 cells for plat-E cells. At the day of transfection, 10 μl of lipofectamine was pipetted into 100 μl D-MEM medium, and 2 μg of endofree DNA were mixed with 100 μl of D-MEM. The two solutions were mixed cautiously and incubated at RT for 20 minutes. Meanwhile, cells to be transfected were washed 2x with D-MEM, and then were covered with 800 μl D-MEM. Then the DNA-Lipofectamine complexes were pipetted onto the cells, the plate was shaked, and was subsequently incubated at 37°C and 10% CO_2 for 5.5h. After that, the complexes were aspirated, and the cells were covered with 2 ml fresh medium. After 12-24h, medium was exchanged again to remove incomplete virions. At the second and third day after transfection, virus-containing medium was harvested and directly used for transduction, or stored at 4°C for up to 5 days.

4.3.13 Retroviral Transduction of B-Cell Precursors

Cells to be transduced were harvested, counted, and set to a concentration of more than 4x10^6/ml. 2x10^5 cells per transduction were pipetted into a 2 ml conical tube, then viral supernatants (see section 4.3.12) were centrifuged for 5 minutes at 1157 g, and 1 ml thereof was added to each tube. According to the cell type, the necessary interleukins were added at the required concentration. The cells were then submitted to spin-infection for 3h at 1157 g at 30°C. After spin-infection, the supernatant was aspirated, and the cells were transferred to 6-well plates or 25 cm^2 dishes containing irradiated OP9 feeder layers. One day after transduction, the cells were selected with the appropriate selection agent if necessary.

4.3.14 Selection of Retrovirus-Containing Cells by Treatment with Antibiotics

Retrovirally transduced cells were replated on fresh irradiated OP9 feeder layers (resistant to the antibiotics to be used) in 6-well plates at a concentration of 2x10^5 per well in 3 ml medium containing the appropriate cytokines. One day after transduction, the appropriate selection agent was added. Necessary concentrations for selecting provirus-containing cells were titrated separately for each cell type. For FL preB-I cells, the following concentrations were used: hygromycin: 0.9-1 m/ml; puromycin: 1-2.5 μg/ml, histidinol: 1.25 mM, bleocin: 20 μg/ml. After 3-4 days, selection was normally completed (as determined by the death of untransduced control cells). Antibiotic-resistant OP9 feeders were generated by retroviral transduction or plasmid-transfection.

4.3.15 Limiting Dilution

Cells to be analysed by limiting dilution were washed 2x in SF-IMDM medium to get rid of remaining growth factors, counted by trypan blue exclusion (5 μl cells, 45 μl trypan blue), diluted to a concentration of 1x10^5 and recounted (10 μl cells, 10 μl trypan blue). Cells were then set to 1x10^4/ml (starting concentration: 1000 cells/well), or 5x10^3/ml (starting concentration: 500 cells/well). The twofold counting of the cells should decrease counting errors in the beginning, since counting of cell numbers below 1x10^4/ml is not feasible any more using a Neubauer chamber. Cells were prepared in 50 cm polypropylene tubes and serially diluted 1:3 down to less than 1 cell per 100 μl in 50 ml conical tubes. Cells were then aliquoted into 96-well flat bottom plates, 100 μl into each well. For each dilution, at least 16 wells, but usually 32 wells were used. Then, 100 μl medium containing twofold concentrated growth factors and/or doxycycline were added to each well. The plates were then sealed to minimize evaporation of medium of the outer wells and incubated for 6-7 days under the appropriate conditions.

To calculate the frequency of growing cells, wells with no growing clones were counted. Clones were defined as colonies containing more than 20 living clustered cells. According to Poisson, the frequency F_0 of wells actually containing no growing cell in a suspension of cells of theoretically m dividing cells per well is calculated as follows: $F_0 = e^{-m}$. Consequentially, in a theoretical suspension of 1 cell per well ($m = 1$), we get $F_0 = e^{-1} = 0.368$. So, at a percentage of 37% negative wells, we can assume that an average of 1 cell per well has started to grow. To extrapolate that point, the log of the fraction of negative cultures was plotted as a function of cell number per well, and the number of growing cells per well was calculated from the equation derived from the linear regression of the resuling data. For a given linear regression of $y = mx + b$, the number x of cells needed for one to grow out was calculated as follows: $x = (ln(37) - b)/m$.

4.3.16 *In vitro* Differentiation of B-Cell Progenitors

Fetal liver preB-I cells and bone marrow derived preB-I cells grown *in vitro* were washed 2x and resuspended in SF-IMDM medium with 2% FCS. After counting, cells were seeded at a concentration of 5x10^5 to 1x10^6 per 6-well dish in 3 ml SF-IMDM medium containing 2% FCS and optionally 1.5% IL-5 supernatant, 1.5% IL-4-supernatant, and 4 μg/ml 0.2 μ filtered αCD40 antibody (clone FGK-45). Cultures were incubated for 3 to 6 days and analysed by FACS for the surface markers IgM, IgD, MHCII, c-kit, CD25, CD93, CD43 and CD23.

4.3.17 Preparation of Thymic T-Cells for Co-Transplantation with B-Cells

C57Bl/6 mice were killed by cervical dislocation, sprayed with 70% ethanol, and pinned on styrofoam plates covered with aluminium foil. The thorax was opened at the sternum. The ribs were cut at the base of the sternum, and the sternum was lifted up carefully to expose the thymus. Both lobes of the thymus were excised and put into cold IMDM-medium. The thymus was crushed between two sterile frosted glass slides until a homogenous cell suspension was obtained. After 1x washing of the cells in 20 ml SF-IMDM medium, cells were resupended in 10 ml medium and counted.

Residual CD19+ cells were removed from the thymic cell suspension by MACS-bead depletion (Miltenyi), see section 4.3.18. Per mouse to be transplanted, 1-2x10^6 cells were used for CD19-magnetic-bead coupling and subsequent B-cell depletion.

For transplantation, T cells were mixed with preB-I cells, centrifuged, and resuspended at a concentration of 2-3x10^5 thymic cells and 5x10^6 B cells per transplantation volume of 100 μl per mouse as described in section 4.4.3.

4.3.18 Depletion of CD19$^+$ B-Cells with Miltenyi MACS Beads

For 1x10^7 cells, cells were centrifuged and resuspended in 90 μl FACS buffer (2% FCS in PBS, sterile). After addition of 10 μl of mouse CD19-MACS-microbeads (Miltenyi), the cell suspension was incubated for 15-30 minutes at 7°C (not on ice). The stained cells were washed 1x with 2 ml FACS buffer and resuspended at 5x10^7 cells per 500 μl. After that, the suspension was filtered through a MACS preseparation filter to exclude clumps. Meanwhile, a MACS LD-column precooled in the fridge was put onto the magnet in a laminar flow hood and was prewetted with 2 ml of FACS buffer. Then, a fresh 15 ml conical tube was put under the column, and the cells were loaded onto the column. After the suspension had passed through, the column was washed with 2 ml medium (at least 2 ml, also if less than 1x10^7 cells were taken), and after passage of residual medium, the depleted cells were counted and stored on ice.

4.3.19 Enrichment of *ex vivo* CD19$^+$ B-Cells using Miltenyi MACS Beads

The MS MACS columns were precooled in the fridge for at least 15 minutes. Meanwhile, splenic, bone marrow, and peritoneal cells were washed 1x with 4°C cold MACS buffer (PBS, 0.5% BSA, 2 mM EDTA) and were resuspended at 1x10^7 cells per 90 μl cold MACS buffer. Per 90 μl cell solution, 10 μl α-mouse CD19-beads were added. After mixing, the cells were incubated in the fridge (not on ice) for 15-30 minutes. Then, the cells were centrifuged at 260 g for 5 minutes and washed 1x with 2 ml MACS buffer per 1x10^7 cells. The cells were then resuspended at 5x10^7 cells per 500 μl and filtered using a MACS preseparation filter. The precooled MS column, which is usable for up to 2x10^8 total cells and up to 1x10^7 labelled cells, was fixed on the appropriate magnet and washed 1x with 500 μl MACS buffer. The suspension was then loaded onto the MS column, and flow-through was collected in a tube (unlabeled cells). The column was then washed 3x with 500 μl cold MACS buffer. After that, the tube containing the unlabeled fraction was removed, and a new sterile tube was prepared. The column was removed from the magnet, and 1 ml MACS buffer was added. Then the plunger (included in the kit) was inserted into the column and the solution containing labeled cells was quickly pushed into the sterile tube. The cells were washed 1x in medium, counted, and used for experiments.

4.4 Animal Work

4.4.1 Preparation of Bone Marrow Progenitor Cells

6 to 14 weeks old animals were killed by cervical dislocation and sprayed with ethanol. On a sterile pad coated with aluminium foil, both femora and tibiae were dissected, and the end joint of each bone was removed. The bones were flushed with a syringe containing ice-cold IMDM-medium. The cells were suspended by pipetting, centrifuged at 260 g for 5 minutes at 4°C, washed 1x in IMDM-medium, resuspended in 10 ml SF-IMDM medium containing irradiated OP9 feeder layers, 2% FCS, and counted. $4x10^6$ cells were seeded into a 75 cm^2 flask in 25 ml SF-IMDM medium containing 2% FCS supplemented with the appropriate cytokines.

4.4.2 Cell Preparation of Spleen and Peritoneal Cavity

Mice were killed by cervical dislocation, sprayed with 70% ethanol, and pinned on styrofoam plates covered with aluminium foil. The skin on the abdomen was cut with a longitudinal section without touching the abdominal membrane.

Peritoneal lavage was done with a plastic pipette. A little hole was cut into the abdominal membrane, and a pipette filled with 5 ml of cold SF-IMDM medium was inserted into the hole. Medium was pipetted up and down several times before the cell suspension was transferred to 50 ml conical tubes. This procedure was repeated 3 times. The cells were washed once with 5 ml of medium, resuspended in 500 μl of medium and counted.

For preparation of the spleen, the abdominal membrane was cut, and the spleen was dissected and directly transferred into 5 ml of ice-cold SF-IMDM medium. The spleen was submerged in a petri dish in 10 ml of medium, cut twice with a scalpel and crushed between two frosted glass slides until a homogenous cell suspension was obtained. The cells were washed 1x with 20 ml SF-IMDM medium. Cells were then resuspended in 50 μl medium to minimize clumping and then incubated in 1 ml 4°C cold erythrocyte lysis buffer for 3 minutes on ice. After incubation, 10 ml of medium were added to stop the reaction. The cells were then centrifuged at 260 g for 5 minutes at 4°C, resuspended in 4-5 ml medium and counted.

4.4.3 Transplantation of PreB-I Cells into the Tail Vein of Mice

Mice to be transplanted were γ-irradiated sublethally 1 day before transplantation with 4 Gy. Cells were harvested as explained in section 4.3.8. After 1x washing in 20 ml 4°C cold IMDM medium without serum and phenol red, the cells were resupended at a density of $5x10^7$ per ml in the same medium and filtered with a 30 μm MACS preseparation filter. The suspension was subsequently stored on ice. Mice to be transplanted were heated with an infrared lamp for 3 minutes. Then 100 μl of filtered cell suspension were transplanted into the tail vein after disinfection of the site of puncture. After that, mice were observed on a daily basis during the first week, and thereafter every third day. The experimental setup was approved by the LAGeSO under the permission number G0099/08.

4.4.4 Immunisation of Mice

Antigen (KLH) was dissolved in PBS at a concentration of 25 mg/ml.

For immunisation with IFA adjuvant, KLH stock solution was diluted to 1 mg/ml in PBS. This solution was mixed 1:1 with IFA as described later. Per mouse and shot, 100 μl of immunisation agent were needed (=50 μg KLH). Since during the process of emulsification the amount of liquid is halved, for

10 mice 1 ml KLH in PBS and 1 ml IFA were used. The emulsion was prepared in 2 ml conical tubes. Maximum 500 μl of KLH in PBS was drawn up in a 1 ml syringe (Braun, Injekt-F, no rubber parts) charged with a 18 gauge needle and injected quickly into the same amount of IFA. The two solutions were emulsified by pipetting up and down until the solution turned into a sticky non-flowing white mass. The emulsion was subsequently drawn up into a new 1 ml syringe using the 18 gauge needle. The needle was then exchanged to a 26 gauge needle (0.45x12 mm) for immunisation. Mice were immunized into the base of the tail vein or IP with 100 μl of emulsion. Immunized mice were examined daily during the next week for signs of indisposition.

For immunisation with TiterMax Gold adjuvant, the general procedure was done as for IFA, with the exception that the initial emulsification started with 250 μl of 2 mg/ml KLH in PBS which was pushed into 500 μl of Titermax Gold until an emulsion had formed, and then the other half of KLH was mixed with the emulsion. 50 μl thereof (50 μg KLH) were injected subcutaneously into the base of the tail or into the neck fold. The experimental setup was approved by the LAGeSO under the permission number G0099/08, "Erweiterungsantrag".

4.4.5 Bleeding of Mice

Mice were warmed under an infrared lamp for 2-3 minutes in order to dilate the tail veins. Mice were then immobilised in a plastic container with an indentation for the tail. The tail was disinfected with Skinman soft disinfection solution, and a small cut was made to incise the basal tail vein. Blood was collected into a 1.5 ml conical tube. For ELISA, around 50-200 μl of blood were collected. Blood flow was then stopped by pressing a sterile swab onto the incision. Collected blood was incubated at RT for 4 hours to coagulate, then the blood samples were centrifuged at 1700 g for 15 minutes at 4°C. Serum was then collected in a 0.5 ml conical tube and frozen at -20°C. The experimental setup was approved by the LAGeSo with the permission number G0099/08.

4.4.6 Feeding of Doxycycline in the Drinking Water

Doxycycline hyclate was dissolved in water at a concentration of 0.2 g/l, and the pH was set to 3 with HCl. Then, 10 g sucrose were added per liter, and the solution was sterile filtered at 0.2 μm. Bottles were protected from light by covering with aluminium foil to preseve the light-sensitive doxycycline. Every 3-7 days, the bottles were exchanged. Feeding of doxycycline was approved by the LAGeSo under the permission number G0099/08.

5 Results

5.1 Vector Design

The aim of the experimental approach was to allow inducible overexpression of proto-oncogenes in murine B-cells to be quick and stable, yet tightly controlled to ensure that the uninduced cells behave as normally as possible. Two inducible gene expression systems were tested to conditionally express the two proto-oncogenes, *Pim1* and c-Myc (Myc): the estrogen receptor-system (ERt system) [84], and the TetON system. In the estrogen receptor-system, the proto-oncogene to be overexpressed is fused to a mutated ligand binding domain (ERt) of the estrogen receptor which can only bind to the antagonistic estrogen-analog 4-OH tamoxifen. This ERt domain is normally bound to proteins of the HSP90 family and, thereby, is kept back in the cytoplasm. Only after encountering tamoxifen, the fusion protein is able to enter the nucleus after a conformational change of the estrogen receptor part and subsequent release of the HSP90 anchor. This system has the advantage, that only 1 vector per transgene has to be inserted into the target cells. The drawback of the ERt system is, that it only works for proteins that are active solely in the nucleus, since the fusion protein is constitutively expressed and present in the cytoplasm. Furthermore, fusion proteins do not always function as expected due to conformational changes or hiding of active sites/domains by the ERt domain.

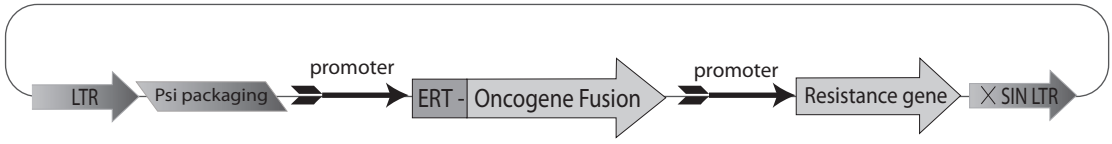

Figure 5.1 – ERt vector containing the retroviral elements needed for efficient packaging of RNA into virions and viral integration into host cells (grey), a constitutively active promoter driving the expression of the ERt-fusion protein (red), and a second promoter driving a resistance gene conferring resistance against puroMycin or hygroMycin to transduced host cells.

The fusion protein consisting of the Myc protein with the C-terminally attached ERt domain was inserted into a retroviral self-inactivating (SIN) vector based on the MoMulV virus (figure 5.1). Retroviral transduction of genes is the only way to introduce transgenes into B lineage cells, since homologous recombination of DNA vectors only works in stem cells, and transfection is only transient.

SIN vectors have a 3'LTR (long terminal repeat) which lacks the main part of the so called U3 region. This U3 region normally encompasses the viral promoter and enhancer (figure 5.2). During incorporation of the retroviral DNA into the host genome, the U3- and R-regions of the 3'LTR replace those of the 5'LTR (figure 5.3). Hence, during integration of SIN vectors into the host genome, the functional 5'LTR promoter is replaced by the incomplete 3'LTR which has no promoter functionality anymore. Removal of the viral promoters and enhancers on both LTRs prevents constitutive expression of the virally transfected genes of interest by the viral promoters. In addition, cellular (onco-)genes in the vicinity of the integration site of the retrovirus cannot be activated anymore by viral promoters or enhancers.

The "AATAAA" sequence at the end of the R region within the 3'LTR is used as a weak poly A signal by the cellular transcription machinery. Stronger poly A sequences inside the retroviral backbone inhibit transcription of complete viral genomic RNA and therefore inhibit production of complete virions necessary to infect and transduce target cells.

As a target for transduction, fetal liver preB-I-cells were chosen, since these cells can easily be main-

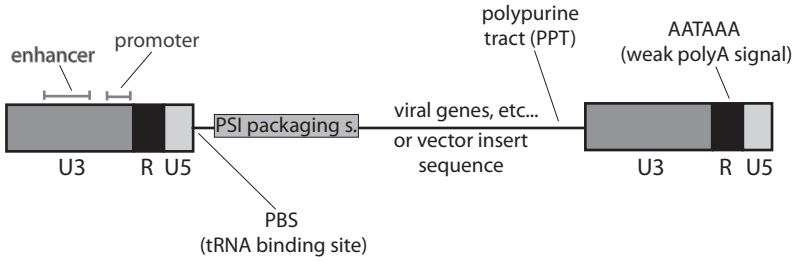

Figure 5.2 – Important elements in retroviral vectors. The LTRs are necessary for transcription of RNA and integration of viral DNA into host cells. The PSI packaging signal is necessary to efficiently pack complete viral RNA into virions. During mRNA-processing, this signal is removed by splicing. This mechanism prevents incomplete, spliced RNA to be packed into virions. PBS: the start of reverse transcription. This sequence is partially complementary to certain tRNAs which serve as a template. The PPT is only slowly degraded by RNAse H during reverse transcription and therefore serves as a primer for the generation of plus-strand DNA. LTR: long terminal repeat, U3: unique 3'LTR RNA, U5: unique 5'LTR RNA. (adapted from an illustration in the book "Retroviruses" by J.M. Coffin et al, 1997, p439)

tained *in vitro* in the presence of bone marrow derived stromal feeder layers (OP9) when supplemented with IL-7. Stably transduced cells were selected for the presence of genes conferring resistance to antibiotics such as puroMycin, hygroMycin or histidinol. Only dividing cells, in our case fetal liver preB-I-cells, can be transduced efficiently with retroviruses. Hence, normal resting B-cells are resistant to retroviral transduction. Retrovirally transduced resting mature B-cells were obtained by *in vitro* or *in vivo* maturation of stably transduced preB-I-cells. Thus, the effects of Pim1 and Myc overexpression could be studied in immature as well as mature stages of B-cells.

Initial tests with preB-I-cell lines transduced with ERt-*Myc* and ERt-*Pim1* vectors showed a high background activity of this system in the analysed B-cells (data not shown). Therefore, the alternative TetOn vector system was developed.

The Tet expression systems require two core components:

1. a regulatory fusion protein containing a viral transactivator domain fused to parts of the bacterial tet repressor, thereafter named tTA or r(t)TA. The transactivation domain is derived from the vp16 protein of *Herpes simplex* virus, and the tet repressor component is taken from the tet repressor protein of *E. coli*.

2. a modified, tetracycline- or doxycycline-responsive promoter consisting of a minimal, inactive CMV (Cytomegalovirus) promoter preceeded by a set of seven copies of the short tet resistance operator sequence of Tn10 transposon (TetO) called TRE (tetracycline-response element) [105], hereafter named ΔCMV promoter. This promoter only drives expression of the gene after the rtTA has bound to its TetO sequence.

In the TetOff system, the tTA can bind the TRE element in the absence of tetracycline and hereby brings the transactivator component of the tTA in close proximity to the minimal CMV promoter, which acitvates transcription of the appropriate transgene. Addition of tetracycline (or doxycycline) leads to a conformational change of the TetO-sequences of the tTA and therefore inhibits further binding of the tTA to the TRE element of the ΔCMV promoter. Upon dissociation from the TRE, the transactivator cannot activate transcription of the transgene anymore. The major drawback of the TetOff system is the need to supply tetracycline over a prolonged period of time if transgene expression is not desired. On the

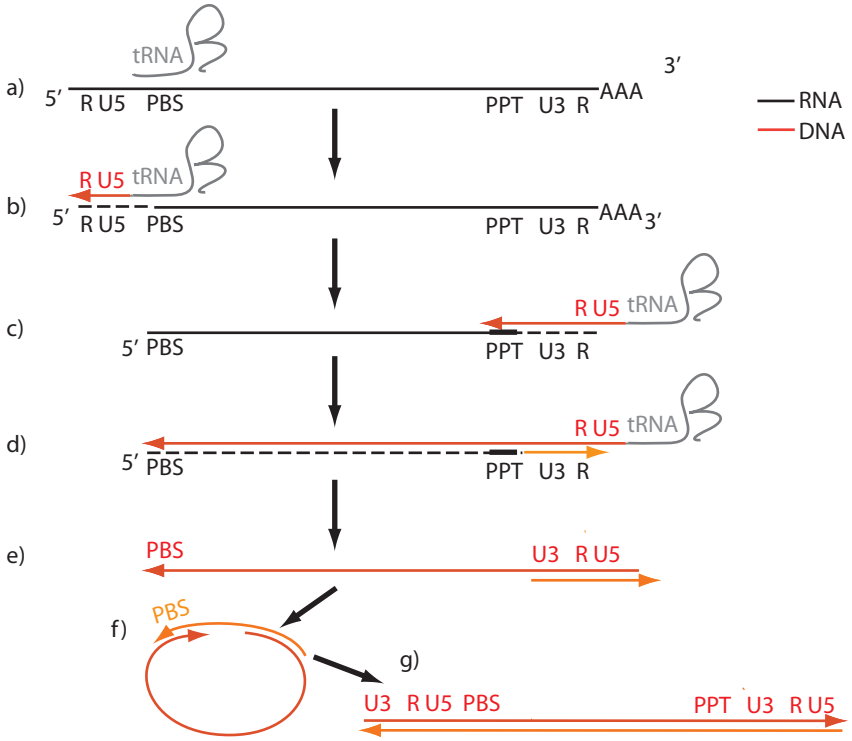

Figure 5.3 – Process of reverse transcription of the retroviral genome. ***a:*** *minus-strand DNA synthesis is initiated using the 3'end of a partially unwound tRNA which is annealed to the primer-binding site (PBS). (b) DNA synthesis is mediated by the reverse transcriptase. (c) RNAse H digests the 5'end of the RNA/DNA duplex. Thereafter, first strand transfer causes the newly formed ssDNA to be annealed to the 3'end of the viral genomic RNA. This is mediated by the identical R sequences at both ends of the RNA genome. (d) minus-strand DNA synthesis resumes, and the RNA template is subsequently degraded by RNAse H. Degradation of RNA is slowed down at the PPT (polypurine tract). This "RNA-primer" serves as a starting point for plus strand DNA synthesis.* ***e,f:*** *the newly formed partially double-stranded DNA anneals at the complementary PBS segments in the plus- and minus-strand DNA, and lacking portions of plus- and minus-strand DNA are generated, each of them serving as templates for the other strand (adapted from an illustration in the book "Retroviruses" by J.M. Coffin et al, 1997, p123).*

other hand, regulation of transgene expression is very tightly regulable and highly inducible.

The TetOn system, on the other hand, includes an additionally mutated tTA called rtTA or reverse transactivator (figure 5.4). This protein cannot bind tetracycline anymore but only recognizes doxycycline. The rtTA can only bind to the ΔCMV promoter if doxycyline is present. Hence, doxycyline only needs to be added if transgene expression is desired. A drawback of this system is, that it might not be quite as tightly turned off in the absence of the inducer as the TetOff system and does not yield expression levels as high as the TetOff system [94]. Anyhow, since we did not want to treat cell cultures and mice with antibiotics for longer periods of time to inhibit transgene overexpression, the TetON system was chosen.

The first attempts to build a TetON vector system were done with the standard rTA vector derived from the pTetOn plasmid (Invitrogen). This rTA is known to have some residual background activity in the absence of doxycycline and is unstably expressed in some eukaryotic cells due to a bad codon usage and splicing activities inside the sequence. Due to these problems, the final vectors were built with the enhanced and mutated rtTA-M2 reverse transactivator designed in H. Bujard's Lab. This codon-optimized version is devoid of splice donor/acceptor sites and contains mutations which reduce background

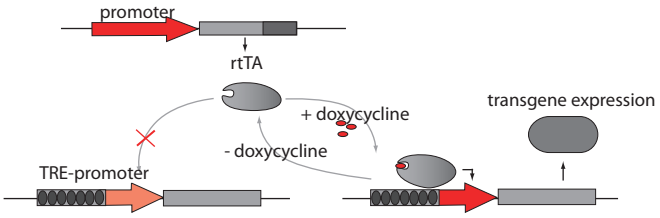

Figure 5.4 – Mode of operation of the TetON system: the reverse transactivator part (rtTA) is transcribed from a constitutively active promoter. In the absence of doxycycline, the rtTA cannot bind the TRE element within the inducible promoter. Hence, the transactivator cannot activate transcription of the inducible transgene. If doxycycline is added, the rtTA can bind the TRE element and subsequently transactivates transcription of the inducible transgene. The reaction is reversible.

transactivation of the rtTA [132].

rtTA-TetON vector

The MoMulV-based self-inactivating vector pSuperRetroPuro was cut with XhoI and ClaI and was subsequently dephosphorylated using calf intestinal phosphatase (CIAP). A linker with sticky ends fitting

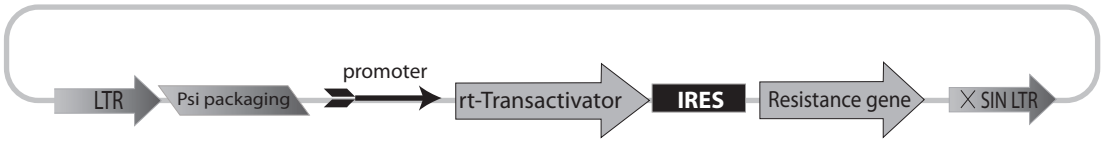

Figure 5.5 – rtTA-TetOn-vector

onto the sticky ends of the above backbone was created by annealing two complementary oligonucleotides (m068, m074). The linker contained the restriction endonuclease recognition sites EcoRI, NruI, ClaI, HpaI, HincII, BamHI and MluI. The linker ligated to the backbone resulted in the plasmid pSR-L. pSR-L was cut with XhoI and EcoRI, and the pgk-promoter amplified by PCR from pSuperRetroPuro with primers m136 & m137 was inserted into pSR-L to result in pSRL-P. Then, the reverse transactivator rtTA-M2 (kind gift from Hermann Bujard, University of Heidelberg) was cut out from its origin plasmid pUHD15-1 4535 with EcoRI and BamHI and cloned into pSRL-P via the EcoRI and BamHI sites. After that, the IRES sequence (=intra-ribosomal entry site) was cut out from the construct pSRL5P Vpmut IRES nsEGFP kindly provided by Dr. Ozan Guezelbey (former Lab member) with BamHI and NcoI. The histidinol resistance gene amplified from the vector pSV2 His using the primers m095 and m147 was cloned into the TOPO-TA vector and sequenced. The histidinol resistance gene was subsequently cut with BspHI (compatible with NcoI) and BamHI. Both the IRES sequence and the histidinol resistance gene were ligated in one step into the BamHI site on the pSRL-P plasmid, which resulted in the plasmid pSRL-P-rtTAM2-His, thereafter called rtTA-TetOn, see figure 5.5.

pSR-TREtight vector For the transgene expression vector, the pSR-L vector mentioned above was used as backbone. The resistance cassette containing a pgk-promoter and puromcycin resistance was amplified by PCR with the primers m069 and m070, and cloned into p-SR-L via the MluI site (=pSR-LP). For the inducible cassette, the P_{tight} promoter from the pTRE-Tight vector was used. P_{tight} contains a modified Tet response element (TRE_{mod}), which consists of seven direct repeats of a 36bp sequence which contains the 19bp tet operator sequence. This ΔCMVtight promoter has even less background activity than the normal ΔCMV promoter. In order to exclude that a possible residual background activity of

the 5'LTR promoter could lead to unwanted expression of the transgene in the absence of doxycycline, the cassette consisting of the ΔCMVtight promoter and the transgene was put into the vector in reverse orientation (figure 5.6). The TREtight cassette consisting of the TREtight promoter and a multiple cloning site was cut out with XhoI and NheI and subsequently cloned into pSR-LP via XhoI and AvrII, which is compatible to NheI.

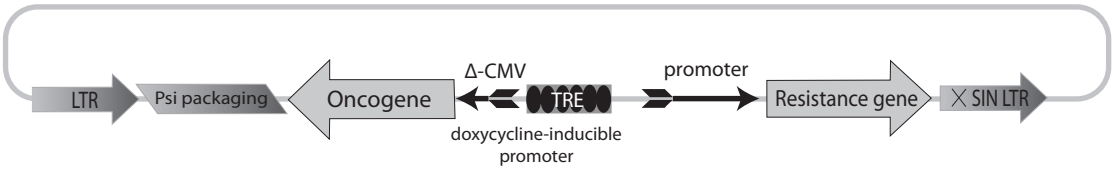

Figure 5.6 – Retroviral SIN vector carrying Pim1, Myc or egfp under the control of the doxycycline-inducible TetON promoter as well as a resistance gene conferring resistance to hygroMycin or puroMycin with its own eucaryotic promoter (promoter of phosphoglycerate kinase (pgk)). The cassette containing the doxycycline-inducible gene was cloned into the retroviral backbone in reverse orientation to inhibit activation of the transgene in the absence of doxycycline by possible residual promoter activity of the SIN-retrovirus.

Myc was amplified by PCR with the primers m101 and m102 from the plasmid pBJ4-cMyc and subsequently cloned into pSR-LP-treTight via BamHI and HindIII sites. The *Myc* gene chosen is a hybrid of the mouse and human *Myc*, as shown in figure 5.7. Regions underlined in green, i.e. the MBI and MBII, are taken from the mouse Myc, and the rest is taken from the human variant. The differences between mouse and human Myc are minimal and are not assumed to result in differential functions. Furthermore, murine Myc can even be replaced by the Myc-family member N-Myc, which results in normal development, normal lymphocyte compartments and normal B lymphocyte activation compared to WT mice [85]. The advantage of a hybrid transgenic *Myc* is, that RNA levels of only the endogenous *Myc*, of only the transgenic *Myc*, or of total *Myc* can be measured just by selecting the according primers for RT-PCR. Pim1 was amplified by PCR from the vector *Pim1*-CS41 using the primers m099 and m100 and subsequently cloned into pSR-LP-TreTight via BglII and HindIII sites. *Egfp* was amplified by PCR from the vector pSRL5P Vpmut IRES nsEGFP with the primers m122 and m123 and subsequently cloned into pSR-LP-treTight via BamHI and HindIII sites.

5.2 Confirmation of Inducible Transcription and Translation of Transgenes

5.2.1 Detection of EGFP Protein in TetOn-*egfp* Transgenic PreB-I Cells by FACS

In order to confirm the functionality of the vectors carrying the different components of the TetON system, the FLd18Ber3 cell line was stably transduced with the rtTA-TetOn vector (fig. 5.5) and the TetOn-*egfp* vector (fig. 5.6) and selected for cells carrying both vectors by adding the respective antibiotics.

To titrate the amount necessary to induce overexpression of transgenes, cells were seeded in 6-well plates onto OP9 feeder layers in the presence of doxycycline at different concentrations ranging from 20 ng/ml up to 5 μg/ml. After 24 hours, cells were harvested and subjected to FACS analysis. Upregulation of EGFP expression was already detected at 20 ng/ml, but reached steady state levels at around 1 μg/ml (figure 5.8a). To minimize effects of longterm doxycyline treatment on preB-I-cells, this concentration of doxycyline was taken for induction of transgenes in the following experiments.

To assess the speed of transgene upregulation, 1 μg/ml doxycycline was added to the preB-I-cells

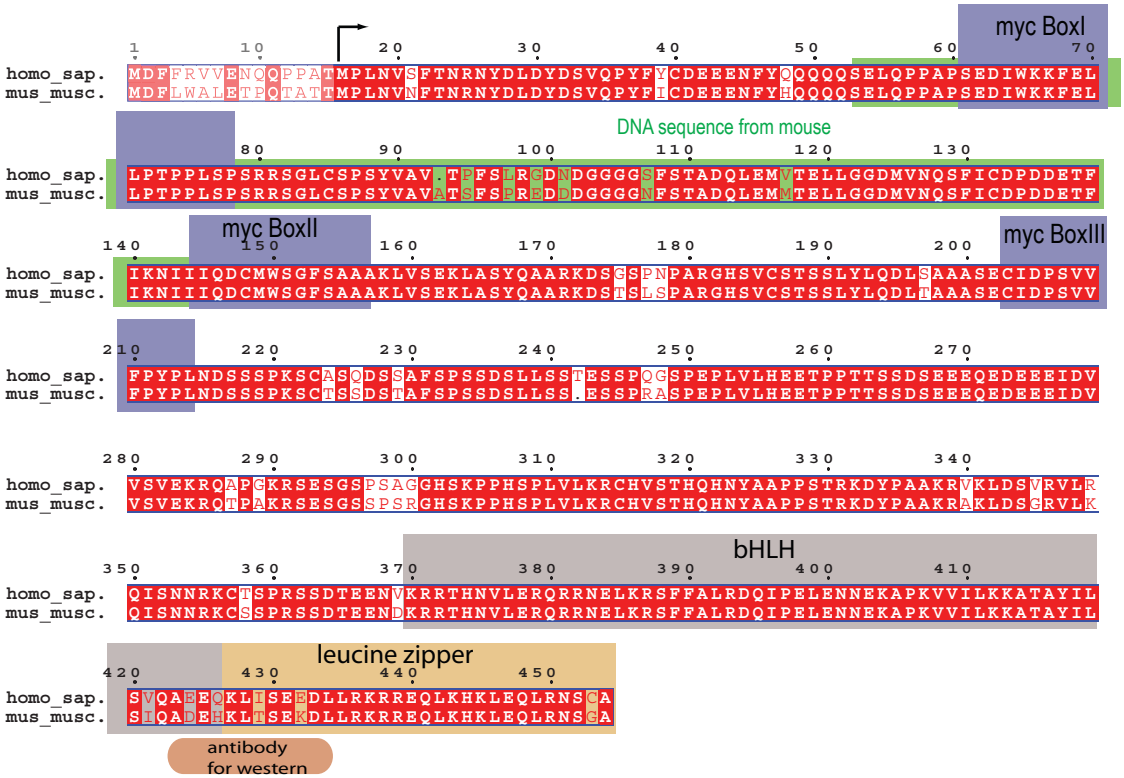

Figure 5.7 – Protein sequence of the hybrid Myc consisting of murine (green) and human sequences. Myc boxes I-III are highly conserved sequences within the Myc family. bHLH: basic helix-loop-helix domain (DNA-binding), leucine zipper: dimerisation domain. The first 15 base pairs are only present in the longer Myc version, which is not used in this work.

harbouring the rtTA-TetOn and TetOn-egfp vectors. At different time points, cells were harvested and analysed by FACS for EGFP expression. EGFP expression was already detected 2 hours after activation of the TetOn-*egfp* gene and subsequently rose to steady state levels during the following 18 hours (see picture 5.8b).

Transgene downregulation after removal of doxycycline was assessed using cells carrying the TetON-*egfp*. Cells were supplemented with doxycycline for 3 days. Then, doxycycline was removed, and at different timepoints after removal of doxycycline (0-9 hours, 72 hours), expression of EGFP was measured by FACS analysis (figure 5.8c). EGFP expression declined sharply 3 hours after removal of doxycycline. Thereafter, EGFP fluorescence further decreased more slowly. After 3 days, no EGFP protein was detectable anymore.

Though the half lifes of different proteins are variable, the fact that the expression of a relatively stable protein such as EGFP is partially downregulated after only 3 hours and completely abolished after 3 days allows the conclusion that the TetON system in general allows relatively fast downregulation of *de novo* protein production. Since Myc protein and mRNA are quite instable, downregulation of Myc protein might be even faster.

In summary, doxycycline concentrations needed for maximal transgene expression were at least 1 μg/ml. Transgene proteins were detected already after 2 hours, and maximal levels of protein were only reached after 24 hours.

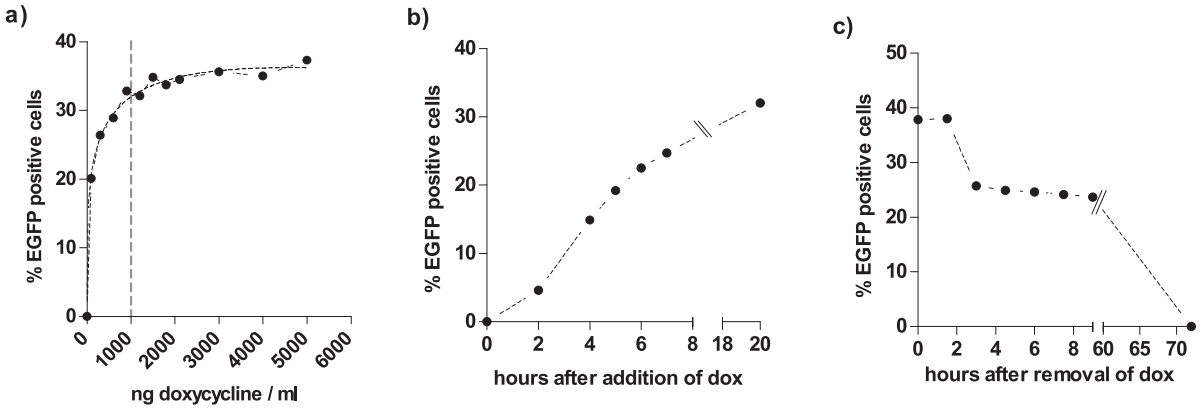

Figure 5.8 – Testing the TetOn system using a preB-I-cell line carrying the TetOn-egfp vector. ***a:*** *Titration of doxycyline. Cells were induced for 24 hours with 1 µg/ml doxycycline before FACS analysis. Dotted grey line: concentration chosen for subsequent exeriments. (b) EGFP overexpression after start of doxycycline treatment (1 µg/ml) with time after induction. (c) Decline of EGFP expression after removal of doxycycline.*

5.2.2 Detection of Upregulated *Myc* and *Pim1* mRNA by RT-PCR

For detection of *Myc* mRNA, the FLd18Ber3 cell line transduced with the rtTA-TetOn vector and the TetOn-*Myc* vector was cultured in the presence or absence of 1 µg/ml doxycycline. At different time points after activation of *Myc*, total RNA was extracted and subjected to RT-PCR. mRNA of the transgenic *Myc* was already detected after 30 minutes and subsequently rose during the following 18 hours (figure 5.9a).

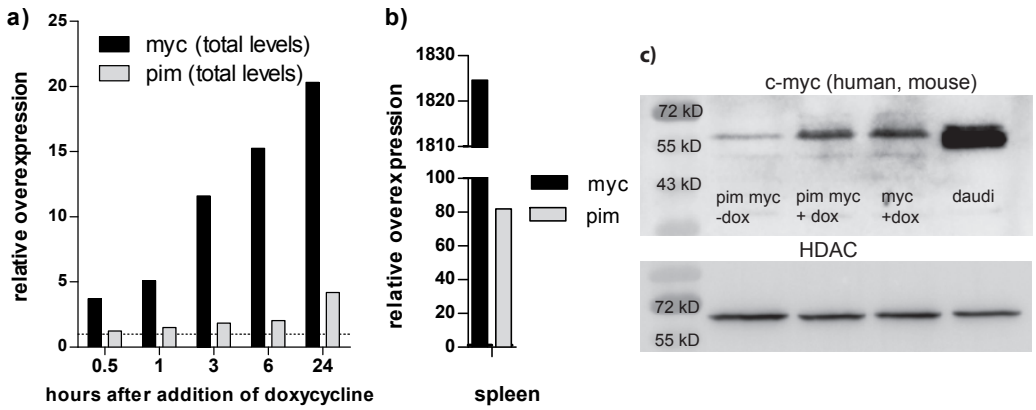

Figure 5.9 – Upregulation of mRNA of Pim1 and Myc after addition of doxycyline. ***a:*** *RT-PCR of preB-I-cells harvested at different time points after transgene activation. (b) Inducibility of transgene overexpression in transplanted B-cells harvested from spleen 5 after transplantation. (1 mouse). Expression levels are shown as relative values compared to uninduced cells, which are set to one. (c) Analysis of Myc protein overexpression by SDS PAGE and Western blotting. Myc protein is readily upregulated after addition of doxycycline in both preB-I-cells carrying only a transgenic Myc gene as well as in preB-I-cells carrying transgenic Myc and Pim1 genes together. The human Daudi cell line is derived from a Burkitt's lymphoma patient and is frequently used as a positive control for Myc expression.*

B-cells carrying a TetOn-*Myc* and -*Pim1* could still upregulate both transgenes 5 months after transplantation into RAG1$^{-/-}$ mice. To show this, 5x10^6 cells of the FLd18Ber3 cell line carrying the rtTA and a Tet-ON-*Pim1* and -*Myc* were transplanted into irradiated RAG1$^{-/-}$ mice. Five months later, splenic and the bone marrow cells were extracted, CD19$^+$ cells were sorted using MACS beads and cultured *in-vitro* in SF-IMDM medium containing 2% FCS and 1% conditioned medium containing IL5 for enhanced survival in the presence or absence of 1 μg/ml doxycyline. After 3 days, total RNA was extracted, and upregulation of *Myc* and *Pim1* mRNA in the doxycycline-treated cultures was confirmed by RT-PCR (figure 5.9).

5.2.3 Detection of Transgenic Myc by Western Blot

Using Western blot analysis, we confirmed induction of Myc protein after addition of doxycycline. FLd18Ber3 preB-I-cells carrying the TetOn *Myc* and *Pim1* were cultured in the presence or absence of doxycycline for 3 days. 3x10^7 cells of each culture were lysed in 300 μl Ripa buffer containing protease inhibitors, and 100 μg of total protein per lane were used as described in section 4.1.18. As a positive control, 7x10^6 cells of the human DAUDI cell line known to overexpress Myc at very high levels were lysed in 300 μl Ripa buffer and subjected to SDS page. As a loading control, antibodies against HDAC1 were used on a parallel blot. The anti-Myc antibody used detects human Myc and, to a lesser extent, murine Myc.

These experiments show that Myc protein overexpression is detected in doxycycline-treated cells, though at much lower levels than in the DAUDI cell line (figure 5.9c).

5.2.4 Degree of Leakiness of the TetOn Vector-Mediated Gene Expression

Expression of genes which are controlled by the TetOn promoter should only be detected in the presence of doxycycline. Though, it is known that the transcriptional control of the TetON system is not 100% tight. Hence, background expression of genes integrated by the TetON vector into the host genome in the absence of doxycycline was assessed as follows: Fetal liver preB-I-cells carrying the rtTA-TetOn and the TetOn-tight *Myc+Pim1* vectors as a negative control for EGFP expression and cells carrying the rtTA-TetOn and the TetOn-tight-egfp vectors were compared for EGFP-expression in the absence and presence of doxycycline. As expected, the cells carrying integrated *Pim1* and *Myc* genes had no background EGFP-expression in the absence of doxycycline. By contrast, 3-5% of the cells carrying the integrated *Egfp* showed EGFP fluorescence intensities above the treshhold defined by the EGFP fluorescence of the negative control (figure 5.10a). Though, there was only a very slight shift of the EGFP fluorescence intensity in the presence of the EGFP vector. In the presence of doxycycline, about 35-50% of the cells carrying egfp showed EGFP fluorescence intensities above the threshold as defined by the cells not carrying egfp, with no distinct peak of EGFP fluorescence intensity.

We can conclude that the integrated inducible vectors show low, but measurable background activity in the absence of doxycycline in a small fraction of cells.

5.2.5 Stability of Doxycycline in Cultivation Medium at 37°C

In order to test the stability of doxycycline in culture media, medium containing the standard amount of doxycycline (1 μg/ml) was preincubated in the 37°C incubator in open tubes to allow gas exchange for 2 and 6 days, respectively. Then, the preincubated medium or fresh medium with or without doxycycline was added to TetOn-egfp FL preB-I-cells which had been grown on stromal cells and IL-7 in the absence of doxycycline for 2 days. After one day of incubation, cells were subjected to FACS analysis. As shown

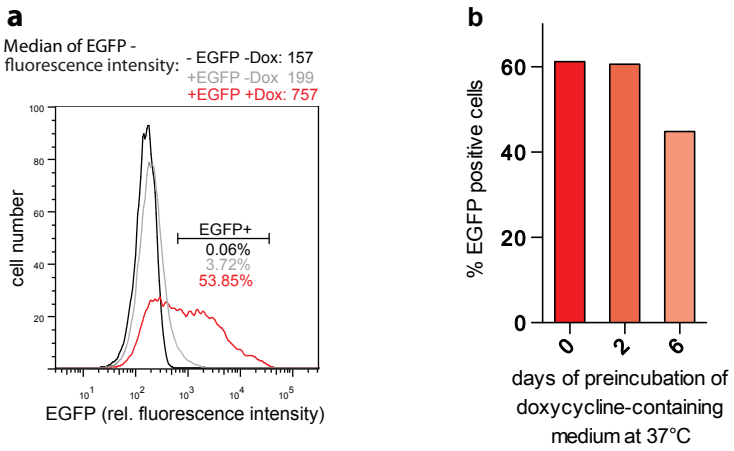

Figure 5.10 – (a) Leaky expression of EGFP in preB-I-cells transfected with the TetOn-egfp vector in preB-I-cells. PreB-I-cells carrying either TetON pim/Myc or TetON-egfp were cultured in the absence or presence of doxycycline for 24 hours before FACS analysis. Compared to cells which did not carry egfp (black line), egfp-carrying cells showed slightly enhanced EGFP fluorescence intensities in the absence of doxycycline (grey line). In the presence of doxycycline, egfp-carrying cells showed a wide range of EGFP-fluorescence intensities (red line), where 50% of the cells had higher EGFP-fluorescence intensities than the negative control. (b) Stability of doxycycline in medium at 37° C. Doxycycline was preincubated for 2 or 6 days in medium at 37° C or added freshly to medium before being added to TetON-egfp-transgenic preB-I-cells. After 24 hours, EGFP expression was measured by FACS analysis.

in figure 5.10b, no reduction of EGFP expression in cells induced with doxycycline preincubated for two days was detected as compared to EGFP expression in the presence of freshly added doxycycline. Doxycycline preincubated at 37°C for 6 days only led to a 20% reduction of EGFP expression. Therefore, each subpassage of cells after 3-4 days was done with medium containing fresh doxycycline.

5.3 Effect of Pim1+Myc-Overexpression on Transgenic PreB-Cells *in vitro*

5.3.1 *in vitro* Differentiation of Normal PreB-I Cells

In a first experiment, the maturation behaviour of normal, untransfected fetal liver preB-I-cells was characterized in order to have reference points for further maturation experiments with preB-I-cells overexpressing Pim1 and Myc. Maturation of preB-I-cells can be induced by removal of IL-7. FLd18Ber3 cells grown on OP-9 feeder layers supplemented with IL-7 were washed and re-cultured in the absence of IL-7. Maturing cell cultures were kept in medium either supplemented with IL5 or with IL5+αCD40 antibodies for 8 days. The cells were counted and subjected to FACS analysis every day.

Without any ligands added, survival of cells decreased drastically from 100% to below 10% between days 1 und 3. After 8 days, there were virtually no viable cells left (figure 5.11c). Addition of IL5 enhanced early survival or proliferation, whereas αCD40+IL-4 led to a proliferation of cells at later stages of development between day 4 and 5 (figure 5.11c). C-kit, a marker for preB-I-cells, was downregulated during the first 2 days, whereas CD25, a marker for preB-II-cells, was upregulated (figure 5.11a). IgM and IgD were first detected around day 3. The percentage of IgM^+ and IgD^+ cells rose subsequently to around 80 and 40% of all viable cells, respectively. These results of B-cell differentiation and apoptosis are comparable to the original observations with preB-I-cells [112]. The markers CD23 and MHCII were

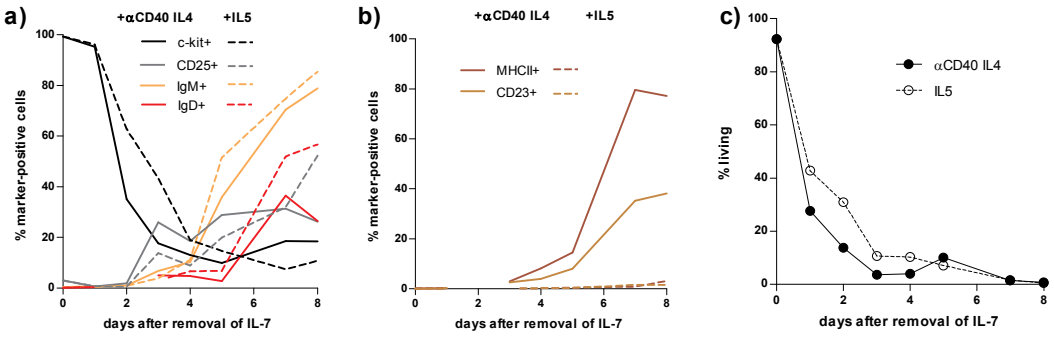

Figure 5.11 – Change in expression of surface markers and survival during in vitro differentiation of preB-I-cells upon removal of IL-7. (a) Timeline of expression of B-cell maturation markers after removal of IL-7. (b) Differential upregulation of MHCII and CD23 in cells matured with IL5 only or with αCD40+IL-4. (c) Survival of cells during in vitro maturation.

only upregulated in the cultures supplemented with αCD40+IL-4 (5.11b), which is in line with the results obtained by Takeda et al. who found that IL-4 is a trigger for CD23 and MHCII expression [130]. Virtually all of the cells retained CD93 [88] on their surface, which suggests that maturation did not proceed *in vitro* to fully mature, $CD93^-$ B-cells.

In summary, normal fetal liver preB-I-cells previously cultured in the presence of IL-7 can be matured *in vitro* in the absence of IL-7 in about 3 to 6 days to IgM^+, partially IgD^+, but still $CD93^+$ B-cells with concomitant apoptosis of the maturing cells.

5.3.2 *in vitro* Growth Behaviour of FL PreB-I Derived Cells Overexpressing Pim1 and/or Myc

To examine the effects of Pim1 and Myc overexpression in FL preB-I-cells *in vitro*, the Fld18Ber3 cell line transduced with the rtTA-TetOn vector and the TetOn-*Pim1* and/or TetOn-*Myc* vectors were first cultured on stromal cells in the presence of IL-7 at $3.3x10^5$ cells/ml. Then, IL-7 was removed from the medium of preB-I-cells, and different growth factors and stimulants were added to the medium in the presence or absence of doxycycline. After 3 to 4 days, cells were counted and, thereafter, recultured in fresh media at $1.3x10^5$ living cells/ml. Induced overexpression of Pim1 alone did not lead to IL-7-independent proliferation or survival in preB-I-cells (figure 5.12a). Also, induced overexpression of Myc alone did not render the cells IL-7 independent for further proliferation. In medium without IL-7 or any other growth factors, they were induced to apoptosis. However, addition of IL5 or αCD40+IL-4 to the cells induced the survival of doxycycline-induced cultures for more than two weeks (figure 5.12b). Finally, if Myc and Pim1 were overexpressed together in the same cells, they proliferated IL-7-independently (figure 5.13). The proliferating cells which originally had shown a preB-I phenotype gradually lost c-kit on the cell surface, acquired CD25, and kept CD93 during this Myc and Pim1-induced, IL-7-independent proliferation (figure 5.14).

In summary, induced overexpression of Pim1 alone had no effect upon proliferation or survival of preB-I-cells in the absence of the preB-I growth factor IL-7. Also, overexpression of Myc alone was not sufficient to induce proliferation of preB-I-cells in the absence of IL-7. However, induced Myc overexpression in the presence of IL5 or αCD40+IL-4 led to survival of a constant number of cells for at least two weeks in culture. This could be due to an enhanced proliferation rate of cells which still have not overcome

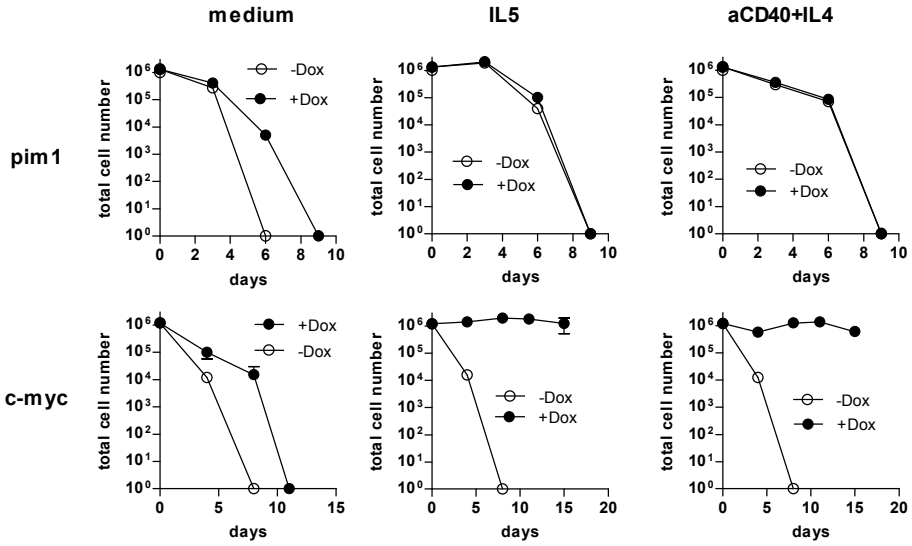

Figure 5.12 – Growth curves of FL Ber3 cell lines expressing either Pim1 or Myc in media containing different growth stimuli

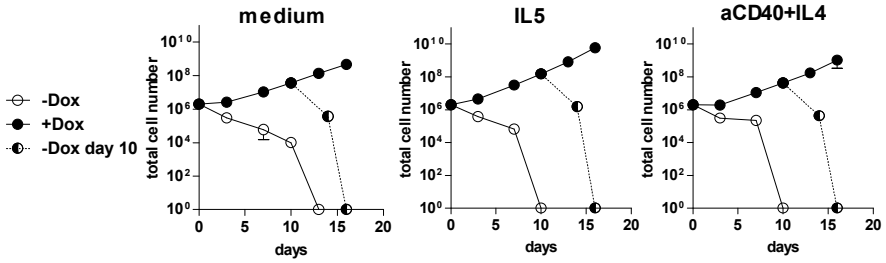

Figure 5.13 – Growth of the doxycycline-induced, Pim1 + Myc-expressing FL preB-I-cell line + Pim1 +Myc with doxycycline in the absence of IL-7, cultured either in medium alone, in medium supplemented with IL5, or in medium supplemented with αCD40+IL-4.

apoptosis, or it could be a consequence of enhanced survival of the cells. This problem is adressed below. Finally, overexpression of Pim1 and Myc together induced growth factor independent proliferation of fetal liver preB-I-cells. However, in contrast to preB-I-cells proliferating in the presence of IL-7 on stromal cells, these cells appear not to remain fixed in their preB-I state of differentiation, since they lost c-kit and acquired CD25 expression.

In order to analyse the combined influence of Pim1 and Myc in later stages of B-cell differentiation, namely in IgM^+CD93^+ immature B-cells, FL-preB-I-cells transduced with TetOn-*Pim1* and TetOn-*Myc* were induced to maturation *in vitro* by the removal of IL-7 in the presence of IL5. After 4 days, doxycycline was added to the cultures to induce Pim1 and Myc. The few surviving cells were grown *in vitro* for 5 days in the presence of IL5 and doxycycline. Then, IgM^+ cells were enriched with a FACS sorter and cultured in the presence of doxycycline and IL5 for additional 4 days to obtain sufficient numbers of IgM^+ cells for further growth tests. Since overexpression of Pim1 and Myc might not be sufficient to induce proliferation of IgM^+ B-cells, different cytokines known to enhance B-cell proliferation or activation were

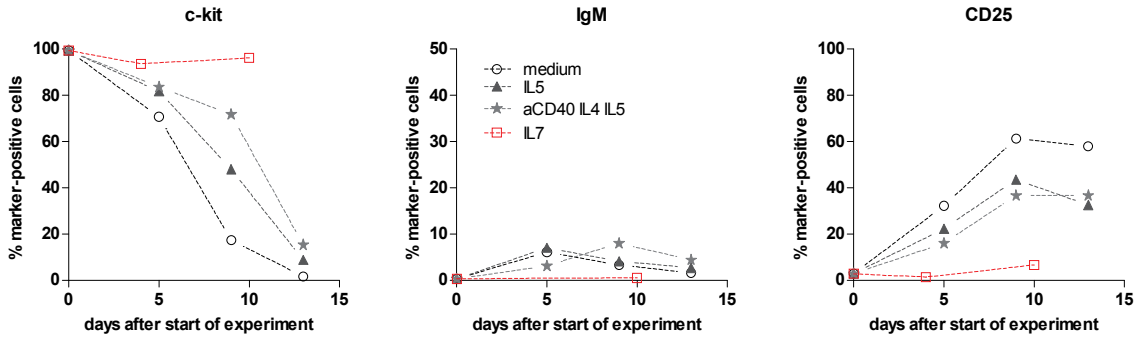

Figure 5.14 – Phenotype of Pim1+Myc-expressing fetal liver preB-I-cells cultured in the presence of doxycycline, in medium supplemented with the cytokines and mitogens as indicated in the legend.

added. The sorted cells kept their IgM$^+$ phenotype and proliferated growth factor independently (figure 5.15). Only the addition of IL5 further improved the proliferation of these Pim1+Myc-expressing IgM$^+$ B-cells, while the addition of IL-2, IL-4, IL6, αCD40, and αCD40+IL-4 had no effect. The addition of LPS had a small inhibiting effect.

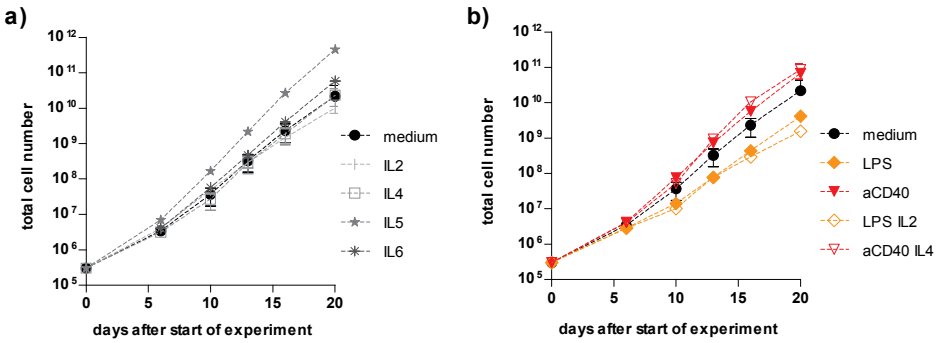

Figure 5.15 – Proliferation of in vitro matured, sorted IgM$^+$ fetal liver cells in the presence of doxycycline to promote expression of Pim1 and Myc (a) in the presence of interleukins, and (b) in the presence of mitogens

In short, some of the IgM$^+$ immature cells could be induced to proliferate *in vitro* even in the absence of B-cell growth and survival factors. The IgM$^+$ phenotype was stable for at least 2 weeks and the few remaining IgM$^-$ cells did not overgrow the cultures. However, since the cultures continued to contain IgM$^-$ cells, it is likely that these cells also proliferated, although this was not tested as with the IgM$^+$ cells shown in figure 5.15. In conclusion, the combined induction of expression of Pim1 and Myc activates the mitotic cell cycle of the series of B-lineage differentiation stages from large preB-I to immature B-cells, i.e. in cells like IgM$^+$ immature cells that would normally develop as non-mitotic resting cells.

5.3.3 Limiting Dilution Analysis of Clonable, Pim1+Myc-Induced PreB-I Cells

To estimate the percentage of preB-I-cells being able to respond to inducible Pim1 and Myc overexpression *in vitro*, limiting dilution analysis was performed. *Pim1+Myc* doubletransgenic preB-I-cells grown in IL-

7 on feeder layers were set up in 96-well plates at different cell numbers per well in medium without IL-7, but containing other growth factors. The cultures were analysed 1 week later. Positive cultures were defined as wells with more than 20 living cells. Plating efficiencies of the different cultures were normalised to the plating efficiency of preB-I-cells on OP9 feeder layers supplemented with IL-7, which was set to 1/1.

In the absence of doxycycline, only preB-I-cells supplemented with IL-7 proliferated with a plating efficiency of almost 1 in 1. Cells stimulated with doxycycline could even grow without addition of growth factors, though only around 1 in 90 cells started to proliferate in medium + doxycycline alone (table 1). When IL5 was added, the frequency of proliferating clones increased to 1 in 35. Also, αCD40 increased the cloning capacity of the doxycycline induced cells to 1 in 60-80.

	no Dox	**mean**	**Dox**	**mean**
OP9 IL-7	1/1, 1/1, 1/1	1/1	1/1	1/1
medium	undetectable		1/64, 1/104, 1/115	1/94
αCD40 IL-4	undetectable		1/69	1/69
αCD40 IL-4 IL5	ND		1/47	1/47
IL5	undetectable		1/27, 1/20, 1/53	1/33
IL-4	undetectable		1/91	1/91

Table 1 – Frequency of clonable cells as determined by limiting dilution of the FLd18Ber3 cell line expressing Pim1 and Myc in the presence of different growth factors. Cells carrying TetOn-Pim1 and TetOn-Myc were grown in the conditions mentioned, and after 7 days, positive wells (containing colonies of more than 20 living cells) were counted. undetectable: none in 800 cells grew.

In summary, in the preB-I-cell pool expressing Pim1 and Myc, only around 1 in 90 cells, i.e. around 1%, were clonable in IL-7-free medium. Growth factors such as αCD40 antibody and IL5, but not IL-4 enhanced cloning efficiency to up to 1 in 35 cells. This is much less than estimated from growth curves of Pim1+Myc-overexpressing preB-cells growing in the absence of IL-7 as shown in figure 5.16

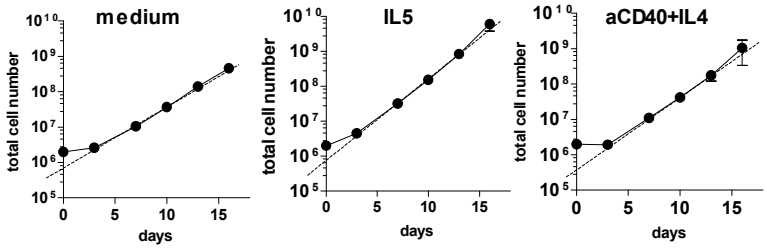

Figure 5.16 – Estimation of approximate numbers of cells growing out from Pim1/Myc-transgenic fetal liver preB-I cells cultured in the absence of IL-7 while overexpressing Pim1 and Myc.

Analyses of preB-I-cells expressing Pim1 and Myc presented in section 5.3.2 have shown that differentiation of these cells in the absence of IL-7 appeared delayed, see figure 5.14. To further characterize this phenomenon, B-cells overexpressing Pim1 and Myc were grown in medium alone or supplemented with growth factors such as IL5, or αCD40+IL-4+IL5 for 6 days to select for Pim1+Myc-responding cells. Most of the non-responders should have died during that period of time. Thereafter, the cells were replated on OP9 feeder layers in the presence of IL-7 and subjected to limiting dilution analysis.

The percentage of cells still able to proliferate under these standard preB-I conditions (in the absence of doxycycline) was taken as a measure for cells which retained the preB-I-cell stage. Hence, the higher

the frequency of cells proliferating again on OP9/IL-7, the higher the percentage of cells being still in the preB-I-cell stage after 6 days of cultivation in the absence of IL-7.

from	to	
	IL-7 OP9	**mean**
Dox	1/457, 1/286, 1/413	1/385
Dox+IL5	1/84, 1/8, 1/11	1/34
Dox+αCD40+IL-4+IL5	1/54, 1/9, 1/11	1/25

Table 2 – Frequency of clonable cells as determined by limiting dilution analysis: Ability of cells which had been overexpressing Pim1 and Myc in different media to grow back on OP9 feeders supplemented with IL-7 in the absence of doxycycline. After 7 days, positive wells (containing colonies of more than 20 living cells) were counted.

Limiting dilution analysis revealed that a large part of the cells cultured in medium + doxycycline alone did not retain their status of differentiation, since only 1 in 385 cells was able to replate on OP9/IL-7. By contrast, 1 in 34 cells cultured in the presence of IL5 and doxycycline replated on OP9/IL-7, and 1 in 25 cells cultured in the presence of αCD40+IL-4+Il5 replated on OP9/Il-7 (table 2). These data suggest that preB-I-cells overexpressing Pim1 and Myc in the absence of IL-7 are not all arrested at the preB-I-cell stage. Surprisingly, growth factors such as IL-5 or αCD40 kept a higher number of cells at the prebI-cell stage, suggesting that growth factors inhibit maturation to later stages of B-cell development.

In conclusion, the limiting dilution analyses of the frequencies of cells capable of clonal proliferation showed that

1) Doxycycline-dependent induction of Pim1 and Myc together in preB-I-cells without supplemented cytokines or ligands around 1% of the estimated 25% of cells that proliferated in mass cultures (figure 5.16). Of these, one in around 400 cells, i.e. 1 in around 40'000 originally induced preB-I-cells could be reversed after 6 days to proliferate again in the absence of doxycycline-induced Pim1 and Myc expression to proliferate like preB-I-cells on stromal cells in the presence of IL-7.

2) Addition of IL-5 (with or without αCD40, IL-4 or other cytokines) increased the doxycycline/Pim1-Myc-induced frequencies by more than a factor of 2 to 1 in 35-45 cells, of which, after 6 days, again 1 in around 35, hence around 1 in around 1000-1500 originally induced preB-I-cells could be raplated as preB-I-cells on stromal cells in the presence of IL-7. Therefore, IL-5 kept around 40-50 times as many cells in a preB-I-cell like state during the 6 days Pim1+Myc-induced , IL-7-independent proliferation of the preB-I-cells. Nevertheless, these analyses show that the vast majority of all Pim1+Myc-induced proliferating preB-I-cells lost their preB-I state and matured to preB-II and sIgM$^+$ cells which, when reversed by the removal of doxycycline, had lost their capacity to grow on stromal cells in IL-7.

5.3.4 Cell Cycle Analysis of PreB-cells Overexpressing Pim1 and/or Myc

Myc can induce mitotic cell cycle progression in density-arrested rodent fibroblasts. In rat and mouse fibroblasts, overexpression of an estrogen-regulated version of the Myc protein resulted in re-entry into and progression through the cell cycle [32] [126].

Hence, the ability of the transgenic Myc to induce cell cycle progression in IL-7 deprived, doxycycline-induced cultures was tested by staining of DNA with propidium iodide (PI). PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. Cells in G2/M phase have twice the amount of DNA as cells in G0/G1 phase, and cells in S phase have an amount of DNA which lies in between. Hence, the intensity of PI staining is directly proportional to the phase of the cell cycle (see figure 5.17 for an example graph).

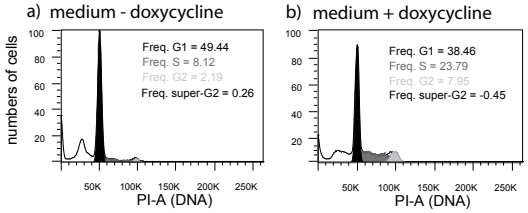

Figure 5.17 – PI-staining of Pim1/Myc-transgenic preB-I-cells in medium without (a) or with (b) doxycycline for 1 day. Analysis was done on living single cells, see Materials and Methods, page 34. Black curve: G1, light grey: G2/M phases, and dark grey curve: S phase

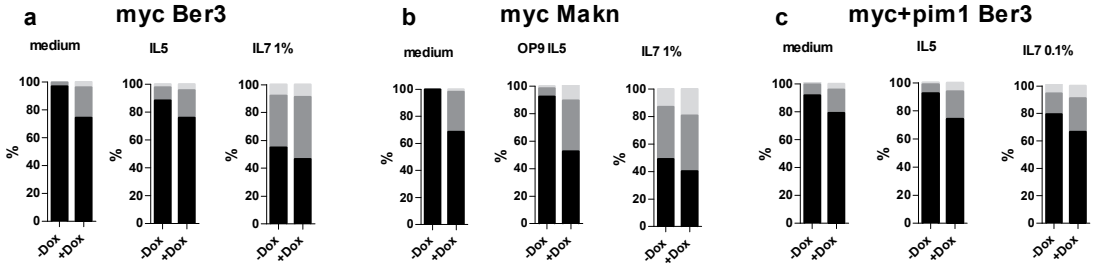

Figure 5.18 – Cell cycle analysis of two preB-I-cell lines carrying Myc ((a) Fld18Ber3-rtta1-Myc, (b) Makn-rtta-Myc), and (c) a cell line carrying Myc + Pim1 (Fld18Ber3-rtta1-pim/Myc) as demonstrated in figure 5.17.

PreB-I-cells were cultured on OP9 feeders in medium alone or in the presence of IL-5 or IL-7 at different concentrations in the presence or absence of doxycycline. After 2 days, cells were harvested, fixed in ethanol, and subsequently stained for DNA (section 4.2.4). As shown in figure 5.18a and b, induced overexpression of Myc in fetal liver preB-I-cells leads to enhanced cell cycle entry, even in medium without any additional growth factors. Cell Cycle entry is also visibly enhanced in standard cultivation conditions of fetal liver preB-I-cells, namely on a feeder layer in the presence of IL-7. In section 5.3.2, it was shown that overexpression of Myc alone was not sufficient to induce IL-7 independent proliferation of preB-I-cells. Combining this result with the above cell cycle analysis, we can conclude that the inability of *Myc* transgenic preB-I-cells to grow IL-7 independently is not due to a failure to enter mitosis. More likely, it is a result of increased proliferation without inhibiting apoptosis.

Cells overexpressing Pim1 + Myc together also show the same pattern, i.e. cell cycle entry is enhanced upon overexpression of Myc and Pim1 for 2 days in cultures kept in medium alone as well as in cultures supplemented with IL-5, or IL-7 (figure 5.18c).

The ratios of cells being in S-phase divided by cells being in G2/M phase ($\frac{\%(S)}{\%(G2-M)}$) did not differ between cells transgenic for *Myc* alone and cells expressing Pim1 and Myc together or between doxycycline-induced and -uninduced cells.

In short, cell cycle progression is enhanced in both *Myc* transgenic and *Myc+Pim1* transgenic preB-I-cells in medium with or without growth factors, IL-7 or IL-5, present.

5.4 Overexpression of Pim1 and Myc in Immature and Mature B-Cells *ex vivo* and *in vivo*

5.4.1 Phenotype of Transplanted FL preB-I-Cells in Different Murine Organs

RAG KO mice were sublethally irradiated with 4 Gy, and 1 day later received 5x10^6 fetal liver preB-I-cells carrying transgenic, but uninduced *Pim1/Myc*. These triple rtTA-*Pim1/Myc* and double rtTA-*Myc* transgenic cells were taken to test their phenotype after transplantation into RAG KO mice in the absence of doxycycline in different hematopoietic organs at 2 months after transplantation.

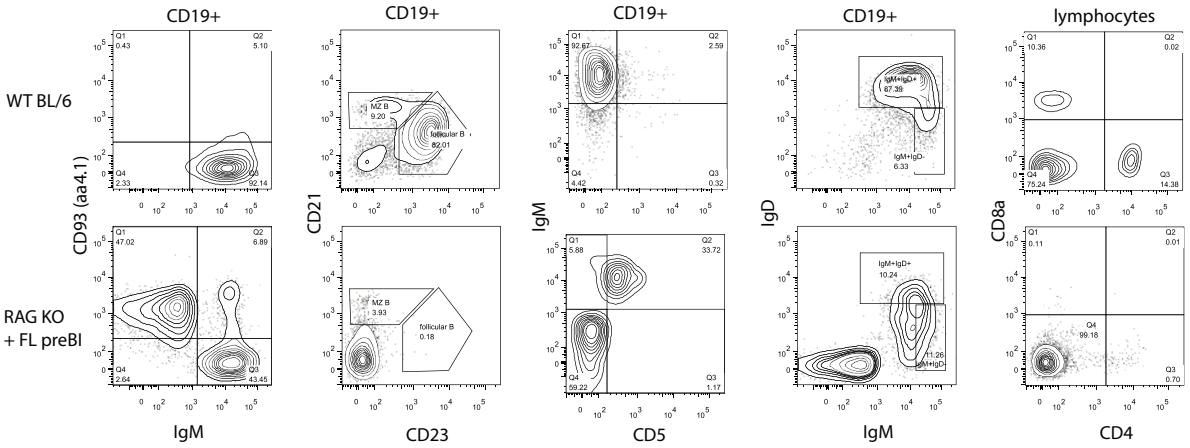

Figure 5.19 – Representative FACS plots showing the phenotype of gated splenic CD19$^+$ cells in a BL/6 wild type mouse (top row) and a RAG KO mouse transplanted with 5x10^6 FL preB-I-cells carrying transgenic Pim1/Myc in the absence of doxycycline 2 months after transplantation (bottom row).

The thymi and lymph nodes of transplanted RAG KO mice were very small and hardly visible. T-cells were absent. The transplanted B-cells mainly populated the spleen and the peritoneal cavity. More than 95% of the CD19$^+$ cells in spleen and peritoneal cavity were donor-derived as confirmed by transplantation of CD45.1$^+$ fetal liver preB-I-cells into CD45.2$^+$ RAG KO recipient mice (data not shown). The bone marrow was only scarcely populated by the transplanted preB-cells, as the main CD19$^+$ population in the bone marrow was host-derived.

	BM	**spleen**	**peritoneum**
BL6	6.55x10^6	21.7x10^6	
RAG KO	1.87x10^6	0.05x10^6	
RAG KO pim/*Myc* 2 w	2.04x10^6 ± 0.8x10^6	0.63x10^6 ± 0.36x10^6	0.08x10^6 ± 0.02x10^6
RAG KO pim/*Myc* 4 w	1.64x10^6 ± 0.84x10^6	1.94x10^6 ± 1.60x10^6	0.57x10^6 ± 0.55x10^6
RAG KO pim/*Myc* 8 w	1.99x10^6 ± 0.88x10^6	13.7x10^6 ± 9.8x10^6	0.85x10^6 ± 0.48x10^6
RAG KO *Myc* 2 w	0.14x10^6 ±0.07x10^6	0.91x10^6 ±0.140x10^6	0.006x10^6 ±0.006x10^6
RAG KO *Myc* 4 w	0.37x10^6 ±0.18x10^6	1.10x10^6 ±0.32x10^6	0.08x10^6 ±0.005x10^6
RAG KO *Myc* 8 w	1.44x10^6 ±0.31x10^6	1.75x10^6 ±0.61x10^6	0.63x10^6 ±0.42x10^6

Table 3 – Total numbers of CD19$^+$ cells in BM (1 hind leg), spleen, and peritoneum. Numbers are derived from 2-3 mice each. w=weeks.

In the spleen, B-cell numbers rose continually within eight weeks after transplantation. CD19$^+$ cell numbers in the different organs depended on the cell line. The Fld18Ber3-rtta1-*Pim1/Myc* cell pool

expanded more rapidly in transplanted mice and yielded higher numbers of splenic B-cells than the Fld18Ber3-rtta1-*Myc* cell pool. Two weeks after transplantation, the pim/*Myc* bearing B-cell pool yielded 4.75x the numbers of splenic B-cells of the *Myc*-bearing B-cell pool. Four weeks after transplantation, 7.5x more splenic B-cells in mice transplanted with *Pim1*/*Myc*-transgenic B-cells than in mice transplanted with *Myc*-transgenic B-cells were detected. Eight weeks after transplantation, 30x more splenic B-cells in mice transplanted with *Pim1*/*Myc*-transgenic B-cells were found than in those transplanted with *Myc*-transgenic B-cells (see table 3).

	total IgM (μg/ml)	**total IgG (μg/ml)**
BL6	480-600	1200-3100
RAG KO	0	0
RAG KO pim/*Myc* 2 w	105-120	0.45-0.75
RAG KO pim/*Myc* 5 w	135-160	61-67
RAG KO pim/*Myc* 9 w	175-185	225-380

Table 4 – Serum levels of total IgM and IgG in RAG KO mice transplanted with 5x10^6 FL preB-I Pim1/Myc transgenic cells, 2, 5 and 9 weeks after transplantation. Values are derived from 2-4 mice for each group.

Two, four and eight weeks after transplantation, the populations within the spleen included between 35% and 75% mature IgM$^+$CD93$^-$ B-cells as well as IgM$^+$CD93$^+$ transitional B-cells and up to 40% IgM$^-$CD93$^+$ cells. In contrast, BL/6 WT mice had more than 90 % mature B-cells and less than 10% immature and transitional B-cells. Furthermore, only B-cells with a MZ-like phenotype (CD21$^+$) could be detected in transplanted RAG KO mice, but practically no follicular CD21^{lo}CD23$^+$ B-cells. In contrast, splenic B-cells of WT Bl/6 mice included around 80% follicular B-cells and 9% MZ B-cells (figure 5.19). Mature B-cells derived from transplanted fetal liver preB-I-cells mainly had a B1-phenotype (CD5$^+$).

Mice transplanted with fetal liver preB-I-cells readily generated approximately one third of wild type serum IgM, but low serum IgG levels (table 4).

5.4.2 Effect of Overexpression of Myc only in B-Cells *in vivo*

To assess whether B-cells overexpressing Myc starting from the preB-I-cell stage would expand *in vivo*, 5x10^6 doxycycline-induced preB-I-cells were transplanted into RAG1$^{-/-}$ host mice fed with doxycycline in the drinking water, and B-cell numbers and maturation stages were assessed 2 and 4 weeks after transplantation. Four weeks after transplantation, doxycycline was removed from the drinking water of the mice, and the effect of Myc downregulation on the B-cell populations was assessed 8 weeks after transplantation. For a outline of the experiment, see figure 5.20.

Two mice per group (doxycycline-treated, untreated) were analysed 2 weeks after transplantation, and 3 mice per group were analysed at four and eight weeks after transplantation.

PreB-I-cells overexpressing Myc alone did not expand significantly in the bone marrow or spleen of RAG1 KO mice over time (figure 5.20). Four weeks after transplantation, there was only a minimal increase in CD19$^+$ immature B-cells of bone marrow and spleen. The only compartment which was increased in mice carrying Myc overexpressing B-cells was the peritoneal cavity. This increase was only visible after 8 weeks (5.20).

One of the transplanted mice analysed 2 weeks after transplantation had threefold higher B-cell numbers in the bone marrow and spleen (figure 5.20, mouse marked with exclamation mark). Some of the transplanted preB-cells in this mouse might have acquired another deregulated oncogene which

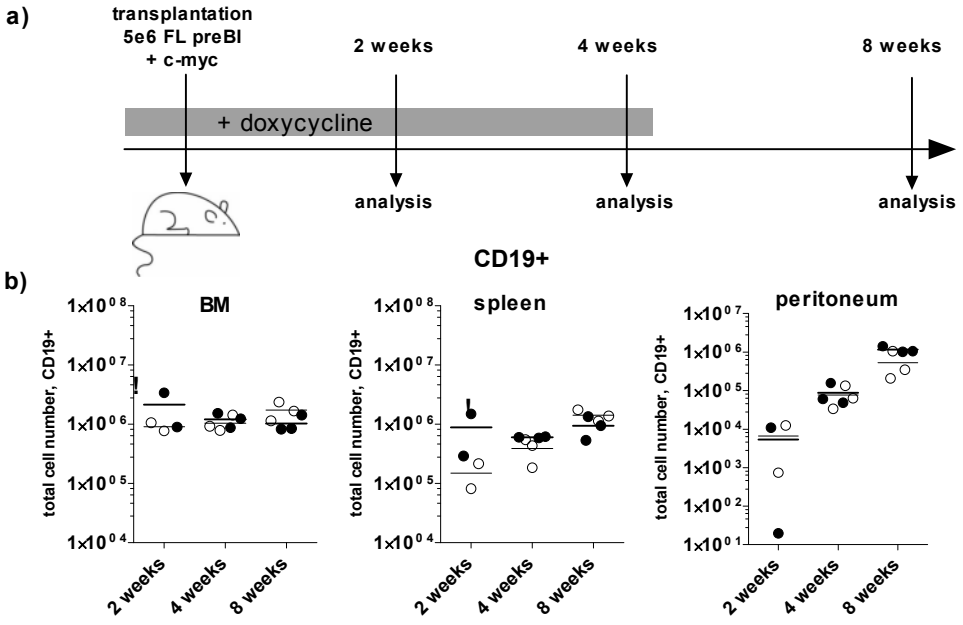

Figure 5.20 – Overexpression of Myc in preB-I-cells transplanted into RAG1 KO mice does not lead to increased B-cell numbers in spleen and bone marrow.(a) Experimental outline. RAG1 KO mice were fed with doxycycline (0.2 g/liter) starting 3 days before transplantation. Two days later, mice were sublethally irradiated and then were transplanted one day after irradiation with 5x10^6 preB-I-cells carrying TetOn-Myc. Expression of these transgenes in the cells was induced one day before transplantation in vitro in doxycycline-containing medium. As a control, mice fed with normal acidified drinking water without doxycycline were transplanted with uninduced preB-I-cells carrying Myc. Doxycycline treatment in doxycycline-induced mice was continued until 4 weeks after transplantation. Thereafter, doxycycline was omitted from the drinking water. Mice were analysed 2, 4 and 8 weeks after transplantation. (a) Experimental outline, (b) total B-cell numbers from the bone marrow from 1 hindleg and from total spleen were calculated. ○: mice carrying uninduced B-cells. ●: Mice carrying B-cells overexpressing Myc. !: mouse with unusually high numbers of B-cells (see text)

cooperated with Myc, which might have caused this unusual B-cell expansion not seen in all other transplanted mice.

The sizes of the different B-cell subpopulations of immature and mature B-cells were not significantly different in doxycycline-treated versus untreated mice (figure 5.21).

In summary, overexpression of Myc alone in preB-I-cells was not sufficient to lead to significantly increased B-cell compartments in the bone marrow and spleen of transplanted RAG1 KO mice over time.

5.4.3 *In vivo* Expansion of B-Cells Overexpressing Pim1 together with Myc

The same experimental setup as described in section 5.4.2 was followed to examine the effect of *Pim1* and *Myc* double-transgenic cells. I.e., 5x10^6 FLpreB-I-cells containing TetOn *Myc* and *Pim1* were transplanted into sublethally irradiated RAG1 KO mice either treated with doxycycline or untreated. B-cell numbers and maturation stages were assessed two and four weeks after transplantation. Four weeks after transplantation, doxycycline was removed from the drinking water of the remaining mice, and the effect of *Pim1* and Myc downregulation on the B-cell populations was assessed eight weeks after trans-

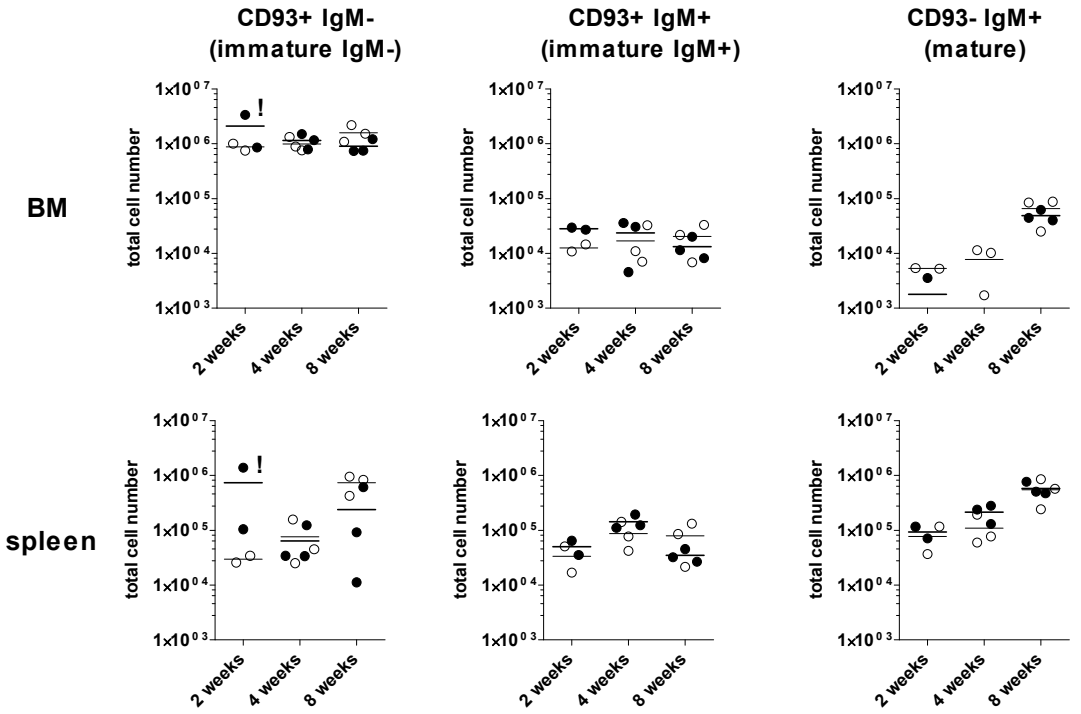

Figure 5.21 – B-cell subsets transgenic for Myc in the bone marrow (upper panel) and spleen (lower panel) in the presence of doxycycline. Total cell numbers of the indicated B-cell phenotype were compared 2, 4 and 8 weeks after transplantation in mice -/+ doxycycline. •: mice carrying B-cells which overexpress Myc, ∘: mice carrying normal B-cells. Cell numbers are calculated from total spleen and bone marrow from 1 complete hindleg.

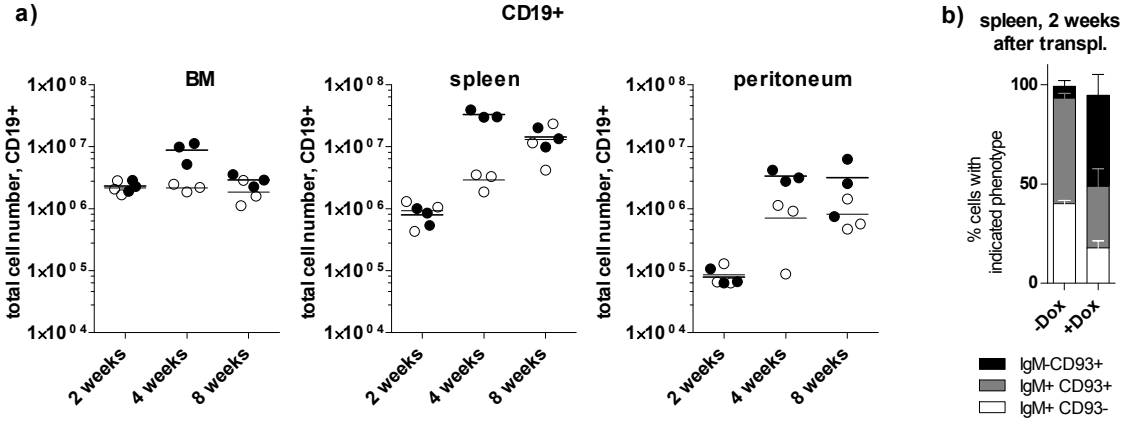

Figure 5.22 – In vivo expansion of B-cells transgenic for Myc and Pim1. The same experimental setup as in 5.20a was used. Per time point and treatment, 3 mice were analysed. (a) Total B-cell numbers from the bone marrow from 1 hindleg and from total spleen were calculated. ∘: mice carrying normal B-cells, i.e. doxycycline-uninduced cells. •: Mice carrying B-cells overexpressing Myc and Pim1 (induced by the addition of doxycycline). (b) Percentage of B-cells with the indicated phenotype in the spleens 2 weeks after transplantation.

plantation. Three mice per group (doxycycline-treated, untreated) were analysed 2, 4 and 8 weeks after transplantation.

Total numbers of CD19$^+$ cells in spleen, bone marrow (1 femur and tibia per mouse) and peritoneal lavage were assessed. Mice which received doxycycline showed no higher numbers of CD19$^+$ B-cells in

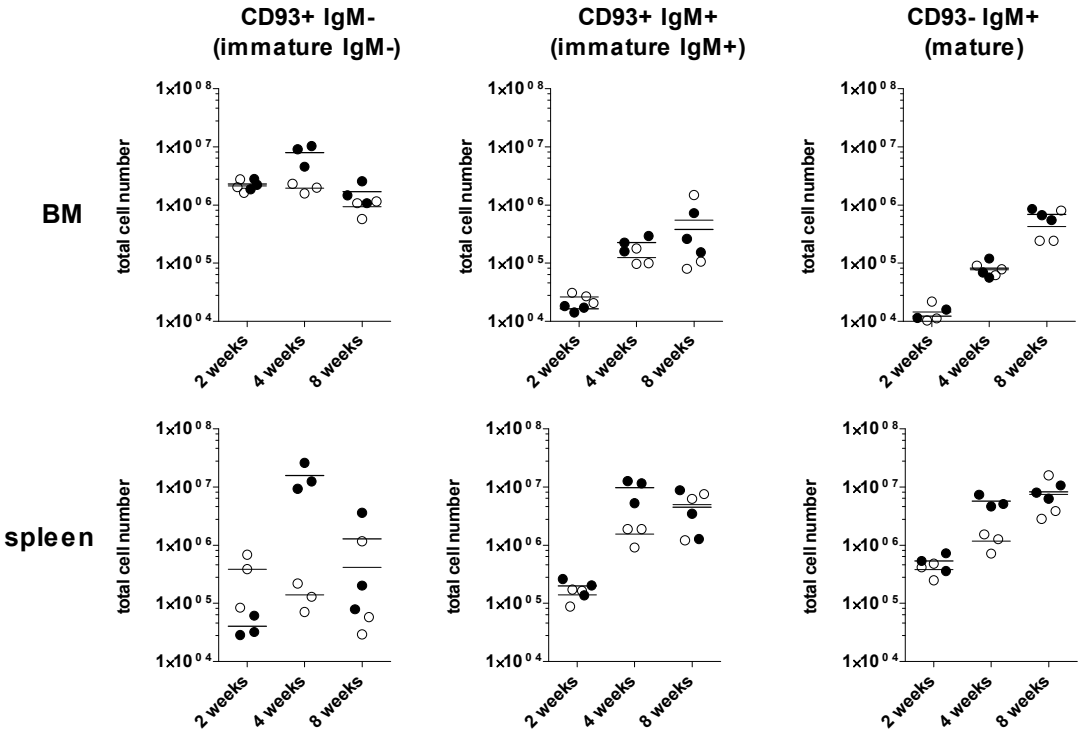

Figure 5.23 – In vivo expansion of B-cell subsets transgenic for Myc and Pim1 in the bone marrow (upper panel) and spleen (lower panel). Total cell numbers of the indicated B-cell phenotype were compared 2, 4 and 8 weeks after transplantation in mice -/+ doxycycline. •: mice carrying B-cells which overexpress Myc+Pim1, ∘: mice carrying normal B-cells. Cell numbers are calculated from total spleen and bone marrow from 1 complete hindleg.

the bone marrow 2 weeks after transplantation. However, a marked increase of CD19$^+$ cells in the bone marrow was observed 4 weeks after transplantation, i.e. 4x more than in uninduced mice (figure 5.22). After the removal of doxycycline at 4 weeks, numbers of CD19$^+$ cells in the bone marrow declined to almost normal levels within 4 weeks (figure 5.22).

In the spleen, numbers of CD19$^+$ cells did not differ from those of control mice without doxycycline after 2 weeks, but the ratio of immature versus mature cells was shifted towards immature, IgM$^-$ cells in the spleen, indicating that immature cells started to expand (figure 5.22c).

Notably, after 4 weeks there was a strong expansion of CD19$^+$ B-cells in the spleens of doxycycline-induced mice. Compared with uninduced mice, more than 10 fold higher numbers of CD19$^+$ B-cells were found (figure 5.22b). Four weeks after removal of doxycycline from the drinking water, i.e. 8 weeks after transplantation, numbers of CD19$^+$ B-cells reverted back to the numbers of splenic B-cells in uninduced mice.

In the peritoneum, an expansion of B-cells was found after 4 weeks that was around 4.5-5 fold the number of B-cells found in the peritoneum of doxycycline-uninduced mice. In contrast to the bone marrow and the spleen, the numbers of peritoneal B-cells remained at increased levels up to 4 weeks after removal

of doxycycline, i.e. at 8 weeks after transplantation.

The BM and splenic B-cells expanding under the influence of induced Myc and Pim1 were stained for CD93 (marker for immature cells), IgM, and CD19. FACS analysis revealed 3 populations of $CD19^+$ B-cells: $CD93^+IgM^-$ cells, which include preB-I and preB-II-cells, $CD93^+IgM^+$ immature cells, and $CD93^-IgM^+$ mature cells.

In the bone marrow, the immature IgM^- preB-II-like fraction was the population which was increased most in numbers 4 weeks after transplantation (figure 5.23). This fraction had 4 times the numbers of cells compared to control mice. Also in the spleen, this immature IgM^- preB-II-like population increased most after 4 weeks, with 100x the numbers of cells found in control mice. Numbers of immature IgM^+ cells were also increased around 6 times, and the mature fraction of B-cells in the spleen was enlarged around 5 times compared to control mice. After 8 weeks, i.e. 4 weeks after removal of doxycycline, cell numbers of almost all fractions declined to levels comparable to control mice.

In summary, Myc + Pim1 overexpressing preB-I-cells transplanted into RAG KO mice fed continuously with doxycyline showed enhanced cellular expansion when compared to transplanted preB-I-cells not overexpressing Pim1 and Myc, i.e. in the absence of doxycycline *in vivo*. Differences in B-cell numbers were visible 4 weeks after transplantation. The B-cell populations which expanded most in the BM and spleen were the IgM^- preB-II-like cells. After removal of doxycycline, i.e. 8 weeks after transplantation, B-cell numbers dropped to normal levels, with the exception of mature B-cells in the BM, which were 2 times as numerous as those in control mice, and B-cells in the peritoneal cavity, which were still 4 times as numerous in doxycycline-induced mice compared to control mice.

5.4.4 Increased Cell Size of B-Cells Overexpressing Myc *in vivo*

Myc has been reported to incease cell size of immature and mature B-cells of Eμ-*Myc*-transgenic mice [75]. To test if Myc also has an influence upon B-cell size in the Myc-overexpression system used here, RAG KO mice were transplanted with transgenic preB-I-cells carrying either *Myc*, or *Pim1* and *Myc* together. One group of 3 mice was treated with doxycycline via the drinking water to induce overexpression of transgenes, and another group of mice received normal acidified drinking water (controls). After 1 month, B-cells of the bone marrow, spleen, and peritoneal cavity were analysed by FACS, and cell size was compared between the doxycycline-induced and uninduced mice (figure 5.24). The forward scatter of light (FSC) produced by cells passing through the laserlight-beam, which is measured in a FACS instrument, is proportional to the cell volume. Hence, bigger cells have higher FSC values. B-cells overexpressing Myc in the spleen and peritoneal cavity had higher mean FSC values than B-cells which did not overexpress Myc. In the bone marrow, there was only a very slight, insignificant increase of FSC in Myc-overexpressing B-cells compared to control B-cells. B-cells overexpressing Myc together with Pim1 had higher FSC values than control B-cells in the spleen and also the bone marrow. In the peritoneal cavity, there was a visible, but insignificant increase in cell size.

In summary, overexpression of Myc leads to an increase of cell size in transplanted B-cells harvested from the spleen, bone marrow and peritoneal cavity.

5.4.5 *Ex vivo* Proliferation of Donor-Derived Splenic B-Cells 4 Months after Transplantation

As shown above, immature IgM^- and IgM^+ B-cells overexpressing Pim1 and Myc together proliferate *in vitro* and *in vivo*, but at the same time are hindered to fully mature. *In vitro* maturation of normal fetal liver preB-I-cells does not generate fully mature $CD93^-$ B-cells. Hence, to elucidate the effects of

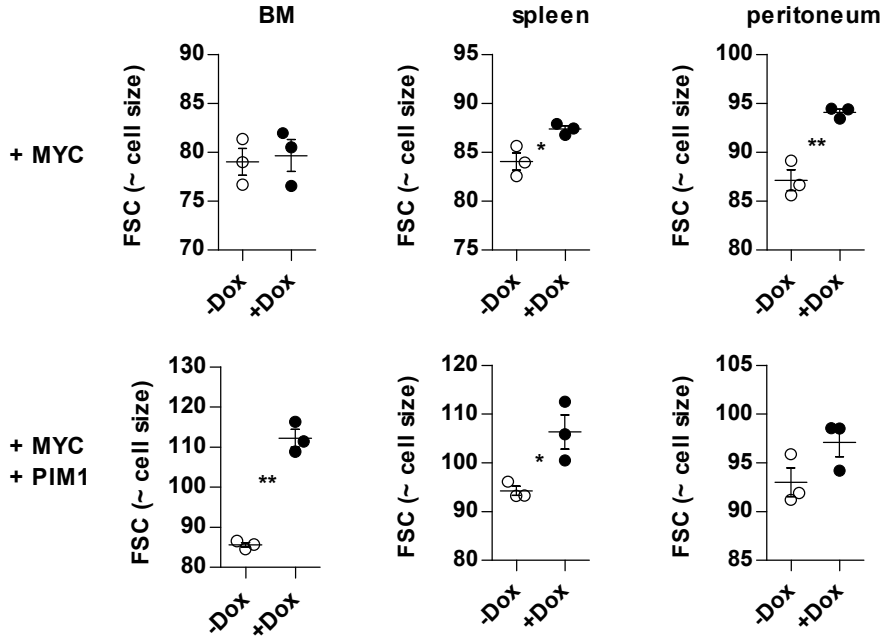

Figure 5.24 – Expression of Myc leads to an increase in cell size of transgenic B-cells in vivo. RAG KO mice were transplanted with 5x10^6 transgenic preB-I-cells either able to overexpress only Myc (top row) or Myc and Pim1 (bottom row). One group of mice was treated with doxycycline in the drinking water, while the second group was left untreated. After 1 month, mice were sacrified, and the average forward scatter (FSC) of gated B-cells (as a measure for cell size) was determined by flow cytometry. ∘: mice w/o doxycycline, •: mice treated with doxycycline.(: p≤<0.05, **: p≤<0.01, 2-tailed, unpaired t-test)*

Pim1 and Myc overexpression upon fully mature B-cells, fetal liver preB-I-cells carrying the TetON-*Pim1* and TetON-*Myc* transgenes were transplanted into RAG KO hosts in the absence of doxycycline to allow complete maturation *in vivo*. Splenic B-cells and bone marrow B-cells were harvested 4 months later and tested for the presence of mature B-cells. B-cells were tested in *in vitro* cultures for proliferation in the presence of doxycycline and different growth factors.

B-cells were purified using CD19-MACS beads as described in section 4.3.19, and set up at 2x10^5 cells per 3 ml medium in different conditions with and without doxycycline. Since only certain stages of B-cells might be responsive to induced Pim1 and Myc expression, B-cells were stimulated *in vitro* with a range of known B-cell activating agents. Conditions tested included "naive" B-cells which were cultured in medium alone, B-cells activated with LPS and IL-2 or IL-5, which leads to plasma cell formation, B-cells activated with αCD40 and different cytokines, which induces maturation and switching, and B-cells activated with both LPS and αCD40 in the presence of different cytokines. Some cultures were additionally supplemented with IL-21 [100] and IL6 which might enhance plasma cell differentiation if only plasmablasts or plasma cells were responsive to overexpression of Pim1 and Myc.

No difference in proliferation of doxycycline-induced splenic and BM B-cells as compared to non-induced cells could be found in almost all culture conditions tested (figure 5.25). The only condition showing enhanced proliferation in the presence of doxycycline was the one with LPS, αCD40 and IL-5 5.25.

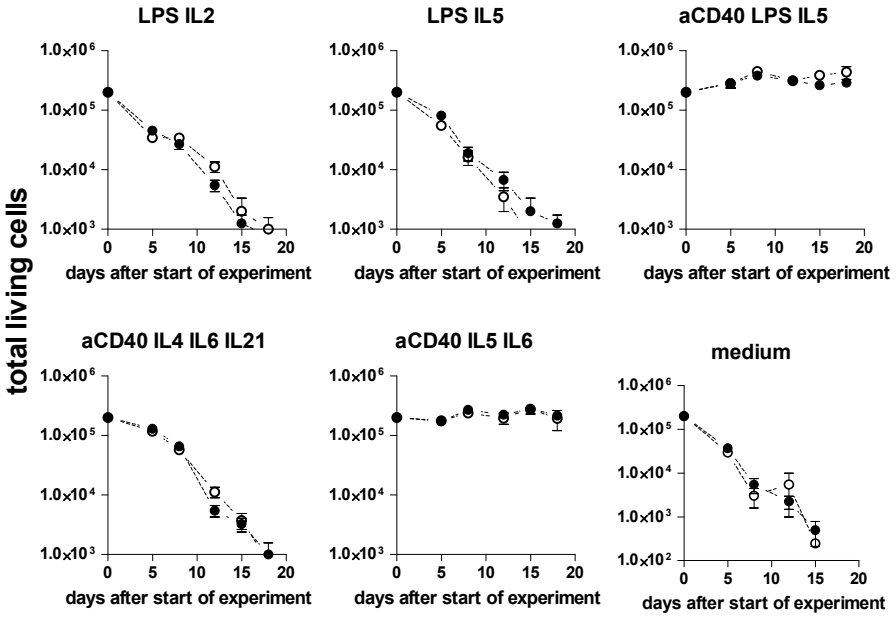

Figure 5.25 – Lack of influence of Pim1 and Myc overexpression on ex-vivo splenic B-cell cultures. Four months after transplantation of FL preB-I-cells carrying Pim1 and Myc into RAG1$^{-/-}$ mice, spleens were extracted. Enriched splenic B-cells were cultured in vitro in the absence (○) or presence (●) of doxycycline and supplemented with different survival- and growth factors. Every 3-5 days, cells were counted and replated in fresh medium.

This apparent unresponsiveness to the actions of Pim1 and Myc in the transplanted B-cells could have resulted from loss of oncogene expression. However, overexpression of Pim1 and Myc in isolated splenic B-cells could still be induced *in vitro* 5 months after transplantation with the addition of doxycycline, as shown in figure 5.9b. Hence, it was not the lack of overexpression of Pim1 and Myc in splenic and BM B-cells which did not allow ligand-independent or -dependent long-term proliferation in contrast to *in vitro* matured IgM$^+$ cells and preB-cells, but an apparent insensitivity of the *ex vivo* isolated, transduced B-lineage cells to Pim1- and Myc expression.

In conclusion, these experiments indicate that in the presence of doxycycline, there was no change in the proliferative response of MACS-sorted splenic B-cells.

5.4.6 *In vitro* Culture of Transplanted Splenic, Bone Marrow and Peritoneal B-Cells one Month after Transplantation

Unreactivity of splenic B-cells to induction of Myc and Pim1 overexpression in the experiment done above might not only be a consequence of intrinsic Myc/Pim1-unreactivity of mature B-cells. Possibly, after 4 months of residence *in vivo*, only limited, unreactive subsets of the originally transplanted *Pim1* and *Myc* transgenic B-cells could have survived. Also, enrichment of B-cells using CD19-MACS beads could have led to damage of the cells. Therefore, the following experiments were conducted one month after transplantation without MACS-bead separation. Splenic, BM and peritoneal cells of two mice were washed and counted. FACS analysis revealed the percentage of CD19$^+$ B-cells. Cultures were set up at 5x10^5 B-cells in 3 ml medium supplemented with various growth- and survival factors for B-cells, with or without doxycycline added. In order to allow exclusive growth of transgenic B-cells, 1.5 μg/ml puroMycine was added, the antibiotic used for selection of transgenic B-cells carrying the TetON-*Pim1*

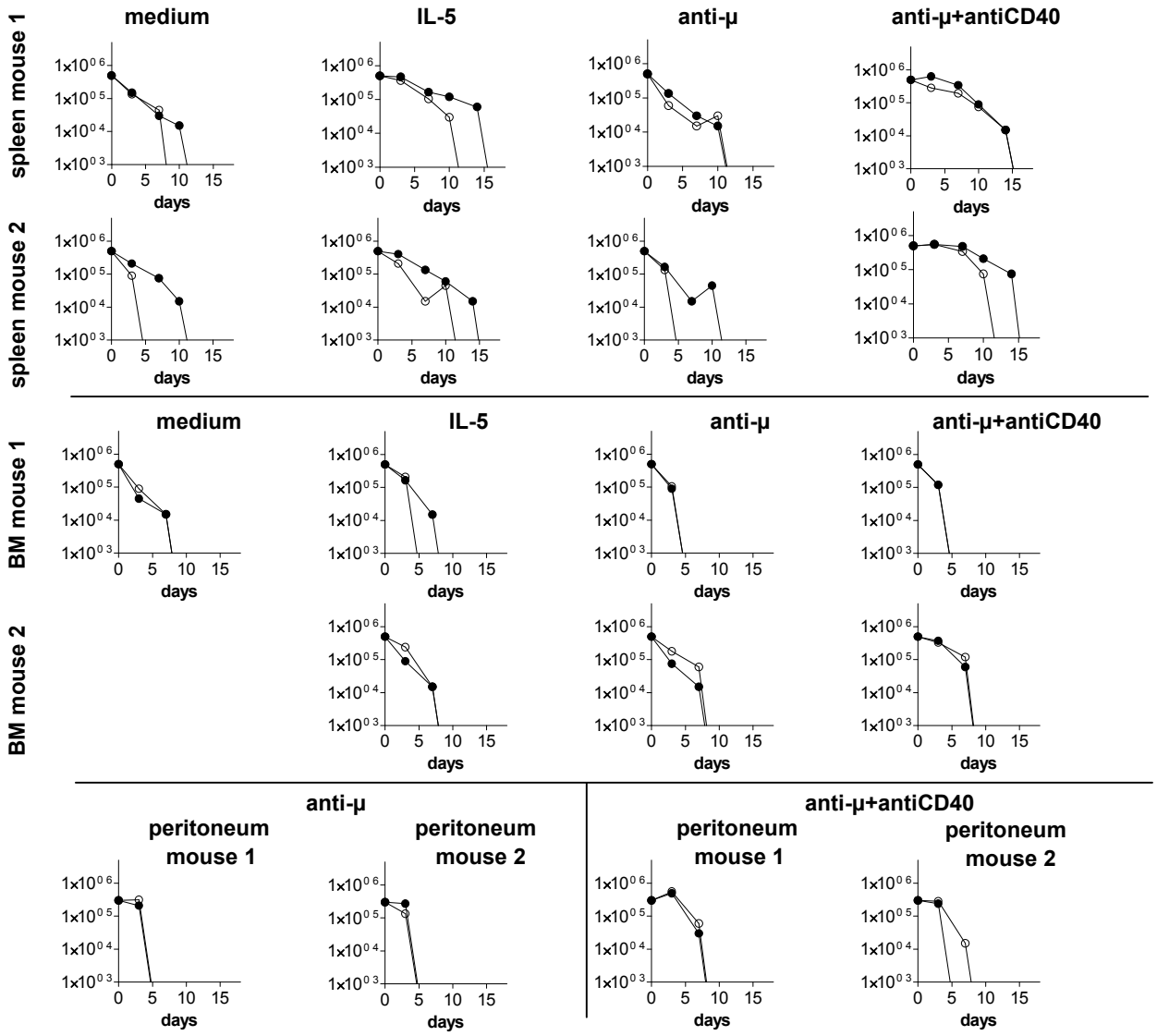

Figure 5.26 – Influence of Pim1 and Myc overexpression onto ex vivo B-cell cultures in the presence of mitogens and survival factors (as indicated in the figure). Total BM, splenic and peritoneal cells were set up at 5x10^5 CD19$^+$ cells (percentage of B-cells assessed by FACS) in 3 ml medium and replated every 3-4 days. ∘ : in the absence of doxycycline. • : in the presence of doxycycline.

vector. Cells were counted and replated in new medium every 3-4 days.

As shown in figure 5.26, none of the cultivation conditions led to sustained proliferation of B-cells in the absence or presence of doxycycline neither in splenic B-cells, nor in peritoneal or BM-B-cells. However, splenic B-cells cultured with IL-5 or αCD40 + IL-4 in the presence of doxycycline showed a prolonged survival of Pim1+Myc-induced cells, but none of the cultures survived for longer than 18 days (figure 5.26).

5.4.7 Detection of *in vitro* Proliferation of B-Cells by CFSE Staining

Another method to detect proliferation of cells is staining with the dye CFSE. As explained in section 4.2.5, the compound CFDA-SE can be covalently bound to the inner cell components in the form of CFSE and is evenly distributed among daughter cells during mitosis. The number of divisions an individual cell has undergone can be assessed by FACS analysis.

FL preB-I-cells carrying TetON-*Pim1* and TetON-*Myc* were transplanted into RAG KO mice, and 1 month later, splenic and bone marrow cells were harvested. Total splenic and BM cells were labelled with CFSE and cultured at 2x10^5 $CD19^+$ B-cells per 3 ml medium supplemented with various growth factors and stimulants in the presence or absence of doxycycline.

After 3-4 days, surviving cells in culture were stained with DAPI and antibodies against CD19, IgM and CD93 and analysed by FACS.

Influence of Cultivation Conditions on the Percentage of B-Cells

The percentage of $CD19^+$ B-cells in the cultures was assessed as a measure for selective expansion or survival of B-cells. Hence, an increasing percentage of $CD19^+$ B-cells can reveal two things: First, if the conditions allow B-cell specific expansion. Second, if Pim1 and Myc influence *ex vivo* proliferation of splenic and bone marrow B-cells, the percentage of $CD19^+$ B-cells would accordingly increase or decrease in doxycycline-treated cultures.

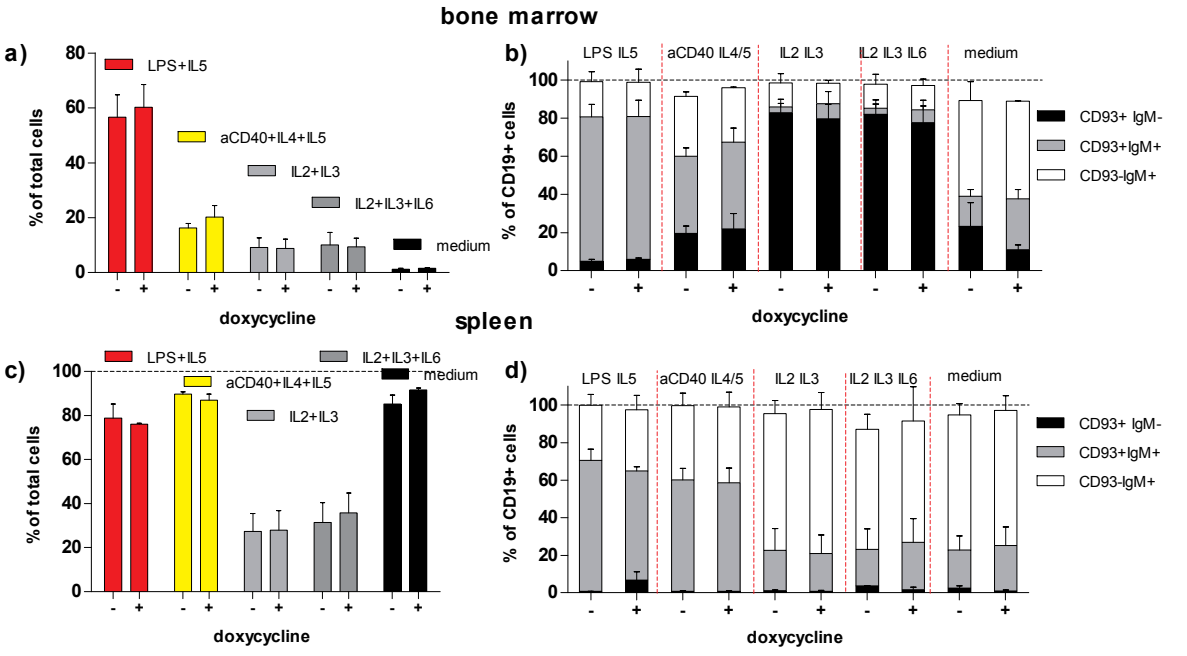

Figure 5.27 – Influence of Pim1 and Myc overexpression in ex-vivo bone marrow and splenic cell cultures upon the percentage of B-cells in the presence of different B-cell mitogens and growth factors. Cells were cultured -/+ doxycycline. After 4 days, the percentages of $CD19^+$ cells in the bone marrow (a) and spleen (c) were assessed by FACS analysis. In addition, the percentages of immature and mature subsets of B-cells were compared between the different cultivation conditions -/+ doxycycline, in bone marrow (b) and spleen (d).

In bone marrow cultures, the highest percentage of $CD19^+$ B-cells was found in the cultures supplemented with LPS + IL-5 followed by the cultures stimulated with αCD40 + IL-4 + IL-5, which resulted

in around 60% and 20% B-cells, respectively, compared to 2% in medium alone (figure 5.27a). Cultures receiving IL-3 showed a smaller percentage of B-lineage cells compared to cultures held in medium alone. IL-3 selectively increased the numbers of surviving immature IgM$^-$ cells, whereas LPS+IL-5 and αCD40 + IL-4 + IL-5 selectively expanded the IgM$^+$ immature B-cells (figures 5.27b, 5.28, and data not shown).

Splenic B-cell cultures had the highest percentages of B-cells if cultured in medium alone or in the presence of αCD40 + IL-4 + IL-5 (90%) and in the presence of LPS + IL-5 (80%) (figure 5.27c). In contrast to the bone marrow B-cells, addition of IL-3 + IL-2 did not increase the percentage of immature IgM$^-$ B-cells (figure 5.27d).

Addition of doxycycline, and thus, overexpression of Pim1 and Myc, did not significantly change the percentages of CD19$^+$ cells. Only very slight differences could be detected, with a tendency to higher percentages of B-cells in the doxycycline-treated cultures.

Comparing the Percentage of Proliferating Cells

To assess if overexpression of Pim1 and Myc induce proliferation in BM and splenic B-cells, the percentages of B-cells which had undergone at least one division were compared between control cultures not receiving doxycycline and doxycycline-induced cultures. In the bone marrow, the CD19$^+$IgM$^+$ and

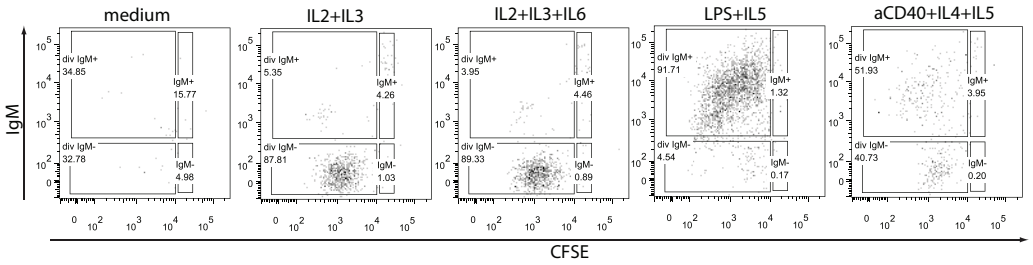

Figure 5.28 – Dot plots showing bone marrow cultures stained with CFSE (x-axis) and IgM (y-axis). Bone marrow B-cells were cultured for 4 days in the presence of the indicated stimulants. Then, FACS analysis was performed to reveal possible effects of Pim1 and Myc upon the numbers of divisions after 4 days. CD19$^+$, living cells are shown.

CD19$^+$IgM$^-$ fractions were analysed separately. See figure 5.28 for representative FACS plots showing IgM$^+$ and IgM$^-$ BM-B-cells analysed for CFSE-content. Splenic B-cells were analysed in total (IgM$^+$ and IgM$^-$ together), since there were only few IgM$^-$ cells. CFSE content of the different subsets was analysed by FACS (figure 5.29(a) and (b) for bone marrow and (c) for spleen).

IgM$^-$ BM cells: In medium alone, around 80% of bone marrow B-cells being IgM$^-$ 4 days after isolation had divided at least once. In bone marrow cultures supplemented with growth factors and interleukins named in figure 5.29a), almost all IgM$^-$ cells had undergone at least 1 division. Therefore, no differences between uninduced and doxycycline-induced cultures could be found. Furthermore, the majority of these IgM$^-$ cells were most probably host-derived, and hence, would not react to doxycycline.

IgM$^+$ BM cells: Culture conditions which led to at least one division in almost all of the B-cells (LPS + IL-5, LPS, αCD40 + IL-4 + IL-5) did not reveal any differences in percentage of cells undergoing at least one division. In contrast, BM B-cells grown in αCD4040 + IL-4 without IL-5, or IL-3 alone, or IL-5 alone, or IL-6 alone, or IL-2 + IL-3 or IL-2 + IL-3 + IL-6 all showed a slight increase in cells which had undergone at least one division (figure 5.29b and d; figure 5.28).

Splenic B-cells: In spleen cell cultures, there was no difference in the percentage of CD19$^+$ cells which had undergone at least 1 division between the doxycycline induced and the uninduced cultures, which is in line with the results from the growth curves of splenic B-cells shown above.

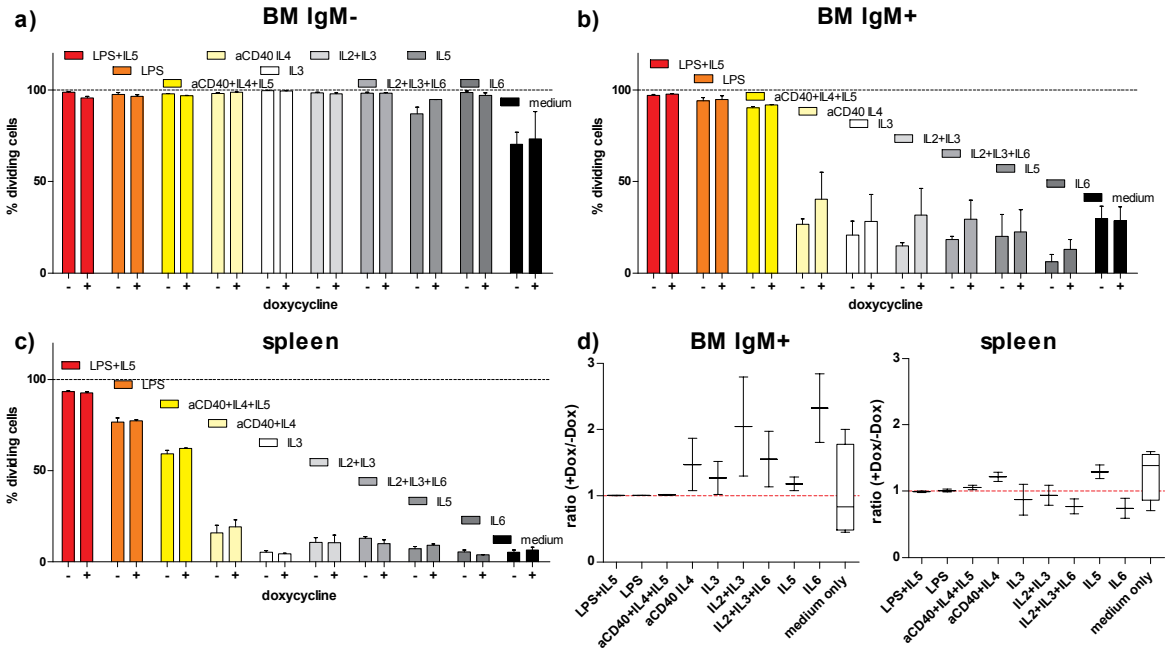

*Figure 5.29 – Influence of Pim1 and Myc overexpression in ex-vivo cultured bone marrow and splenic B-cells derived from 2-4 mice upon cell-cycle entry. Bone marrow and spleen cells were stained with CFSE and cultured for 4 days in the presence of different mitogens and growth factors. Percentages of B-cells and B-cell subsets which had undergone at least 1 division were compared between Pim1+Myc overexpressing cultures and control (doxycycline-uninduced) cultures (***a-c***). (d) Min/Max plots showing the quotient* $\frac{\%\ of\ cells\ divided\ at\ least\ 1x\ with\ dox}{\%\ of\ cells\ divided\ at\ least\ 1x\ without\ dox}$.

Comparing the Numbers of Divisions in Splenic Cultures -/+ Doxycycline

Overexpression of Myc and Pim1 might not only induce cell cycle entry in previously nonproliferating cells, but the two proto-oncogenes might also enhance or prolong proliferation. Therefore, the CFSE-labeled splenic B-cells were analysed upon how many times the individual cells divided, and doxycycline-induced cultures were compared to doxycycline-free cultures. There was no relevant difference in the distribution in the numbers of divisions between doxycycline-induced and -uninduced cultures (figure 5.30 a). Also in the bone marrow, there was no difference in the pattern of numbers of divisions of doxycycline-induced versus uninduced cells (figure 5.30 b).

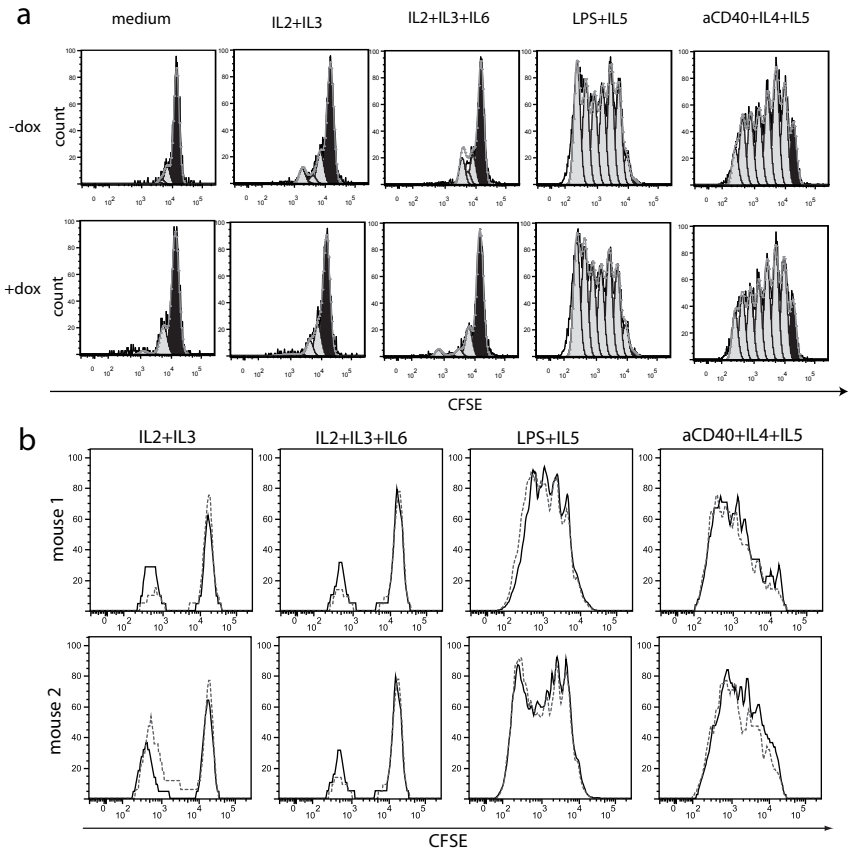

Figure 5.30 – (a) Influence of Pim1 and Myc overexpression in ex vivo splenic B-cell cultures on the number of divisions made after 4 days of culture. Splenic B-cells labeled with CFSE were cultured in the presence of the indicated growth factors and stimulants. Then, $CD19^+$ living cells were analysed by FACS for CFSE content with the FlowJo-Proliferation Software Module. Up to 8 populations of cells could be detected which had undergone 0-7 divisions. There was no difference in the distribution of the numbers of divisions between doxycycline-induced and -uninduced cells. (b) Ex vivo CFSE-staining of BM B-cells: Histogram overlays showing IgM^+ bone marrow cultures stained with CFSE. Bone marrow B-cells were cultured for 4 days in the presence of the indicated stimulants. Then, FACS analysis was performed to reveal possible effects of Pim1 and Myc upon the numbers of divisions after 4 days. black line: w/o doxycycline; grey line: with doxycycline

5.5 Overexpression of Pim1 and Myc in Activated B-Cells *in vivo*

5.5.1 B- and T-Cell Compartments of RAG1$^{-/-}$ Mice Transplanted with FL-preB-I Cells and Thymocytes

In the previous experiments, transplantations of preB-I-cells were done under conditions where mature B-cell compartments would be generated in the absence of T-cells. We therefore attempted to generate B-cell compartments in mice in which T-cells were present.

RAG KO mice transplanted with fetal liver preB-I-cells did not develop T-cells which could coactivate B-cells during immune responses against T-cell dependent protein antigens. Hence, after initial

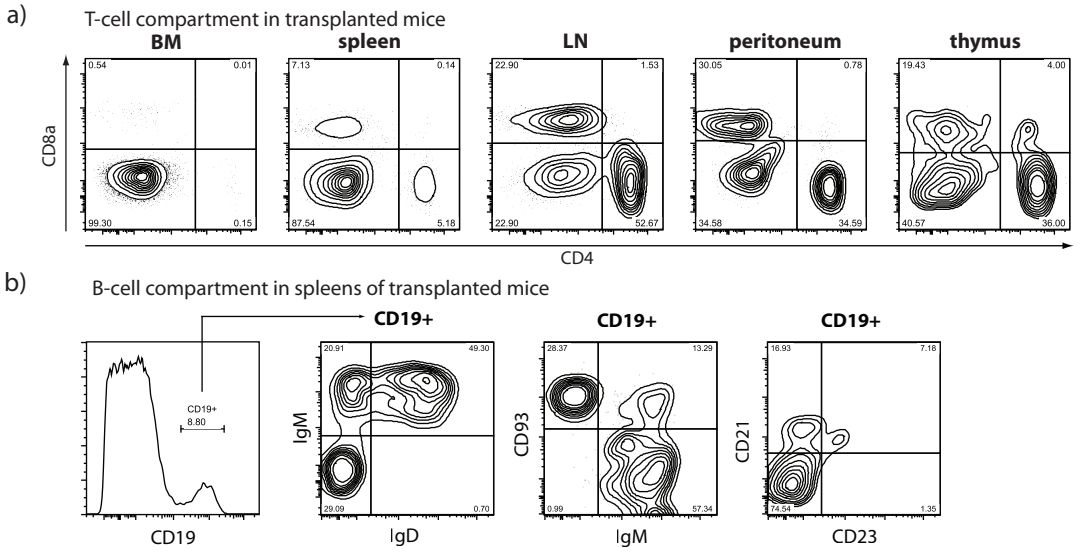

Figure 5.31 – (a) Reconstitution of the T-cell compartment of RAG KO mice by co-transplantation of thymocytes depleted of B-cells. Two months after transplantation, CD4$^+$ and CD8$^+$ T-cells were still readily detected in spleen, lymph nodes, peritoneal cavity and bone marrow. Thymi were barely detectable and contained practically no doublepositive T-cells as expected. (b) Phenotype of splenic B-cells in RAG KO mice transplanted with Pim1/Myc transgenic preB-I-cells together with thymocytes, 2 months after transplantation (in the absence of doxycycline).

co-transplantation tests conducted with PAX5-/- FL proB-cells, CD4$^+$CD25$^+$ splenic T-cells and B-cell-depleted DN2/DN3 thymocytes and total thymocytes as a source for T-cells, total thymocytes depleted of CD19$^+$ cells were chosen to be co-transplanted as an easy source for T-cell reconstitution.

	spleen	**thymus**
total CD4 single-positive cells	3.74x10^6 ± 1.93x10^6	5.43x10^4 ± 5x10^4
total CD8 single-positive cells	2.39x10^6 ± 1.78x10^6	6.6x10^4 ± 6x10^4
total CD4/CD8 double-positive cells	-	6x10^3 ± 6x10^3

Table 5 – Total numbers of T-cell subsets in spleen and thymus 8 weeks after co-transplantation of preB-I-cells and 3x10^5 CD19-depleted thymocytes. Numbers are derived from 3 mice each.

Thymocytes readily reconstituted the CD4$^+$ single-positive and CD8$^+$ single-positive T-cell compartments in the easily detectable lymph nodes, the spleen, the peritoneum, and the bone marrow (figure 5.31a and table 5). The thymus was barely detectable and contained few T-cells with practically no double-positive T-cells. T-cells repopulated the hosts long-term and were still detectable 8 months after

transplantation. The B-cell compartments of these mice were comparable to those of mice which did not receive cotransplanted thymocytes (section 5.4.1 on page 58, and figure 5.31b).

5.5.2 Overexpression of Myc and Pim1 in Activated B-Cells *in vivo* in the Presence of T-Cells

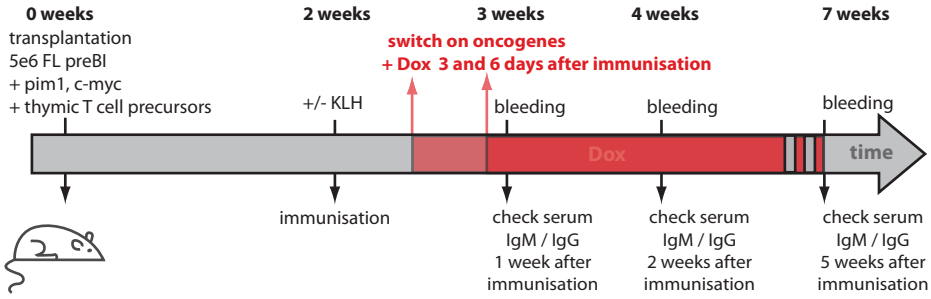

Figure 5.32 – Experimental setup: Generation of in vivo activated B-cells in RAG KO host mice reconstituted with Pim1/Myc transgenic B-cells and wild type thymocytes by immunisation with the T-cell dependent antigen KLH (keyhole limpet hemocyanin) and subsequent overexpression of Pim1/Myc in activated B-cells.

To assess the effect of Pim1+Myc overexpression on mature activated B-cells, 5x10^6 preB-I-cells carrying *Pim1+Myc* and 3e5 B-cell-depleted thymocytes from a C57Bl/6 wildtype mouse were co-transplanted into sublethally irradiated RAG1$^{-/-}$ mice to allow concomitant B- and T-cell reconstitution. Two weeks after transplantation, these mice were challenged with the T-cell dependent antigen KLH. Three and 6 days after immunisation, doxycycline was added to the drinking water of the mice to induce overexpression of Pim1 and Myc in B-cells at different stages of the KLH-induced immune response. Co-transplanted control mice encompassed immunised mice which did not receive doxycycline, as well as unimmunised mice which received only doxycycline (3 weeks after transplantation), and finally, mice which were left unimmunised and were not treated with doxycycline. For an outline of the experimental setup, see figure 5.32.

One week, 2 weeks and 5 weeks after immunisation, peripheral blood from the tail vein was collected, and serum was prepared. The sera were tested for KLH-specific and total IgM and IgG levels by ELISA.

As shown in figure 5.33, total IgM levels were slightly higher in KLH-immunised mice 2 and 5 weeks after immunisation. KLH-specific IgM levels increased only marginally in KLH-immunised mice with time after immunisation. Differences in IgM levels between unimmunised and immunised mice were highest 5 weeks after immunisation. In contrast to the preB-I-cell-transplanted RAG1 KO mice, control wildtype B57Bl/6 mice immunised with KLH had increased levels of total IgM as well as highly increased KLH-specific IgM antibody levels one and two weeks after immunisation. Unimmunised transplanted RAG KO mice receiving doxycycline showed only marginally higher total Ig-levels 1 and 2 weeks after immunisation in the serum compared with untreated unimmunised control mice. By contrast, immunised mice receiving doxycycline starting from 3 or 6 days after immunisation had increased total IgM levels 1, 2 and 5 weeks after immunisation (figure 5.33 right column). Total IgM levels were comparable to those in immunised wildtype mice. KLH-specific IgM levels were also higher than in control mice receiving only doxycycline or only KLH.

Total IgG levels of immunised mice were slightly higher than those of control mice starting 2 weeks after immunisation, and KLH-specific IgG levels were not changed. Five weeks after immunisation, total and KLH-specific IgG antibody levels in all groups of mice had a high variance (figure 5.34). Mice receiving doxycycline in addition to KLH-immunisation showed slightly increased total IgG levels especially at week

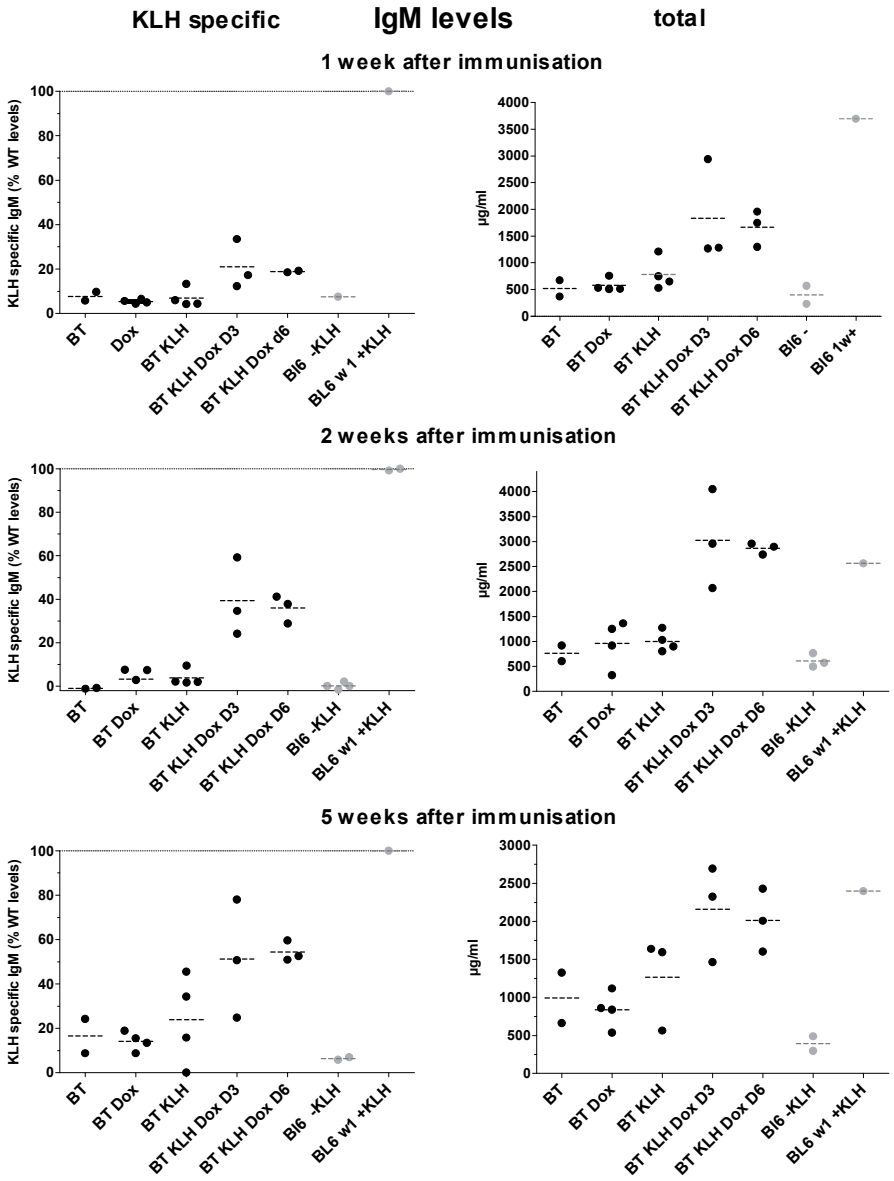

Figure 5.33 – ELISA (IgM) of sera taken from immunised RAG1 KO mice carrying Pim1/Myc transgenic B-cells. IgM antibody levels in the serum were measured 1, 2, and 5 weeks after immunisation. For KLH-specific IgM (left column), serum IgM levels were normalised to KLH-specific IgM serum levels of WT mice 1 week after immunisation (=100%). BT: RAG KO mice were reconstituted with Pim1/Myc-transgenic fetal liver preB-I-cells and thymocytes. KLH: mice were immunised 2 weeks after transplantation with KLH (keyhole limpet hemocyanin). D3, D6: 3 or 6 days after immunisation, mice were fed with doxycycline to switch on Myc and Pim1 in B-cells. BL6: wild type mice, either unimmunised, or 1 week after immunisation with KLH (grey dots).

2 after immunisation. KLH-specific IgG levels were also increased in the second week after immunisation in this group of mice.

In summary, KLH-immunisation of RAG KO mice transplanted with fetal liver preB-I-cells and thymocytes yielded only low quantities of antigen-specific IgM and barely detectable antigen-specific IgG in the absence of doxycycline-induction, i.e. only 5% of WT levels of specific IgM and less than 1% of WT

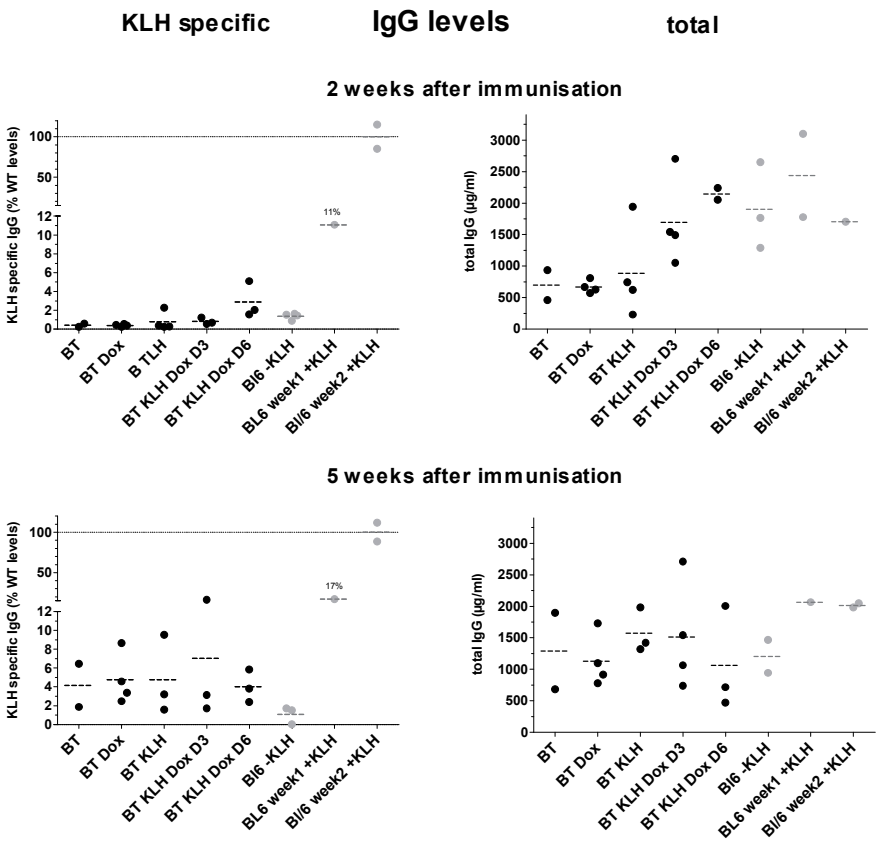

Figure 5.34 – ELISA (IgG) of sera taken from immunised RAG1 KO mice carrying Pim1/Myc transgenic B-cells. Some mice were immunised 2 weeks after transplantation with KLH (keyhole limpet hemocyanin). Three and 6 days after immunisation, some mice were fed with doxycycline to switch on Myc and Pim1 in B-cells. IgG antibody levels in the serum were measured 2 and 5 weeks after immunisation. For KLH-specific IgG (left column), serum IgG levels were normalised to KLH-specific IgG serum levels of WT mice 2 weeks after immunisation (=100%).

levels of KLH-specific IgG, which was barely above background levels of unimmunised mice.

Although immunisation did not yield KLH-specific IgG-antibodies, activation of the proto-oncogenes *Pim1* and *Myc* had an effect upon B-cells in immunised mice. Total IgM levels were only significantly increased in mice which were immunised and additionally received doxycycline to activate Pim1 and Myc. Also KLH-specific IgM levels increased in that manner. The same was true for IgG; total IgG and KLH-specific IgG only rose well above background levels if mice were immunised and Pim1 and Myc were activated after immunisation. KLH specific IgM levels were highest in mice which were fed with doxycycline 3 days after immunisation, thus at an early timepoint of the immune response, probably at a timepoint where switching to other isotypes did not yet start. On the other hand, KLH specific IgG levels were highest when immunised mice were fed with doxycycline 6 days after immunisation, thus during or after switching. According to this, overexpression of Myc and Pim1 together in early activated B-cells leads to enhanced IgM production, probably by enhancing or prolonging proliferation and/or Ig secretion.

5.5.3 Does a T-cell Dependent Antigen also Elicit B-Cell Immune Responses in the Absence of T-Cells?

As shown above in section 5.5 on page 71, immunisations of RAG KO mice harbouring *Myc/Pim1* transgenic B-cells and thymic T-cells only showed marginally increased KLH-specific IgM serum levels but did not yield relevant KLH-specific IgG levels. Therefore, a control experiment was conducted to evaluate if immunisation with KLH was indeed T-cell dependent or wether immunisation with KLH would also yield enhanced IgM serum levels in RAG KO mice only harbouring transgenic B-cells. As

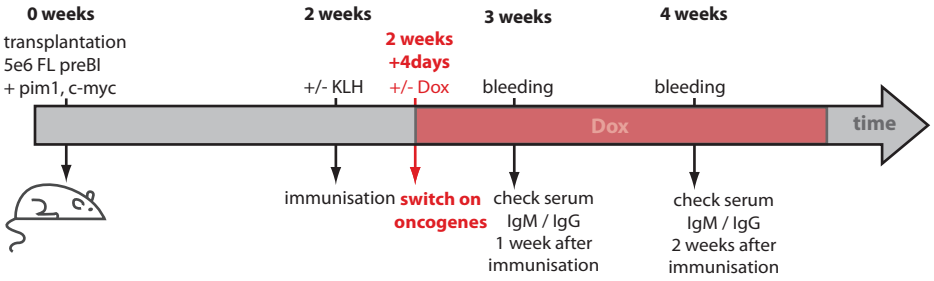

Figure 5.35 – Outline of the control experiment which clarified wether the increased IgM and IgG serum levels in KLH-immunised transplanted mice harbouring Pim1+Myc-overexpressing B-cells are dependent on the presence of T-cells

shown in the outline in figure 5.35, sublethally irradiated RAG KO mice were transplanted with $5x10^6$ fetal liver preB-I-cells carrying TetON-*Myc* and -*Pim1*. Two weeks after transplantation, 2 groups of mice were immunised with 50 μg KLH in 50 μl TiterMax Gold into the neck fold. Four days after immunisation, 1 group of immunised mice and 1 group of unimmunised transplanted mice were fed with doxycycline in the drinking water. Antibody serum levels were tested for total IgM and IgG and KLH specific IgM and IgG 1 and 2 weeks after immunisation. In general, total IgM levels of RAG KO mice harbouring only transgenic B-cells were up to 2x lower than those of RAG KO mice harbouring transgenic B-cells plus thymic T-cells, and total IgG cells were even more than 10x lower in T-cell free mice. As shown in figure 5.36, total IgM levels were not increased in immunised mice compared to control mice after 1 and 2 weeks. IgG levels were very low in all mice and did not remarkably rise after immunisation with KLH. Remarkably, IgM-antibody levels did not increase in RAG KO mice transplanted with fetal liver preB-I-cells expressing Myc and Pim1 after immunisation. It can be concluded that the increased IgM serum levels in immunised mice transplanted with FL-preB-I-cells expressing Pim1 and Myc together after immunisation is at least partially dependent on T-cells or T-cell derived factors.

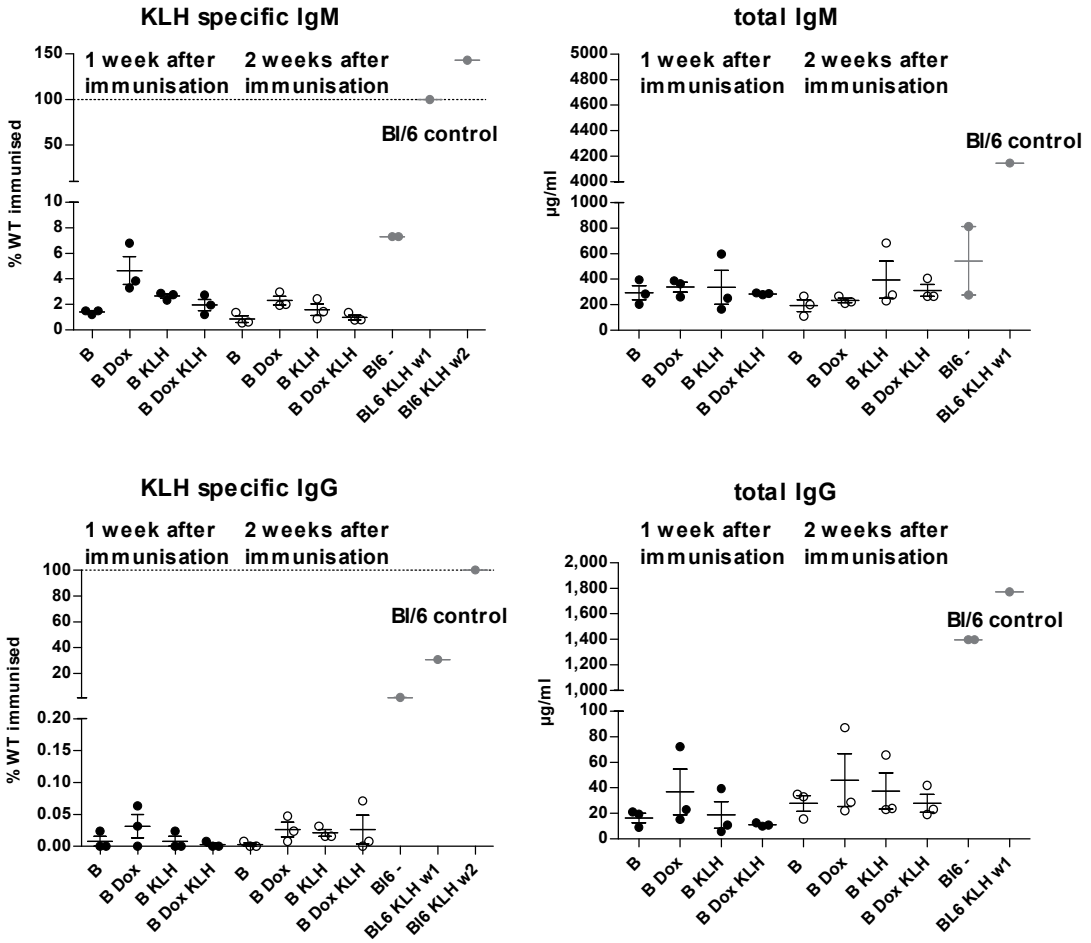

Figure 5.36 – Serum IgM and IgG-levels of RAG KO mice transplanted with Pim1/Myc transgenic B-cells in the presence of T-cell dependent antigen

6 Discussion

6.1 Establishing the System for Overexpression of Transgenes

6.1.1 Conditional Overexpression of Proto-Oncogenes in Murine B-Cells Using a Retroviral TetON System

The retroviral TetON vector system used in this thesis work is suitable to conditionally overexpress proto-oncogenes at different stages of murine B-cell development *in vitro* as well as *in vivo*.

The vector system includes 2 components:

- Two vectors carrying the genes of Pim1 and Myc under the control of doxycycline-inducible promoters.
- One vector carrying the rtTA (reverse transactivator) which is a fusion protein of a transactivation domain and a domain which binds the doxycycline-inducible promoters.

6.1.2 The Pros and Cons of Using a Retroviral TetON System in B-Cells

Normal B-cells and their progenitors cannot integrate DNA fragments by homologous recombination. Transfection of DNA by an electrical pulse or with the aid of liposomes is also not feasible, since it remains transient. Hence, proliferating B-lineage cells lose the transfected DNA and, thus, its possible effects rapidly. Hence, stable integration of foreign DNA into B-cells can only be achieved either by the generation of transgenic mice or by the use of retroviral vectors.

Because of the long time it takes to generate the appropriate transgenic mice carrying doxycycline-inducible forms of *Pim1* and *Myc* as well as the transactivator rtTA, such mice were not made. Instead, retroviral transduction of transgenes into host cells was used. It allows to generate a larger number of constructs and to transfect different combinations of these constructs rapidly. However, the transfected cells are transgenetically much more heterogenous because they carry one, or often more than one, retroviral inserts at different sites in the cellular genome.

Retroviruses are thought to integrate into the host genome in a sequence-unspecific, yet non-random manner [14]. Target site selection varies among different retroviruses. Preferred sites of integration are often actively transcribed and are therefore freely accessible [119]. Also, distortions of the DNA structures owing to the wrapping of DNA around nucleosomes can enhance integration of a retrovirus into a specific site. In the case of the mouse leukemia virus (MLV), integration occurs near the 5'end of a transcription unit in 20% of the cases [14]. Integration of the viral DNA could therefore destroy the integrity of a gene, either by integrating into the locus or by supplying its own promoters or viral enhancers to drive a previously silent cellular gene. Examples for this mechanism are the activation of Pim1 [25] or the activation of Myc in mouse T-cell lymphomas [22] by enhancers of the Moloney murine leukemia virus. This transforming activity of retroviruses is even used for studies which aim to find new proto-oncogenes.

Also, early attempts to use retroviruses in gene therapy have failed due to the generation of retrovirally induced leukaemias and neoplasias [26]. For example, in a trial conducted in 1999, 11 children suffering from serere X-linked immunodeficiency were treated with a MLV-based vector. Nine out of them showed clinical improvement, but during the following years, 4 of the patients developed T-cell leukemia. A sequence analysis of the T-cells from the patients demonstrated clonal expansion of T cell clones that contained an insertion of the vector in the vicinity of the LMO2 proto-oncogene which led to the activation of the LMO2 gene by the LTR enhancer [41].

In order to avoid secondary transformations induced by the integration of retroviruses and subsequent activation of cellular proto-oncogenes in the vicinity of retroviral promoters and enhancers, self-inactivating vectors (SIN) were used in this work. SIN vectors delete the enhancers and promotors upon integration into the cellular DNA. While SIN vectors are known to have lower titers (at least 10 times lower) than normal retroviral vectors ([21], p. 444), and therefore are more difficult to transduce into host cells, they proved to be sufficiently infective for preB-I cells (20-40% efficiency).

But one problem also remains when SIN vectors are used: Enhancers, suppressors or promoters of the host genome in the vicinity of the retroviral integration site could promote, or partially silence the transcription of genes inside the provirus. In the TetON vector expression system, this could lead to transcription of the doxycycline-driven proto-oncogene in the absence of doxycycline. Therefore, to minimize effects of cellular promoters, enhancers and suppressors upon the proviral proto-oncogenes, the expression cassette containing the proto-oncogene with the TRE-promoter was put into the vector in reverse orientation. Still, nearby cellular promoters and enhancers could lead to transcription from the minimal CMV promoter which is part of the TRE promoter. Hence, residual background transcription activity of the transgene is possible and has to be considered when interpreting the data.

Many onco-retroviruses can only infect proliferating cells, since they can only enter the nucleus during mitosis, when the nuclear membrane is disassembled ([21], p165). In addition, the selection of cells which carry the transgenes with selection markers such as antibiotic resistance is only successful if proliferating cells can be kept in culture for at least one week. In the case of B-cells, this means that only stages which can be cultivated *in vitro* are promising targets for retroviral infection. So far, the only B-cell stage which can be kept proliferating for extended periods of time is the preB-I-cell stage. PreB-I-cells taken from the fetal liver can be cultivated for weeks in the presence of bone marrow stromal feeder layers and IL-7. In contrast, the bone marrow-derived preB-I-cells grown in IL-7 on OP9 feeders, only proliferate for 20-30 days under these conditions before they die. Therefore, these cells were not used in this work.

Later stages of B-cells have to be derived from efficiently transduced preB-I cells, either *in vitro* by removing IL-7, or *in vivo* by transplanting preB-I cells which give rise to mature splenic and peritoneal B-cells.

Each transduced cell might integrate the viral DNA at a different place. Some of the loci are better places for transcription than others due to surrounding repressor- or enhancer elements. Also, some loci might be closed by remodelling of chromatin during differentiation of the cells. Furthermore, in the work of this thesis, up to three retroviral constructs had to be integrated into a single cell. The use of IRES (internal ribosomal entry site) sequences, which allow transcription of two genes from one single promoter, would allow the selection of cells which transcribe both the transgene and the selection marker behind at the same time. This trick was used in the expression vector for the rtTA, which has a constitutively active promoter. Hence, (almost) every cell which is resistant to histidinol also expresses the rtTA. For the inducible proto-oncogenes, this technique is not realizable, since this would mean that selection of efficiently transduced cells would have to be done in the presence of doxycycline. Consequently, the cell would also overexpress the transgene (oncogene) at an undesired time point. Hence, for the vectors carrying inducible transgenes, a second promoter was inserted into the retroviral backbone which allows constitutive transcription of the selection marker. However, that does not guarantee that every positively selected cell is really able to induce transgene expression. The result is a multitude of cells which are all more or less inducible for each of the transgenes, as shown in figure 6.1. Very likely, some of the cells which should express Pim1 and Myc only induce the expression of one of the proto-oncogenes. Also, the effectiveness of induction of the expression of both transgenes is most probably different from cell to cell. Therefore, in contrast to a transgenic mouse, where transgenic expression can be better controlled in each cell by controlled insertions of the constructs, retrovirally transduced cells are a mix of differentially

inducible cells (figure 6.1).

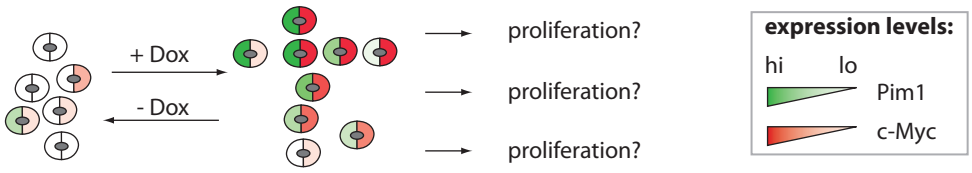

Figure 6.1 – Possible patterns of Myc- and Pim1-overexpression by the TetON expression vectors.

On the other hand, exactly this heterogeneity of expression levels in different cells is not necessarily a disadvantage. The effects of proto-oncogene overexpression in cells might also be dependent on the level of overexpression. Low levels of Myc and/or Pim1 overexpression might be too low for induction of proliferation, whereas too high levels could lead to genomic instability, as shown for Pim1 [109] and Myc [35] or apoptosis, in the case of Myc [142]. Also, the ratio of Myc to Pim1 overexpression might be important for induction of proliferation. In addition, levels of Myc and Pim1 necessary for induction of proliferation could be different in B-cells at different stages of development. Hence, the retroviral TetON system used here provides exactly this multitude of combinations of Pim1 and Myc inducibility.

6.1.3 Confirmation of Inducible Transcription and Translation of Transgenes

Overexpression of transgenes in preB-I cells was induced *in vitro* after addition of doxycycline to the culture medium. Only 30 minutes after addition of doxycycline, preB-I cells carrying the vectors showed increased mRNA levels of Myc and Pim1. Overexpression of transgenes was also increased in *ex vivo* transgenic splenic and bone marrow B-cells after induction with doxycycline.

Overexpression of Myc after doxycycline induction was also confirmed by SDS page and western blot. The Daudi cells used as a positive control are clonal and therefore all have the same Myc translocation. Hence, they should show a uniform expression of Myc, whereas the transduced, doxycycline treated FL preB-I cells expressing Myc consist of a pool of differentially inducible cells, as explained earlier. This heterogeneity in terms of Myc expression might result in the observed lower overall expression of Myc compared to the Daudi cell line.

Confirmation of Pim1 overexpression on the protein level by SDS page/western blot did not succeed with the Pim1 specific antibody used in these studies. Hence, upregulation of Pim1-mRNA and induction of growth-factor-independent proliferation only in transgenic preB-I cells carrying Myc and Pim1 together had to be taken as a proof for successful Pim1 expression after doxycycline-induction.

Upon induction of expression of EGFP in stably transduced preB-I cell lines, EGFP protein was detectable after 2 hours and subsequently rose during the next 22 hours. Finally, about 35-50% of the cells carrying EGFP showed EGFP fluorescence intensities above the threshold, as defined by the cells without EGFP-vector, with no distinct peak of EGFP fluorescence intensity. The lack of a distinct “peak” of EGFP-positive cells might be a consequence of the heterogenous cell population with cells carrying different copy numbers of retroviral inserts as well as cells carrying silent or badly transcribed vectors as discussed above.

Induction of transgenes *in vivo* was achieved by adding doxycycline to the acidified drinking water of mice carrying transgenic B-cells.

Doxycycline concentrations normally used for *in vivo* induction of TetOn systems range from 0.02 g/liter up to 2 g/liter. Mice fed with 2 g/liter doxycycline showed severe signs of dehydration after only

a couple of days [17]. Therefore, for the *in vivo* experiments conducted in this work, only 0.2 g/liter were administered.

Over the course of the *in vivo* experiments, mice receiving doxycycline did not show signs of indisposition.

The addition of sucrose to doxycycline-containing drinking water to improve palatability is widespread, but controversial. Comparative studies done with reporter mice receiving doxycycline either with or without 5% sucrose showed that the addition of doxycyline alone did not affect the water uptake of mice, which lies in between 4-5 ml per day. On the other hand, addition of 5% sucrose lead to excessive water uptake of around 14 ml per mouse and day (around 3 times the normal uptake) and concomitant reduction of food-intake. Furthermore, the sweetened water was often contaminated with moulds [59]. Hence, to ensure the uptake of sufficient amounts of doxycycline and, at the same time, not to allow growth of mould, only 1% sucrose was added to the drinking water in the studies here.

6.1.4 Reversibility of Induced Overexpression of Transgenes

The induction of transgene expression was reversible. PreB-I cells carrying an inducible EGFP transgene already showed a reduction of EGFP-expression 2 hours after removal of doxycycline, followed by a very slow decline during the next 6 hours. After 3 days, no residual EGFP-expression was detectable anymore.

The time it takes to completely switch off transgene expression after removal of doxycyline is dependent on the stability of the transgenic RNA and protein. In the case of EGFP, the half life of the protein has been shown to be around 26 hours [23]. In contrast, the half-life of Myc RNA and protein in mammalian cells have been measured to be only around 20-30 minutes [46] [38] [124], and the half-life of Pim1 protein in Bcr-Abl tumour cells has been shown to be 1.3 hours [122]. Hence, it can be reasoned that the time it takes to completely switch off expression of Pim1 and Myc is much shorter than that of EGFP. Firefly luciferase has a half-life of around 3 hours [131] which makes it a better candidate to estimate the time it takes to induce or shut off expression of Myc and Pim1 than the long-lived EGFP. Experiments done with CHO cells carrying a doxycycline-repressible (TetOff-) firefly-luciferase have shown that luciferase-activity started to increase 30 minutes after removal of doxycycline from the medium [24]. Vice versa, luciferase-activity in doxycycline-treated cells started to drop as soon as 30 minutes after addition of doxycycline and reached baseline levels already after 9 hours.

6.1.5 Leakyness of the Doxycycline-Inducible Expression System

FACS analysis of preB-I-cell lines carrying the rtTA-TetON vector and the TetON-EGFP vector showed measurable, but only weak EGFP expression in the absence of doxycycline in a small portion of cells. Hence, within the polyclonal population of EGFP-transfected cells, integration of the EGFP-vector might have occurred at genomic sites that allowed EGFP expression at low levels even in the absence of the inducer of gene expression, e.g. in the absence of doxycycline. As mentioned above, some murine leukemia viruses often integrate in the 5' end of a transcription unit, i.e. in the vicinity of the corresponding promoter and/or enhancer. Such an enhancer could activate the inactive minimal CMV promoter which is part of the TetON-expression vectors used here in the absence of doxycycline (figure 6.2a). Even the enhancer elements of the promoter which drives the resistance gene inside the proviral cassette could possibly influence expression of the ΔCMV promoter in the absence of doxycyline (figure 6.2b). A direct influence of a promoter upon the inducible transgene is more unlikely, since the inducible expression cassette is integrated into the vector in reverse orientation, and therefore, transcription started by this cellular promoter would need to proceed through the whole resistance cassette before reaching the inducible

transgene (figure 6.2c).

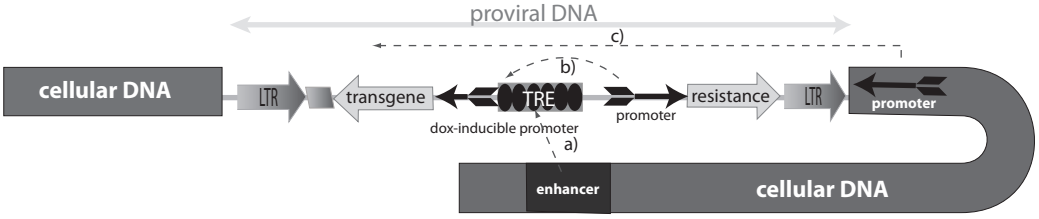

Figure 6.2 – Possible causes for leaky expression of the retrovirally integrated inducible transgene in the absence of doxycycline: Enhancers of cellular promoters in the vicinity of the provirus (a) or within the provirus (b) could activate the minimal CMV promoter. It is unlikely that a promoter in the vicinity of the provirus transcribes the inducible transgene, since transcription would collide with that of the resistance gene (c).

Leaky, but low overexpression of the oncogenes Pim1 and especially Myc in the absence of doxycycline can be neglected in short-term experiments, since there is not enough time to select for the few cells which exhibit background-Myc/Pim1 expression with concomitant enhanced survival. But in long-term experiments such as transplantations, the few cells with unwanted overexpression of the proto-oncogenes might accumulate in the host over time, which might mask the effect of doxycycline-dependent induction of Pim1+Myc in the cells.

6.1.6 The Use of Fetal Liver-Derived preB-I-Cells versus Bone Marrow-Derived preB-I-Cells for the Generation of B-Cells *in vitro* and *in vivo*

As stated above, only fetal liver-derived preB-I-cells can be cultivated *in vitro* for extended periods of time [112]. Fetal liver-derived preB-I-cells have been reported to repopulate preferentially the B1 compartment [55]. On the other hand, bone marrow-derived preB-I cells have been shown to repopulate mainly the B2 compartment [50], but proliferated in the presence of IL-7 and stromal cell feeder layers for only 20-30 days in our hands. Furthermore, bone marrow-derived hematopoietic cells are not as readily transduced with the vectors used in this thesis than fetal liver-derived preB-I cells (personal observation, data not shown). Hence, for repetitive transductions and selection of transduced cells *in vitro*, fetal liver-derived preB-I cells had to be taken.

Transplantation of fetal liver preB-I cells grown in IL-7 on OP9 feeders into RAG KO hosts indeed replenished almost exclusively the B1-B/MZ-B-cell compartment in peritoneal cavity and spleen as judged by expression patterns of CD5, IgD, CD21, and the lack of CD23 expression in splenic B-cells. Co-transplantation of thymocytes at the day of transplantation of preB-I cells did not support the generation of B2-B-cells. Upon immunisation of B/T-transplanted RAG KO mice with the T_H-dependent antigen KLH, there was no relevant increase in KLH-specific IgM and IgG-antibodies in the serum of those mice within 1-5 weeks after immunisation compared to wild type BL/6 mice. Reasons for this inability to properly react to T-cell dependent antigens might be the following: First, the pool of transplanted B-cells might not harbour the fitting antibody to recognize KLH-epitopes, simply because the number of transferred, surviving cells is too small, as it is not replenished from precursors in the bone marrow. Second, the B-cells might not be able to process the signals of activated T_H-cells or not be able to react to T-cell dependent antigens, because $CD23^+CD21^{lo}$ B-cells are needed for T-cell dependent responses, and, last but not least, the transferred T-cells might be unable to respond properly to the antigen.

At the moment, different transplantation schemes with B-cell precursors from the bone marrow or fetal liver, as well as T-cell precursors or T-cells, are tested in our group to find a satisfying transplantation

protocol which warrants proper reconstitution of all B- and T-cell compartments necessary for T-cell dependent B-cell responses in RAG KO mice.

6.2 *In vitro* and *in vivo* Maturation of Fetal Liver PreB-I Cells

As discussed earlier, mature, resting B-cells cannot be transduced retrovirally, and lipofection only transfers the vectors temporarily into the cells. Therefore, all maturation stages of B-cells later than the preB-I-cell stage had to be derived from those successfully transduced fetal liver preB-I cells.

Maturation of fetal liver preB-I cells is simply triggered by the removal of IL-7 from the cultivation medium. Within 3 days, 80% of the cells lost expression of the preB-I cell marker c-kit, and within 6 days, around 60% of the surviving cells were IgM^+ and 30% were IgD^+. Though, more than 95% of the cells retained CD93 (aa4.1) in their surface, which indicates that the cells are not fully mature. Furthermore, more than 90% of the cells died within the first 3 days of maturation. Hence, the generation of B-cells with a fully mature phenotype ($CD93^-$ IgD^+) was not possible *in vitro*.

To obtain fully mature B-cells, successfully transduced preB-I cells were transplanted into the tail veins of irradiated RAG KO mice. The cells mainly populated the spleen and the peritoneal cavity, but scarcely the bone marrow (most probably the few mature B-cells found there were recirculating cells). In the spleen, cell numbers rose continually within eight weeks after transplantation. $CD19^+$ cell numbers in the different organs depended on the cell line. Two, four and eight weeks after transplantation, the populations within the spleen included up to 50% mature B-cells $IgM^+IgD^+CD93^-$ as well as IgM^+CD93^+ transitional B-cells and up to 40 % IgM^-CD93^+ cells. In contrast, BL/6 WT mice had more than 90 % mature B-cells and less than 10% immature and transitional B-cells. Furthermore, only B-cells with a MZ-like phenotype ($CD21^+$) could be detected in transplanted RAG KO mice, but practically no follicular $CD21^{lo}CD23^+$ B-cells as explained earlier, whereas WT B1/6 mice had around 80% follicular B-cells and 9% MZ B-cells.

This lack of follicular B-cells was not caused by a lack of T-cells, since co-transplantation of thymic T-cell precursors did not rescue the follicular B-cell phenotype, though the T-cell precursors matured to $CD4^+$ or $CD8^+$ single-positive T cells in the spleen, lymph nodes, peritoneal cavities, and bone marrow, where they persisted for at least 8 months. Mice transplanted with fetal liver preB-I cells readily generated satisfying IgM- and low IgG- serum antibodies. In the presence of co-transplanted T cells, serum levels of IgM and IgG were increased around 4 times and 10 times, respectively.

Immunisation of RAG KO mice transplanted with fetal liver preB-I cells with the T_H-dependent KLH generated a low IgM-serum response, and an only very limited specific IgG-serum response as explained above. In contrast, wildtype mice readily generated KLH-specific serum IgG upon immunisation.

Hence, reconstitution of RAG KO mice with fetal liver preB-I cells was poor and failed to generate B-cell compartments which could switch and hypermutate to antigen-specific IgG responses. Still, it provided a first experimental system to test the effects of Pim1 and Myc overexpression *in vivo* and to generate fully mature B-cells *in vivo*, which could then tested for effects of Pim1 and Myc *in vitro*.

6.3 Overexpession of Pim1 and Myc in B-Cells at Different Stages of Development

6.3.1 Overexpression of a Single Transgene, Pim1 or Myc, in PreB-I-Cells

Normal mouse fetal liver preB-I-cells proliferate and are kept in their preB-I state of differentiation in the presence of IL-7. Upon removal of IL-7, preB-I-cells begin to differentiate to IgM^+ cells within 3 to 6

days. The majority of the cells are induced to apoptosis and die. After 6 days, there is hardly any living cell left.

Overexpression of murine Pim1 in preB-I-cells with the TetOn system used in this thesis does not lead to an increased survival or proliferation of preB-I or differentiating B-cells. PreB-I cells remain dependent on IL-7. This observation is in line with the results obtained by van Lohuizen et al. with Eμ-Pim1 transgenic mice, which revealed no abnormalities in hematopoietic tissues of healthy mice [133].

Overexpression of Myc alone in preB-I cells led to stable cell numbers over time (for at least two weeks) if the cells were supplemented with growth factors such as IL5 or αCD40+IL4. In the absence of these growth factors, the cells did not survive longer than 5-8 days. This effect of Myc-overexpression could be a consequence of enhanced proliferation, enhanced survival, or a combination of both, as well as a combination of enhanced proliferation with increased apoptosis.

Cell cycle analysis of preB-I cells overexpressing Myc for 2 days in the presence or absence of IL-7 indicated that Myc indeed increases the percentage of cycling cells. Addition of the B1-B-cell growth factor IL5 led to even higher percentages of cycling cells, whereas the B2-B-cell stimulants αCD40+IL4 did not increase the cell cycle entry of Myc-transgenic cells compared to Myc-transgenic cells cultivated in medium without growth factors.

These data indicate that overexpression of Myc increases cell cycle entry also in the absence of growth factors. This has also been observed by other groups. For example, in an EBV-transformed resting human B-cell line, overexpression of Myc alone induces cell cycle entry and proliferation [102]. Also, overexpression of Myc in density-arrested and serum-starved rat fibroblasts has been shown to induce proliferation. The duration of the productive cell cycles is hereby not changed, but only the frequency of cell cycle entry [57]. A detailed analysis of these cells showed that Myc overexpression indirectly activates preexisting cyclinE-CDK2 kinase activity which is necessary to proceed through the G1 phase [126]. Furthermore, Myc has been shown to upregulate expression of Cdc25a phosphatase, which activates cyclin-CDK2 complexes by dephosphorylating CDK2 [40].

On the other hand, it has been shown by several groups that Myc can induce apoptosis in different cell lines, which can be inhibited by the addition of the right kind of cytokines. Rat fibroblasts overexpressing Myc in the absence of growth factors are induced to apoptosis independently of the cell cycle state at much higher percentages than without Myc overexpression [52]. The addition of growth factors can inhibit Myc-induced apoptosis. Myc-dependent apoptosis was also reported in hematopoietic cells upon deprivation of growth factors [6]. Hence, the observed increased cycling together with a lack of increasing cell numbers over time might well be a consequence of a balance between enhanced proliferation and increased apoptosis.

In conclusion, even the combination of Myc overexpression and addition of survival factors was not sufficient to lead to an increase in cell numbers of preB-cells over time *in vitro*. Though overexpression of Myc alone was sufficient to induce cell cycle-entry and -completion of fetal liver preB-I cells, the generated cells were not able to survive long-term in the absence of IL-7.

6.3.2 Overexpression of Pim1 and Myc together in PreB-I-Cells *in vitro*

In the work presented here, it could be shown that overexpression of Myc and Pim1 together in fetal liver preB-I cells *in vitro* leads to proliferation of the cells with a resulting increase of cell numbers over time. Proliferation as such of Pim1/Myc transgenic cells is independent of growth factors such as IL-5 or αCD40+IL-4. However, the extent of proliferation and/or survival was cytokine-dependent, as cultures supplemented with IL-5 and also with αCD40+IL-4 reached higher cell numbers over time than those

cultivated in medium alone.

Cell cycle studies done with preB-I cells *in vitro* showed that cells which overexpress both Myc and Pim1 have higher percentages of cells in S- and M-phase than normal (doxycycline-uninduced) preB-cells. However, the percentages of Pim1+Myc overexpressing cells which were in S and G2/M phase were comparable to those of Myc-only transgenic cells. Interestingly, the cell cycle phosphatase Cdc25a, which is able to dephosphorylate and therefore activate the cyclin dependent kinase CDK2, forms a complex with Pim1 and acts as its substrate. Phosphorylation of Ccd25a by Pim1 increases Cdc25a-phosphatase activity [95]. Furthermore, Cdc25a activity has been shown to be limiting in serum-starved cells, and is also known to form a complex with the growth-factor-inducible protein kinase raf [126].

Taken together, though Myc and Pim1 can cooperate during cell cycle progression as shown by other groups, Myc alone is sufficient to lead to cell cycle entry in mouse fetal liver cells cultivated *in vitro* in the presence of 2% fetal calf serum. Overexpression of Pim1 in addition to Myc in mouse preB-cells *in vitro* did not further increase the percentage of cells in S/M/G2 phase, but led to an increase in cell numbers over time, most probably due to enhanced survival. AnnexinV-stainings, which will allow to estimate the percentage of pre-apoptotic cells, will reveal if the failure of Myc overexpression to increase preB-cell numbers over time is really due to enhanced apoptosis.

The phenotype of proliferating cells in the absence of IL-7 only slowly shifts from the c-kit$^+$ preB-I cell stage to the c-kit$^-$CD25$^+$ preB-II cell stage, and only few cells acquire IgM on the surface. These data propose that the cells are somehow inhibited to further differentiate. This is in line with the observation that Myc must be downregulated from the transition of preB-cells to mature B-cells and in plasmablasts to acquire the plasma cell stage [81]. The few IgM$^+$ cells which are found over time might be generated from preB-cells which do not express sufficient levels of Myc and Pim1, either from the beginning on, or during cultivation by silencing of the retroviral cassette.

6.3.3 Overexpression of Pim1 and Myc in IgM$^+$ Cells *in vitro*

*W*ith the inducible proto-oncogene overexpression system used in this thesis, it could be shown that *in vitro* differentiated IgM$^+$ immature B-cells start to proliferate if Myc and Pim1 are switched on. Proto-oncogene-induced proliferation is independent of growth factors, since immature IgM$^+$ B-cells also grew in the absence thereof. Anyhow, addition of the B1-B-cell growth factor IL-5 and also addition of αCD40 -/+ IL-4 led to increased total cell numbers compared to cultures without the addition of growth factors. On the other hand, addition of LPS or IL-2 led to diminished total IgM$^+$ B-cell numbers compared to the cultures without growth factors. IL-2 had been shown to expand a subfraction of IgM$^+$ plasmablasts *in vitro* taken from germinal centres of human tonsils [60]. LPS on the other hand drives plasma cell differentiation. It is not clear why IgM$^+$ cells stimulated with these plasma cell-inducing factors proliferate worse. A possible reason might be that either overexpression of Pim1 and Myc in B-cells triggered to become plasmablasts does not promote *in vitro* proliferation as well as in preB-I-cells and IgM$^+$ immature B-cells, or the stimulated cells might be more susceptible to (Myc-induced) apoptosis.

This growth-factor independent proliferation of Pim1+Myc-overexpressing IgM$^+$ immature B-cells is a further argument that overexpression of Myc hinders B-cell maturation. If normal B-cell maturation was not inhibited, preB-I cells overexpressing Pim1 and Myc in the absence of IL-7 would be expected to mature to IgM$^+$ B-cells during proliferation. Since also IgM$^+$ immature B-cells grow under these conditions, their percentage would slowly increase over time. But an increase in IgM$^+$ cells was not observed.

6.3.4 Limiting Dilution Analysis of Clonable, Pim1+Myc-Induced PreB-I-Cells

All experiments presented in this thesis were done with a pool of cells carrying resistance markers provided by the retroviral vectors. This doesn't necessarily mean that all cells were able to overexpress Pim1 and Myc sufficiently to lead to growth factor independent proliferation as discussed earlier. Different levels of rtTA-expression, or rare homologous recombination events during incorporation of the retroviral backbone, may have led to individual cells not being able to overexpress one of the proto-oncogenes adequately. Studies with different cell pools expressing TetON EGFP showed inducibility of EGFP-expression in ranges of 30% - 85% of the cells (data not shown). To get a rough estimate which fraction of preB-I-cells is induced to proliferate in the absence of IL-7 by overexpression of Pim1 and Myc, limiting dilutions were performed. Around 1 in 100 cells successfully produced a clone of at least 20 cells within 7 days in medium without growth factors. In the presence of growth factors such as IL-5 or αCD40+IL4, the frequency of successful clones rose to 1 in 30-70. This is far less than the frequency of successfully proliferating cells estimated from the growth curves of *Pim1+Myc* transgenic preB-I cells in the absence of growth factors (1 in 2-8). Why that? A possible reason is that many cell lines are not clonable at all though they grow well in bulk cultures. Hence, for a cell, living alone in 200 μl of unconditioned fresh medium might be a hostile environment due to lack of soluble cell-metabolites and cell-cell interactions, which makes it more difficult to survive and proliferate.

In short, Pim1 and Myc overexpression lead to growth factor-independent proliferation with concomitant increase of total cell numbers in around 1-10% of the original preB-I cells, depending on the conditons.

To determine if the low percentage of responding Pim1+Myc-expressing cells is the result of a low frequency of cells which can really induce Pim1 and Myc sufficiently, or if it is rather the effect of stochastic cell death, or even dependent on the appropriate ratio of Pim1- and Myc overexpression (or a mixture of all), transgenic preB-I cells have to be cloned and thereafter need to be subjected to limiting dilution. Cloning efforts done until now did not result in clones which were able to proliferate in limiting dilution assays.

6.3.5 Transplantation of PreB-I Cells Overexpressing Myc and/or Pim1 into RAG KO Mice

It could be shown that preB-cells overexpressing Pim1 and Myc together started to proliferate *in vitro* growth factor independently and were hindered to differentiate to IgM^+ cells. To elucidate what happens to those preB-cells *in vivo*, preB-I cells overexpressing Pim1 and Myc together were transplanted into RAG KO mice fed with doxycycline via the drinking water. In mice receiving doxycycline (= overexpressing Pim1 and Myc), the immature IgM^- and IgM^+ B-cell compartment was enlarged compared to doxycycline-uninduced mice. The enlarged preB-cell compartment could be observed in the peritoneum, in spleen and in the bone marrow and was especially obvious 4 weeks after transplantation. In spleen, also the mature B-cell compartment was enlarged 4 weeks after immunisation. Since *in vitro* experiments have indicated that Myc-overexpressing cells might not mature to IgM^+ cells, the observed increase of splenic mature B-cells in doxycycline-treated mice might be a cause of silencing of Myc-overexpression or variabilities in doxycycline-serum levels of the mice. The enlargement of the immature B-cell compartment is not surprising, since Eμ-*Myc* Eμ-*Pim1* doubletransgenic mice develop preB-cell tumours *in utero* [135].

After downregulation of Pim1/Myc overexpression, cell numbers fell down to those of doxycycline-uninduced mice within 4 weeks in the bone marrow and spleen, but not in the peritoneal cavity, where the cell numbers were around 4 times those of uninduced control mice. The most prominent B-cell phenotype

4 weeks after downregulation of Pim1 and Myc expression was IgM^+CD93^-. Hence, the majority of cells which had expanded under the influence of Pim1 and Myc overexpression disappeared after switching off expression of Pim1 and Myc, probably due to the lack of appropriate niches for the B-cells or due to the inability to properly mature and subsequent negative selection. Single cell PCRs of the expanded immature IgM^- B-cell compartment will reveal if these cells already have out-of-frame VDJ_H or VJ_L-rearrangements and are therefore lost after Pim1/Myc-downregulation.

In the case of RAG KO mice transplanted with *Myc*-single-transgenic FL-preB-I cells, no abnormal outgrowth of B-cell populations could be detected in the presence of doxycycline. This was not as expected, since Eμ-*Myc* transgenic mice have been shown to harbour enlarged pro- and preB-cell compartments already before birth in the fetal liver and later in the bone marrow and the spleen, although this expansion of early B-cells was not accompanied by a dramatic increase of total B-cells [75]. Though, there are a number of differences between the two systems. First, the Eμ-enhancer is thought to be active already prior to H-locus rearrangement [69]. Hence, expression of Eμ-*Myc* commences much earlier in B-cell development than that of TetON-Myc introduced into preB-I cells. Secondly, transplantations of FL preB-I cells into irradiated hosts only supplies one wave of maturing B-cells and does not replenish the bone marrow with long-term hematopoietic stem cells as existing in transgenic Eμ-*Myc* mice. Hence, only a limited pool of (pre)B-cells is available. Thirdly, the transplanted cell pool does not contain 100% equally Myc-inducible cells, whereas Eμ-*Myc* mice most probably harbour B-cells which all have the same active translocation. Furthermore, inducible Myc-expression in host mice transplanted with transgenic preB-I-cells is dependent on the uptake of doxycycline and the serum levels of active doxycycline over time. It is inevitable that there is some variation of doxycycline-concentration in the serum of these mice, which might lead to death of some of the cells due to subsequent variations in Myc protein levels.

Though there was no detectable increase in B-cell numbers in mice transplanted with Myc-expressing preB-I cells, the size of Myc-expressing cells in the spleen and the peritoneum of these mice was larger than that of mice which received control preB-I cells. Myc has been shown to increase protein synthesis and cell size of murine lymphocytes at all stages of development independently of the cell cycle phase [75] [62] [120]. Hence, the enlarged size of transplanted B-cells in the presence of doxycycline indicates that Myc-overexpression was indeed successful in these cells and, hence, the unresponsiveness of the cells was not simply due to a failure to overexpress Myc. In the bone marrow, no increase in cell size could be observed. This might be a consequence of the low number of transgenic B-cells present in the bone marrow compared to the number of host-derived proB/preB-cells existing RAG KO mice combined with the inability to outgrow the host-derived B-cell precursors. In contrast to the mice transplanted with Myc-expressing preB-I cells, the mice which received *Pim1/Myc*-transgenic B-cells also had B-cell precursors with a detectably larger size in the bone marrow in addition to those in the spleen and peritoneal cavity, most probably because the frequency of transgenic cells in the pool of BM $CD19^+$ cells, which was normally low, had increased due to the advantage in proliferation and/or survival of *Pim1/Myc* transgenic cells.

6.3.6 Mature B-Cells and Pim1+Myc Overexpression

As shown above, overexpression of Pim1 and Myc together in fetal liver preB-I cells and immature IgM^+ cells leads to proliferation of those cells with concomitant reduced maturation *in vitro* and *in vivo*.

But what about mature B-cells?

Pim1+Myc-transgenic mature B-cells extracted from spleens, bone marrow and peritoneal cavities of transplanted RAG KO mice could not be induced to sustained *in vitro*-proliferation with subsequent increase in total cell numbers upon stimulation of Pim1 and Myc overexpression. Still, subtle effects of

Pim1+Myc-overexpression could be detected. The cultures of splenic B-cells supplied with doxycycline survived longer.

CFSE-stainings of such cultivated bone marrow and spleen cells also did not show a doxycycline-induced increase in proliferation. There were only subtle changes in the number of cells which had proliferated at least once within 4 days or the average number of proliferations done within that timespan in bone marrow and splenic B-cells induced with doxycycline.

In short, B-cells taken from spleen, bone marrow or peritoneal cavity from transplanted RAG KO hosts could not be induced to sustained *in vitro* proliferation by overexpression of Pim1 and Myc, either without external stimulation or with the stimuli used in this work.

This does not yet mean that Myc/Pim1 overexpression in mature B-cells does not have any influence upon these cells, since not every *ex vivo* cell type can be adapted to proliferate *in vitro*.

Efforts to generate activated B-cells by cotransplanting thymocytes together with *Pim1+Myc* transgenic B-cells and subsequent immunisation of those mice with a T-cell dependent antigen were hampered by the inability of the transplanted B- and T-cells to properly react to the antigen. While a very minor antigen-specific antibody response either of the IgM or of the IgG isotype was detected, overexpression of Pim1 and Myc in transplanted B-cells resulted in increased total and KLH-specific IgM and IgG levels only in immunised mice, and not in unimmunised mice receiving doxycycline or KLH-immunised mice. Furthermore, in a control experiment conducted with mice which were transplanted with transgenic preB-cells, but without thymocytes, the same immunisation scheme in combination with doxycycline treatment did not result in enlarged IgM and IgG antibody levels.

Thus, a so far uncharacterized B-cell type or maturation stage which is generated or activated during an immune response in the presence of T-cells or T-cell-derived growth factors might have a proliferative or survival advantage if Pim1 and Myc are overexpressed. This proposed advantage seems to be short-term, since the difference in total IgM and IgG serum levels in Pim1+Myc overexpressing immunised mice did not further increase 5 weeks after immunisation as compared to 2 weeks after immunisation.

Further experiments aiming at characterising this Pim1+Myc-responsible activated B-cell type should be done with transgenic mice carrying inducible Pim1 and Myc genes to assure proper and stable expression of these transgenes. It might also be necessary to generate the proper B2-B-cell and T-cell compartments, maybe by using progenitor cells other than those generated from fetal liver. Fetal liver-derived preB-I-cells have been shown to generate insufficient B2-B-cell compartments which would be necessary to allow relevant T-cell dependent antibody responses if transplanted using the protocols presented herein. Since the retroviral vectors used so far could not efficiently transduce bone marrow B-cell precursors grown in the presence of IL3, IL6 and SCF, which generate B1- and B-2-B-cell compartments as well as the T-cell compartment, such vectors have to be designed.

With transgenic B-cells which are able to efficiently reconstitute also the B2-B-cell compartment, and which can cooperate in T-cell dependent antigen responses if transplanted into RAG KO mice, future experiments can be conducted to elucidate if, indeed, there is a B2-B-cell fraction which can respond to Pim1 and Myc overexpression with short-term or sustained proliferation. Changes in the compartment size of mature follicular B cells, germinal center B-cells (as characterized by FAS/CD95, GL7, CD86 (B7.2) and PNA), MZ cells ($CD21^+$), plasmablasts, and other subsets can then be distinguished by FACS analysis and by immunofluorescence stainings of frozen spleen sections.

Myc as a proto-oncogene is involved in a whole set of neoplasias of immature, mature, and activated B-cells. Burkitt's Lymphomas are predefined by the presence of a translocation of the Myc gene to the Ig_H gene (found in 80% of cases) or to the Igκ or Igλ gene (in the remaining 20% of cases) [36], bringing Myc expression under B-lineage control. Burkitt's lymphoma cells typically express monotypic surface

IgM, and the proliferation fraction is nearly 100%. In multiple myeloma (plasma-cell-derived neoplasia in the bone marrow), Myc protein levels are elevated due to a mutation which makes the mRNA more active (Willis et al. in [92]). Furthermore, Myc is often deregulated in Eμ-bcl2 mice which developed plasmacytomas (Harris et al. in [92]). BCL2 and Myc also cooperate in follicular lymphoma [65]. Finally, Myc and the oncogene V-ABL of the Abelson murine leukemia virus together generate plasmacytomas and preB-cell lymphomas in mice (Harris et al. in [92]).

Thus, Myc is very well able to cooperate in the neoplastic transformation of mature lymphocytes. From my results it appears that Pim1 is either not sufficient to cooperate with Myc in mature fetal liver-derived B-cells *in vitro* and *in vivo*, or that other partner oncogenes are needed for full transformation of mature fetal liver-derived B-cells.

7 Conclusion and Outlook

In conclusion, overexpression of Myc alone in fetal-liver-derived B-cells *in vitro* and *in vivo* does not efficiently lead to growth factor-independent proliferation though it increases cell cycle entry in preB-cells. Experiments done so far imply that the Myc-intrinsic ability to induce cell cycle entry and apoptosis at the same time might be skewed to apoptosis in some stages of B-cells. The scheme in figure 7.1 illustrates a

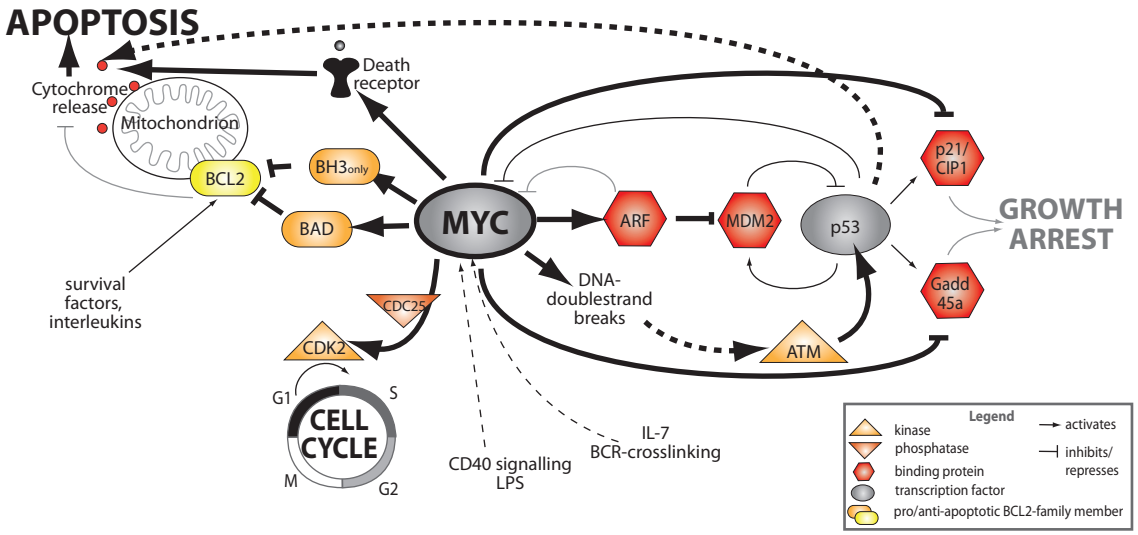

Figure 7.1 – Model of how Myc influences the balance between cell growth/proliferation and apoptosis. Results from several publications (see text) are pooled. Myc activates the ARF/MDM2/p53 pathway, which promotes growth arrest or apoptosis. At the same time, Myc blocks proteins which are important for growth arrest, and therefore promotes apoptosis. Furthermore, Myc activates pro-apoptotic BCL2-family members and sensitizes the cell for proapoptotic stimuli via death receptors. On the other hand, Myc upregulates proteins necessary for cell cycle entry and cell growth. Survival factors, mitogens, and death signals fine-tune this network, which results either in cell death or cell cycle entry.

simplistic model of how Myc might be implicated in tuning the intricate balance between cell cycle entry, growth arrest and apoptosis. Myc has been shown to indirectly activate p53 protein [123], which is a main switch between growth arrest and apoptosis. Myc downregulates ARF2 via FoxO-transcription factors, and hereby leads to activation of p53 by indirectly blocking the activity of ARF [58]. In the presence of Myc, p53 activity is skewed to induction of apoptosis [63]: Expression of P21/CIP1 and GADD45a, which are both activated by p53 and normally promote growth arrest, are blocked by Myc. Furthermore,

Myc leads to increased sensibility to death signals and activates a range of pro-apoptotic members of the BCL2 family *Hoffman2008*, which also block their antiapoptotic counterparts. In normal cells, Myc activity is tightly regulated by negative feedback loops, and might be induced only in certain settings such as stimulation of the BCR, or CD40 signalling, or LPS stimulation [68], and, in immature B-cells, upon IL-7-signalling [98]. Many of these triggers also induce the expression of pro-survival proteins [101]. However, in cells where Myc is deregulated for instance by translocation to a different locus, e.g. the Ig_H locus with its promoter, Myc can no longer be controlled by negative regulation anymore, and a cell might find itself in an environment which does not provide the necessary survival-signals.

And this is where PIM1, and probably a range of other Myc-cooperating proto-oncogenes such as BCL2, BCL6, and V-ABL, might come in. As shown in the model in figure 7.2, PIM1, in addition to stabilizing Myc protein and activating the phosphatase CDC25a which is essential for cell cycle entry [95], PIM1 is also able to block pro-apoptotic BCL2-family members [1]. Interestingly, PIM1 is also upregulated in response to αCD40- and LPS-signalling [141]. Hence, expression of PIM1 might be sufficient - at least in some B-cell differentiation stages - to shift the balance in favour to cell cycle progression and against induction of apoptosis.

Future experiments with PIM1+Myc transgenic B-cells will also include the generation of clonal preB-I-cell populations, each with a defined capacity to overexpress PIM1 and Myc. These clones can then be examined upon induction of proliferation at different immature B-cell stages, which will reveal if growth factor-independent proliferation is a clonal feature, i.e. might be dependent on the levels of Pim1/Myc overexpression or their ratio, or if it is a"stochastic" process, i.e. of a clonal population of preB-cells, only a certain percentage is able to proliferate.

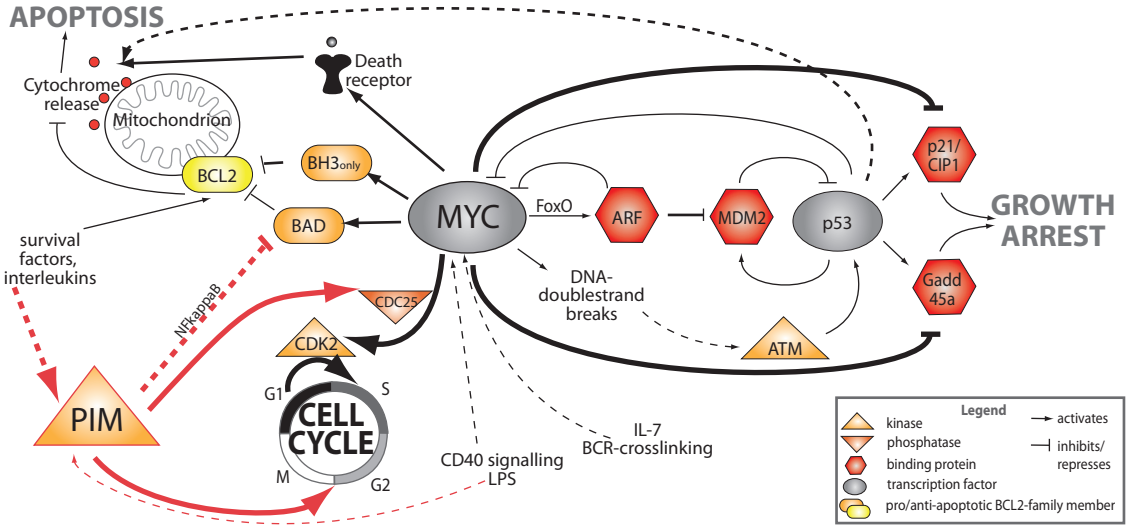

Figure 7.2 – Model of how PIM and Myc influence the balance between cell growth/proliferation and apoptosis. Results from several publications cited in this thesis are pooled. Pim1 might override the pro-death signals which Myc triggers by activating proapoptotic bcl2-family members. Furthermore, PIM is in involved in the activation of the cell cycle

Pim1 might not always be the right partner for Myc to generate B-cells which prefer proliferation to apoptosis. The results shown here imply that Pim1 and Myc might mainly cooperate to generate B-cell tumours with immature characteristics, since mature B-cells only showed marginal effects upon Pim1+Myc-overexpression *in vitro* and *in vivo*. Since it is not only Pim1 which regulates survival signals, it might well be that the general balance in more mature cells is skewed more drastically towards the apoptosis program, and PIM might simply not be strong enough to overcome this. Another possibility

is, that some important proteins which are activated by Pim1 in immature B-cells are not present or less abundant in mature B-cells.

Future experiments will include overexpression of Myc-mutants often found in Burkitt's Lymphoma which do not induce apoptosis anymore. Other cooperation partners for Myc instead of Pim1 which might also lead to a survival advantage of mature B-cells will also be tested as soon as usable T- and B-cell-reconstitution of host mice for antigen-specific responses is established.

Candidates for Myc-cooperating proteins which might allow sustained proliferation of cells are NFκB-signalling pathway components such as NEMO and IKK2, BCL-6, TCL-1, and PIM-2 and other survival factors or BCL-2 family members. Also, the effect of Pim1 and Myc overexpression in B-cells derived from other mouse lines might be interesting, since expression levels of proliferation- and apoptosis-related genes might be variable in different hosts.

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11/02-11/03	**Practical training** at the Institute for medical Microbiology, Basel, CH
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02/04-11/05	**Technical assistant** (Biozentrum Basel, CH)
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Lectures

During my studies, I attended lectures of the following lecturers: Prof. Dr. David Senn; Prof. Dr. Hanspeter Beck; Prof. Dr. Reto Brun; Prof. Dr. N. Weiss; Prof. Dr. M. Tanner; Prof. Dr. Volker Schmid; Prof. Dr. Gerd Pluschke; Prof. Dr. Urs Jenal; Prof. Dr. H. Rowell; Prof. Dr. Stephen C. Stearns; Prof. Dr. Christian Körner; Prof. Dr. U. Gisi; Dr. Jürg Oetiker; Prof. Dr. Andres Wiemken; Prof. Dr. Jürg Stöcklin; Prof. Dr. Walter Gehring; Prof. Dr. Gottfried Schatz; Prof. Dr. Helmar Burkhart.