New insights into the molecular and cellular requirements of lymphocyte development

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Dekan

For my family

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

Marie Skłodowska Curie

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2. Abbreviations

µH	µ heavy chain
AGM	aorta-gonad-mesonephros
AID	activation-induced cytidine deaminase
ALP	all-lymphoid progenitor
AML	acute myeloid leukemia
Bcl2	B-cell lymphoma 2
BCR	B-cell receptor
BLP	B-cell biased lymphoid progenitor
BM	bone marrow
BrdU	Bromodeoxyuridine
CD	Cluster of Differentiation antigen
CFU	colony-forming unit
CFSE	Carboxyfluorescein succinimidyl ester
cγ	“common” IL-2R gamma
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
DC	dendritic cell
DL1	Delta-like 1
DL4	Delta-like 4
DN	double-negative
DNA	deoxyribonucleic acid
DNP-KLH	dinitrophenyl-keyhole limpet hemocyanin
Ebf1	early B-cell factor 1
EPLM	early progenitor with lymphoid and myeloid potential
ES	embryonic stem
ETP	earliest thymic progenitor
Flt3	Fms-like tyrosine kinase 3
Flt3Ltg	human Flt3L transgenic

Flt3-ITD	Flt3-internal tandem duplication
FL	Flt3 ligand (manuscript 1)
FL	fetal liver (manuscript 3)
FoB	follicular B cell
FTOC	fetal thymic organ culture
GC	germinal center
Hhex	hematopoietically expressed homeobox
HSC	hematopoietic stem cell
IgH	immunoglobulin heavy
IgL	immunoglobulin light
IL	interleukin
IL-7R	IL-7 receptor
ILC	innate lymphoid cell
ISP	immature single positive
Jak	janus kinase
LMPP	lymphoid primed multipotent progenitor
LPS	lipopolysaccharide
MAIT	mucosal-associated invariant T
M-CSF	macrophage colony-stimulating factor
MEP	megakaryocyte-erythroid progenitor
miRNA	microRNA
MPP	multipotent progenitor
MZB	marginal zone B cell
N	non-templated nucleotide
NH	Nup98-HoxB4
NK	natural killer
NKT	natural killer T
NP	4-hydroxy-3-nitrophenylacetyl
Pax5	Paired box protein 5
pDC	plasmacytoid DC
Rag	recombination-activating gene
RNA	ribonucleic acid

RNAseq	RNA sequencing
RTOC	reaggregated thymic organ cultures
SCF	Stem-cell factor
SCID	severe combined immunodeficiency
STAT	signal transducer and activator of transcription
TCR	T-cell receptor
TdT	terminal deoxynucleotide transferase
TF	transcription factor
TI	T-cell independent
Treg	regulatory T cell
TSLP	thymic-stromal lymphopoietin
TSLPR	TSLP receptor
TSP	thymus-settling progenitor
WT	wild type
YFP	yellow fluorescent protein

3. Summary

The development of functional lymphocytes fending off pathogenic intruders yet tolerating self is dependent on the instructive or permissive action of soluble and/or membrane-bound factors. This is best exemplified by the loss, reduction, or functional impairment of some lymphocyte populations in mouse or man lacking designated cytokines, their receptors or important signal mediators.

The Early Progenitor with Lymphoid and Myeloid potential (or EPLM) has been identified in our laboratory several years ago [1]. This cell population combines two different fate options, namely lymphoid and myeloid, which were long thought being mutually exclusive. However, now we were able to show that the total EPLM population can be further subdivided into subpopulations with differentiation biases. This finding follows the trend that multipotentiality of a given progenitor cell population is often based on heterogeneity amongst the population, rather than single cells having all the fate options still utilizable. EPLM co-express the receptors for Fms-like tyrosine kinase 3 ligand (Flt3L) and interleukin-7 (IL-7) and the $Ly6D^{+}$ subpopulation of EPLM includes the direct progenitor of committed B-cell precursors. By generating an almost complete set of Flt3L and IL-7 deficient as well as transgenic mice and the respective combinations, we analyzed the involvement of Flt3L and IL-7 in the B-cell commitment process. Strikingly, excess Flt3L, which provides the system with tremendous numbers of functional precursors, rescued the B-cell defect in the absence of IL-7. Even if the anti-apoptotic factor Bcl2 was ectopically expressed, B-cell commitment as well as development were possible in $IL\text{-}7^{-/-}$Bcl2tg mice. These results contradict previous reports describing IL-7 as a non-redundant cytokine [2]. We provide evidence that IL-7 acts in a permissive way mediating survival of $Ly6D^{+}CD19^{-}$ EPLM precursors and proliferation of committed B-cell progenitors. The commitment event itself appears to be independent of the action of IL-7.

Under physiological conditions, hematopoiesis occurs in the specialized microenvironment of the bone marrow. Now we show the successful circumvention of stromal cell dependency in *in vitro* culture systems for the long-term propagation of mouse progenitor lymphocytes. B-lymphocyte precursors grew in suspension cultures supplemented with Flt3L, IL-7, and stem cell factor (SCF). This result suggests that signals mediated by SCF and Flt3L substitute those delivered by stromal cells, since up to now pro-B cell propagation was only possible in the presence of OP9 stromal cells and IL-7. T-lymphocyte precursors depend on plate-bound Notch1 ligand Delta-like 4 as well as soluble IL-7 and SCF. Such feeder-free proliferation and also differentiation systems have several advantages. Firstly, they provide control over the dose, strength, as well as duration of cytokines and other ligand signaling for instance. Thereby the molecular requirements for lymphocyte development can be further addressed. Subsequently, one could study the molecular and epigenetic changes accompanying the development and differentiation of mouse lymphocytes *in vitro*. Contaminations originating from stromal cell nucleic acids or proteins can now be completely ruled out. Secondly, in these cultures the progenitor cells remain accessible to manipulations, such as overexpression or shRNA-mediated knock-down of gene transcripts encoding proteins of interest. Since these progenitor lymphocytes maintain *in vivo* reconstitution capacity and build a functional immune system upon injection into sublethally irradiated recombination deficient hosts, the effect of mutations can be immediately observed *in vitro* but also *in vivo*. Thirdly, an efficient *in vitro* system for the expansion of functional progenitor cells is only likely to become approved for potential therapeutic applications in humans if no co-culture settings have been applied. The next step would be to extend the applicability of this system to human progenitor lymphocytes. This would be an ideal tool to address the still poorly defined requirements for human hematopoiesis and one could potentially use these cells in a therapeutic setting.

The fourth project that is discussed within this thesis addresses the B-cell co-receptor molecule CD19. As a positive regulator of B-cell receptor (BCR) signaling divergent expression of CD19 affects B-cell development. In order to address this

subject in further detail we employed the highly effective Nup98-HoxB4 (NH) hematopoietic stem cell (HSC) immortalization system developed by Ruedl et al. [3]. We ectopically expressed CD19 or a CD19 mRNA directed shRNA in these immortalized HSCs and reconstituted sublethally irradiated recipient mice. We observed diminished developmental progression from pre/proB cells to immature B cells due to transgene over-expression of CD19. Also reduction of CD19 expression resulted in reduced numbers of mature B cells with a developmental block at the immature to mature B cell transition. These results indicate that aberrant CD19 levels interfere with selection of B cells into the mature B-cell compartment. Tonic signals mediated by the BCR are indispensable for positive selection of B cells whereas a strong signal causes negative selection [4-10]. CD19 acts as a positive regulator of BCR signaling [11]. Therefore, high expression of CD19 might mimic a strong BCR signal, in this way mediating the developmental block. Low levels of CD19 might interfere with or dampen the tonic BCR signal required for positive selection of non-autoreactive immature B cells. Our results clearly indicate that a certain minimal threshold level of CD19 expression has to be reached but must not be widely exceeded, to mediate normal B-cell development. This highlights the important regulatory role of CD19 in fine-tuning the BCR signal response.

The projects described in this thesis address the molecular requirements for mouse lymphocyte development. Using several mutant and transgenic mice, we could clearly show that commitment to the B-cell lineage is possible without the action of IL-7. Moreover, we successfully developed stromal cell free *in vitro* culture systems to obtain large numbers of functional lymphocyte progenitors capable of *in vivo* reconstitution. A second *in vitro* culture system we successfully implemented is the Nup98-HoxB4 system. Multipotent HSCs can be expanded, manipulated, and the effect of a manipulation can be monitored upon *in vivo* reconstitution of a fully functional immune system. We found that the selection of mature B cells is critically dependent on the amount of CD19 expressed by B-cell progenitors.

4. Introduction

4.1 Early Hematopoiesis and a changing dogma

Hematopoiesis is the term used to describe the process of blood cell formation. There are many different kinds of blood cells but historically, these are broadly classified as “erythrocytes”, meaning they are red cells, “thrombocytes” meaning they are involved in thrombus formation, “myeloid cells”, meaning they are found in and are derived from the bone marrow, and “lymphoid cells” meaning they are found in lymphoid organs and circulate in lymphoid vessels. That the bone marrow was also a producer of some lymphoid cells was only discovered in the 1950s. Myeloid and lymphoid cells constitute respectively the innate and adaptive arms of the immune system fending off invading pathogens and transformed cells. Erythrocytes and thrombocytes are essential mediators of oxygen transport and blood coagulation, maintaining the integrity and functionality of the organism. The short lifespan of most hematopoietic cells implicates the necessity for their continuous replenishment from multipotent HSCs that reside in specialized cellular niches in the bone marrow. James Till and Ernest McCulloch originally demonstrated the existence of multipotent stem cells in the early sixties and thereby became the fathers of stem cell science [12, 13]. They studied the effect of radiation on the bone marrow of mice and found multipotent cells in hematopoietic tissue that proliferate and form numerable colonies upon injection into heavily irradiated mice suffering from bone marrow failure [14]. Ultimately, research on HSCs has led to their purification and characterization using phenotypic markers by Spangrude, Heimfeld, and Weissman in 1988 [15].

Hematopoiesis, meaning the formation of all cellular blood components, is one of the best-understood developmental processes. By definition, HSCs are capable of self-renewal as well as directed differentiation via distinct lineage precursors to form all cell types of the hematopoietic system. Subdivisions have been made in order to discriminate between long-term and short-term reconstitution capabilities upon

transplantation. Three models use differential cell-surface markers to best classify these HSC populations [16]. Already in 1996, Osawa et al. demonstrated long-term reconstitution of the lymphohematopoietic system from a single HSC highlighting the power of this cell type [17]. The balance between controlled self-renewal and differentiation needs to be tightly regulated, emphasized by the high incidence of hematologic diseases caused by interference in this process.

The characterization of oligopotent hematopoietic precursors supported the assumption of a stepwise differentiation process, in the course of which the hematopoietic cells gradually loose differentiation capabilities and become committed to a certain lineage. Downstream of the HSC compartment, where self-renewal capacity has been lost but multipotentiality retained, cells are described as multipotent progenitors (MPPs). Loss of megakaryocyte and erythroid potential characterizes the so-called lymphoid primed multipotent progenitor compartment (LMPP), where cells retain the capacity to give rise to myeloid and lymphoid cells [18]. At this early stage of differentiation, expression of lymphoid restricted genes such as *Rag*1 or *Rag2*, *Dntt* or *CD127* can already be correlated with lymphoid priming [19, 20]. Further downstream of LMPPs, in most models of hematopoiesis, progenitors become restricted to give rise to only lymphoid or myeloid cells, respectively. According to their initial lineage-restricted differentiation capability, these progenitor populations were termed common lymphoid progenitor (CLP) and common myeloid progenitor (CMP) [21, 22]. This classification into myeloid and lymphoid development was believed to be irrevocable and the two emerging lineages were thought to evolve in parallel proscribing any further bi-potential progenitors. This stepwise differentiation model of hematopoiesis was summarized as a hierarchical scheme with HSCs at the apex, generating intermediate precursor cell populations downstream and with the mature (end-stage) hematopoietic cell types at its end (Figure 1). In this branched model of hematopoiesis, progenitors with both lymphoid and myeloid capacity would seem to be excluded.

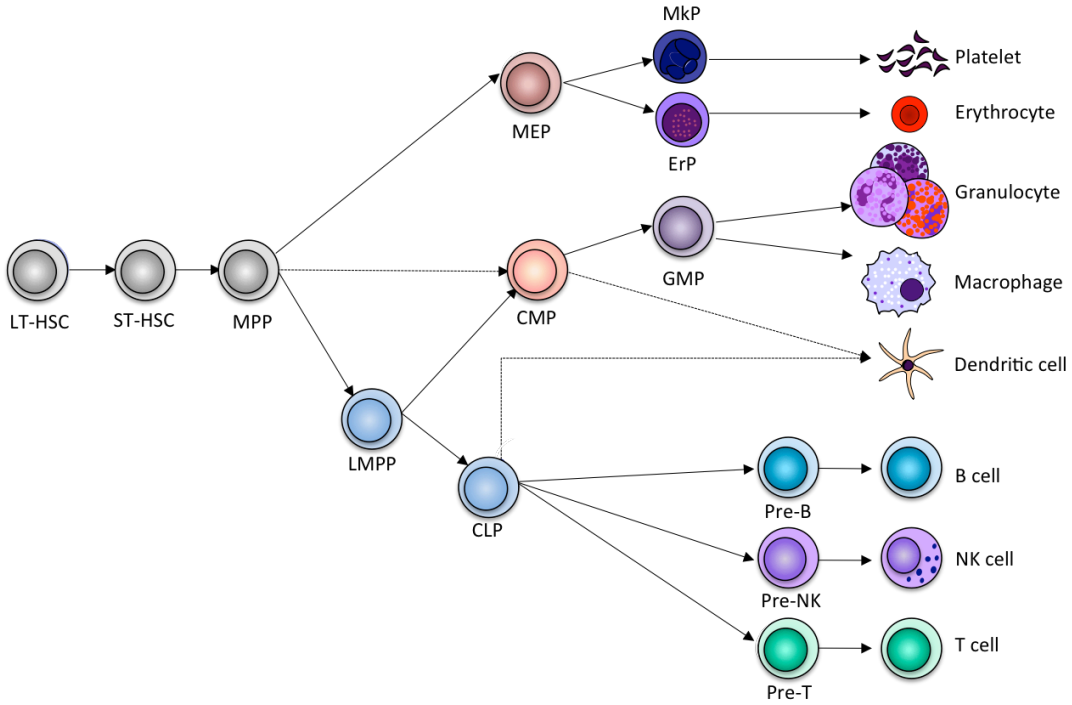

Figure 1: Classical hematopoietic scheme. Classical schematic representation of hematopoietic development with the HSC compartments at the apex and mature cell types resembling the end of the differentiation process. Arrows indicate differentiation into a more restricted population. In this model the lymphoid and myeloid branch of hematopoiesis are separated with the exception of dendritic cells. Moreover, the differentiation process is unidirectional with clear-cut developmental potentials assigned to the well-defined progenitor-cell populations. LT-HSC: long-term hematopoietic stem cell, ST-HSC: short-term HSC, MPP: multipotent progenitor, LMPP: lymphoid primed MPP, MEP: megakaryocyte/erythroid progenitor, ErP: erythrocyte progenitor, GMP: granulocyte/macrophage progenitor, MkP: megakaryocyte-committed progenitor, CLP: common lymphoid progenitor, Pre-B/NK/T: precursor B/NK/T cell.

The hematopoietic precursor populations placed in these schemes were initially described as homogenous and strictly compartmentalized pools of cells. Hematopoietic development was viewed as a unidirectional process and end-cell types were supposed to be generated via a series of precisely defined differentiation steps ultimately resulting in commitment. However, emerging evidence is challenging this constrained description of hematopoiesis with restricted routes of differentiation that anticipates specification as an irrevocable decision. New models of hematopoiesis favor a progressive, asymmetric loss of lineage potential, moving away from the strict compartmentalization and allowing for more plasticity [23].

4.1.1 Plasticity during hematopoietic development

Initial and strong indication for more plasticity within hematopoiesis came from studying pro-B cells generated in mice in which the *Pax5* gene had been deleted [24]. The *Pax5* gene encodes a master-transcription factor implicated in B-

lineage commitment and mice deficient in expression of this gene display a strict block in B-cell development [25]. Interestingly, *in vitro* propagated Pax5-deficient pro-B cells maintained plasticity with regards to lymphoid as well as myeloid differentiation capabilities both *in vitro* and *in vivo* [26]. Later on, a cell population matching these differentiation characteristics was described in the bone marrow of wild type mice. This cell population was called the early progenitor with lymphoid and myeloid potential – or shortly EPLM [1]. Even committed precursor B cells from normal, wild-type mice regain multipotentiality upon conditional loss of Pax5 [27] and a similar regain of multipotentiality has also been shown upon loss of Ebf1, resulting in plasticity among $CD19^{+}$ progenitors [28]. Interestingly, coincident heterozygous deletions of both Ebf1 and Pax5 caused T-lineage conversion within committed B-lymphoid progenitors [29]. In summary, these discoveries showed that the inactivation of a lineage commitment factor is able to activate the intrinsic multilineage potential of cells. Lineage plasticity has also been shown for the megakaryocyte/erythroid and granulocytic/macrophage lineages. Myelomonocytic differentiation depends on the transcription factor PU.1 [30] and the importance of the transcription factor GATA-1 for erythroid and megakaryocytic differentiation has been revealed by GATA-1 deficient mice [31-33]. These two proteins antagonize each other and enforced expression of GATA-1 in myeloblasts transforms them into megakaryocyte-erythroid progenitors (MEP), and enforced expression of PU.1 reprograms MEP cells into myeloblasts (see also Figure 2) [34, 35].

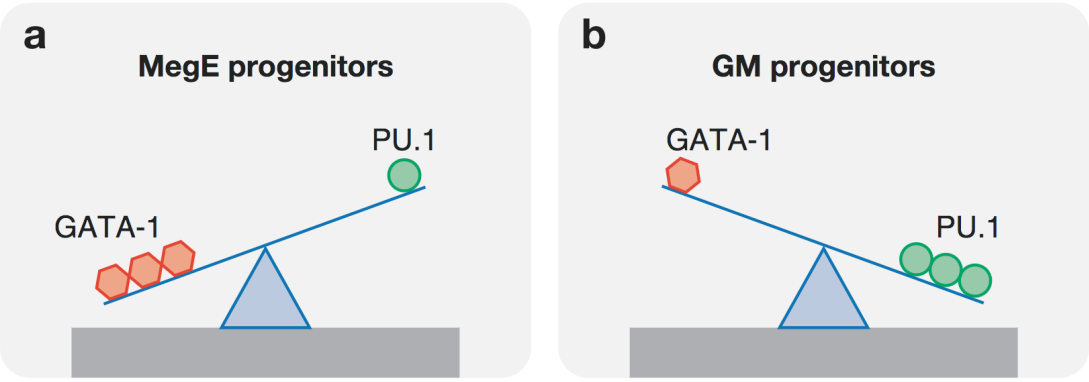

Figure 2: changing the balance of two lineage hematopoietic transcription factors induces reversible reprogramming of committed myeloid cells. Excess GATA-1 specifies MegE cell fate, and PU.1 in excess specifies GM cell fate. MegE: Megakaryocyte/erythroid. GM: Granulocyte/Macrophage. Figure taken from Laiosa, C.V., M. Stadtfeld, and T. Graf, Determinants of lymphoid-myeloid lineage diversification. Annu Rev Immunol, 2006. 24: p. 705-38.

This shows that just by changing the balance of two lineage transcription factors one can reprogram committed myeloid cells [36].

The identification of other progenitors with combined developmental potentials besides the EPLM provides further evidence for a less hierarchical model of hematopoiesis without the restricted lympho-myeloid diversification. In particular, cells sharing B/macrophage, T/macrophage, or natural killer (NK)/T differentiation capability have been described [37-40]. For the earliest thymocyte population a substantial B, macrophage, DC, and NK cell potential besides the T cell potential was demonstrated [41, 42]. Moreover, using transplantation, a dual origin from lymphoid and myeloid progenitor cells has been shown for plasmacytoid dendritic cells (pDCs), which revokes the strict compartmentalization of mature hematopoietic cells [43, 44]. Also transcriptionally identical human DCs were successfully derived from CLPs and CMPs [45]. These examples of lymphoid-myeloid bi-potential clearly contradict the obligatory bifurcation of hematopoiesis in lymphoid and myelo-erythroid lineages [46] and reveal a substantial plasticity amongst progenitor cells.

4.1.2 Heterogeneity

There is accumulating evidence that multipotent progenitor cell populations identified over the years are more heterogeneous than previously thought. For the CMPs differential cell-surface expression of Slamf1 (CD150), Endoglin (CD105), and Itga2b (CD41) was shown to be correlated with individual developmentally restricted lineage potentials for the granulocyte/macrophage, erythroid, and megakaryocytic lineages respectively [47]. Using single-cell RNA sequencing the groups of Amos Tanay and Ido Amit elegantly showed that myeloid progenitors consist of a mixture of transcriptionally primed cells [48], thereby supporting the initial sub-grouping based on cell-surface marker expression. *In vivo* lineage tracing experiments likewise illustrated heterogeneity within CMPs [49]. CLPs, characterized phenotypically by high surface expression of CD127, low-level expression of cKit (CD117) and Sca1 and absent expression of lineage markers were further sub-grouped after their initial description [21]. Already by 2000, expression of terminal deoxynucleotide

transferase (TdT) within the lineage negative fraction of bone marrow cells indicated CLP heterogeneity [50]. When Flt3 (CD135) was found to be expressed on multipotent progenitors downstream of the HSC [51], re-analysis of the CLP compartment revealed absolute lymphoid multipotentiality only within the $Flt3^+$ proportion. CLPs that have lost Flt3 expression mostly contained B-restricted progenitors [52]. Later on, Ly6D was identified and used to assign B-cell restricted progenitors within the $Flt3^+$ CLP population. Therefore, $Ly6D^+$ CLPs were termed BLPs (B-cell biased lymphoid progenitor), whereas $Ly6D^-$ CLPs were named ALPs – all lymphoid progenitors, since they retain T- as well as NK-cell potential [53]. Different combinations of expression levels of the surrogate light chain component lambda5 (λ5) and the Rag1 protein within the $Flt3^+$ CLP compartment were also shown to be useful in demonstrating restricted lineage potentials [54, 55]. Thus, a multitude of indications suggests that classic multipotent progenitors identified in the bone marrow of both mice and to a lesser extent in man are composed of a mixture of cells with more constrained lineage potentials. This again emphasizes the plasticity within the hematopoietic system and implicates a developmental continuum with fluent passages linking the so-far strict progenitor cell populations along the hematopoietic scheme. This raises the question of whether it will ever be possible to unambiguously identify a stage of hematopoiesis at which lineage commitment becomes irrevocable. Taken together, the above results would seem to force a rethink of current and propose alternative models of hematopoiesis.

4.1.3 The pairwise model of hematopoiesis

The identification of progenitor cells challenging the lymphoid/myeloid dichotomy and the increasing evidence for plasticity resulted in a pairwise model of hematopoiesis being proposed (Figure 3) [16, 56]. The multipotent HSC remains centered, whereas the developmental potentials of the hematopoietic progenitor populations are now illustrated as colored arches arranged in a circle around the HSC. With advancing development these arches become shorter indicating restriction of potentials and ultimately commitment. The waiver of lineage branching points and arrows allows for multiple alternative routes resulting in particular end-cell types, reflecting, for example, the development of identical DCs with dual origin

[45]. The placement of lineages in near-neighbor relationships illustrates the description of most bi-potent hematopoietic cell populations that can also cross the lymphoid-myeloid divide.

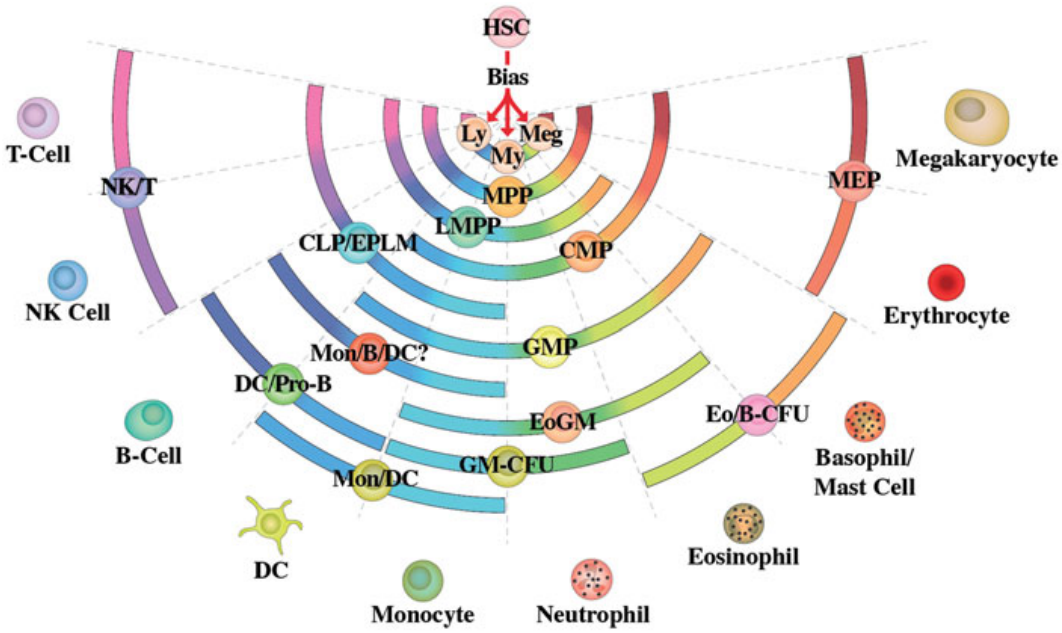

Figure 3: The pairwise model of hematopoiesis. This revised model of hematopoiesis places closely related lineages next to each other, without strictly compartmentalizing the scheme into different sub-lineages as the classical model is doing. The colored arches represent the known developmental potentials of individual progenitor cell populations, which allows for more than one route ultimately leading to the very same end-cell type. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DC/Pro-B, dendritic cell and B lymphocyte progenitor; Eo/B-CFU, eosinophil and basophil progenitor; EPLM, early progenitor with lymphoid and myeloid potential; GMP, granulocyte and macrophage progenitor; LMPP, lymphoid-primed multi-potent progenitor; MEP, megakaryocyte and erythrocyte progenitor; Mon/B/DC?, monocyte, B lymphocyte and dendritic cell? progenitor; Mon/DC, monocyte and dendritic cell progenitor; NK/T, natural killer cell and T lymphocyte progenitor; HSC, hematopoietic stem cell; Ly, lymphoid bias; Meg, megakaryocyte bias; My, myeloid bias. Figure taken from Brown, G., et al., Versatility of stem and progenitor cells and the instructive actions of cytokines on hematopoiesis. Crit Rev Clin Lab Sci, 2015. 52(4): p. 168-79.

One advantage of the pairwise model is that it enables flexibility and the return to multipotentiality, as was shown for $Pax5^{-/-}$ pro-B cells [26]. The contingencies of hematopoietic development are all represented and are now illustrated as a continuum. Despite the multitude of evidence pointing towards a less restricted lineage dichotomy, compartmentalization and unidirectionality within hematopoietic development, so far no one has shown the extent to which alternative routes are used *in vivo*.

4.1.4 Stem cell transplantation as model for steady-state hematopoiesis

Recent advances question our approaches of measuring stem cell activity and the way we believe hematopoiesis is working. Because the mainstay of research used to ascertain the functional properties of a given bone marrow progenitor population were transplantation experiments, the deduced models of hematopoiesis mostly describe blood cell formation under stress conditions. Myeloablated hosts with purified cell populations injected into the blood circulation are far apart from steady-state hematopoiesis. Nevertheless, these models suggested that only a small number of HSCs contribute to blood cell homeostasis [57]. Now, Sun et al. as well as Busch et al. provided strong evidence that in an unperturbed system, classical long-term HSCs have only limited contribution to blood cell formation. Instead thousands of lineage-restricted as well as multipotent clones are successively recruited into hematopoiesis, each of them with only minimal contribution, as it is illustrated in Figure 4 [58, 59].

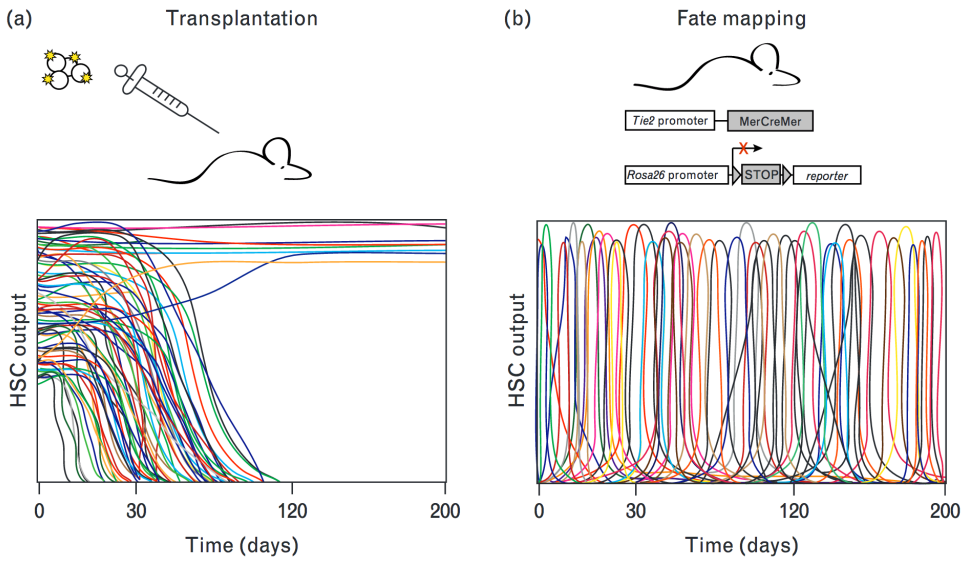

Figure 4: Schematic representation of HSC contribution to hematopoiesis tested in transplantation or by fate mapping. (a) Transplantation of HSCs results in transient multiclonal contribution by many different HSCs during the first weeks. However, multiclonality fades, as most of HSC clones are lost within the first 4 months. Long-term engraftment is achieved by few dominating HSC clones. (b) A fate map mouse model based on Tamoxifen induced Cre/loxP-mediated recombination of the Rosa26 reporter locus marks Tie2^{+} HSCs and their progeny with YFP. Using this system, Busch et al. showed polyclonal contribution of many HSCs in steady-state hematopoiesis [59]. Individual HSC clones participate only rarely. (a) and (b) Each colored line represents an individual HSC clone and waves indicate periods of activity. HSC: hematopoietic stem cell, Mer: mutated estrogen receptor site, MerCreMer: Cre recombinase fused to two Mer sites. Figure and part of the text taken from Busch, K. and H.R. Rodewald, *Unperturbed vs. post-transplantation hematopoiesis: both in vivo but different.* Curr Opin Hematol, 2016. 23(4): p. 295-303.

Sun et al. used an elegant clonal marking system based on a DNA transposon, which is randomly integrated into the genome, thereby generating a specific genetic tag for the corresponding cell and its progeny, whereas Busch et al. approached steady-state hematopoiesis by YFP labeling of $Tie2^{+}$ HSC and mathematical modeling. These studies revealed that many transient clones initially drive hematopoiesis following transplantation. However, the majority of them do not engraft. Instead, in the long run hematopoiesis following transplantation is maintained by only few HSC and hence is oligoclonal. Steady-state hematopoiesis has polyclonal character with stem and progenitor cells downstream from HSC as the main driver [59]. Such distinct results make it reasonable not only to re-think our current model of hematopoiesis, but also our experimental approaches of addressing the lineage-potentials as well as contributions of progenitor cell populations. Post-transplantation hematopoiesis seems to reflect the physiology of steady-state hematopoiesis only to a limited extent [60].

4.2 Lymphocyte development

In adult mammals, B-lymphocyte development takes place in the bone marrow and to a lesser extent also in the spleen, whereas T-cell development is largely restricted to the thymus. The thymus does not contain self-renewing stem cells and in order to maintain thymopoiesis is absolutely dependent on the continuous replenishment with precursors migrating from the bone marrow [61]. T- as well as B-lymphocytes express clonally-distributed antigen receptors, which have been tested for autoreactivity during their development. The enormous variety of potential antigen-recognition specificities amongst receptor molecules is generated by the random recombination of gene segments orchestrated by the recombination-activating gene products, the Rag1 and Rag2 enzymes [62, 63]. Individual stages of B- and T-lymphocyte development can be characterized by cell-surface and intra-cellular markers, cell cycle profile, and rearrangement status of immunoglobulin heavy and light chain genes for B cells or the α and β chain genes for T cells (See Figure 5 and 6) [64-67]. In mice, two different nomenclatures using slightly different

markers were proposed to describe B-cell developmental stages: the so-called Philadelphia nomenclature [68] and the Basel nomenclature [69]. In this introduction, the Basel nomenclature will be used. Through the coordinated expression or down-regulation of lineage-related proteins, the B- or T-cell fate of hematopoietic progenitors becomes specified and ultimately committed. Specification means the establishment of lineage-specific gene expression signatures, and commitment implies the loss of the capability to differentiate into alternative lineages [70].

4.2.1 B-cell development

From very early on in their development, expression of several transcription factors, including Ikaros, E2A, FoxO1, and Ebf1 establishes an epigenetic landscape permitting B-cell specification [71, 72]. Consequently, Ebf1 and in turn mainly Pax5 can activate the B-cell specific transcriptional program and repress alternative lineage opportunities, thereby accomplishing B-cell commitment [73-76]. The first cell that under physiological conditions is committed to the B-lineage is the pre-BI cell (see Figure 5). It is phenotypically characterized by the expression of CD19 and CD117 (cKit) and genotypically by their immunoglobulin heavy (IgH) chain loci being both D_H-J_H rearranged [66].

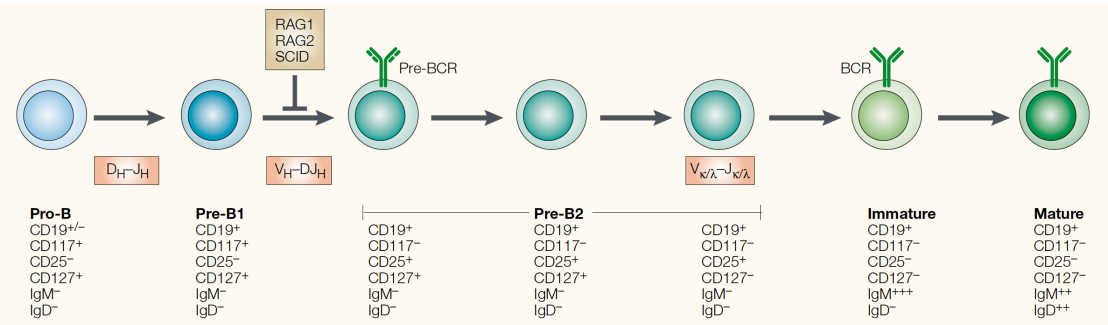

Figure 5: Schematic representation of early stages of B-cell development in analogy to T-cell development as depicted in Figure 6. B cells develop in sequential stages identified by differential expression of cell surface and intra-cellular markers, the rearrangement status of the IgH and IgL chain gene segments, and the cell-cycle profile. Cell-surface expression of CD19, CD117, CD25, CD127, IgM, and IgD is indicated for all stages from pro-B to mature B cells. Moreover the rearrangement events are indicated and expression of pre-BCR and BCR. In RAG1 or RAG2 deficiency or in SCID mutants B-cell development is blocked at the pre-BI to pre-BII transition. D_H: heavy chain diversity gene segment, J_H: heavy chain joining gene segment, V_H: heavy chain variable gene segment, IgM/IgD: immunoglobulin M/D, RAG1/2: recombination-activating gene 1/2, BCR: B-cell receptor, $V_{\kappa/\lambda}$: κ/λ light chain variable gene segment, $J_{\kappa/\lambda}$: κ/λ light chain joining gene segment. Figure taken from Ceredig, R. and T. Rolink, A positive look at double-negative thymocytes. Nat Rev Immunol, 2002. 2(11): p. 888-97.

A successful V_H to DJ_H rearrangement results in initially cytoplasmic and later (see below) surface expression of a μ-heavy (μH) chain, which together with the surrogate light chain proteins VpreB and λ5 builds the pre B-cell receptor (preBCR) [77-80]. These $preBCR^+$ cells are classified as large pre-BII cells. They have lost expression of cKit and gained expression of CD25 [65]. Only cells expressing a μ heavy (μH) chain protein capable of pairing with the surrogate light chain components build a functional surface-expressing preBCR. The preBCR is responsible for the proliferative expansion of large pre-BII cells and also for the positive selection of these progenitors [66, 81, 82]. Further rearrangements at IgH chain loci are suppressed by means of transient down-modulation of the recombination machinery, reduced germline transcription and histone acetylation [83-86]. These mechanisms contribute to ensure allelic exclusion of antigen-receptor genes, which mediates mono-specificity of mature lymphocytes [87]. PreBCR signaling extinguishes its own expression by directly silencing transcription of the *VpreB* and *Igll1* genes [88]. Subsequently, the cells stop proliferation and enter the small pre-BII stage. Here, the rearrangement machinery becomes re-expressed and targets the immunoglobulin light (IgL) chain gene loci κ and λ. Once a functional gene product is generated it builds the BCR on the cell surface, again subject to the condition that pairing of IgL chains with the μH chain is possible. At this stage the B cell is called an IgM^+ immature B cell. If a correctly rearranged light chain cannot pair with the μH chain or happens to forms a BCR with autoreactive specificity, secondary rearrangements are initiated in order to resolve this issue. This process was identified independently by Rolink, Weigert, and Nemazee and has been termed receptor editing [89-91].

IgM^+ immature B cells leave the bone marrow and migrate to the spleen for their final maturation steps. In order to proceed in their development, the right dosage of BCR signaling is required. This so-called tonic or basal BCR signal is only generated by a non-autoreactive BCR composed of functionally paired IgH and IgL chains. An autoreactive BCR would generate a much stronger signal, whereas cells expressing only non-pairing immunoglobulin chains lack a signal [92]. If signaling via the BCR is manipulated, this positive selection fails as is the case in CD19-deficient

mice [93]. The developmental disturbances shown by many mice with mutations in BCR signal strength regulators further underline the importance of appropriate signaling [6, 8, 94-96]. In the spleen IgM^+ immature B cells recently arrived from the bone marrow are termed transitional B cells and can be discriminated from mature B cells by expression of CD93, their short half-life as well as their sustained susceptibility to anti-IgM induced apoptosis [97-100]. The final developmental step is the differentiation of transitional B cells into one of the mature B-cell populations: marginal zone B cells, follicular B cells, or B-1 B cells.

4.2.2 T-cell development

T-cell specification and commitment strongly depend on the thymic microenvironment, or more specifically on the Notch1 ligand Delta-like 4 expressed by thymus stromal cells [101]. The importance of Notch signaling for T-cell commitment has been shown by conditional inactivation of Notch1 in hematopoietic precursors [102] or one of its ligands, Delta-like 4 [101, 103] in the thymus; this led to ectopic B-cell development. Moreover, expression of Notch1 in the bone marrow environment resulted in ectopic T-cell development [104]. Downstream of Notch signaling, key T-cell specification transcription factors such as Bcl11b, GATA-3, and Tcf1 are induced [105-108]. The earliest thymic progenitors (ETPs) are characterized phenotypically as $Lin^-cKit^+Flt3^+CD44^+CD25^-$. Based on the controlled sequence of expression of the T-cell co-receptor genes CD4 and CD8, T-cell development can be roughly categorized (see Figure 6).

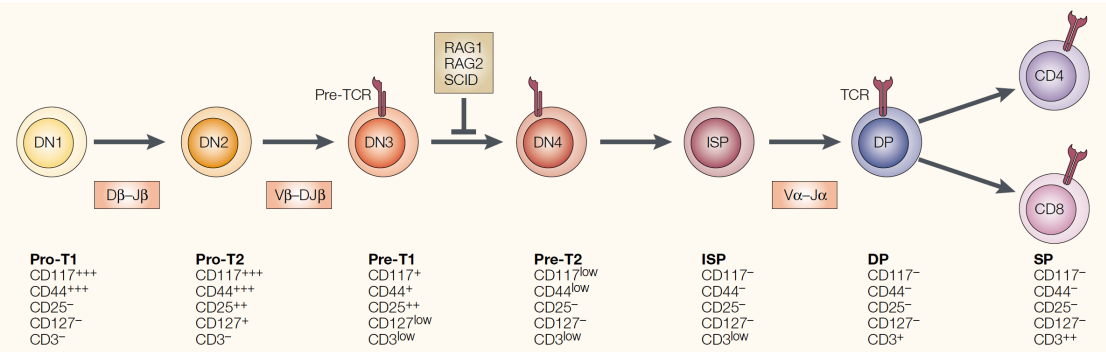

Figure 6: Schematic representation of early stages of T-cell development in analogy to B-cell development as depicted in Figure 5. T cells develop in sequential stages identified by differential expression of cell surface and intra-cellular markers, the rearrangement status of the TCRβ and TCRα chain gene segments, and the cell-cycle profile. Cell-surface expression of CD117, CD44, CD25, CD127, and CD3 is indicated for all stages from pro-T1/DN1 to mature CD4 or CD8 SP T cells. Moreover the rearrangement events are indicated as well as

expression of pre-TCR and TCR. In RAG1 or RAG2 deficiency or in SCID mutants T-cell development is blocked at the pre-T1 to pre-T2 transition. Two corresponding nomenclatures are used, describing equal developmental stages. Dβ: β-chain diversity gene segment, Jβ: β-chain joining gene segment, Vβ: β-chain variable gene segment, RAG1/2: recombination-activating gene 1/2, TCR: T-cell receptor, V_α: α-chain variable gene segment, J_α: α-chain joining gene segment, DN: double-negative with regards to CD4 and CD8 expression, ISP: immature single positive with regards to CD8 expression, DP: double-positive with regards to CD4 and CD8 expression, SP: single positive with regards to CD4 or CD8 expression, respectively. Figure taken from Ceredig, R. and T. Rolink, A positive look at double-negative thymocytes. Nat Rev Immunol, 2002. 2(11): p. 888-97.

In the thymus Flt3 (CD135) expression can be used to subdivide the earliest thymocyte subpopulation with any remaining B-lineage potential being particularly associated with CD135 expression [42, 109, 110]. ETPs and the earliest developmental stages lack expression of both CD4 and CD8 markers and are therefore termed double-negative (DN) thymocytes. This episode can be further subdivided using the cell surface markers CD44, CD25, and CD117 into four consecutive stages. The $CD44^+CD25^-CD117^{hi}$ DN1 stage, the $CD44^+CD25^+CD117^{hi}$ DN2 stage, the $CD44^-CD25^+CD117^{low}$ DN3 stage, and the $CD44^-CD25^-CD117^-$ DN4 stage [67, 111-113]. Thymocytes proceed along these stages and recombine the V_β, D_β, and J_β segments of the TCRβ-chain. Expression of the pre T-cell receptor (preTCR) together with the surrogate α-chain protein PreTα characterizes the DN3 stage [114, 115]. Following proliferative expansion, mouse thymocytes pass a $CD8^+$ immature single positive (ISP) stage and then up-regulate both, CD4 and CD8 to become CD4/CD8 double positive cells. At that double-positive stage the V_α and J_α gene segments of the TCRα chain are rearranged. Once a TCRαβ complex is expressed on the surface, positive and negative selection generates mature CD4 single-positive or CD8 single-positive T cells [116].

4.3 IL-7 and Flt3L – Cytokines guiding lymphocyte development

Hematopoiesis depends on external signals provided by the combined presence of soluble factors and stromal cells, which guide the maintenance, survival, proliferation, and differentiation of progenitors. The bone marrow contains specialized stromal cells, which make direct cell-cell contact with progenitors and secrete cytokines [117]. Many of these cytokines are extremely potent and generally act over short ranges. They are frequently made available to progenitors by being incorporated into the extra-cellular matrix that stromal cells produce. The mode of

action of these cytokines can be either 1) instructive, directly inducing a certain lineage differentiation by actively switching lineage-specific genes on or off, or 2) permissive, by selectively allowing proliferation or survival of progenitors destined to become cells of a particular lineage [118, 119]. Whether cytokines play an instructive or permissive role in hematopoiesis is extremely controversial. Two cytokines, which have been of particular interest for T- and B-lymphocyte development, are IL-7 and Flt3L. Their respective receptors are co-expressed immediately before commitment and mutant mice show disturbances of lymphoid development (see Figure 7) [2, 120-122].

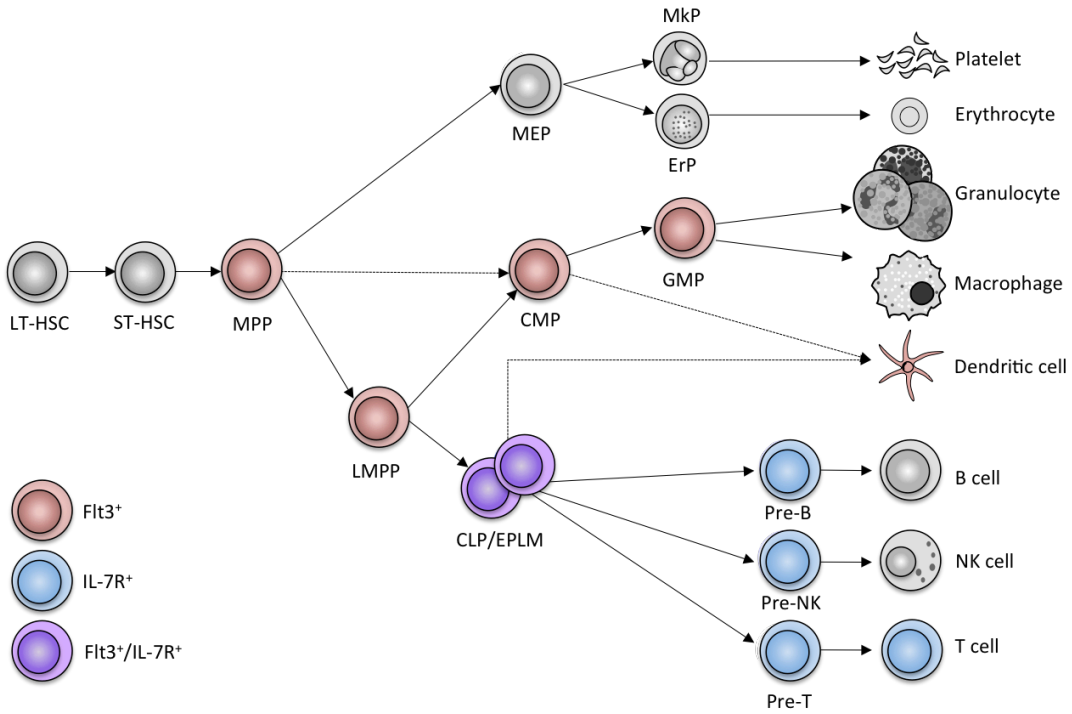

Figure 7: Cytokine receptor expression on hematopoietic cell populations. Simplified representation of hematopoietic development in analogy to the classical hematopoietic scheme in Figure 1. Coloration of cells is used to represent functional expression of the cytokine receptor Flt3 (red), CD127/IL-7Rα (blue), or both (purple). Thereby it becomes clear that the closely related CLP/EPLM progenitors are the only population sharing Flt3L and IL-7 responsiveness. LT-HSC: long-term hematopoietic stem cell, ST-HSC: short-term HSC, MPP: multipotent progenitor, LMPP: lymphoid-biased MPP, MEP: megakaryocyte-erythrocyte progenitor, ErP: erythrocyte progenitor, MkP: megakaryocyte-committed progenitor, GMP: granulocyte-macrophage progenitor, CLP: common lymphoid progenitor, EPLM: early progenitor with lymphoid and myeloid potential, Pre-B/NK/T: precursor B/NK/T cell.

4.3.1 IL-7

IL-7 was originally identified as a cytokine secreted by bone marrow stromal cells that supported progenitor B-cell survival [123]. Later, it was shown that IL-7 also had activity on thymocytes and T-cell survival [124]. After that, a specific receptor for IL-7, comprising a ligand-specific IL-7 receptor (IL-7R) α chain (CD127) and the "common" IL-2Rγ (cγ) chain, was identified [125]. Binding of the IL-7 cytokine to the IL-7 receptor induces signaling via Jak1 and Jak3, which consequently activate the signal transducer and activator of transcription (Stat) 5 protein [126, 127]. Deletion of the gene encoding the *IL7r* or the *IL7* cytokine results in a leaky arrest of T-cell development at the DN2 stage and absence of γδ T cells [2, 120, 128], whereas B-cell development is blocked at the pro-B cell stage. Cumulatively this results in severely reduced numbers of T and B lymphocytes in the periphery, which is indicative of a non-redundant role of IL-7, at least in mice. Since humans with mutations in the *IL7R* gene display a normal B-cell phenotype the role of IL-7 in human B-cell development remains unclear. This is discussed in chapter 4.4.2.

Published reports showing the rescue of the T-cell defect by transgenic expression of the pro-survival protein Bcl2 in *IL7r*$^{-/-}$ mice suggested that IL-7 acts in a permissive way during early T-cell development [129, 130]. Further evidence for this conclusion was provided by deletion of the pro-apoptotic proteins Bax or Bim, leading to a similar restoration of T-cell development in the absence of IL-7R signaling [131, 132]. In contrast to this clear rescue initial reports indicated that B-cell development is not re-established by transgenic expression of Bcl2 in *IL7r*$^{-/-}$ mice [133, 134]. Therefore it was assumed that IL-7 might work instructively, committing cells to the B-cell lineage. The identification of Stat5 binding sites within the promoter regions of Ebf1 and Pax5, transcription factors indispensable for B-lymphoid commitment, supported this hypothesis [2, 135, 136]. However, recent advances have provided clear evidence for IL-7 operating permissively for B-cell development. The proof for binding of Stat5 to regulatory elements of the Pax5 gene under physiological *in vivo* conditions is still pending [137]. Even the regulation of Ebf1 by Stat5 was shown to be only indirect [138]. Also ectopic expression of Bcl2 could partially rescue the B-cell developmental arrest that is observed in

conditionally Stat5 deficient mice [139]. The same restoration has been shown now also for Vav-Bcl2 *IL7r*$^{-/-}$ mice, where there is recovery of pre-BI cell numbers [139]. Moreover, these recovered B cells expressed normal levels of E2A, Ebf1 and Pax5. In an *in vitro* system, stimulation with IL-7 failed to induce expression of the endogenous Ebf1 gene, indicating that IL-7R signaling or active Stat5 alone are not sufficient to induce transcription [140]. In summary, these data provide enough evidence to state that IL-7R mediated activation of Stat5 might be sufficient, but is not necessary for the induction of Ebf1 and Pax5 transcription factors. Also in B-cell development IL-7 seems to act in a permissive way through regulating cell survival and proliferation rather than as an instructive cytokine [141].

4.3.2 Flt3L

Fms-like tyrosine kinase 3 (Flt3), or CD135, is a receptor tyrosine kinase cloned by homology with other receptor tyrosine kinases described simultaneously by Matthews et al. in Cell [142] and Rosnet et al. in Oncogene [143]. The Flt3 ligand (Flt3L) was cloned by Lyman et al. [144]. Several hematopoietic cell populations express Flt3 that is first detectable within the LSK compartment [145]. Later in hematopoietic development, expression is sustained by progenitors with myeloid and/or lymphoid potential, but not by progenitors of the megakaryocyte/erythrocyte lineage (see Figure 7) [18, 146-150]. Recently, using a Flt3L over-expressing transgenic mouse line (Flt3Ltg), our laboratory revealed the instructive action of Flt3L in hematopoietic development, inducing myeloid and lymphoid lineages at the MPP stage at the expense of megakaryocyte/erythrocyte development [151]. With one known exception, namely DCs [150], expression of Flt3 is extinguished upon lineage commitment. During B-cell development, expression of Pax5 directly antagonizes expression of Flt3 [152]. So far, the only ligand binding Flt3 and inducing downstream signaling seems to be Flt3L [144]. The membrane-bound form of Flt3L can also be released as a soluble homodimeric protein and both forms have been shown to activate the receptor [153, 154].

Due to its high expression in a variety of hematologic malignancies and especially its prominent involvement in acute myeloid leukemia (AML), Flt3 and also

its ligand have been extensively studied. In 1996 the group of Misawa first detected an internal tandem duplication of exon 14 of the *FLT3* gene (FLT3-ITD) amongst patients suffering from AML, thereby suggesting an involvement of these mutations in the pathogenesis of the disease [155]. Subsequently, many groups confirmed these data and additional types of mutations were identified [156, 157]. In a mouse model expressing FLT3-ITD in the hematopoietic system exclusively, the majority of mice developed a myeloproliferative syndrome reminiscent of human AML [158].

Mice with a targeted disruption of the *Flt3* gene were described having normal mature hematopoietic cell populations [121]. More interestingly, they show deficiencies in early B-lymphoid progenitor cell populations, suggesting a compensatory effect mediated by other cytokines neutralizing the loss of B-cell precursors after the Flt3L responsive stage. In bone marrow transplantation experiments, the intrinsic Flt3 deficiency revealed a further impairment in T-cell and myeloid reconstitution [121, 159]. Loss of Flt3L in mice resulted in reduced numbers of leukocytes in the bone marrow, peripheral blood, lymph nodes, and spleen of adult mice. In particular, in the bone marrow there was a reduction in myeloid as well as B-cell progenitor cell populations and reductions in NK cells and DCs in the periphery. In contrast, thymic cellularity, blood hematocrit, as well as platelet numbers were not affected [122]. Since neither the ablation of Flt3 nor Flt3L resulted in a complete loss of any hematopoietic cell population, it is likely that Flt3 mediated signaling exerts its function in concert with signaling from other cytokines such as SCF, or IL-7 and compensatory mechanisms presumably cause these relatively mild phenotypes [140, 147, 157, 160]. Evidence for such complementarity comes from analysis of Flt3L IL7r double-deficient mice, which show loss of all stages of fetal and adult B-cell development [160]. Moreover, in these mice both fetal and adult thymopoiesis was abrogated thereby demonstrating an indispensable role for Flt3L in IL-7R-independent lymphopoiesis [161].

Flt3L is best known for its ability to stimulate the expansion of $CD34^+$ hematopoietic progenitors and DCs *in vivo* and *in vitro* [146, 162-167]. Daily injections of Flt3L result in a dramatic increase in peripheral DCs and an indirectly mediated increase of functional peripheral regulatory T cells [168]. Moreover NK-cell

numbers are elevated following Flt3L administration and Flt3L has been shown to act as regulator of DC-mediated NK-cell activation, thereby rendering the cytokine interesting as a potential broad-spectrum anti-tumor agent [169, 170]. Our laboratory also showed expansion of the bone marrow EPLM progenitor cell population upon Flt3L injection [171]. Back then these promising results prompted us to generate a transgenic mouse continuously over-expressing human Flt3L (Flt3Ltg) under the control of the β-actin promoter [151]. Flt3Ltg mice confirmed the importance of Flt3L for dendritic cell homeostasis. They display large numbers of dendritic cells and other cells of the myeloid lineage, resulting in splenomegaly and blood leukocytosis. Due to a rapid reduction of erythroid progenitors, these mice quickly develop anemia and a reduction in platelet numbers called thrombocytopenia. In summary these data strongly suggested an instructive action of Flt3L on multipotent progenitors, inducing their development into myeloid/lymphoid lineages and suppressing the megakaryocyte/erythrocyte fate.

4.4 Fetal and adult hematopoiesis

Cells of the hematopoietic system have to be continuously replenished from hematopoietic stem and progenitor cells. For example, in man, throughout adult life, the bone marrow produces approximately 10^6 red blood cells per second. However, the intrinsic properties of stem cells change with development and relatively little is known about the regulatory mechanisms guiding these modifications. For example fetal HSCs vary significantly from adult HSCs in terms of gene expression, developmental potential, self-renewal, and regulation [172, 173]. The striking difference between fetal and adult HSC is best exemplified by competitive reconstitution experiments revealing the advantage of fetal liver derived hematopoietic progenitors over adult bone marrow derived ones [110, 174]. In mice, hematopoiesis from adult HSC begins at about 3-4 weeks of age. One example of the factors differentially expressed by fetal and adult HSC is the transcription factor Sox17, which has been shown by Kim et al. to distinguish fetal liver from adult HSCs [173]. More recently Damnernsawad et al. highlighted the importance of Kras for

adult hematopoiesis, while its role in fetal hematopoiesis remains questionable [175]. Since fetal HSCs retain their fetal-like differentiation capability even in an adult microenvironment, these features would seem to be cell intrinsic [176].

In vertebrates the sites of hematopoiesis change during ontogeny. This starts in the yolk sac, later moving to the aorta-gonad-mesonephros (AGM) region, the placenta, the fetal liver, and finally the bone marrow [177]. From embryonic day 7.5, a first wave of hematopoiesis originates in the yolk sac. However, only from E10.5 onwards, when HSCs have been autonomously generated in the AGM region [178] does definitive hematopoiesis, defined as the capability of complete and long-term hematopoietic repopulation of irradiated adult recipient mice, occur. From there the fetal liver and later the bone marrow is seeded. The bone marrow is where all hematopoietic stem cell and progenitor populations reside during adulthood [177, 179, 180]. Once the thymus anlage is formed from the third pharyngeal pouch it attracts thymocyte progenitors via chemokine gradients [181, 182]. Recently, Ramond et al. have shown that the colonization of the fetal thymus occurs in two waves. Between E12 and E15 these early thymus-settling progenitors (TSPs) are T-cell restricted with a clear preference for fast differentiation into mature T cells over population expansion. From E16 onward, TSPs resemble LMPPs in their possible differentiation spectrum, proliferate extensively, and have lost the potential to give rise to embryonic γδ T cells [183].

4.4.1 Lymphocytes with innate features

Fetal lymphopoiesis is known to give rise to some of the specialized subsets of so-called “innate-like” B and T lymphocytes [184-188]. One of the characteristics of innate-like cells is their expression of a restricted set of semi-invariant, germ-line-encoded, mostly autoreactive, antigen receptors with limited diversity. This restricted repertoire is achieved by preferential usage of a limited number of variable gene segments during Rag1/2 mediated gene rearrangements [189]. These features place them developmentally at the interface between innate and adaptive immunity and also anatomically since they occupy specialized locations.

Innate-like B cells include marginal zone and B-1 B cells, whereas innate-like T cells are γδ T cells, CD1d-restricted natural killer T (NKT) cells, and mucosal-associated invariant T (MAIT) cells. Amongst these, B-1 B cells and γδ T cells are known to arise preferentially during embryogenesis. Nevertheless what all these innate lymphocytes have in common is their ability to recognize conserved antigenic structures on the surface of micro-organisms, setting them apart from conventional T and B cells, whose randomly recombined and clonally distributed antigen receptors are evolutionary perfected to detect the multitude of potential pathogenic structures. Many of these conserved antigenic structures seem to be of self-origin and are exposed upon stress or tissue damage. Innate-like B cells spontaneously secrete antibodies of the IgM isotype class. These “natural” antibodies recognize antigen in a promiscuous manner and show a considerable degree of autoreactivity [190-193]. Because of their broad range of reactivity, natural antibodies are an effective first line of defense against invading pathogens or neoself antigens linked with aging or cellular stress until the more specific adaptive immune response comes into effect [192].

Marginal zone B cells are the only B-cell subpopulation dependent upon Notch2 for their development. Conditional deletion of Notch2 results in a strong decrease in marginal zone B cells [194] rendering mice susceptible to bacterial infections [195]. This emphasizes the importance of these specialized B-cell subsets in the immune system. The BCR genes and corresponding antibody molecules secreted by B-1 B cells lack non-templated nucleotide (N) sequence insertions in the heavy chain. This can be traced back to the lack of TdT enzyme expression during embryogenesis [196, 197]. Only in 2006 Montecino-Rodriguez et al. succeeded in identifying within the fetal bone marrow a $Lin^{-}B220^{low}CD19^{+}$ B-1 B cell specified progenitor cell population. Thereby B-1 B cells could be shown to represent a separate lineage of B cells, which is mostly fetally derived [198]. In adult bone marrow these precursors are much less abundant, resulting in an almost complete lack of ability by adult bone marrow to replenish B-1 B lymphocytes. Therefore mature B-1 B cells show a robust self-renewal capacity ensuring their maintenance throughout life [199]. The “natural memory” phenotype of innate-like lymphocytes

suggests an earlier exposure to antigen that might have occurred during their development. The borderline self-reactivity of their antigen receptors might indicate that innate lymphocytes only just escape negative selection during their development, indicating also that they may have arisen by a process called “agonist selection”. Strong signals mediated by the antigen receptors would be a necessary prerequisite for agonist selection. In accordance with that negative regulators of BCR signaling inhibit the generation of B-1 B cells [200, 201]. It is the expression of these special receptor specificities that drives differentiation into their particular lineage as well as their preferential anatomical localization [202-205]. In the periphery, unrestricted activation is potentially regulated by the expression of inhibitory receptors reminiscent of NK cells [189]. Since some of the innate-like B and T lymphocytes are generated during embryogenesis or shortly after birth, their specialized differentiation requirements might be provided only within the fetal primary lymphoid organ microenvironment.

In transplantation experiments, the generation of some innate-like lymphocyte subsets is an easy, yet powerful, readout to determine adult or fetal features of the transplanted progenitor cells [184, 185]. Pro-B cells isolated from fetal livers and transplanted into severe combined immunodeficiency (SCID) mice generated mature B cells expressing high levels of CD5 and only low levels of IgD, phenotypic features resembling B-1 B cells. These experiments indicated that the B-1 B-cell differentiation capacity is intrinsic to fetal derived hematopoietic precursors. In contrast, transplantation of pro-B cells isolated from adult bone marrow generated $CD5^{-}IgD^{high}IgM^{+}$ B-2 B cells. These mutually exclusive developmental potentials suggest a developmental switch occurring in B-lymphopoiesis [206]. This developmental switch could be in part mediated by differential expression of the microRNA (miRNA)-binding protein Lin28b, which in turn regulates the transcription factor Arid3a [188, 207]. *Lin28b* mRNA is targeted for degradation by *Let-7* miRNA, whose biogenesis is in turn blocked by Lin28b, resulting in a mutually exclusive expression pattern illustrated in Figure 8 [208].

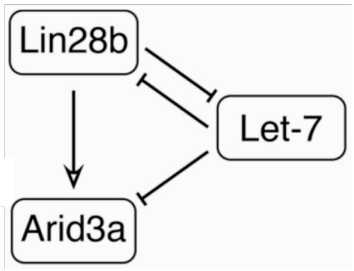

Figure 8: Model for cross-regulation of Lin28b, *Let-7* miRNA, and the transcription factor Arid3a. Lin28b induces expression of Arid3a, whose mRNA is targeted for degradation by *Let-7* miRNA. *Lin28b* mRNA is also targeted for degradation by *Let-7* miRNA, whose biogenesis is in turn blocked by Lin28b, resulting in a mutually exclusive expression pattern. Arid3a is thought to modulate BCR signaling, thereby altering selection of newly formed B cells into the mature B-cell compartment (not shown). Figure taken from Li, Y.S., et al., A developmental switch between fetal and adult B lymphopoiesis. Ann N Y Acad Sci, 2015. 1362: p. 8-15.

Lin28b has been found to be expressed by pro-B cells of fetal origin, which consequently lack *Let-7* miRNA expression. Progenitor B cells isolated from adult bone marrow showed the complementary expression pattern [188, 207]. Forced expression of *Let-7* miRNA in fetal liver derived pro-B cells resulted in B-2 B-cell generation upon transfer into SCID recipient mice, whereas expression of Lin28b in adult bone marrow derived pro-B cells restored fetal like B-cell populations [207]. In the very same study, the authors also showed that intact BCR signaling is an absolute requirement for B-1 B-cell generation from Lin28b-expressing bone marrow cells, thereby further supporting the idea that these innate-like lymphocytes depend on agonist selection. The transcription factor Arid3a was identified as a direct target of *Let-7* miRNA showing a similar expression pattern as Lin28b. Moreover, Arid3a has been implicated in regulating BCR signaling, thereby again suggesting that BCR-signal strength mediated kind of agonist selection may be involved in regulating B-cell population selection [206, 209, 210]. However, Lin28b is not only able to switch B-lymphopoiesis. Ectopic expression of Lin28b in adult bone marrow hematopoietic stem and progenitor cells enabled them to mediate fetal-like reconstitution of not only B-1a and marginal zone B cells, but also γδ T cells, and NKT cells [188].

4.4.2 Differential cytokine requirements during fetal and adult lymphopoiesis

Besides dependency on intact and strong signaling from the antigen receptor, there is also evidence for differential requirements of certain cytokines for fetal lymphopoiesis. For example, in mice, fetal B-cell development is known to be less

dependent on IL-7 [211]. Fetal liver pro-B cells of gene-deleted Stat5$^{-/-}$ and *IL7r*$^{-/-}$ embryos expressed normal levels of Ebf1 and Pax5 [139, 176]. B-1 B lymphopoiesis in particular has also been shown to be largely independent of IL-7 [211]. The exact role of IL-7 in human B lymphopoiesis is still debated. Whereas in mice, in the absence of IL-7, both T and B lymphopoiesis are severely compromised, [212], in man, individuals with mutations in the *IL7R* gene have a severe T-cell deficiency yet display normal numbers of circulating B cells [213]. This has led to the general assumption that human B-cell development is independent of IL-7. However, this conclusion might be misleading, because *IL7R* gene mutations are fatal if not treated with bone marrow transplantation. All patients analyzed with this condition have been infants [214] and therefore their B cells are very likely to be mostly of fetal origin. In a fully human *ex vivo* model system IL-7 independent B-cell development can only be accomplished using umbilical cord blood derived, but not adult bone marrow derived progenitors [215]. The IL-7 responsiveness of mouse adult and fetal T-cell progenitors has also been shown to differ, with the fetal ones being far less dependent [216]. In addition, adult DN1 and DN2 thymocyte differentiation is inhibited by IL-7, whereas fetal DN1 and DN2 thymocytes are not affected [217].

Thymic-stromal lymphopoietin (TSLP) is a cytokine that was originally cloned from a thymic stromal cell line [218] and has been implicated to mediate IL-7 independent fetal B lymphopoiesis [219, 220]. The receptor for TSLP shares the IL-7Rα chain with the IL-7 receptor, but uses the unique TSLPR chain instead of the cγ chain for signaling [221, 222]. *IL7r*$^{-/-}$ mice lack IL-7 as well as TSLP mediated signaling and show a 10-fold more severe reduction in B-cell numbers compared to IL-7 deficient mice [219, 220]. Interestingly, the B lymphopenic phenotype of cγ$^{-/-}$ mice is exacerbated by additional deficiency of the *Tpte2* gene, encoding the solely used TSLPR chain [223]. This further supports the important role of TSLP in IL-7 independent lymphopoiesis. The responsiveness of fetal derived B-cell progenitors to TSLP, which is not measurable in adult pro-B cells, is indicative of a unique fetal requirement for TSLP [219, 220]. The importance of TSLP is best exemplified by its transgenic expression in IL-7$^{-/-}$ animals. Here, thymic cellularity and architecture were normalized [224]. Besides TSLP, Flt3L has been implicated to figure

prominently in fetal lymphopoiesis. Thus, Sitnicka et al. revealed an indispensable role of Flt3L in IL-7Rα independent fetal T-lymphopoiesis [161]. Subsequently, Jensen et al. proposed that Flt3L was responsible for sustained fetal B lymphopoiesis in the absence of IL-7 [225]. In summary there is an ongoing debate regarding whether TSLP, Flt3L, or both cytokines participate in fetal lymphopoiesis in particular.

Mice are born after a gestation period of about 21 days at a time when the cellular components of their immune system are only beginning to be generated. Therefore, the fetal and neonatal primary lymphoid organ microenvironments provide a peculiar niche matching the requirements for the rapid generation of specialized lymphocyte subsets. There is clearly strong developmental pressure acting on the immune system to rapidly establish a first line of defense to be functional when protection by placentally transferred maternal antibodies disappears [226].

4.5 Culture systems

Hematopoiesis is the generic name for the coordinated differentiation of multipotent HSCs via several intermediate stages towards a mature blood cell type. This process only takes place in certain anatomical locations and in adult mammals is localized in the bone marrow. Lymphocyte production takes place mostly in two anatomical locations also called primary lymphoid organs; the bone marrow for B cells and the thymus for T cells. The thymus is generally seeded by bone marrow-derived progenitors distinct from HSC. Primary lymphoid organs provide a defined microenvironment for lymphocyte development. This microenvironment comprises several elements including stromal cells, the proteinaceous factors they express and/or secrete as well as extracellular matrix molecules with which stromal cells surround themselves. The protein factors can be membrane-bound or soluble and stem and progenitor cells depend on these for their survival, proliferation, or commitment.

In order to study the requirements or general features of hematopoietic development, researchers depend on *in vitro* culture systems imitating these microenvironments. Initial studies of hematopoiesis were performed *in vivo* and used colony-forming unit (CFU)-spleen assays emerging in the spleens of irradiated recipient mice upon injection of bone marrow cell suspensions as measurement for HSCs and their pluripotency [13]. However, later on it was found that some of these spleen colonies were only transient and probably coming from hematopoietic progenitors and not real HSCs [227]. In 1966 Bradley and Metcalf described the growth of mouse bone marrow colonies *in vitro* in soft agar [228]. Within the agar they embedded feeder cells derived from adult kidney or embryos that provided the growth factors needed to observe growth of colonies. However, the authors already proposed that "addition of possible humoral factors to the culture medium may provide more precise techniques suitable for the examination or direction of differentiative trends in bone marrow cells" [228]. Down to the present day CFU assays in semi-solid methylcellulose-based medium supplemented with appropriate cytokines are used to measure the frequency of hematopoietic stem and progenitor cells and to identify cytokines or other factors that influence hematopoiesis. Thereby, the CFU is classified based on morphologic and phenotypic criteria of the end-cell types it produced. The experimentally determined number of CFUs has been shown to correlate positively with overall survival after transplantation of the corresponding sample [229].

4.5.1 *In vitro* B-cell cultures

For B-cell development, bone marrow derived stromal cell lines have been available for some time and are readily used in combination with a defined set of cytokines in order to promote B lymphopoiesis *in vitro* [230]. In 1982 such an *in vitro* co-culture system for the propagation of B cells was established by Whitlock and Witte [231]. In these so-called Whitlock-Witte cultures, bone marrow suspensions are seeded into flasks and after some time, adherent cells grow out and subsequently serve as a feeder layer for the growth of freshly seeded B-lymphocyte progenitors. Later, several stromal cell clones supporting B lymphopoiesis and/or myelopoiesis were established and extensively used. The use of stromal cell based

systems contributed decisively to the discovery that IL-7 is an important cytokine and growth factor for early B cells and became a helpful tool to look at B-cell colonies [232, 233]. Whereas the PA6 stromal cell line supports maintenance of the earliest B-cell progenitors but not their maturation, ST2 stromal cells are capable of supporting the whole process of B-cell differentiation [234-236]. In addition, S17 stromal cells together with the cytokine IL-7 promote B-cell growth [232, 237]. However, these stromal cells also support myeloid as well as erythroid differentiation from single HSCs [238]. The NIH-3T3 fibroblast line alone does not support proliferation of bone marrow derived precursors. However in the presence of additional exogenous factors, B-cell differentiation can be supported. By being able to add defined factors, a degree of controllability on the system is achieved.

The stromal cell line mostly used in recent times is OP9 [239, 240]. OP9 cells were derived from the bone marrow of macrophage colony-stimulating factor (M-CSF or CSF-1) deficient *op/op* mice [241]. In the absence of M-CSF, B-cell development from hematopoietic progenitors is much more efficient with OP9 cells because macrophage growth is not supported; macrophage growth can impair lymphocyte maintenance [242, 243]. A major drawback of stromal cell cultures is the impossibility to ultimately define the minimal signal requirements for B-cell development. Additional stromal cell derived factors as well as their relative availability cannot be accurately controlled. Nevertheless, stromal cell co-cultures helped to clarify many important issues of early B-cell development. For example firstly that the high κ/λ light chain ratio, which is distinctive of the mouse peripheral B-cell repertoire, is already evident in the antigen-independent transition from pre-B to B cells [244]. Secondly, the essential role of Pax5 in suppressing alternative lineage choices, thereby committing precursors to the B-cell lineage was shown using such cultures [25]. Thirdly, the expression dynamics of the *Rag* gene products during rearrangement of the immunoglobulin gene segments were investigated [83]. Moreover it could be shown that mouse B cells could undergo switch recombination even when the heavy chain locus was still in germline configuration. The finding that this process is dependent on the end-joining repair system was one of the cornerstones in subsequently identifying the key enzyme activation-induced cytidine

deaminase (AID) [245]. Despite these landmark discoveries and in order to better define the minimal requirements needed for long-term propagation of progenitor B cells *in vitro*, we developed a stromal cell free culture system [manuscript in preparation].

4.5.2 *In vitro* T-cell cultures

Even though B-cell propagation was for a long time successfully achieved in monolayer stromal cell cultures, for T cells, only complex 3D culture systems were applicable in mimicking T-cell differentiation *in vitro*. This was firstly because the factor determining T-cell commitment at the expense of other lineage fates had not been identified and secondly because the three-dimensional architecture of the thymus had been shown to be required for maintaining expression of this commitment factor even in thymic stromal cell monolayer cultures [246]. Thirdly and more obviously, available stromal cell lines seemed incapable of providing the appropriate signals.

Fetal thymic organ cultures (FTOC) were for a long time the technique of choice for addressing T-cell development *in vitro* [247, 248]. Even though rather cumbersome to set up, several major findings were made using FTOC. Subsequently, reaggregated thymic organ cultures (RTOCs), where thymic stromal cells and potential progenitors were centrifuged together, efficiently supported T-cell development and were extensively used [249]. Recently, the group of Ellen Robey has applied thymus slice cultures to study T-cell development *in situ* [250]. The latter technique has the advantage that the structural integrity of the thymic microenvironment with medullary and cortical zones remains intact.

In 2002 Juan Carlos Zúñiga-Pflücker and colleagues showed for the first time the efficient and long-term commitment and propagation of T cells in a simple stromal cell monolayer system [251, 252]. Use was made of the well-established OP9 stromal cell line, but in addition, since the Delta-Notch system had been identified as instructing T-cell lineage choice, OP9 cells were transduced with the Notch-ligand Delta-like 1 (DL1) [102, 104]. This generated the OP9-DL1 stromal cell line, which efficiently promoted the generation of T cells from fetal liver stem cells and

embryonic stem (ES) cells and has been extensively used since then. However, the disadvantage of co-cultures to dissect the detailed signaling requirements for the differentiation process is the uncontrolled delivery of additional signals by stromal cells. Also the density and distribution of DL1 on transduced cells cannot be controlled. In order to overcome these limitations we developed a stromal cell free *in vitro* culture system for the long-term propagation of fetal liver and adult bone marrow derived pro-T cells (see Figure 9) [253].

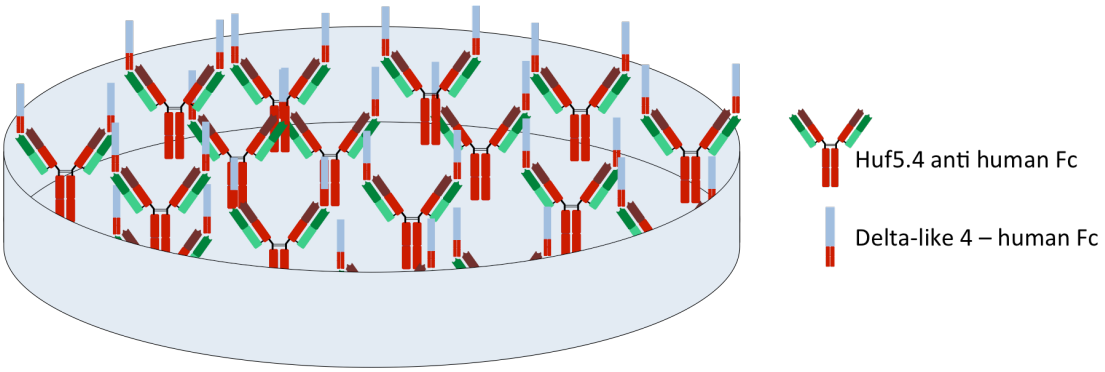

Figure 9: Schematic representation of the stromal cell free culture system for the long-term propagation of fetal liver and adult bone marrow derived pro-T cells [253]. On bacterial grade tissue culture plates 10µg/ml Huf5.4 anti-human IgG-Fc antibody is coated in PBS. After washing 2µg/ml Delta-like 4-human IgG1 Fc fusion protein is bound by Huf5.4 and consequently uniformly oriented. Hematopoietic progenitors sorted from adult bone marrow or fetal liver tissue detect the Delta-like 4 ligand with their Notch receptor, differentiate to the T-cell lineage and expand long-term as pro-T cells resembling *ex vivo* sorted T-cell progenitors.

The extracellular domain of mouse Delta-like 4 (DL4) (amino acids 1-521) was fused to the Fc part of human IgG1. Using an anti-human IgG-Fc antibody (hybridoma Huf5.4 – generated in house) the DL4 fusion protein was presented in the correct orientation to be detected by hematopoietic progenitor cells in tissue culture plates [253]. This allows for the long-term growth of functional pro-T cells *in vitro*.

4.5.3 *In vitro* culture assays – the right tool to address *in vivo* hematopoiesis?

Regardless of which *in vitro* culture system is used for the propagation or differentiation of progenitor cells, it is unclear to which extent the findings reflect the actual *in vivo* process. The addition or lack of designated cytokines or adhesion molecules in *in vitro* cultures might impose a bias for the outgrowth or loss of a certain subpopulation. Thereby the cloned stromal cells used and the factors they

produce in combination with media supplements predetermine the range of possible outcomes, and obviously excluding others. It might be an illusion to assume one is able to perfectly mimic the *in vivo* microenvironment by providing all the potential growth and/or differentiation stimuli that make an endogenous hematopoietic niche unique. Stromal cell co-culture systems used for the propagation or differentiation of progenitor cells, also for example in limiting dilution analyses, can only facilitate a certain range of lineage fates. Thereby one can address the frequency of potential progenitors with a certain lineage potential, but the actual contribution of their potentials to hematopoiesis *in vivo* remains elusive. The same holds true for colony assays, even though in these systems stromal cells are absent. Even the use of uncloned (polyclonal) bone marrow derived stromal cells as in Whitlock-Witte cultures, cannot reproduce the spatio-temporal patterning and migration activity of progenitors, which go through different niches. There, the composition, local availability, as well as concentration of factors differ. Besides fibroblasts, macrophages, adipocytes, osteoblasts and osteoclasts are all components of the bone marrow stromal niche. Moreover, the marrow vasculature plays a major role in hematopoiesis [254, 255]. In summary, a simple cloned stromal cell line can perhaps never reflect all these different features. Importantly, whether assays should be carried out at the local “physiological” low (hypoxic) oxygen concentration or in the standard atmospheric oxygen concentration is another issue.

Even if a specific combination of factors makes lineage commitment, differentiation, proliferation, or survival of a cell type possible *in vitro*, this provides no proof that *in vivo* under physiological conditions the same mechanisms function. Nevertheless, the cultures described above permit the direct comparison of the lineage potentials of different progenitor subpopulations. Thus, “positive” and “negative” control populations can be included in experiments. Importantly, to obtain large numbers of lymphocyte progenitors which have proven functionality *in vivo* the use of *in vitro* expansion cultures is a fair solution [253]. To address lineage potentials and differentiation requirements the stromal cell based or the colony-forming assays are a suitable way to compare different populations. However it is totally unclear whether they reflect what is actually going on *in vivo*. For example, *in*

vitro differentiation assays have shown that the earliest thymic lymphocyte populations possess robust myeloid potentials besides the T-cell potential [41, 42]. However, the IL-7r fate-map mouse almost excluded contribution of ETPs to the thymic myeloid lineage *in vivo* [256]. Also many other bone marrow hematopoietic precursors such as HSCs, MPPs, LMPPs, CLPs, and EPLM [1, 15, 18, 21, 145] have been shown to possess T-cell potential. Though, the T-cell fate could have been induced artificially *in vitro* and might represent a non-physiological differentiation pathway. For a moment the use of *in vivo* approaches such as transplantation assays to address the mechanisms of hematopoiesis might seem to resemble more closely the endogenous process. However, the transplantation of progenitor cells in myeloablated hosts induces stress-induced hematopoiesis, which might be still very different from steady-state hematopoiesis (see 1.1.3) [58].

4.6 CD19 – The B-cell co-receptor molecule

CD19 is a cell-surface molecule exclusively expressed by B cells upon commitment. For that reason it is used as a pan-lineage marker [257]. CD19 starts to be expressed early on during B-cell development as a direct target gene of the transcription factor Pax5 [73]. It exerts its regulatory function in terms of B-cell activation as part of the BCR-signaling machinery [11]. CD19 participates in the B-cell co-receptor complex together with CD21, CD81 (TAPA-1), and CD225 (Leu-13) [258-261]. It mediates recruitment of regulatory molecules, such as Vav, PLCγ2, PI3K, or Lyn [262-270] to the cell membrane after stimulation. Possibly, CD19 brings these factors in close proximity to the BCR, thereby enhancing signaling activity in a similar manner to CD4 and CD8 and the TCR [271, 272]. Only upon terminal differentiation into antibody-secreting plasma cells CD19 expression is abolished [257, 273], whereas memory B cells retain CD19 on their surface. Most likely, the shutdown of CD19 expression in plasma cells is a direct consequence of repressed *Pax5* transcription [274].

The two extracellular immunoglobulin (Ig)- like domains of CD19 are connected to the 242 amino acid long cytoplasmic domain via a transmembrane

region [275]. The 95kDa glycoprotein belongs to the immunoglobulin superfamily [276, 277]. The nine highly conserved tyrosine residues of the intracellular part are inevitable for signal transduction [259, 260, 278]. Figure 10 depicts a schematic drawing of the domain structure of the CD19 protein.

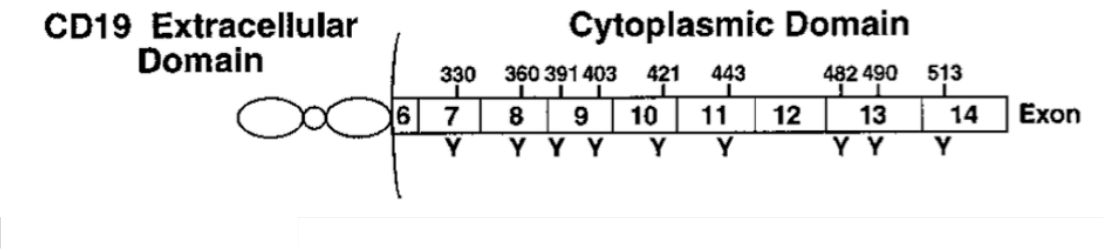

Figure 10: Schematic representation of murine CD19 protein structure. The extracellular domain is composed of two immunoglobulin-like domains. Intracellularly, nine highly conserved tyrosine residues are unevenly distributed over 9 exons. Figure taken from Fujimoto, M., et al., *CD19 regulates Src family protein tyrosine kinase activation in B lymphocytes through processive amplification.* Immunity, 2000. **13**(1): p. 47-57.

In the early nineties, the group of Thomas Tedder generated a transgenic mouse expressing the human CD19 gene [279] as well as a mouse lacking CD19 [272]. Robert Rickert generated his own CD19-deficient mouse model with similar results [280]. In both studies, antigen-independent B-cell development was found normal, whereas mature recirculating B cells were decreased [272, 280]. Moreover, the unconventional B-1 B-cell compartment was lost. Transgenic expression of CD19 resulted in a dose-dependent decline in mature B-cell numbers [279].

4.6.1 CD19-deficient mice

The gene encoding for CD19 was targeted by homologous recombination in embryonic stem cells [272]. The resulting loss of CD19 expression in mutant mice caused no severe differences in the development of conventional B cells in the bone marrow. However, peripheral B cells were significantly reduced in numbers, especially in the peritoneal cavity where they were decreased by 75%. More detailed analysis confirmed that B-1 B cells, the prominent cell type in the peritoneal cavity, were drastically reduced. This strongly indicates an important role for CD19 in their development and/or maintenance [95, 272, 280]. Marginal zone B cells were also reduced [201]. The remaining mature B cells responded with less proliferation to mitogenic stimulation and reduced serum immunoglobulin levels were detected.

Upon sublethal irradiation, the numbers of auto-reconstituted B cells were decreased in CD19$^{-/-}$ mice. This was first evident in the large cycling pre-BII cell compartment suggesting an involvement of CD19 in preBCR signaling [281]. Also in mixed bone marrow chimeras, CD19-deficient early B cells showed a competitive disadvantage compared to wild type cells [281]. Bromodeoxyuridine (BrdU) and CFSE labeling revealed a shorter lifespan of CD19-deficient cells *in vivo* [282] and concomitant overexpression of Bcl2 suggested an involvement of CD19 in cell survival. In such mice, the marginal zone compartment was partially rescued and follicular B-cell numbers were increased. However, germinal centers (GC) were still not formed upon immunization with a hapten-protein-conjugate, which implies that Bcl2 cannot substitute for antigen induced differentiation signals [282].

Generally, CD19-deficiency severely impairs BCR-signaling capacity [283] resulting in poor reactivity to most transmembrane signals. For example, the proliferative capacity in response to lipopolysaccharide (LPS) stimulation or surface-IgM crosslinking was reduced compared with wild type littermates [272]. The impaired response of CD19$^{-/-}$ mice against the T-cell independent (TI)-2 antigen α(1-3)-dextran can be attributed to the reduced numbers of B-1 B cells in these mice [280, 284]. However, since CD19-deficient mice responded normally to immunization with the TI-2 antigen 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll, the response to which is mediated by B-2 B cells, one can conclude that CD19 is not necessary for B-cell activation mediated by TI-2 antigens [280]. Nevertheless, the intense cross-linking of BCR by NP-Ficoll may compensate for the lack of CD19 in these conditions. Upon immunization with the T-cell dependent antigen dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH) serum-immunoglobulin levels where 10% reduced compared to wild type littermates, and even 30% upon re-challenge [272]. Generally, features originating in a GC reaction, such as affinity maturation, memory B cell and long-lived plasma cell generation, were reduced. Total numbers of GCs were decreased and the ones present were smaller in size [272, 280, 285]. Resulting from the hypo-responsive nature of CD19-deficient B cells, their selection into germinal center responses was diminished. The survival of existing memory B cells is also entirely dependent on CD19 [286].

4.6.2 Human CD19 transgenic mice (hCD19tg)

In human CD19 transgenic mice, the construct was exclusively expressed by B cells, most likely due to the high homology between the murine and the human CD19 promoter [24, 73]. These mice display a reduction of mature peripheral B cell numbers. However, a decrease is first detectable at the $B220^{lo}IgM^{+}$ immature B cell stage in the bone marrow [272]. The effect is gene dose-dependent, as heterozygous mice show an intermediate phenotype and B-cell numbers change with varying copy numbers [279]. If the expression level of the human CD19 transgene matches the one on circulating human B cells, one can rescue normal B-cell function as well as development in CD19-deficient mice [287]. CD19 diminishes the activation threshold of surface immunoglobulin, thereby potentially initiating clonal deletion of immature IgM^{+} B cells by imitating a BCR with high affinity for self-antigens [11]. This hypothesis is substantiated by the similarity in phenotype between CD19-deficient mice and those overexpressing the μ- or δ-immunoglobulin heavy chain [288, 289]. Here, a premature and pronounced positive signal during B-cell development results in decreased numbers of peripheral mature cells.

The hyper-reactivity of hCD19tg B cells is reflected in their increased proliferative response to LPS stimulation or anti-IgM treatment [272]. Also immunization with a T-cell dependent antigen results in elevated serum immunoglobulin levels despite the evident B-cell deficiency in these mice [272, 279].

CD19 deletion and its transgenic expression suggest that tight control of CD19 levels is essential for accurate B-cell development. The hypo-responsiveness of CD19-deficient and the hyper-responsiveness of hCD19tg cells imply that CD19 is a direct regulator of B-cell activation thresholds, most likely due to its role as part of the co-receptor complex. The loss of CD19 could potentially result in a loss of sufficient “tonic” signaling activity, resulting in B cells failing positive selection. In contrast, transgenic expression of elevated CD19 levels might mimic an autoreactive BCR, thereby inducing negative selection.

5. Results

I Adult mouse B-cell development in the absence of Interleukin-7 reveals its permissive role in B-cell commitment

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First author - Manuscript in preparation

II A stromal cell free culture system generates mouse pro-T cells that can reconstitute T-cell compartments in vivo

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III Reconstitution of a functional B-cell compartment in immunodeficient mice with pro-B cells propagated with or without stromal cells

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IV The selection of mature B cells is critically dependent on the expression level of the co-receptor CD19

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Conflict of interest

The authors declare no commercial or financial conflict of interest.

Abstract

Hematopoietic cells are continuously generated throughout life from hematopoietic stem cells (HSC), thus making hematopoiesis a favorable system to study developmental cell fate decisions. The main factors incorporating environmental signals to developing hematopoietic cells are cytokines, which exert their function either in a permissive or an instructive way. The cytokines Flt3-ligand (FL) and interleukin-7 (IL7) are important regulators of B-cell development, since their absence leads to compromised commitment to the B-cell lineage, manifested in the dramatic reduction of $CD135^{+}CD127^{+}Ly6D^{+}CD19^{-}$ progenitors and the loss of their B-cell potential. However, the cytokines' precise mode of action in this process has been a subject of debate. In the present study we assessed the rescue of B-cell commitment in mice lacking IL7 while at the same time over-expressing FL. Our analysis showed that increased *in vivo* levels of FL are sufficient to rescue B-cell commitment in the absence of IL7, demonstrating significant expression of *Ebf1* and *Pax5* and restored generation of $CD19^{+}$ B-cell progenitors. Further analysis of $IL7^{-/-}$ mice over-expressing the pro-survival gene *Bcl2*, as well as IL7tg and $FL^{-/-}$ mice, suggests that both FL and IL7 regulate B-cell commitment in a permissive manner; FL by inducing proliferation of $CD135^{+}CD127^{+}Ly6D^{+}CD19^{-}$ progenitors and IL7 by providing survival signals to these progenitors.

Introduction

Hematopoiesis, the generation of all blood cells from hematopoietic stem cells (HSC), is a lifelong process that takes place in the adult bone marrow. Over the last decades, accumulating evidence has suggested that HSC give rise to the different hematopoietic lineages through the generation of oligo-potent progenitors with limited self-renewal capacity and restricted developmental potentials. The activation of lineage-specific gene-transcription programs in these progenitors eventually leads to their commitment to a particular hematopoietic lineage. Cytokines are the most prominent environmental factors that regulate lineage commitment of hematopoietic progenitors and they do so by acting either in an instructive or a permissive manner [1]. In the instructive mode of action cytokines induce a signaling cascade in the target progenitor cell that leads to the initiation of a lineage-specific gene program, typically through up-regulation and/or activation of transcription factors, eventually resulting in commitment to a particular lineage. In contrast, the permissive model advocates that commitment of progenitors to different lineages occurs in a cell-autonomous, stochastic manner and the cytokine acts as a selection rather than a commitment factor, by promoting the survival and/or proliferation of a specific lineage at the expense of other lineages originating from the same progenitor. Elucidating the precise mode of action of cytokines is technically challenging and therefore the instructive versus permissive debate on hematopoietic cytokines remains open [2]. While the permissive model has been favored in the past, recent data have provided solid evidence for the instructive action of several important cytokines such as M-CSF, G-CSF, EPO and Flt3-ligand [3-6]. However, our understanding of how cytokines regulate hematopoiesis remains elusive, as different cytokines can act in various ways and their function might be cell-context dependent [7]. Moreover, most studies to date have addressed cytokine regulated myeloid differentiation and little is known about the role of cytokines in commitment to the lymphoid lineages.

Interleukin-7 (IL7) is considered the most crucial cytokine for the generation of B cells. The importance of IL7 for B-cell development has been clearly demonstrated by the dramatic defect in B-cell generation in mice lacking either the cytokine [8] or its receptor [9]. Interestingly, while human B-cell progenitors are also responsive to IL7 [10], disruption of IL7 signaling caused by mutations seems to not ablate B-cell development in these patients [11, 12]. IL7 has been initially identified as a growth factor for B-cell progenitors [13] and early studies demonstrated that over-expression of the pro-survival gene *Bcl2* in an *in vivo* setting was unable to rescue B-cell development in the absence of IL7 signaling, therefore suggesting that the cytokine acts in an instructive manner to commit progenitors to the B-cell fate [14, 15]. The subsequent findings that uncommitted Common Lymphoid Progenitors (CLP) from $IL7^{-/-}$ mice lacked expression of the transcription factor Early B-cell Factor 1 (Ebf1) [16] and that Ebf1 over-expression could partially restore B-cell generation from these CLP [17], led to the hypothesis that IL7, through Stat5 activation, instructs commitment to the B-cell lineage by initiating Ebf1 expression in uncommitted progenitors. In support of this hypothesis, a putative Stat5 binding site was later identified on the Ebf1 promoter [18]. However, a more recent study has shown that Bcl2 can rescue B-cell generation in a Stat5 conditional knock-out mouse model [19]. Furthermore, it has been demonstrated that the Ebf1-expressing fraction of CLP ($Ly6D^{+}$ CLP) is dramatically reduced in $IL7^{-/-}$ mice [20], therefore providing an alternative possibility for the reduced Ebf1 expression observed in $IL7^{-/-}$ CLP. Interestingly, this $Ly6D^{+}CD19^{-}$ progenitor stage is where B-cell commitment events are initiated at the molecular level [21]. Hence, while the importance of IL7 as a growth factor for committed B-cell progenitors has been well established, it remains unclear whether it instructs oligo-potent progenitors to commit to the B-cell lineage through Ebf1 and Pax5 up-regulation.

Ftl3-ligand (FL), the only known ligand for Flt3 receptor (CD135), is a cytokine important for the generation of many hematopoietic lineages and its function has gained much attention as mutations in FL signaling are commonly found in Acute Myeloid Leukemia (AML) [22]. Committed B-cell progenitors do not express CD135 since expression of the B-cell commitment factor Pax5 leads to Flt3 down-regulation

[23]. However, early studies on $Flt3^{-/-}$ and $FL^{-/-}$ mice showed an impaired ability of bone marrow progenitors from these mice to reconstitute B cells upon *in vivo* transplantation [24, 25], while subsequent experiments demonstrated that FL is essential for maintaining normal numbers of uncommitted B-cell progenitors [26].

We have recently described a FL-transgenic mouse model (hereafter FLtg) expressing high levels of FL *in vivo*, which has enabled us to suggest an instructive role for FL in early stages of hematopoiesis [6]. By breeding these mice with $IL7^{-/-}$ mice we show in the present study that increased FL levels can rescue B-cell commitment in $CD135^{+}CD127^{+}CD19^{-}$ progenitors and restore the numbers of early $CD19^{+}$ B-cell progenitors in the absence of IL7 signaling, suggesting a permissive role for IL7 in B-cell commitment. Further analyses of a combination of mouse genotypes over-expressing or lacking FL and IL7, as well as the pro-survival gene Bcl2, have enabled us to identify a permissive role for both IL7 and FL in B-cell commitment.

Materials and Methods

Mice

For breeding and analysis, age- and sex-matched C57BL/6 $FL^{-/-}$ [25], FLtg [6], $IL7^{-/-}$ [8], $IL7R\alpha^{-/-}$ [9], IL7tg [27], and Bcl2tg [28] mice were used at 6–11 weeks of age. All mice were bred and maintained in our animal facility under specific pathogen-free conditions. Animal experiments were carried out within institutional guidelines (authorization number 1888 from cantonal veterinarian office, Basel).

Antibodies, flow cytometry, and sorting.

For analysis, cells were flushed from femurs of the two hind legs of mice. The procedure was performed in PBS containing 0.5% BSA and 5mM EDTA. The following antibodies were used for flow cytometry (from BD Pharmingen, eBioscience, BioLegend, or produced in house): anti-B220 (RA3-6B2), anti-CD117 (2B8), anti-CD19 (1D3), anti-NK1.1 (PK136), anti-SiglecH (551), anti-CD11c (HL3), anti-CD115 (AFS98), anti-Ly6D (49-H4), anti-CD127 (SB/199), anti-CD135 (A2F10), anti-Sca1 (D7), anti-IgM (M41), anti-CD21 (7G6), anti-CD23 (B3B4), anti-CD4 (GK1.5), anti-CD8 (53.6.7), anti-TCRβ (H57). For detection of Ebf1 and cell cycle analysis, cells were fixed and permeabilized after cell-surface staining using the Foxp3 Fix/Perm buffer set (eBioscience)**,** and subsequently stained with PE-conjugated anti-Ebf1 (T26-818) or FITC-conjugated anti-Ki67 (B56) and DAPI, according to the supplier's protocol. Flow cytometry was done using a BD LSRFortessa (BD Biosciences) and data were analyzed using FlowJo Software (Treestar). For cell sorting, a FACSAria IIu (BD Biosciences) was used (>98% purity).

In vitro limiting dilution assays

Experiments have been performed as previously described [6].

Quantitative real-time PCR analysis

RNA extraction was performed using TRI Reagent® (Life Technologies) followed by cDNA synthesis using using GoScript™ Reverse Transcriptase (Promega). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). The primers used were: *Ebf1:* Ebf1-F: 5'-CAGGAAACCCACGTGACAT-3'; Ebf1-R: 5'-CCACGTTGACTGTGGTAGACA-3', *Pax5:* Pax5-F: 5'ACGCTGACAGGGATGGTG-3'; Pax5-R: 5'-GGGGAACCTCCAAGAATCAT-3', *Foxo1:* Foxo1-F: 5'-AGTGGATGGTGAAGAGCGT-3', Foxo1-R: 5'-GAAGGGACAGATTGTGGCG-3', *Actin:* Actin-F: 5'-CTGTCGAGTCGCGTCCACC-3', Actin-R: 5'-CGCAGCGATATCGTCATCCA-3'.

Statistical analysis

Statistical analysis was performed with Prism 6.0g software (GraphPad Software, Inc.). Two-tailed unpaired Student's t tests were used for statistical comparisons. If not differently indicated, data are presented as mean values ± SD or SEM as indicated. n.s. not significant or $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

Results

Increased in vivo levels of FL rescue B-cell commitment in IL7$^{-/-}$ CD19^{-} progenitors

We have previously reported the characterization of an uncommitted B-cell progenitor, with combined lymphoid and myeloid potential (Early Progenitor with Lymphoid and Myeloid potential – EPLM) [29]. Further sub-fractionation of this population with the use of surface markers SiglecH, CD11c, CD115 and Ly6D enabled us to identify the Ly6D^{+}SiglecH^{-}CD11c^{-}CD115^{-} fraction of EPLM (hereafter Ly6D^{+} EPLM) as the population containing most of the B-cell potential within EPLM, while being devoid of myeloid potential (Servera et al, manuscript in preparation). This EPLM subpopulation is identified as Lin^{-}CD19^{-}CD117^{int}B220^{int}Ly6D^{+}CD135^{+}CD127^{+} (Figure 1A), therefore partially overlapping phenotypically with Ly6D^{+} CLP (Supplementary Figure 1) and pre-pro-B cells [30, 31]. Ly6D^{+} EPLM numbers in IL7$^{-/-}$ and FL$^{-/-}$ mice showed a significant decrease compared to WT control animals; 7-fold for IL7$^{-/-}$ and 13-fold for FL$^{-/-}$ Ly6D^{+} EPLM (Figure 1B and C). A similar dramatic decrease was observed in Ly6D^{+} CLP from both mutant mice (Figure 1B and C), while FL deficiency also affected the numbers of Ly6D^{-} EPLM and CLP (Supplementary Figure 2). Therefore, the Ly6D^{+} EPLM/CLP represents the earliest stage in the B-cell developmental pathway affected by the absence of IL7.

We have recently generated a mouse model expressing high *in vivo* levels of FL [6]. Analysis of the progenitor compartment in these mice showed a dramatic increase in the numbers of EPLM and CLP, with the Ly6D^{+} fractions of these progenitors increased 90-fold and 28-fold relative to WT, respectively (Figure 1D and E). Prompted by this finding we crossed FLtg with IL7$^{-/-}$ mice in an attempt to assess the extent to which increased FL levels could potentially rescue the loss of Ly6D^{+}CD19^{-} progenitors in IL7$^{-/-}$ mice. As shown in Figure 2 (A and B), *in vivo* over-

expression of FL leads to a significant increase in the numbers of FLtg-IL7$^{-/-}$ EPLM and CLP, which reach the levels observed in FLtg mice. Crucially, a full rescue of Ly6D^{+} EPLM and CLP can be seen in these mice, with a striking 470-fold and 31-fold increase in their numbers, respectively, relative to their IL7$^{-/-}$ counterparts (Figure 2 A and B). Furthermore, the numbers of the earliest committed CD19^{+}CD117^{+} pro-B cells were fully restored in FLtg-IL7$^{-/-}$ mice, showing a 251-fold increase compared to IL7$^{-/-}$ mice (Figure 2C, Supplementary Figure 3). However, this rescue was less pronounced in downstream CD19^{+}CD117^{-}IgM^{-} and CD19^{+}IgM^{+} B-cell stages (Figure 2C), since these cells require IL7 to expand. As a result of this rescue in bone marrow B-cell generation, numbers of splenic marginal zone and follicular B cells were significantly increased in FLtg-IL7$^{-/-}$ mice compared to IL7$^{-/-}$ (Figure 2D). T-cell development in the thymus of Fltg-IL7$^{-/-}$ mice was not rescued (data not shown) while an increase in peripheral T cells was observed (Supplementary Figure 4) most likely due to the increased IL2 levels in these mice [32].

In order to assess whether these rescued FLtg-IL7$^{-/-}$ Ly6D^{+}CD19^{-} progenitors have the potential to give rise to B cells *in vitro*, we sorted FLtg-IL7$^{-/-}$ Ly6D^{+} EPLM and plated them on OP9 stromal cells in the presence of IL7, in limiting dilution conditions. As shown in Figure 3A, FLtg-IL7$^{-/-}$ Ly6D^{+} EPLM could generate B cells at similar frequencies as their WT and FLtg counterparts, while the few IL7$^{-/-}$ Ly6D^{+} EPLM we were able to isolate were unable to do so (data not shown). This indicates that increased FL levels restore the generation of these Ly6D^{+} progenitors, rather than merely expanding the few Ly6D^{+} EPLM/CLP found in IL7$^{-/-}$ mice. Moreover, real-time quantitative PCR analysis of Ly6D^{+} EPLM from FLtg-IL7$^{-/-}$ mice revealed significant expression of *Ebf1*, *Pax5* and *Foxo1* transcription factors in the absence of IL7 (Figure 3B). This result was confirmed for Ebf1 expression at the protein level by intracellular FACS staining. As shown in Figure 3 C and D, a significant proportion of FLtg-IL7$^{-/-}$ Ly6D^{+} EPLM expressed Ebf1 protein. Even though the percentage of FLtg-IL7$^{-/-}$ Ebf1^{+}Ly6D^{+} EPLM did not reach WT levels, it was similar to the one found in FLtg mice, which produce IL7. Therefore, *Ebf1/Pax5* expression and subsequent commitment to the B-cell fate can occur without active IL7 signaling present, arguing against an instructive role of this cytokine in B-cell commitment.

CD127 (IL7Rα) is a receptor subunit shared between IL7 and thymic stromal lymphopoietin (TSLP), a cytokine able to rescue B-cell development in the absence of IL7 when present at high levels [33]. Since TSLP is produced by dendritic cells [34], which are dramatically expanded in FLtg mice [6], *in vivo* FL over-expression could lead to increased levels of TSLP which in turn would rescue B-cell development in FLtg-IL7$^{-/-}$ mice. To investigate this possibility we injected IL7$^{-/-}$ or IL7Rα$^{-/-}$ mice with FL (10 daily injections of 10μg each) and assessed the rescue of Ly6D^{+} EPLM and downstream CD19^{+} progenitors in these mice. As shown in Figure 3E, FL injections into IL7$^{-/-}$ mice resulted in a significant increase in total EPLM, Ly6D^{+} EPLM and CD19^{+}CD117^{+} B-cell progenitors, comparable to the rescue observed in FLtg-IL7$^{-/-}$ mice. FL injected IL7Rα$^{-/-}$ mice also demonstrated a significant rescue of Ly6D^{+} EPLM and CD19^{+}CD117^{+} pro-B cells, indicating that the observed rescue of B-cell commitment in Fltg-IL7$^{-/-}$ mice is not mediated through the action of TSLP.

IL7 promotes survival, but not proliferation, of Ly6D^{+}CD135^{+}CD127^{+}CD19^{-} progenitors

Even though our FLtg-IL7$^{-/-}$ mouse model suggests that IL7 is dispensable for B-cell commitment, the dramatic decrease in IL7$^{-/-}$ Ly6D^{+} EPLM/CLP argues for a role of IL7 in the maintenance of this population when FL levels are limiting. This could be either by promoting their survival or their proliferation. To investigate the potential role of IL7 as a survival factor for Ly6D^{+}CD135^{+}CD127^{+}CD19^{-} progenitors we crossed IL7$^{-/-}$ mice with mice expressing the pro-survival gene Bcl2 under the control of the H2k MHC Class I promoter [28]. Analysis of the CD19^{-} progenitor compartment in these mice showed a minor but statistically significant 2.6-fold increase in the numbers of Ly6D^{+} EPLM and a similar 2.2-fold increase in Ly6D^{+} CLP (Figure 4A and B). Cell cycle stage analysis of Ly6D^{+} EPLM of these mice indicated that Bcl2 transgene over-expression had an anti-proliferative effect on Ly6D^{+} EPLM (Supplementary Figure 5), thereby compromising to some extent the rescue of these progenitors' numbers. Importantly, when plated on OP9 stromal cells together with

IL7, Bcl2tg-IL7$^{-/-}$ Ly6D^{+} EPLM could potently give rise to B cells at frequencies similar to WT mice (Figure 4C), indicating that this partially rescued Ly6D^{+} population is functional in giving rise to B cells. Indeed, when analyzing CD19^{+} committed progenitors in the bone marrow we could see a significant 68-fold increase in the earliest CD19^{+}CD117^{+} pro-B cell compartment, compared to IL7$^{-/-}$ (Figure 4D). Due to the anti-proliferative effect of Bcl2 over-expression (Supplementary Figure 5 and [35]), as well as the requirement for IL7 that CD19^{+} cells have for their proliferation, Bcl2tg-IL7$^{-/-}$ CD19^{+}CD117^{+} numbers did not reach WT levels, while downstream CD19^{+} immature B cell populations showed a less pronounced but still significant rescue (Figure 4D). In the periphery of these mice, Bcl2 over-expression increased the numbers of marginal zone and follicular B cells, while T-cell numbers were rescued, as previously reported (Supplementary Figure 6) [14]. We conclude that providing an extra Bcl2-mediated survival signal *in vivo* can partially rescue IL7$^{-/-}$ Ly6D^{+}CD19^{-} progenitors with the ability to commit to the B-cell pathway and results in a significant generation of CD19^{+} progenitors; therefore suggesting a role for IL7 in facilitating the survival of Ly6D^{+}CD135^{+}CD127^{+}CD19^{-} progenitors.

In order to evaluate the potential proliferative effect of IL7 on Ly6D^{+}CD135^{+}CD127^{+}CD19^{-} progenitors, we analyzed a mouse model in which expression of IL7 is driven by an MHC Class II promoter, resulting in increased *in vivo* levels of the cytokine [36]. These mice exhibit a lymphoproliferative phenotype with increased numbers of CD19^{+} B cells [27]. In contrast to bone marrow CD19^{+} cells, Ly6D^{+} EPLM numbers did not increase in response to elevated IL7 (Figure 5A, B and C). In addition, the cell cycle profile of Ly6D^{+} EPLM remained unaltered in IL7tg mice compared to WT (Figure 5D), arguing against a proliferative action of IL7 on these progenitors. In order to exclude the possibility that a proliferative signal by FL present in these mice might compromise the effect of increased IL7 on the cell cycle status of Ly6D^{+} EPLM, we crossed IL7tg with FL$^{-/-}$ mice. As shown in Figure 5E and F, *in vivo* over-expression of IL7 did not result in a significant increase in Ly6D^{+} EPLM or CLP numbers in the absence of FL. In contrast, a 3-fold increase in numbers of CD19^{+}CD117^{+} cells was observed (Figure 5G), which is in agreement with the proliferative effect of IL7 on CD19^{+} B cells. Ultimately this resulted in a small but

significant increase in splenic follicular B cells (Supplementary Figure 7). Moreover, cell cycle analysis of IL7tg-FL$^{-/-}$ Ly6D^{+} EPLM showed no significant change in the cycling profile of these cells compared to their FL$^{-/-}$ counterparts (Figure 5H). Therefore, we conclude that while IL7 acts as a proliferative factor for CD19^{+} committed B cells, it does not do so for their Ly6D^{+}CD19^{-} precursors.

FL induces proliferation of Ly6D^{+}CD135^{+}CD127^{+}CD19^{-} progenitors

As evident in Figure 5H, loss of *in vivo* FL signaling resulted in a significant increase in the percentage of resting Ly6D^{+} EPLM and a simultaneous decrease in the percentage of cycling cells, consistent with a proliferative action of FL on Ly6D^{+} EPLM. Indeed, comparison of Ly6D^{+} EPLM numbers in mice either lacking or over-expressing FL showed a 14-fold reduction in FL$^{-/-}$ Ly6D^{+} EPLM numbers compared to WT, while FLtg Ly6D^{+} EPLM increased 105-fold (Figure 6A). A similar response to FL levels was observed in Ly6D^{+} CLP, as well as Ly6D^{-} EPLM and CLP (Figure 6A). Cell cycle stage analysis of Ly6D^{+} EPLM from these mice showed a significant increase in the percentage of resting cells and a decrease in the percentage of cycling cells when FL signaling is absent, while FLtg Ly6D^{+} EPLM showed the reverse (Figure 6B and Supplementary Figure 8). Thus, our data indicate that FL promotes the proliferation of Ly6D^{+}CD135^{+}CD127^{+}CD19^{-} progenitors.

In order to evaluate if FL additionally regulates the survival of Ly6D^{+}CD135^{+}CD127^{+}CD19^{-} progenitors, we crossed FL$^{-/-}$ mice with Bcl2tg mice. As shown in Figure 6C, Bcl2tg-FL$^{-/-}$ mice showed a minor 2-fold increase in Ly6D^{+} EPLM numbers compared to their FL$^{-/-}$ counterparts (1.8-fold for Ly6D^{+} CLP). Nevertheless, the *in vitro* B-cell potential of FL$^{-/-}$ Ly6D^{+} EPLM progenitors was not improved by Bcl2 over-expression (Figure 6D). Downstream CD19^{+} progenitors also demonstrated a partial but significant rescue (Figure 6E). Our analysis of Bcl2tg-FL$^{-/-}$ mice suggests that the reduction in the Ly6D^{+}CD135^{+}CD127^{+}CD19^{-} progenitors observed in FL$^{-/-}$ mice could only partially be explained by a survival role of FL. Nevertheless, the clear

change in the cycling profile of these progenitors in response to the absence or the over-abundance of FL *in vivo*, as well as the inability of Bcl2 to rescue their *in vitro* B-cell potential, points towards proliferation as being the main effector function of the cytokine at this developmental stage.

FL does not instruct commitment to the B-cell lineage

The striking rescue in B-cell commitment observed in our FLtg-IL7$^{-/-}$ mice could be explained by a potential instructive role of FL when present at high levels *in vivo*. However, as shown in Figure 3A, FLtg and FLtg-IL7$^{-/-}$ Ly6D^{+} EPLM did not show a higher capacity in generating B cells *in vitro*, compared to WT Ly6D^{+} EPLM. More importantly, *Ebf1* and *Pax5* expression in these cells appeared to be reduced compared to WT, with *Ebf1* mRNA being expressed at almost half the amount relative to WT and *Pax5* even less (Figure 3B). Intracellular staining for Ebf1 protein expression showed that the percentage of Ebf1-expressing Ly6D^{+} EPLM was reduced in FLtg mice (Figure 3C and D). Therefore, increased *in vivo* FL signaling seems to result in a decreased Ebf1^{+}Ly6D^{+} EPLM population, rather than a down-regulation of *Ebf1* gene expression. We extended this analysis to FL$^{-/-}$ Ly6D^{+} EPLM and found that while absence of FL *in vivo* leads to a reduction in the numbers of Ly6D^{+} EPLM (Figure 1C), it does not significantly reduce the percentage of Ebf1^{+} cells within the population (Figure 7A and B), consistent with a permissive rather than instructive role of FL. Finally, the decrease in the Ebf1^{+} fraction of Ly6D^{+} EPLM upon exposure to high levels of FL was reflected in the increased ability of these progenitors to give rise to T cells *in vitro*, as manifested by the high frequency of T-cell clone generation when FLtg Ly6D^{+} EPLM were plated on OP9DL1 stromal cells in the presence of IL7 (Figure 7C). The above data suggest that FL does not instruct commitment to the B-cell lineage through up-regulation of *Ebf1* and *Pax5* expression.

Discussion

Commitment to the B-cell lineage is mediated by the expression of Ebf1 and Pax5 transcription factors and it is initiated prior to CD19 expression, in $CD135^{+}CD127^{+}Ly6D^{+}$ progenitors [30, 31]. In $IL7^{-/-}$ mice this $Ly6D^{+}$ CLP compartment is significantly reduced [20], a finding that we confirmed in the present study for both CLP and EPLM, a $B220^{int/+}$ population partly overlapping with CLP and pre-pro B cells (Figure 1B and C). Thus, the $Ly6D^{+}$ CLP/EPLM progenitors represent the developmentally earliest stage in the B-cell pathway affected by the absence of IL7. Moreover, IL7 has a proliferative effect on committed $CD19^{+}$ B-cell progenitors, therefore making the investigation of its role in B-cell commitment challenging when using $CD19^{+}$ cells as readout. Hence, we assessed the role of IL7 in B-cell commitment by analyzing the $Ly6D^{+}$ CLP/EPLM compartment in different mouse models. Our analysis of FLtg-$IL7^{-/-}$ mice showed a complete rescue of $Ly6D^{+}$ CLP/EPLM both in numbers and in their ability to generate B cells *in vitro* (Figures 2 and 3). Furthermore, in Fltg-$IL7^{-/-}$ mice *Ebf1* and *Pax5* were expressed at similar levels to FLtg mice, thereby indicating that IL7 signaling is not required for their up-regulation at the $Ly6D^{+}CD19^{-}$ stage. These results suggest that IL7 is not acting as an instructive cytokine in B-cell commitment by initiating *Ebf1* and *Pax5* expression at the $CD135^{+}CD127^{+}CD19^{-}$ stage, as previously hypothesized [16-18], but rather as a permissive one.

While early studies showed an inability of Bcl2 over-expression to rescue B cells in the absence of IL7 signaling [14, 15], a more recent investigation suggested a survival role for IL7 in B-cell development based on a Bcl2-mediated rescue of $CD19^{+}$ progenitors in conditional $Stat5^{-/-}$ mice [19]. However, all previous studies have focused on the potential rescue of $CD19^{+}$ committed cells. Moreover, they have used $IL7R\alpha^{-/-}$ mice, thereby excluding the assessment of $CD19^{-}$ progenitor B-cell potential upon exogenous IL7 addition, an important phenotype of IL7 deficiency. By focusing

on Ly6D^{+}CD19^{-} progenitors, where B-cell commitment is initiated, our analysis of Bcl2tg-IL7$^{-/-}$ mice showed a minor but significant rescue in Ly6D^{+} EPLM (Figure 4). Importantly, a full restoration of their ability to give rise to B cells in culture was observed (Figure 4C), pointing towards survival as a main role for IL7 at the CD135^{+}CD127^{+}CD19^{-} stage. In agreement with the aforementioned published data we find a significant but not complete rescue of CD19^{+} progenitors in Bcl2tg-IL7$^{-/-}$, which can be explained partly by the inability of Bcl2 to rescue the proliferative function of IL7 and partly by its own anti-proliferative effect (Suppl. Fig. 4) [35]. Interestingly, analysis of IL7tg mice, expressing high levels of IL7 *in vivo*, showed that IL7 indeed acts as a proliferative factor following commitment to the B-cell lineage and expression of CD19, but not before. Even in the absence of FL, when Ly6D^{+} CLP/EPLM numbers were compromised, excess IL7 was unable to significantly increase their numbers, while it did so for CD19^{+} B-cell progenitors (Figure 5). This result is in agreement with our proposed role for IL7 as a survival factor for Ly6D^{+}CD19^{-} progenitors, since increased *in vivo* levels of a survival but not proliferative factor would not result in increased numbers of progenitors, when survival is not already compromised. Hence, we propose that the main role of IL7 at the CD135^{+}CD127^{+}CD19^{-} stage is to provide survival signals to the progenitors until they commit to the B-cell lineage upon Pax5 and CD19 expression, after which it induces their proliferation (Figure 7D). This survival role becomes particularly critical when FL levels are limiting, thereby explaining the reduction in Ly6D^{+} CLP/EPLM seen in IL7$^{-/-}$ mice.

The rescue in B-cell commitment without active IL7 signaling occurs when FL is expressed above physiological levels. Even though a minor role for FL as a survival factor for CD135^{+}CD127^{+}Ly6D^{+}CD19^{-} progenitors cannot be excluded, the main effect of FL on these progenitors seems to be the induction of their proliferation, as suggested by their expansion and their increased cycling upon FL over-expression, with the reverse phenotype observed upon loss of FL signaling (Figure 6). Moreover, increased FL leads to expansion of Lin^{-}CD117^{+}Sca1^{+} cells (LSK) [6], thereby increasing the developmental input into the CD135^{+}CD127^{+}Ly6D^{+}CD19^{-} progenitor stage. None of the mouse models analyzed in the present study gave any evidence for an

instructive role of FL in B-cell commitment. In contrast, excess FL seems to result in a proportional reduction of the *Ebf*- and *Pax5*-expressing Ly6D$^+$CD19$^-$ progenitors (Figures 3 and 7). One explanation for this reduction could be the increased percentage of cycling FLtg Ly6D$^+$CD19$^-$ progenitors, resulting in a decreased fraction of them initiating the B-cell developmental program. Alternatively, another environmental factor, responsible for initiation of Ebf1/Pax5 expression and B-cell commitment, could be the limiting factor in FLtg mice, thus leading to a smaller fraction of the expanded Ly6D$^+$CD19$^-$ compartment entering the B-cell pathway, in comparison to WT. Notably, increased FL signaling does not increase Stat5 phosphorylation levels in Ly6D$^+$ EPLM (data not shown), therefore excluding the possibility that excess FL can substitute a potential instructive function of IL7 through aberrant Stat5 activation. Our conclusion is that in WT mice FL is mainly responsible for generating enough CD135$^+$CD127$^+$Ly6D$^+$CD19$^-$ progenitors, both by inducing their proliferation and by increasing their developmental input from the LSK compartment (Figure 7D) [37, 38]. As a result, increased *in vivo* levels of FL lead to a dramatic increase in numbers of CD135$^+$CD127$^+$Ly6D$^+$CD19$^-$ progenitors, therefore surpassing the need for the survival role of IL7 at this stage and resulting in a significant fraction of them committing to the B-cell lineage.

The generation of B-cell progenitors observed in our FLtg-IL7$^{-/-}$ mouse model is reminiscent of the apparent IL7 independency of human B lymphopoiesis, as demonstrated by the relatively normal numbers of B cells in patients with mutations in components of the IL7 signaling pathway [11, 12]. It should be noted, however, that all patients with such mutations are neonates and in neonatal IL7$^{-/-}$ mice B-cell development has also been shown to take place [39]. Therefore, the apparent difference in the IL7 dependency of B-cell development between humans and mice could actually reflect the corresponding difference between fetal/neonatal and adult lymphopoiesis. Our data showing increased FL signaling being able to rescue B-cell commitment in the absence of IL7 could provide a potential explanation for this apparent difference. Fetal/neonatal CD135$^+$IL7R$^+$CD19$^-$ progenitors might be subjected to higher levels of FL by their microenvironment and/or show higher sensitivity to FL signaling, in comparison to adult CD135$^+$IL7R$^+$CD19$^-$ progenitors, thus

making the survival role of IL7 redundant. In support of this hypothesis, previous studies have shown that even though fetal B-cell progenitors preferentially respond to TSLP, FL signaling remains an absolute requirement for fetal B lymphopoiesis [40, 41].

Whether cytokines regulate differentiation to different blood lineages in an instructive or a permissive way has been a long standing debate [42, 43]. It is becoming increasingly evident that cell fate regulation by cytokines is quite complex, with the effect of cytokines on progenitors being influenced by their intra-cellular context, and vice versa [7]. Our present data indeed suggest that the action of cytokines can be cell-context dependent. Our previous analysis of early hematopoiesis in FLtg mice indicated an instructive role for FL in promoting differentiation of multi-potent progenitors towards lympho-myeloid and away from erythroid fate [6]. In the present study, we provide evidence that FL acts in a permissive manner when it comes to commitment of $CD135^{+}CD127^{+}CD19^{-}$ progenitors to the B-cell lineage. In addition, we show that while IL7 induces proliferation on committed $CD19^{+}$ B-cell progenitors, it does not do so on $IL7R^{+}CD19^{-}$ progenitors, suggesting that changes in the transcription factor and signaling molecule landscape upon commitment to the B-cell lineage influence the effector function of IL7.

The *Ebf1/Pax5* up-regulation and subsequent B-cell commitment in FLtg-$IL7^{-/-}$ mice that we show herein reveals that IL7 signaling can be dispensable for early B-cell commitment events, therefore suggesting a permissive role for IL7. This, in turn, raises the issue of the potential environmental regulation of B-cell commitment. One possibility could be that another environmental signal from the bone marrow microenvironment - other than IL7, TSLP and FL - initiates *Ebf1* expression in $CD135^{+}IL7R^{+}Ly6D^{+}CD19^{-}$ progenitors resulting in subsequent *Pax5/CD19* expression and B-cell commitment. Alternatively, $CD135^{+}IL7R^{+}Ly6D^{+}CD19^{-}$ progenitors that have not been instructed by other signal(s) to develop to other lineages, could express *Ebf1* in a cell-autonomous, stochastic manner, with a fraction of those obtaining sufficient Ebf1 levels to initiate the B-cell gene program and eventually commit to the B-cell lineage. The intricate transcription factor network that sustains B-cell

commitment through a series of positive feedback regulatory loops [44] provides conceptual support to the latter hypothesis. Further investigation of the molecular B-cell commitment events in relation to environmental signals is required to confirm either of the two aforementioned hypotheses.

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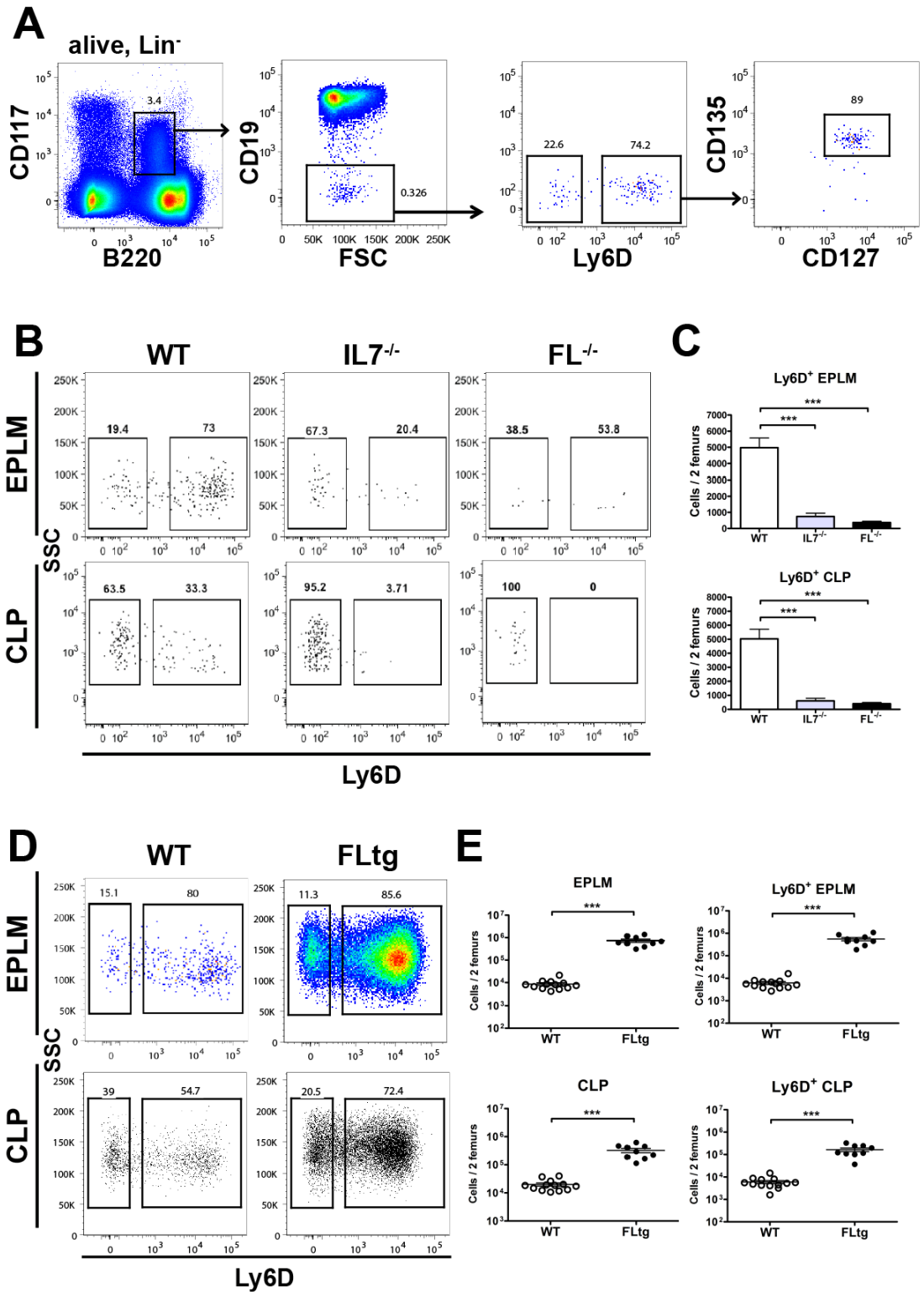


Figure 1: IL7 and FL are necessary for the generation of a normal Ly6D$^+$CD135$^+$CD127$^+$CD19$^-$ compartment. (A) FACS plots showing the gating strategy used for identification of Ly6D$^+$ EPLM and their percentage of CD135 and CD127 expression. Lineage staining: SiglecH, CD115, CD11c, NK1.1, Gr-1. **(B)** Representative FACS plots of EPLM (upper row) and CLP (lower row) from the bone marrow of WT,

$IL7^{-/-}$ and $FL^{-/-}$ mice. **(C)** Absolute numbers of $Ly6D^{+}$ EPLM (upper graph) and CLP (lower graph) from the bone marrow of WT (n=7), $IL7^{-/-}$ (n=5) and $FL^{-/-}$ (n=10) mice. **(D)** Representative FACS plots of EPLM and CLP from WT and FLtg mice. **(E)** Absolute numbers of total EPLM and CLP (left graphs) and $Ly6D^{+}$ EPLM and CLP from WT and FLtg mice.

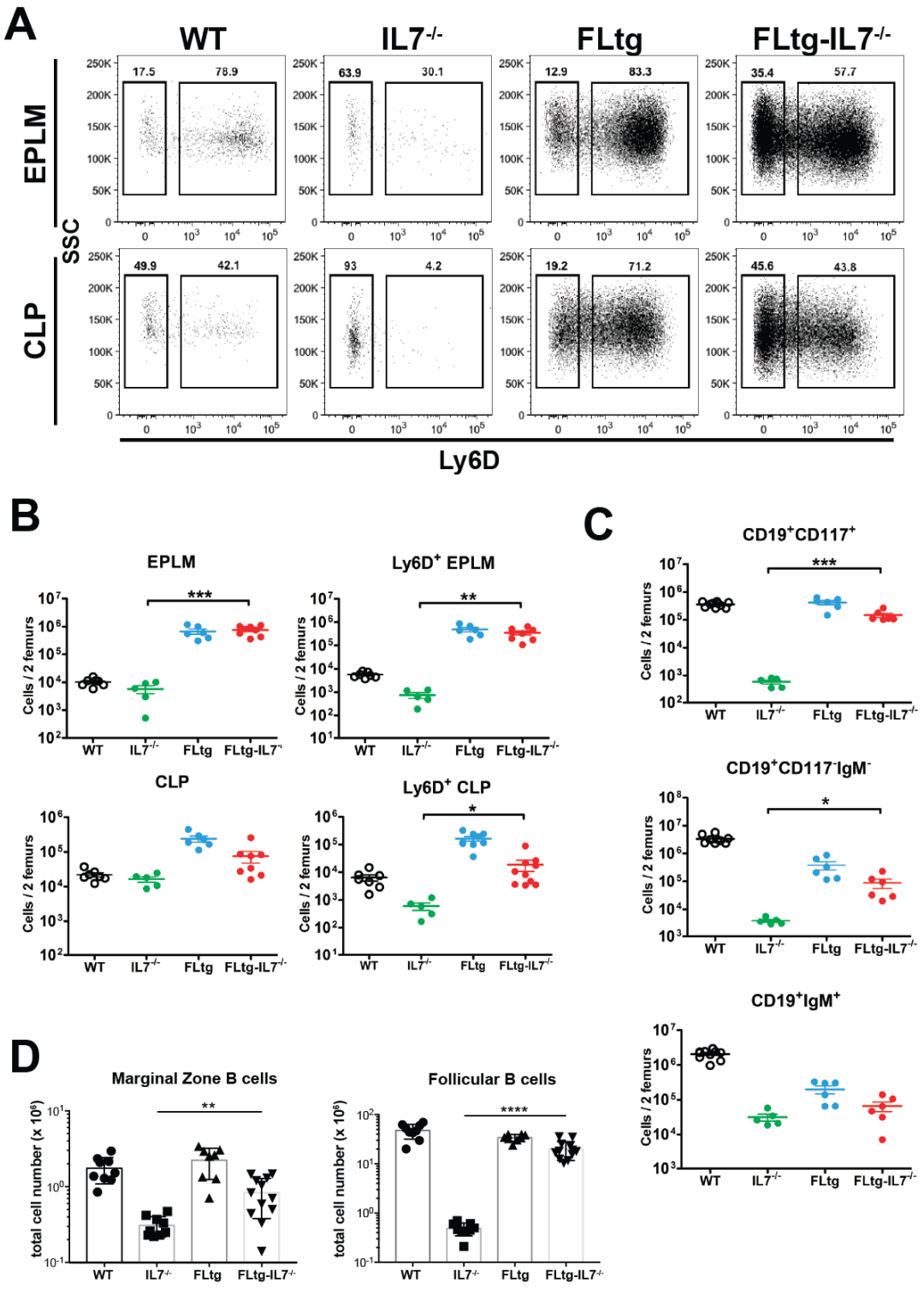


Figure 2: Increased *in vivo* FL levels rescue B-cell generation in IL7$^{-/-}$ mice. (A) Representative FACS plots of EPLM (upper panel) and CLP (lower panel) from WT, IL7$^{-/-}$, FLtg and FLtg-IL7$^{-/-}$ mice. **(B)** Absolute numbers of EPLM (top left), CLP (bottom left), Ly6D^{+} EPLM (top right) and Ly6D^{+} CLP (bottom right) from the mouse genotypes indicated on the x-axes. For each mouse genotype mean ± SEM is shown. **(C)**

Absolute numbers of $CD19^+CD117^+$ (top), $CD19^+CD117^-IgM^-$ (middle) and $CD19^+IgM^+$ (bottom) bone marrow cells from the mice indicated on the x-axes. For each mouse genotype mean ± SEM is shown. **(D)** Absolute numbers of $CD19^+CD21^{high}CD23^{low}$ marginal zone (left) and $CD19^+CD21^+CD23^+$ follicular (right) B cells in the spleens of WT or mutant mice, as indicated on the x-axes. For each mouse genotype mean ± SD is shown.

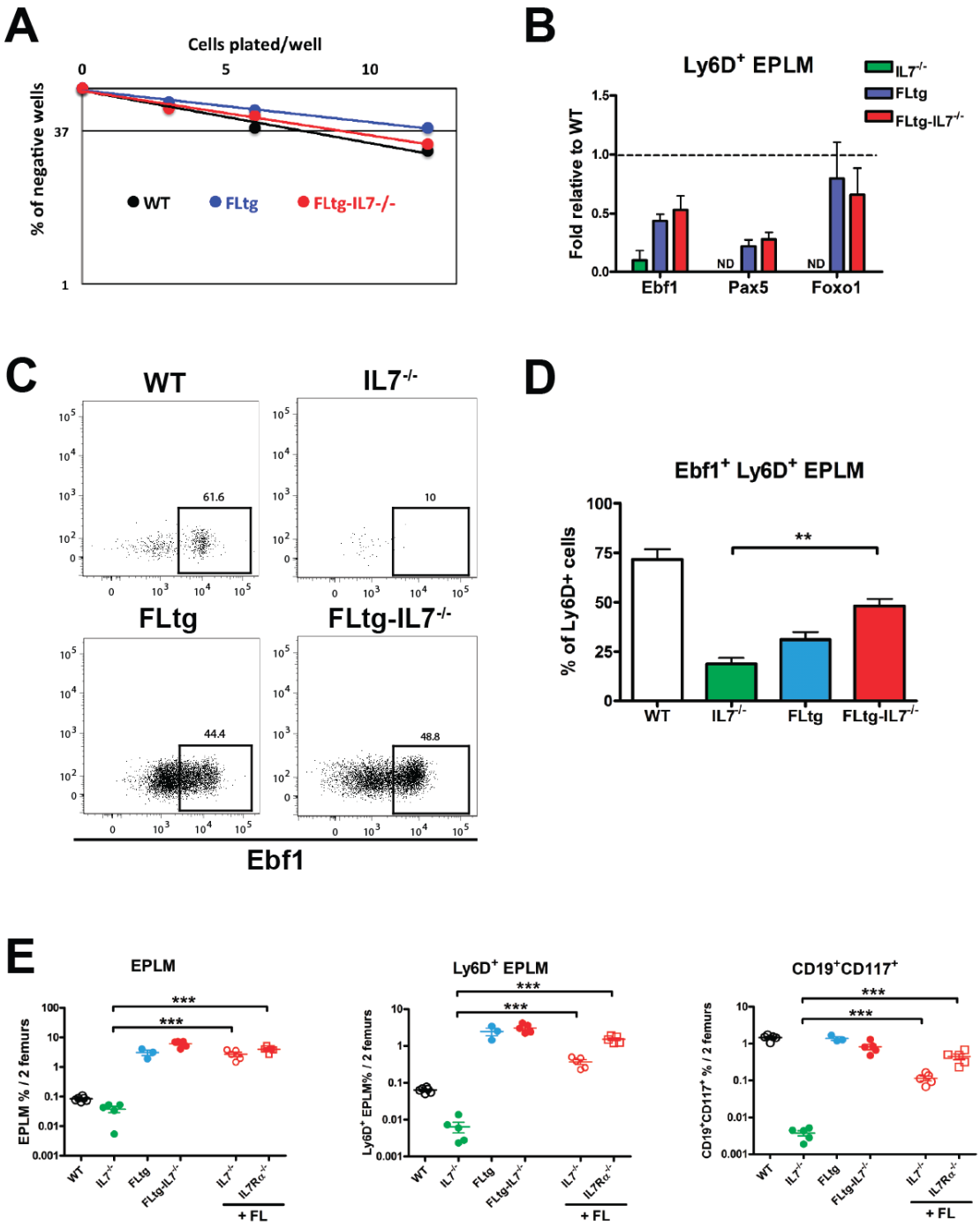


Figure 3: Increased *in vivo* FL rescues B-cell commitment in the absence of IL7 and/or TSLP. (A) *In vitro* limiting dilution analysis of Ly6D$^+$ EPLM B-cell potential. Ly6D$^+$ EPLM were sorted from WT, FLtg and FLtg-IL7$^{-/-}$ mice and plated at the indicated concentrations on OP9 stromal cells together with IL7. One representative out of four independent experiments is shown. **(B)** Real-time quantitative PCR analysis showing expression of *Ebf1*, *Pax5* and *Foxo1* mRNAs in Ly6D$^+$ EPLM sorted from the indicated mouse genotypes. Bars show fold expression relative to WT (set

as 1). Error bars represent the SEM from 3-6 independent experiments. **(C)** Representative FACS plots showing expression of Ebf1 protein within the $Ly6D^+$ EPLM of the indicated mouse genotypes. **(D)** Percentages of Ebf1-expressing $Ly6D^+$ EPLM from WT (n=7), $IL7^{-/-}$ (n=3), FLtg (n=11) and FLtg-$IL7^{-/-}$ (n=6) mice. Bars show mean ± SEM. **(E)** EPLM (left), $Ly6D^+$ EPLM (middle) and $CD19^+CD117^+$ (right) numbers from WT, $IL7^{-/-}$, FLtg, FLtg-$IL7^{-/-}$ mice, as well as from $IL7^{-/-}$ and $IL7R\alpha^{-/-}$ mice injected intra-peritoneally with 10 daily doses of 10 µg FL each (indicated as +FL). Shown is mean ± SEM.

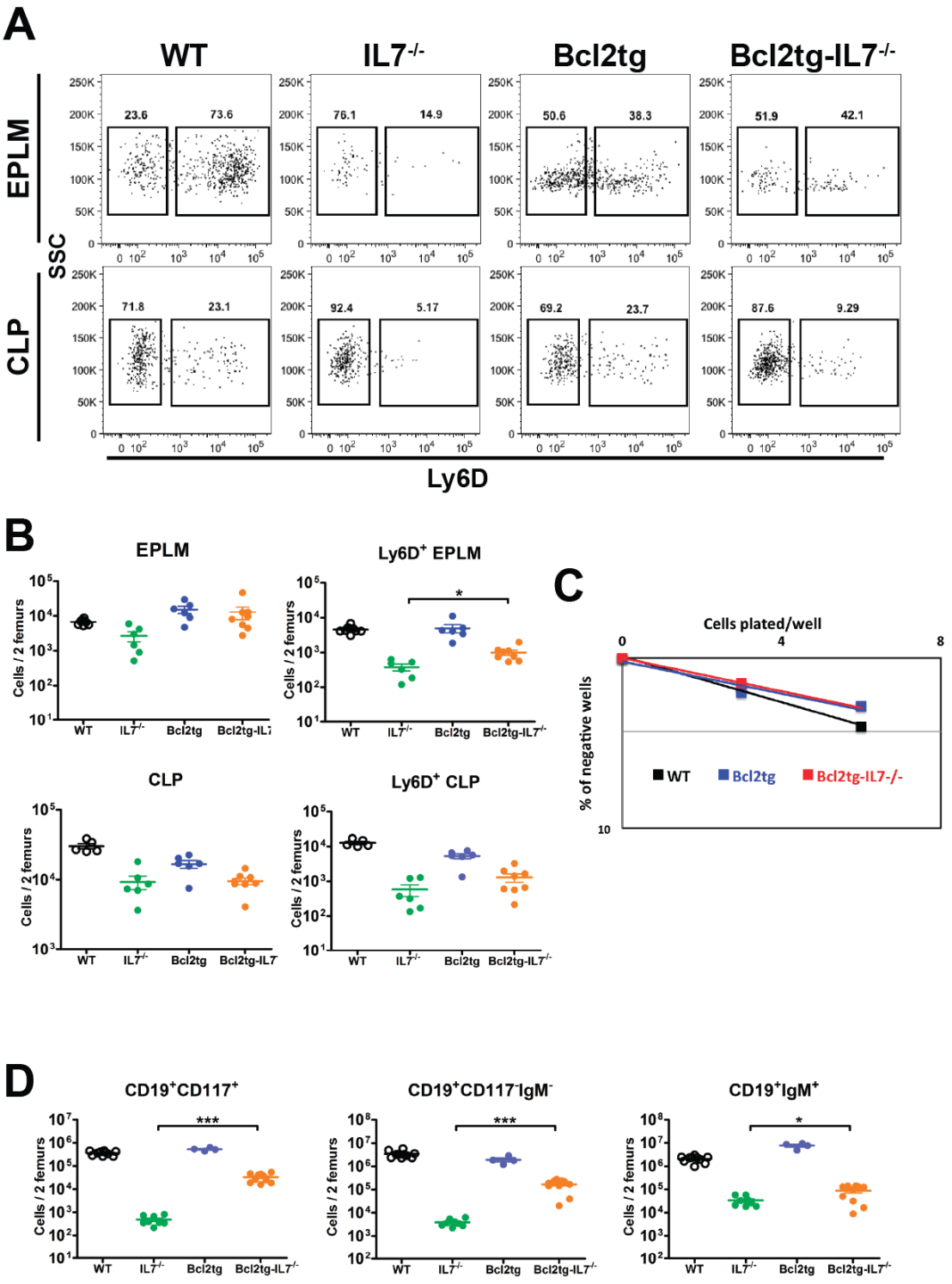


Figure 4: Bcl2 over-expression partially rescues B-cell commitment in IL7$^{-/-}$ mice. (A) Representative FACS plots of EPLM (upper panel) and CLP (lower panel) from WT, IL7$^{-/-}$, Bcl2tg and Bcl2tg-IL7$^{-/-}$ mice. **(B)** Absolute numbers of EPLM (top left), CLP (bottom left), Ly6D^{+} EPLM (top right) and Ly6D^{+} CLP (bottom right) from WT and mutant mice, as indicated on the x-axes. For each mouse genotype mean ±

SEM is shown. **(C)** *In vitro* limiting dilution analysis of $Ly6D^+$ EPLM B-cell potential. $Ly6D^+$ EPLM were sorted from WT, $IL7^{-/-}$, Bcl2tg and Bcl2tg-$IL7^{-/-}$ mice and plated at the indicated concentrations on OP9 stromal cells together with IL7. One representative out of three independent experiments is shown. **(D)** Absolute numbers of $CD19^+CD117^+$ (left), $CD19^+CD117^-IgM^-$ (middle) and $CD19^+IgM^+$ (right) bone marrow cells from WT and mutant mice, as indicated on the x-axes. For each mouse genotype mean ± SEM is shown.

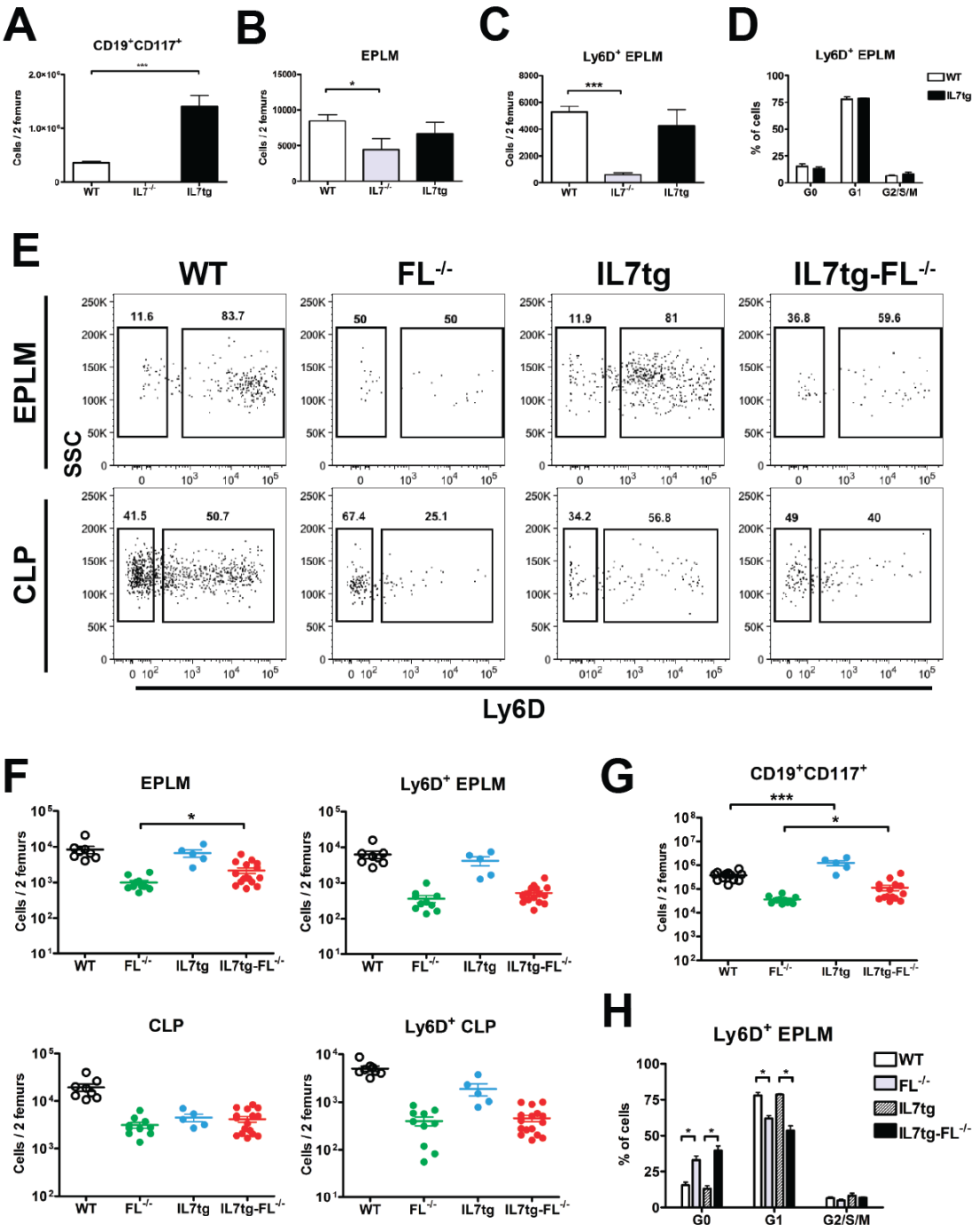


Figure 5: IL7 does not induce proliferation of Ly6D$^+$CD135$^+$CD127$^+$CD19$^-$ progenitors. (A) Absolute number of CD19$^+$CD117$^+$ cells in the bone marrow of WT (n=10), IL7$^{-/-}$ (n=5) and IL7tg (n=8) mice. **(B)** Total number of EPLM in bone marrow of WT (n=14), IL7$^{-/-}$ (n=7) and IL7tg (n=5) mice. **(C)** Ly6D$^+$ EPLM numbers in the bone marrow of WT (n=14), IL7$^{-/-}$ (n=7) and IL7tg (n=5) mice. **(D)** Cell cycle analysis of Ly6D$^+$ EPLM from WT (n=5) and IL7tg (n=2) mice. Graph shows percentages of Ly6D$^+$ EPLM in G0 (Ki67$^-$DAPI$^-$), G1 (Ki67$^+$DAPI$^-$) and G2/S/M (Ki67$^+$DAPI$^+$) stages. Bars in A,

B, C and D show mean ± SEM. **(E)** Representative FACS plots of EPLM (upper panel) and CLP (lower panel) from WT, $FL^{-/-}$, IL7tg and IL7tg-$FL^{-/-}$ mice. **(F)** Absolute numbers of EPLM (top left), CLP (bottom left), $Ly6D^+$ EPLM (top right) and $Ly6D^+$ CLP (bottom right) from WT and mutant mice, as indicated on the x-axes. For each mouse genotype mean ± SEM is shown. **(G)** Absolute numbers of $CD19^+CD117^+$ bone marrow cells from WT and mutant mice, as indicated on the x-axis. For each mouse genotype mean ± SEM is shown. **(H)** Cell cycle analysis of $Ly6D^+$ EPLM from WT (n=5), $FL^{-/-}$ (n=3), IL7tg (n=2) and IL7tg-$FL^{-/-}$ (n=3) mice. Graph shows percentages of $Ly6D^+$ EPLM in G0 ($Ki67^-DAPI^-$), G1 ($Ki67^+DAPI^-$) and G2/S/M ($Ki67^+DAPI^+$) stages. Bars show mean ± SEM.

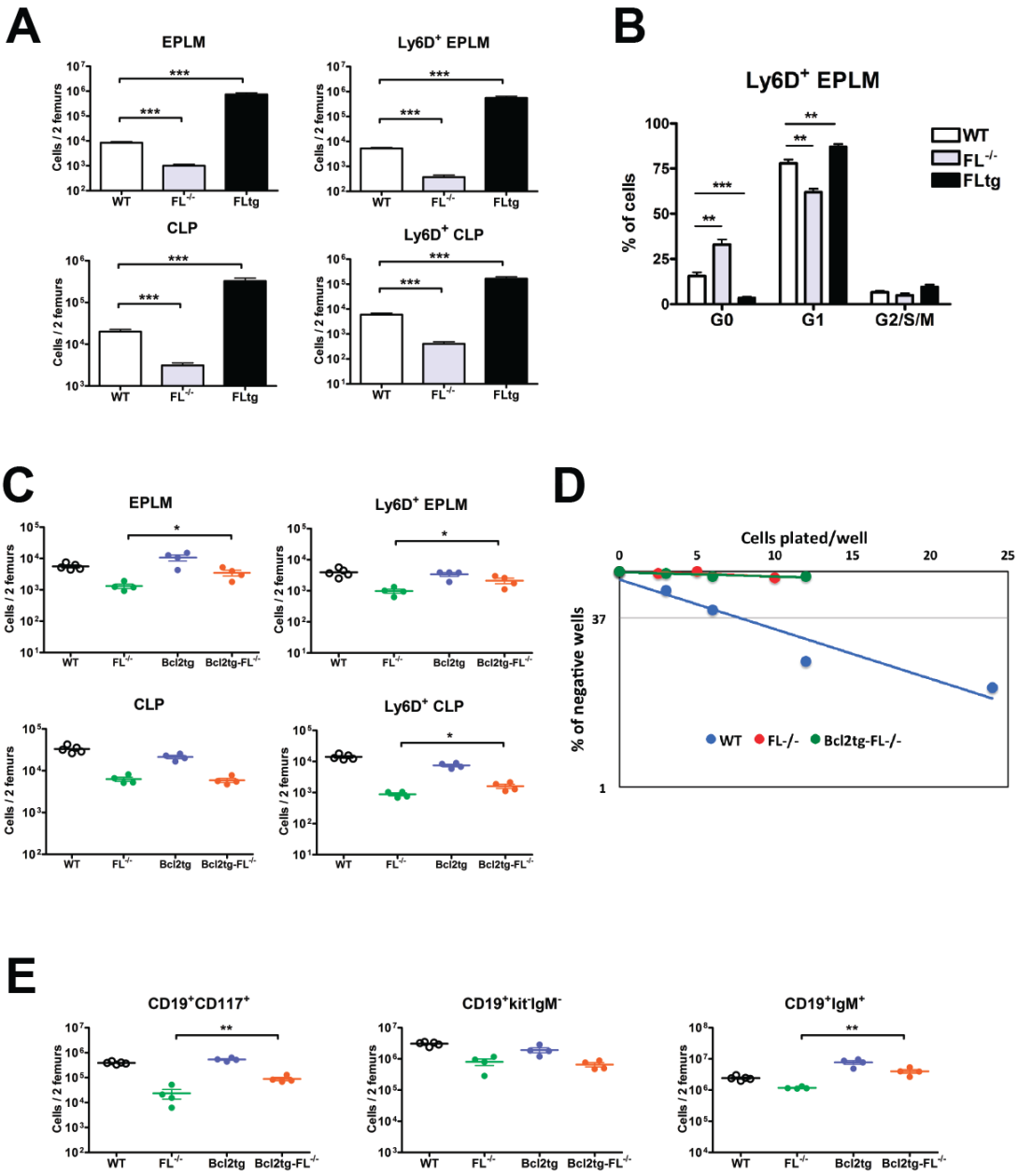


Figure 6: FL promotes proliferation but not survival of Ly6D$^+$CD135$^+$CD127$^+$CD19$^-$ progenitors. (A) Absolute numbers of EPLM (top left), CLP (bottom left), Ly6D$^+$ EPLM (top right) and Ly6D$^+$ CLP (bottom right) from WT (n=14), FL$^{-/-}$ (n=10) and FLtg (n=9) mice. Bars show mean ± SEM. **(B)** Cell cycle analysis of Ly6D$^+$ EPLM from WT (n=5), FL$^{-/-}$ (n=3) and FLtg (n=9) mice. Graph shows percentages of Ly6D$^+$ EPLM in G0 (Ki67$^-$DAPI$^-$), G1 (Ki67$^+$DAPI$^-$) and G2/S/M (Ki67$^+$DAPI$^+$) stages. Bars show mean ± SEM. **(C)** Absolute numbers of EPLM (top left), CLP (bottom left), Ly6D$^+$ EPLM (top right) and Ly6D$^+$ CLP (bottom right) from WT and mutant mice, as indicated on the x-axes. For each mouse genotype mean ± SEM is shown. **(D)** *In vitro*

limiting dilution analysis of Ly6D$^+$ EPLM B-cell potential. Ly6D$^+$ EPLM were sorted from WT, FL$^{-/-}$ and Bcl2tg-FL$^{-/-}$ mice and plated at the indicated concentrations on OP9 stromal cells together with IL7. **(E)** Absolute numbers of CD19$^+$CD117$^+$ (left), CD19$^+$CD117$^-$IgM$^-$ (middle) and CD19$^+$IgM$^+$ (right) bone marrow cells from WT and mutant mice, as indicated on the x-axes. For each mouse genotype mean ± SEM is shown.

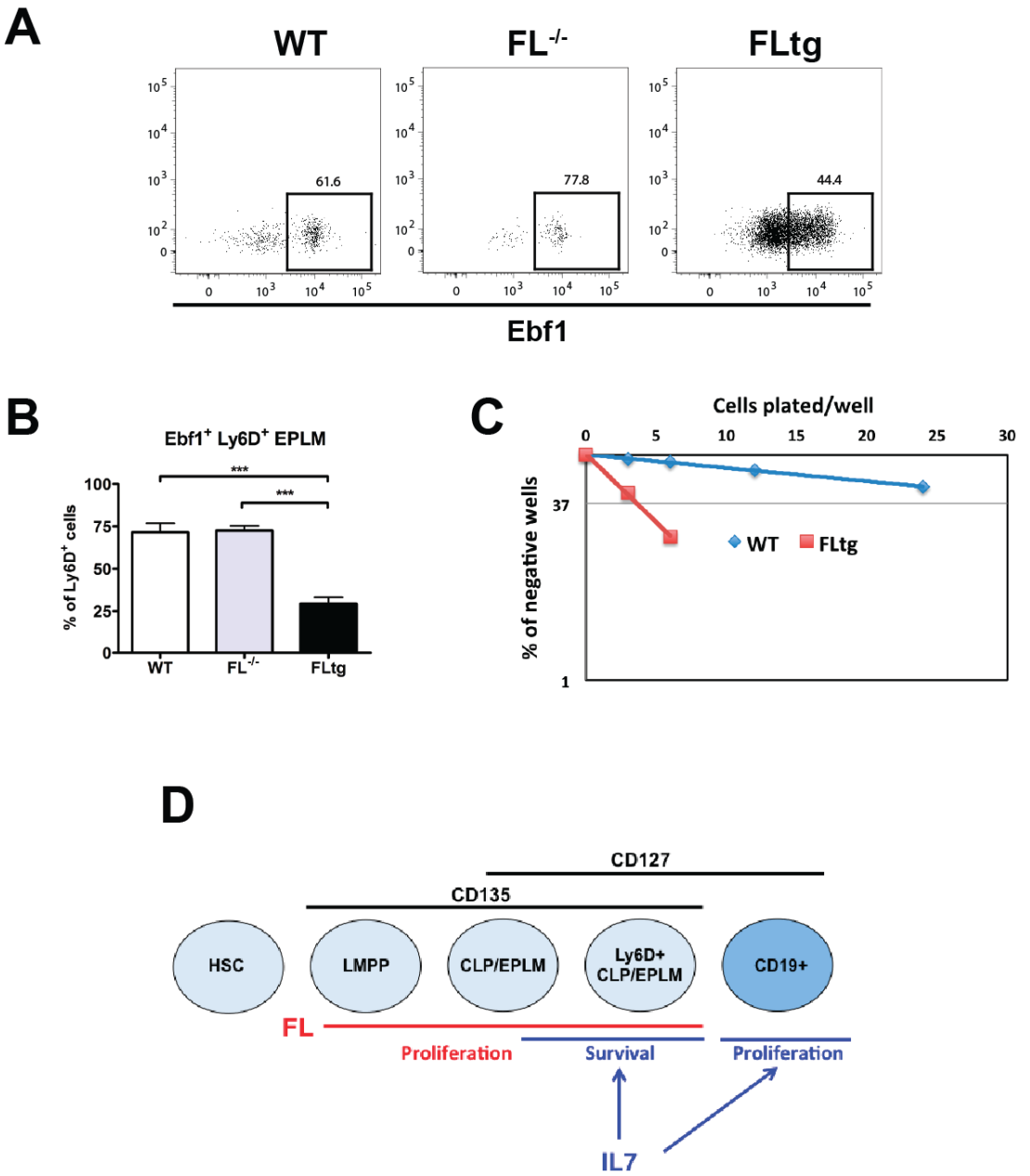
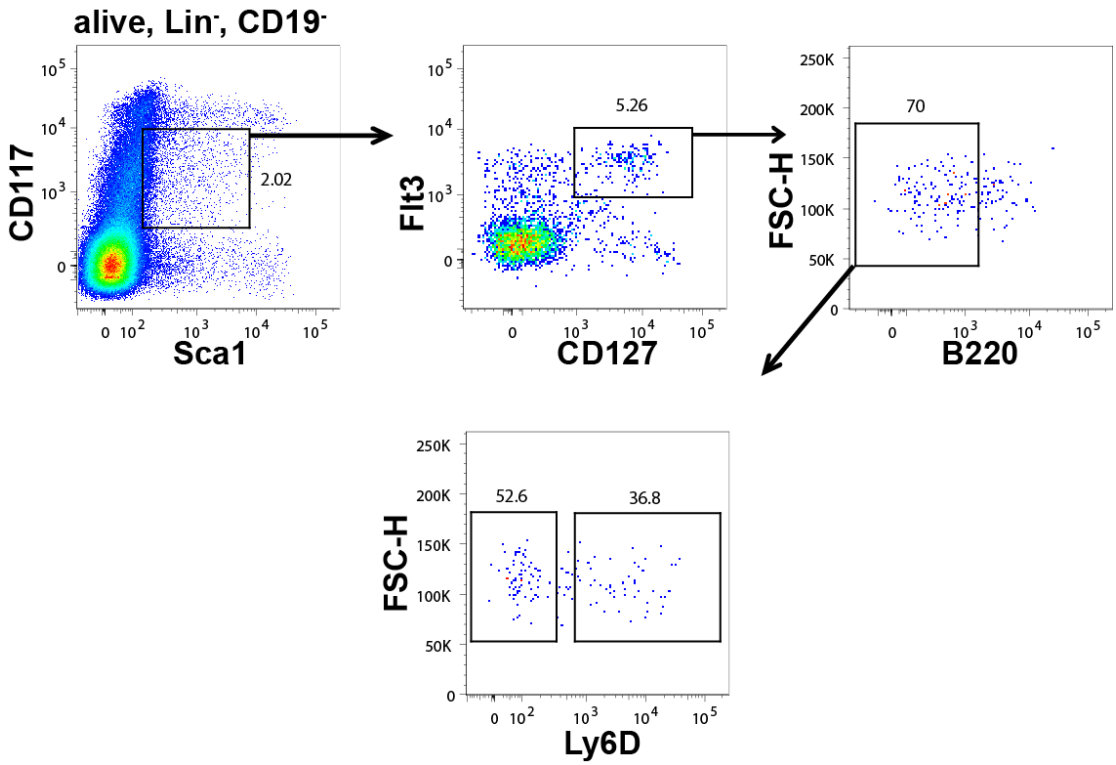
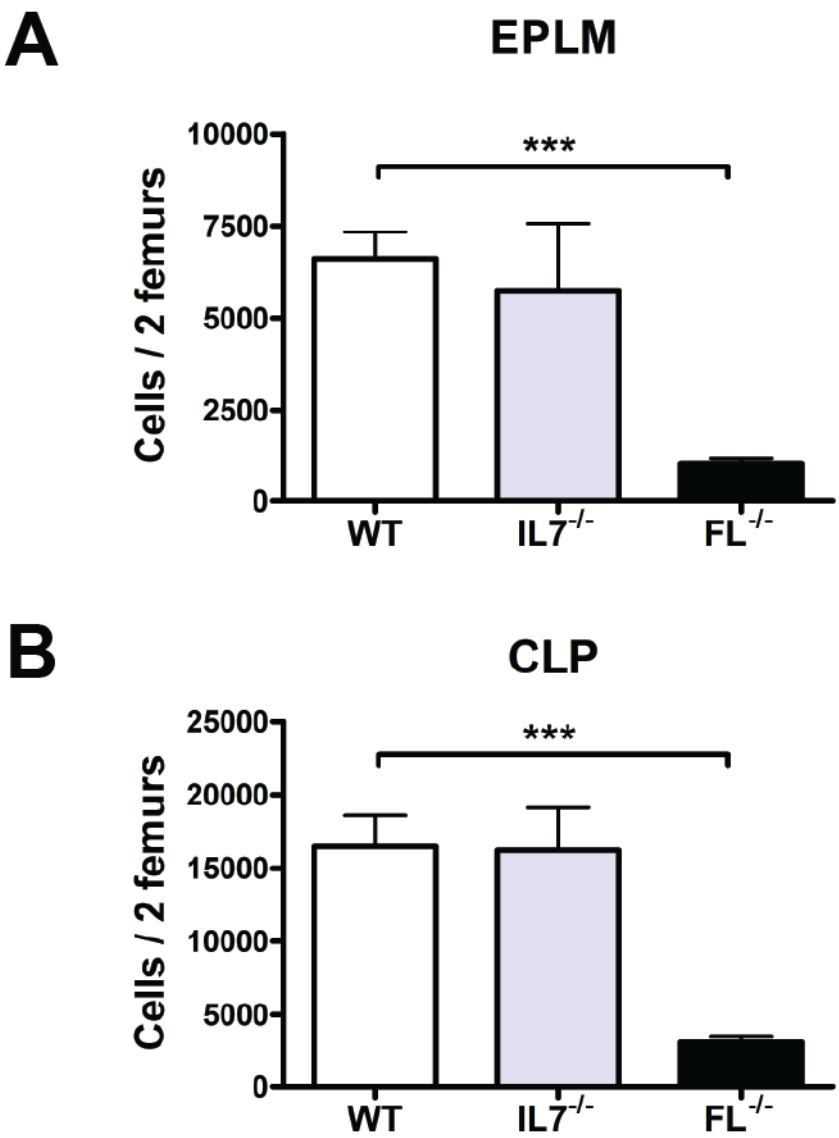


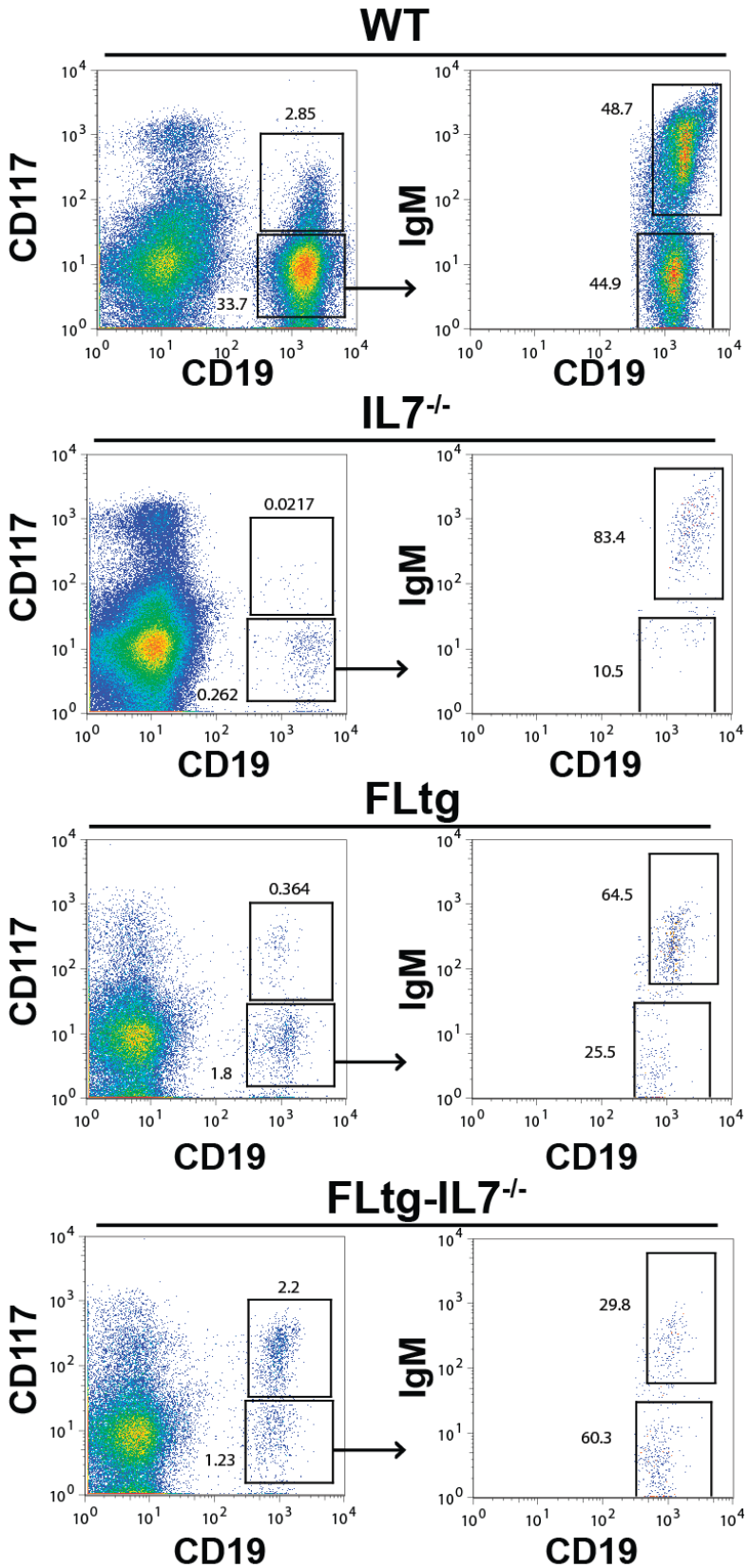
Figure 7: FL does not instruct Ebf1 expression and B-cell commitment. (A) Representative FACS plots showing expression of Ebf1 protein within the Ly6D$^+$ EPLM of WT, FL$^{-/-}$ and FLtg mice. **(B)** Percentages of Ebf1-expressing Ly6D$^+$ EPLM from WT (n=7), FL$^{-/-}$ (n=5) and FLtg (n=12) mice. Bars show mean ± SEM. **(C)** *In vitro* limiting dilution analysis of Ly6D$^+$ EPLM T-cell potential. Ly6D$^+$ EPLM were sorted from WT and FLtg mice and plated at the indicated concentrations on OP9DL1 stromal cells together with IL7. One representative of four independent experiments is shown. **(D)** schematic model for the permissive role of IL7 and FL acting on hematopoietic progenitors and CD19$^+$ committed B-cell precursors.



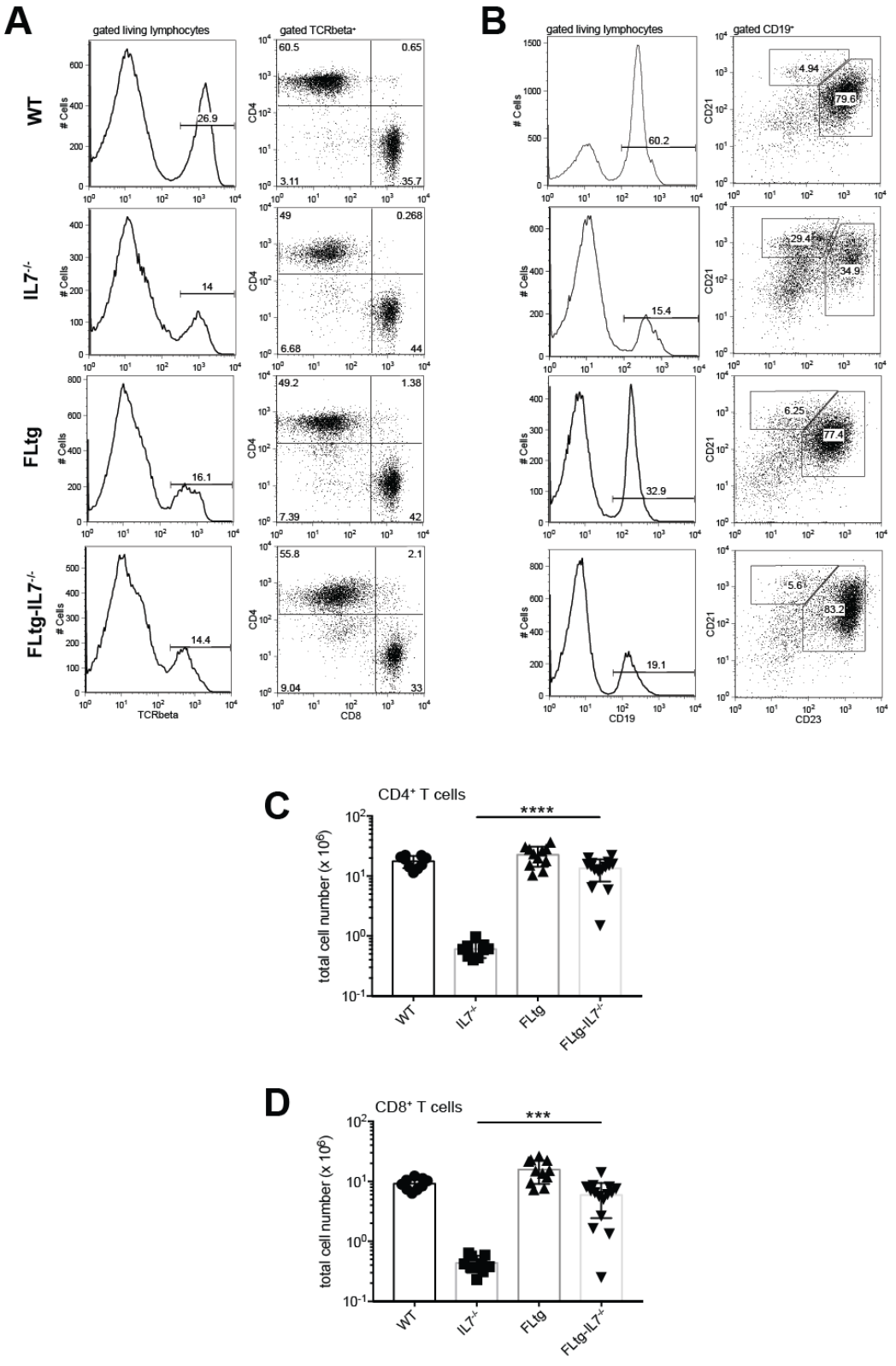
Supplementary Figure 1: Gating strategy for identification of CLP. FACS plots showing the gating strategy used for identification of Ly6D^{+} CLP. Lineage staining: SiglecH, CD115, CD11c, NK1.1, Gr-1.



Supplementary Figure 2: Absolute numbers of EPLM **(A)** and CLP **(B)** progenitors in WT (n=7), $IL7^{-/-}$ (n=5) and $FL^{-/-}$ (n=10) mice. EPLM were stained as shown in Figure 1A and CLP as shown in Supplementary Figure 1. Student's t test. ***P ≤ 0.001. Bars show mean ± SEM.

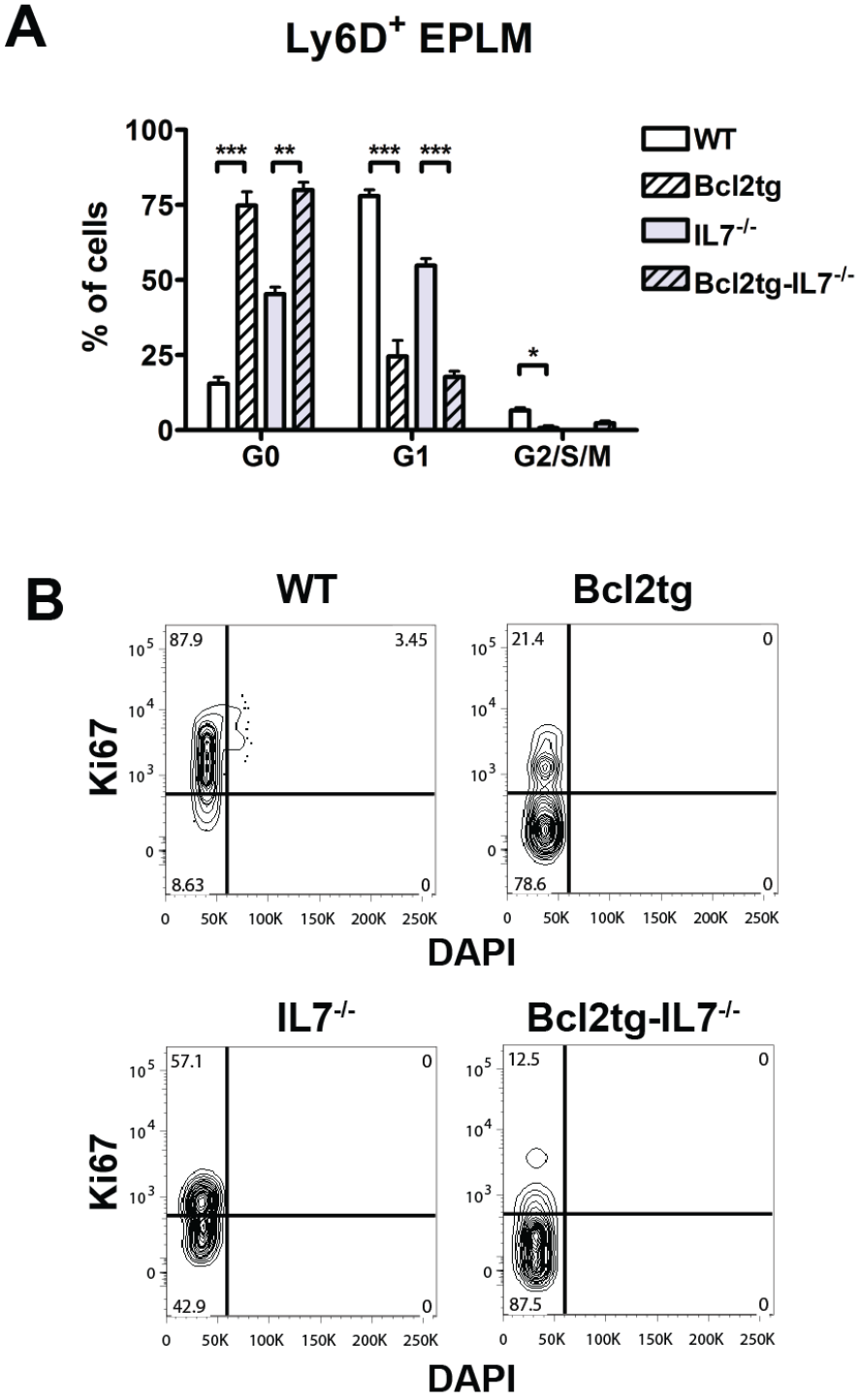


Supplementary Figure 3: Rescue of CD19$^+$ bone marrow B-cell progenitors in FLtg-IL7$^{-/-}$ mice. Figure shows representative FACS plots for the identification of CD19$^+$CD117$^+$, CD19$^+$CD117$^-$IgM$^-$ and CD19$^+$IgM$^+$ bone marrow cells.

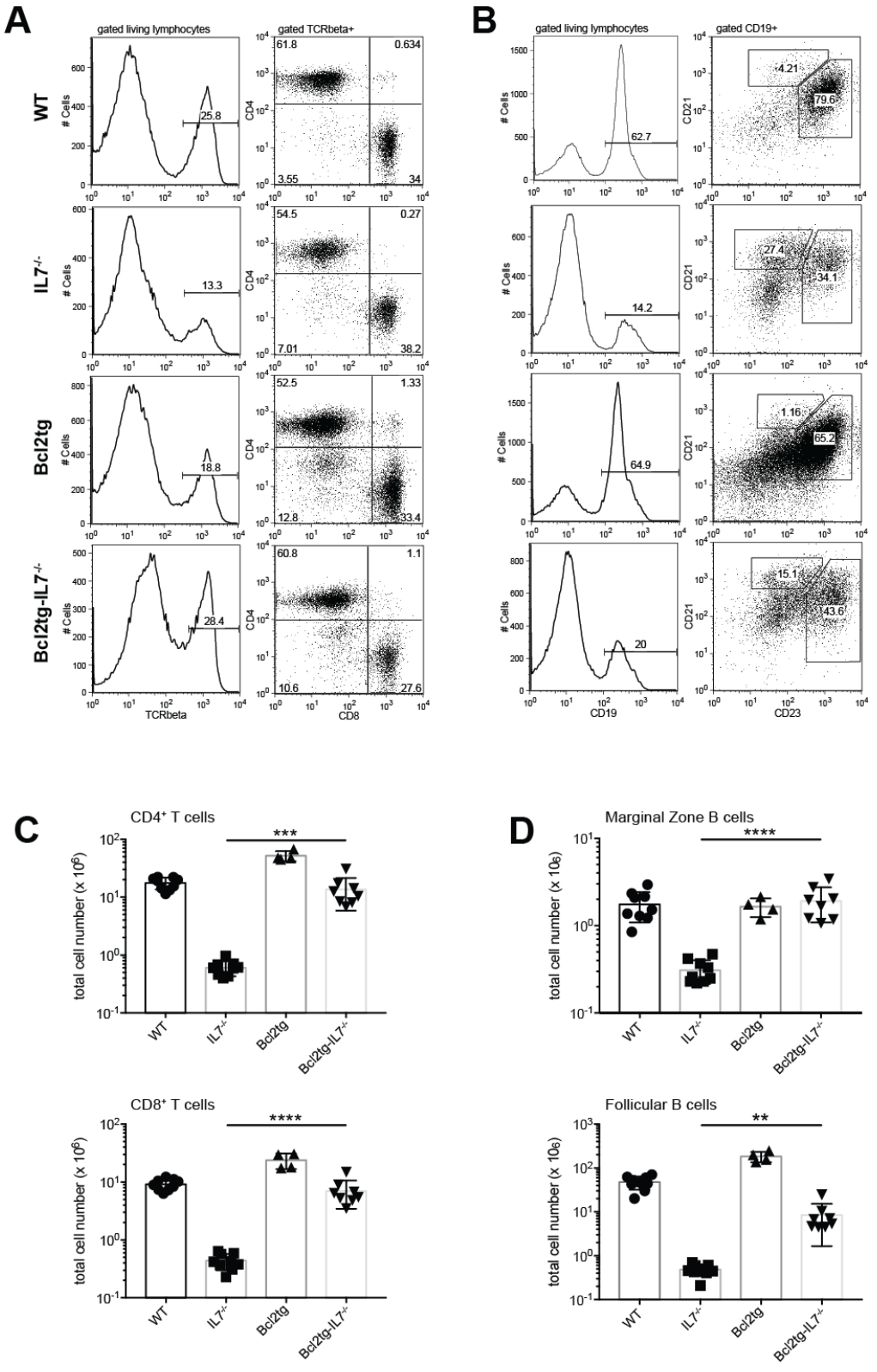


Supplementary Figure 4: T and B cells in the spleens of FLtg-IL7$^{-/-}$ and single mutant mice as indicated. (A) Representative FACS plots illustrating T cells in the spleens of WT (first row), IL7$^{-/-}$ (second row), FLtg (third row), and FLtg-IL7$^{-/-}$ (fourth row) mice. After gating on living lymphocytes TCRβ^{+} cells are further sub-grouped in

CD4 and CD8 positive T cells. **(B)** Representative FACS plots illustrating B cells in the spleens of WT (first row), $IL7^{-/-}$ (second row), FLtg (third row), and FLtg-$IL7^{-/-}$ (fourth row) mice. After gating on living lymphocytes $CD19^{+}$ cells are further sub-grouped in $CD21^{high}CD23^{low}$ marginal zone B cells and $CD21^{+}CD23^{+}$ follicular B cells. **(C and D)** Absolute numbers of splenic $CD4^{+}$ **(C)** and $CD8^{+}$ **(D)** T cells, stained as shown in A, from WT and mutant mice as indicated on the x-axes. ***P ≤ 0.001, ****P ≤ 0.0001. Student's t test; n = 9-15. Data shown above are mean ± SD.

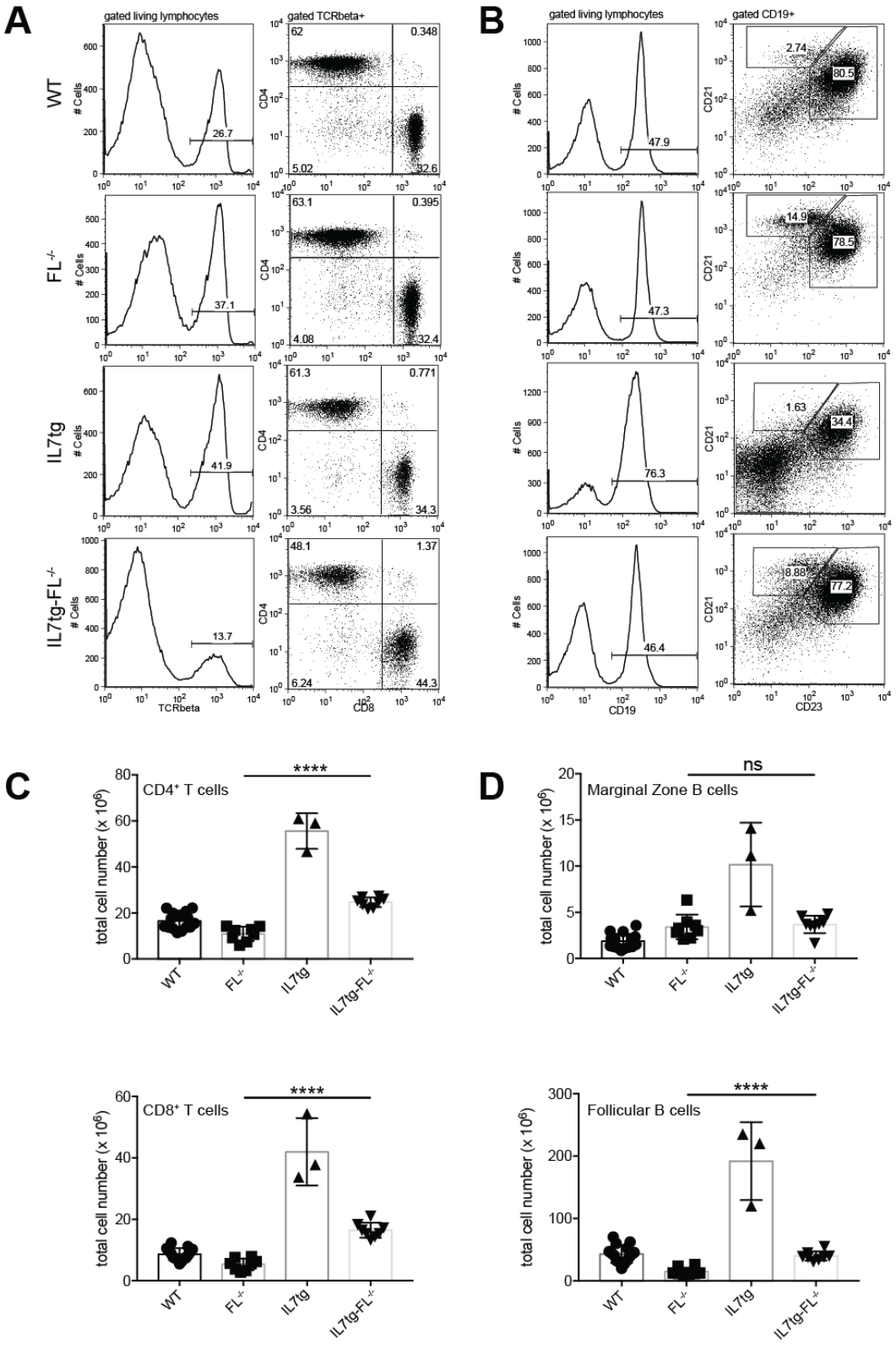


Supplementary Figure 5: Anti-proliferative effect of Bcl2 over-expression *in vivo*. (A) Cell cycle analysis of Ly6D$^+$ EPLM from WT (n=5), Bcl2tg, (n=2), IL7$^{-/-}$ (n=2) and Bcl2tg-IL7$^{-/-}$ (n=4) mice. Graph shows percentages of Ly6D$^+$ EPLM in G0 (Ki67$^-$ DAPI$^-$), G1 (Ki67$^+$DAPI$^-$) and G2/S/M (Ki67$^+$DAPI$^+$) stages. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Student's t test. Bars show mean ± SEM. **(B)** Representative Ki67/DAPI FACS plots of the Ly6D$^+$ EPLM cell cycle analysis collectively presented in A.



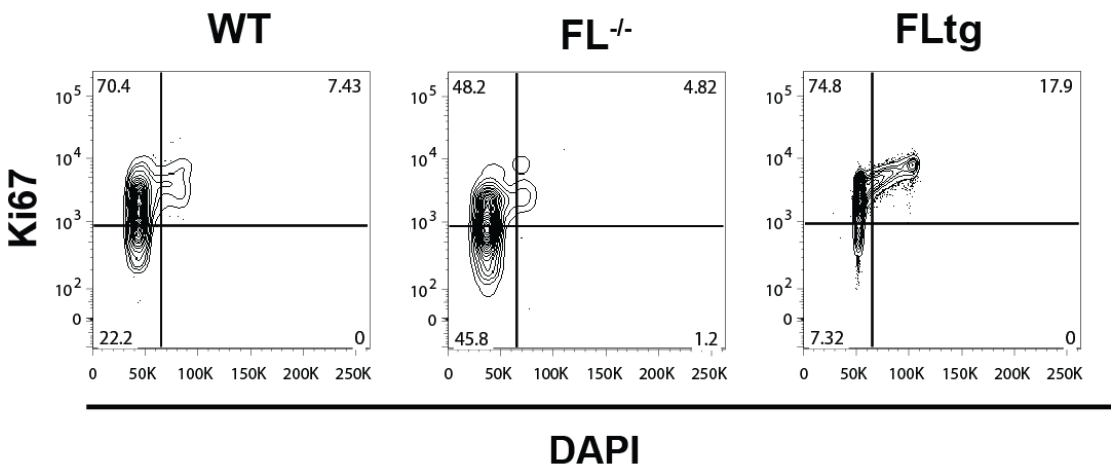
Supplementary Figure 6: Bcl2-mediated rescue of splenic T and B cells in the absence of IL7. (A) Representative FACS plots illustrating T cells in the spleens of WT (first row), IL7$^{-/-}$ (second row), Bcl2tg (third row), and Bcl2tg-IL7$^{-/-}$ (fourth row) mice. After gating on living lymphocytes TCRβ^{+} cells are further sub-grouped in CD4 and

CD8 positive T cells. **(B)** Representative FACS plots illustrating B cells in the spleens of WT (first row), $IL7^{-/-}$ (second row), Bcl2tg (third row), and Bcl2tg-$IL7^{-/-}$ (fourth row) mice. After gating on living lymphocytes $CD19^{+}$ cells are further sub-grouped in $CD21^{high}CD23^{low}$ marginal zone B cells and $CD21^{+}CD23^{+}$ follicular B cells. **(C)** Absolute numbers of splenic $CD4^{+}$ (top) and $CD8^{+}$ (bottom) T cells, stained as shown in A, from WT and mutant mice as indicated on the x-axes. **(D)** Absolute numbers of splenic marginal zone (top) and follicular (bottom) B cells, stained as shown in B, from WT and mutant mice as indicated on the x-axes. **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Student's t test; n = 4-9. Data shown above are mean ± SD.



Supplementary Figure 7: Effect of IL7 over-expression on WT and FL$^{-/-}$ splenic T and B cells. (A) Representative FACS plots illustrating T cells in the spleens of WT (first row), FL$^{-/-}$ (second row), IL7tg (third row), and IL7tg-FL$^{-/-}$ (fourth row) mice. After gating on living lymphocytes TCRβ^{+} cells are further sub-grouped in CD4 and

CD8 positive T cells. **(B)** Representative FACS plots illustrating B cells in the spleens of WT (first row), $FL^{-/-}$ (second row), IL7tg (third row), and IL7tg-$FL^{-/-}$ (fourth row) mice. After gating on living lymphocytes $CD19^{+}$ cells are further sub-grouped in $CD21^{high}CD23^{low}$ marginal zone B cells and $CD21^{+}CD23^{+}$ follicular B cells. **(C)** Absolute numbers of splenic $CD4^{+}$ (top) and $CD8^{+}$ (bottom) T cells, stained as shown in A, from WT and mutant mice as indicated on the x-axes. **(D)** Absolute numbers of splenic marginal zone (top) and follicular (bottom) B cells, stained as shown in B, from WT and mutant mice as indicated on the x-axes. ns not significant or $P > 0.05$, ****$P \leq 0.0001$. Student's t test; n = 3-15. Data shown above are mean ± SD.



Supplementary Figure 8: Effect of *in vivo* FL levels on the cell cycle of Ly6D$^+$ EPLM. Representative Ki67/DAPI FACS plots of the Ly6D$^+$ EPLM cell cycle analysis collectively presented in Figure 6B.

II A stromal cell free culture system generates mouse pro-T cells that can reconstitute T-cell compartments in vivo

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A stromal cell free culture system generates mouse pro-T cells that can reconstitute T-cell compartments in vivo

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T-cell lymphopenia following BM transplantation or diseases such as AIDS result in immunodeficiency. Novel approaches to ameliorate this situation are urgently required. Herein, we describe a novel stromal cell free culture system in which Lineage$^-$Sca1$^+$c-kit$^+$ BM hematopoietic progenitors very efficiently differentiate into pro-T cells. This culture system consists of plate-bound Delta-like 4 Notch ligand and the cytokines SCF and IL-7. The pro-T cells developing in these cultures express CD25, CD117, and partially CD44; express cytoplasmic CD3ε; and have their TCRβ locus partially D–J rearranged. They could be expanded for over 3 months and used to reconstitute the T-cell compartments of sub-lethally irradiated T-cell-deficient CD3ε$^{-/-}$ mice or lethally irradiated WT mice. Pro-T cells generated in this system could partially correct the T-cell lymphopenia of pre-Tα$^{-/-}$ mice. However, reconstituted CD3ε$^{-/-}$ mice suffered from a wasting disease that was prevented by co-injection of purified CD4$^+$ CD25^{high} WT Treg cells. In a T-cell-sufficient or T-lymphopenic setting, the development of disease was not observed. Thus, this in vitro culture system represents a powerful tool to generate large numbers of pro-T cells for transplantation and possibly with clinical applications.

Keywords: BM transplantation · Lymphopenia · Notch ligand Delta-like 4 (DL4) · T-cell development · Treg cell

Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Like all other cells of the hematopoietic system, T cells are derived from HSCs. However, unlike all other hematopoietic lineages, T-cell development occurs only in the thymus. The thymus does not harbor HSCs and in order to maintain T lymphopoiesis, it must be constantly colonized by progenitor cells migrating from the BM [1–4]. Transplantation experiments have shown that various BM progenitors can enter the thymus and generate T cells [5], however the nature of these progenitors under physiological conditions is still debated.

Based on the expression of the surface markers CD4 and CD8, thymopoiesis can be subdivided into four consecutive stages. Developmentally, the earliest cells express neither CD4 nor CD8

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and are called double-negative (DN) cells. DNs are the precursors of cells expressing both CD4 and CD8 molecules, called double-positive (DP) cells. DP cells that survive the positive and negative selection processes in the thymus will generate single-positive cells expressing either CD4 or CD8 that leave the thymus to colonize secondary lymphoid organs.

The DN stages can be further subset using the differential surface expression of CD25, CD44, and CD117. The developmentally earliest DN1 cells are $CD44^+CD117^+$, but do not express CD25. DN2 cells express all three markers, whereas DN3 cells have lost CD44 and CD117 [6–8]. Upon productive rearrangement of *TCR*β gene fragments, the pre-TCR is expressed and DN3 cells undergo proliferative expansion [9, 10] before they enter stage DN4 and lose expression of CD25. Immature single-positive cells are the direct descendants of DN4 cells and express CD8 in mice [6–8]. DP cells have CD4 as well as CD8 on their surface and rearrange the *TCR*α gene segments. Cells that are able to express a functional αβTCR undergo positive and negative selection [11, 12] to become single-positive mature T cells.

Besides IL7-IL7R [13, 14] and SCF-cKit [15, 16] signaling, WNT and Sonic hedgehog [17, 18] are known to be important for T-cell development. However, Notch signaling has been shown to be the most crucial one. Conditional inactivation of Notch-1 [19] or one of its ligands, Delta-like 4 (DL4) [8, 20], resulted in a complete block of T-cell development in the thymus. Several years ago Zúniga-Pflücker et al. introduced a stromal cell based system able to induce T-cell differentiation from fetal-liver-derived HPCs [21, 22]. They retrovirally transduced OP9 BM stromal cells to express the Notch ligand DL 1 (OP9-DL1) and heretofore this system has been the one to be used to understand early stages of thymocyte development. However, additional signals delivered by OP9 cells cannot be excluded and the exact amount of DL1 expression and the intensity of Notch signal delivery are hard to control. Moreover, it is unlikely that pro-T cells derived from coculture settings will ever be approved for therapeutic purposes. Therefore, to identify the minimal requirements necessary for T-cell commitment and differentiation, we developed a stromal cell free culture system. This culture system consists of plate-bound DL4, soluble SCF, and IL-7. Under these conditions, BM-derived, lineage-negative $Sca1^+$ and $CD117^+$ cells (LSKs) differentiate into DN2–DN3 like cells. These cells can then be transplanted to reconstitute the T-cell compartments of both T-cell-sufficient and T-cell-deficient mice and to improve the T-cell compartments in T-lymphopenic recipients.

Results

Development of a stromal cell free culture system to study T-cell differentiation

Stromal cell based culture systems have been useful for analyzing T-cell development in vitro [21, 23]. However, in our hands, several points emerged from using this culture system, the most notable being the heterogeneous response of cloned Pax-5-deficient pro-B cells during their induction to differentiate toward the T-cell lineage [24]. A possible reason for this heterogeneity was the variability in strength and duration of Notch signaling. Therefore, to equalize the delivery of Notch signals, we prepared a soluble DL4-Fc fusion protein [25–27]. This contained mouse DL4 linked to the Fc-region of human IgG_1. However, initial in vitro experiments of adding DL4-Fc fusion protein to progenitor cells were not successful. Therefore, we decided to fix the DL4-Fc fusion protein to the surface of tissue culture plates via an in-house-generated human IgG_1 Fc region-specific mouse mAb (Huf 5.4). Thus, a solution containing 10 μg/mL of the Huf 5.4 DL4-Fc-capturing mAb was used to coat plastic plates before addition of soluble DL4-Fc.

Using the OP9-DL1 stromal cell system, we had previously shown that Pax-5-deficient ($Pax\text{-}5^{-/-}$) pro-B cells differentiated toward the T-cell lineage upregulated CD117 and downregulated CD93 [16]. As shown in Figure 1A, these same cells underwent exactly the same phenotypic changes on plate-bound DL4. Optimal differentiation was achieved when plates were coated with 1 μg/mL or more of DL4-Fc.

The initial finding that nonattached DL4-Fc did not induce the differentiation of $Pax\text{-}5^{-/-}$ pro-B cells toward the T-cell lineage suggested that soluble DL4-Fc might even inhibit T-cell differentiation. To test this hypothesis, $Pax\text{-}5^{-/-}$ pro-B cells were cultured in plates coated with 2 μg/mL DL4-Fc in the presence of IL-7 (100 U/mL) and SCF (20 ng/mL) together with various amounts of soluble DL4-Fc.

As shown in Figure 1B, differentiation could be completely inhibited by soluble DL4-Fc added at concentrations between 1 and 10 μg/mL. Now that the differentiation of a cloned cell line toward the T-cell lineage appeared homogeneous and occurred in a semi-synchronous fashion, gene expression analysis of $Pax\text{-}5^{-/-}$ pro-B cells, cultured 5 days on plate-bound DL4 in presence of IL-7 and SCF, was carried out. As controls, $Pax\text{-}5^{-/-}$ pro-B cells cultured in the presence of IL-7 and SCF only (no stromal cells) were used. Table 1A and B summarize the T-cell genes upregulated more than threefold and B-cell genes downregulated more than threefold. Overall this gene expression analysis revealed 140 genes more than threefold upregulated and 252 genes more than threefold downregulated. The complete dataset of differentially expressed genes is summarized in Supporting Information Table 1 as well as in a Geo Dataset under the accession number GSE46191. These findings strongly support the conclusion that this stromal cell free culture system efficiently induces the development of $Pax\text{-}5^{-/-}$ pro-B cell clones toward the T-cell lineage and moreover might well be an ideal system to identify Notch target genes necessary for T-cell development using a clonal population of responder cells.

Developing T cells from ex vivo isolated progenitors

To further test this stromal cell free culture system, we initiated cultures with sorted undifferentiated hematopoietic progenitors, in this case LSK cells, from BM. Fifty thousand mouse BM LSK cells were sorted and cultured in 1 mL medium supplemented

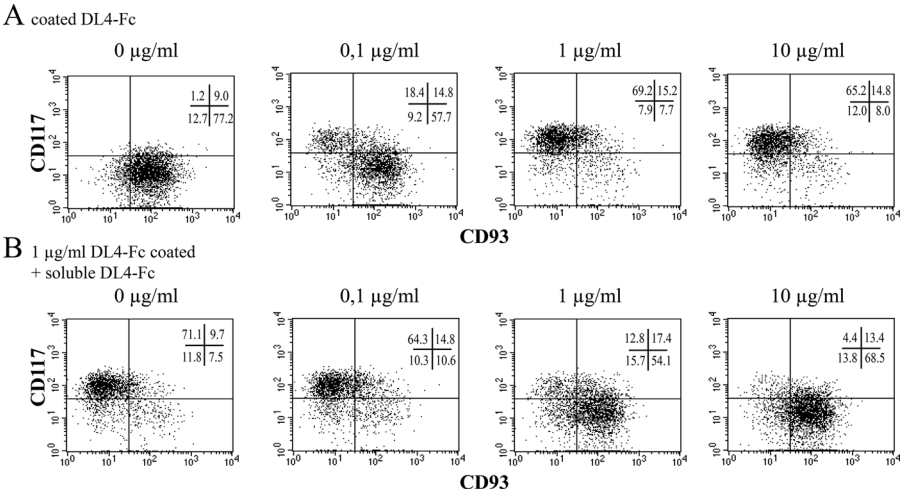

Figure 1. Culturing Pax-5$^{-/-}$ pro-B cells with SCF and IL-7 modulates CD93 and CD117 expression. (A, B) Pax-5$^{-/-}$ pro-B cells were cultured for 5 days (A) on plates coated with 0.0, 0.1 1, and 10 μg/mL DL4-Fc only, or (B) on plates coated with 1 μg/mL DL4-Fc in the presence of SCF; IL-7; and 0.0, 0.1, 1, and 10 μg/mL soluble DL4-Fc. The expression of CD93 and CD117 was evaluated by flow cytometry. One representative experiment of four experiments performed is shown. Percentages of CD117^{+}/CD93^{+} cells are indicated.

with 100 U/mL IL-7 and 20 ng/mL SCF in 1 well of a 24-well plate coated with 1 μg/mL DL4-Fc. After 5–6 days, cells became confluent and were then re-plated in 2 × 4 mL IL-7- and SCF-containing medium into 2 wells of a 6-well plate coated with DL4-Fc. Thereafter, the cells were recultured at 2 × 10^5/mL into fresh DL4-Fc-coated plates every 4 days in medium containing the cytokines.

Phenotypic analysis was performed at various days after onset of cultures but found not to change significantly over time. Thus, as shown in Figure 2A–E, a small fraction of cells expressed high levels of CD44 and low levels of CD25 (4.0% in this example; 4.1% ± 0.8, $n = 6$; Fig. 2A). Yet, another small fraction expressed low levels of CD25 and no CD44 (4.0% ± 0.8, $n = 6$). All the other cells expressed high levels of CD25 and around 60% (59.0% ± 1.2, $n = 6$) of those were also CD44^{high}, whereas the rest were CD44-negative. Moreover, all cells expressed CD117 whose level of expression correlated with that of CD44 (Fig. 2B). Furthermore, CD27, Gata3, Notch1, and Bcl 11b were detectable (Supporting Information Fig. 1). Cytoplasmic staining revealed that over 85% (87.7% ± 0.4, $n = 6$) expressed CD3ε (Fig. 2C). Neither cytoplasmic TCRβ (Fig. 2D) nor cell-surface CD4 or CD8 (data not shown) were detectable in or on these cells. Next, we analyzed Dβ1–Jβ1 TCR rearrangements (Fig. 2E). As expected, all seven rearrangements were readily detectable in total ex vivo isolated thymocytes and no un-rearranged germline band was detectable (lane 1). In marked contrast, conventionally cultured Pax-5$^{-/-}$ pro-B cells did not show any rearrangements but only the germline band (lane 2). In two independently established pro-T-cell lines cultured with plate-bound DL4-Fc (lanes 3 and 4), all seven rearrangements were readily detectable and the germline band was also clearly visible. Based on these phenotypic and rearrangement analyses, our in vitro propagated

Table 1. T-cell-associated genes upregulated (A) and B-cell-associated genes downregulated (B) in Pax-5-deficient pro-B cells cultured for 5 days on DL4-Fc-coated plates in the presence of SCF and IL-7

A			B		
Gene	Fold change up	Transcript ID	Gene	Fold change down	Transcript ID
CD3γ	187.6	NM_009850	*EBF-1*	25.5	NM_007897
Granz. A	156.7	NM_010370	*VpreB3*	23	BC062250
GATA-3	39.4	NM_008091	*Lef1*	14.9	NM_010703
Granz. B	34.3	NM_013542	*VpreB1*	12.5	NM_016982
CD7	30.7	NM_009854	*VpreB2*	9.5	NM_016983
Deltex-1	15.1	NM_008052	*IgH*	6	AF028616
TCF-7	13.9	NM_009331	*BLK*	5.2	NM_007549
CD25	9.2	AK168113	*IgL*	4.7	AF005353
CD27	8.8	NM_001033126	*CD93*	4.1	NM_010740
TCRγ	8.5	BC034889	*CD79B*	3.9	NM_008339
CD3δ	6.8	NM_013487	*CD79A*	3.3	NM_007655
SLP76	6.1	NM_010696			
CD117	3.7	NM_021099			
TCRβ	3.5	AF012139			
Notch-3	3.3	NM_008716			

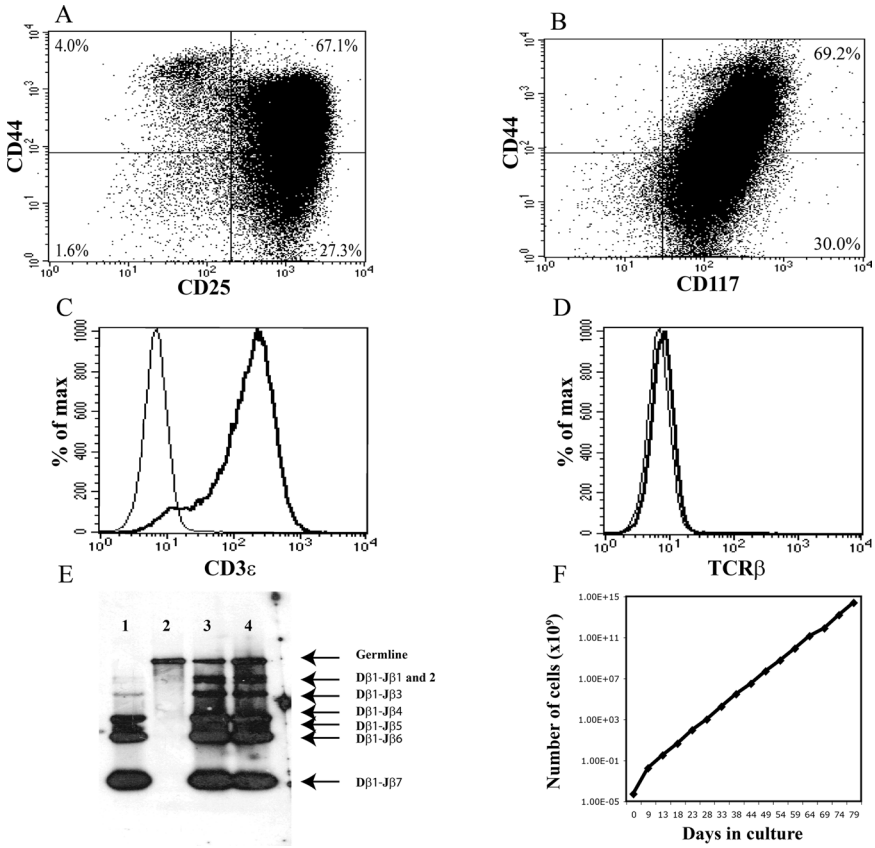

Figure 2. LSKs differentiate into pro-T cells when cultured on DL4-Fc-coated plates in the presence of IL-7 and SCF and can be kept proliferating for long term. LSKs were cultured on DL4-Fc-coated plates in the presence of IL-7 and SCF for 5 weeks. (A) Flow cytometry plot of CD44 and CD25 expression. (B) Flow cytometry plot of CD44 and CD117 expression. (C) Histogram of cytoplasmic CD3ε expression. (D) Histogram of cytoplasmic TCRβ expression. (A–D) One representative of more than ten individuals established pro-T-cell lines is shown. (E) Dβ1–Jβ1 rearrangement analysis of total thymocytes (lane 1), Pax-5-deficient pro-B cells cultured on stromal cells in the presence of IL-7 (lane 2) and LSKs cultured on DL4-Fc-coated plates in the presence of IL-7 and SCF (lane 3 and 4). TCR rearrangement analysis was performed on five individually established pro-T-cell lines. The results of two of them are shown. (F) Proliferation of LSKs cultured on DL4-Fc-coated plates in the presence of IL-7 and SCF. One representative of three established pro-T-cell lines is shown.

pro-T cells seem to closely resemble DN2–DN3 thymocytes isolated ex vivo.

We also analyzed the long-term proliferative capacity of these pro-T cells. A cumulative growth curve of one of over ten of such experiments is shown in Figure 2F. As can be seen, growth was exponential over the 79 days of this experiment, with cells expanding from 10^5 to 10^{24}, a 10^{19}-fold increase with an average overall doubling time of about 1.5 days. This finding indicates that these pro-T cells have extensive self-renewal capacity in vitro.

The above-described findings were made with LSKs as starting cells. However, identical results (phenotype and growth properties) were obtained when CLPs, DN1, or DN2 cells were seeded.

In vivo T-cell reconstitution of T-cell-deficient recipients by T-cell progenitors

Recently it was shown that pro-T cells propagated on OP9-DL1 stromal cells were able to partly reconstitute the T-cell compartments in vivo [28]. To test whether our pro-T cells generated in a stromal cell free system could also reconstitute all T-cell compartments in vivo, we performed the following experiment: CD45.1 CD3ε-deficient mice were lightly irradiated (4 Gy) and reconstituted intravenously with 5×10^6 T-cell progenitors generated from BM LSKs of CD45.2 mice cultured on plate-bound DL4-Fc in the presence of SCF and IL-7 for more than 5 weeks. As shown in Figure 3A, at 3 weeks after transfer, practically all thymocytes were CD45.2-positive. Moreover, these thymi contained all CD4- and CD8-defined subpopulations (Fig. 3B) and TCRβ was brightly expressed on the expected proportions of CD4- and CD8-defined subsets (Fig. 3C). At this time point, about 10–30 $\times$ 10^6 cells could be recovered from these thymi. However, the reconstitution of the thymus was only transient in that by 4–5 weeks DPs had disappeared. Careful kinetic analysis of thymus reconstitution revealed that only very low numbers of donor-derived thymocytes were present at day 14 and that highest numbers were found at days 21–26. Analysis of the peripheral T-cell compartments 5–6 weeks post reconstitution revealed a robust reconstitution of both CD4 (2–4 $\times$ 10^6 per spleen) and CD8 (0.5–1.5 $\times$ 10^6 per spleen) T cells (Fig. 3D). This reconstitution was observed in all mice (over 20 mice analyzed). However, CD4 cells expressing Foxp3 were practically undetectable (Fig. 3E), indicating an absence of Treg cells. Likely as a consequence of this, transplanted mice developed a fatal wasting disease marked by diarrhea and weight loss, 5–6 weeks posttransplantation. Histological analysis of both liver and colon revealed massive lymphocyte infiltrations in these organs (Fig. 3F and G).

Previously, we reported that the development of a similar type of wasting disease could be prevented by co-injection of mature Treg cells [29]. Likewise, using the pro-T-cell transplantation model described herein, development of wasting disease could also be prevented by cotransfer of mature Treg cells. Thus,

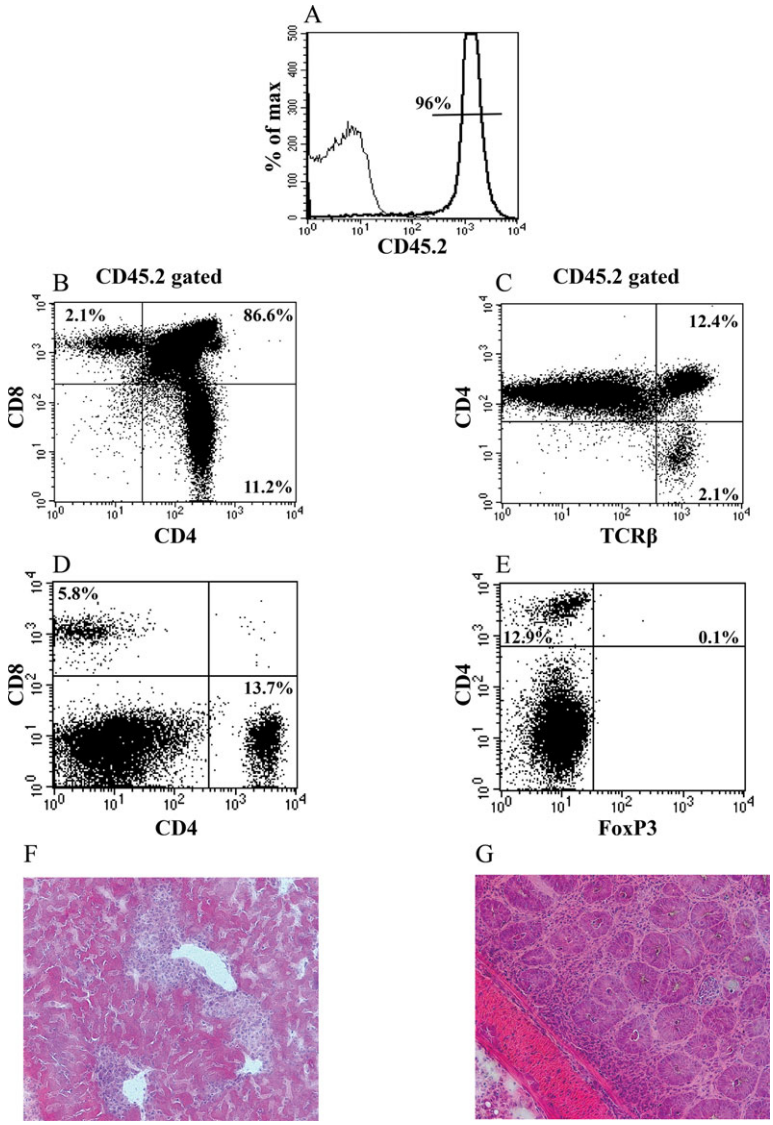

Figure 3. T-cell reconstitution of sublethally irradiated CD45.1 CDε$^{-/-}$ mice by CD45.2 pro-T cell. (A–C) CD45.1 CDε$^{-/-}$ mice were sublethally irradiated, and then 10^7 CD45.2 pro-T cells were injected. Twenty-four days later, thymi of these mice were analyzed for expression of (A) CD45.2 (bold-line histogram) (B) CD4 and CD8 on CD45.2$^+$ cells, and (C) CD4 and TCRβ on CD45.2$^+$ cells by flow cytometry. (D–G) CD45.1 CDε$^{-/-}$ mice were sublethally irradiated and injected with 10^7 CD45.2 pro-T cells. Forty-five days later, spleen, liver, and colon were evaluated by flow cytometry and histology. (D and E) Flow cytometry plots of spleen (not gated on CD45.2) are shown. (D) CD4 and CD8 expression (E) CD4 and Foxp3 expression (F and G) H&E staining of (F) liver and (G) colon sections are shown with a 200× magnification. One representative mouse of 20 reconstituted mice is shown.

4 Gy-irradiated CD45.1$^+$ CD3ε$^{-/-}$ recipients were reconstituted with a mixture of 1×10^5 ex vivo sorted CD4$^+$CD25^{high} Treg cells from C57Bl/6 CD45.1 mice and 5×10^6 CD45.2 B6 T-cell progenitors from in vitro cultures. Mice remained healthy and peripheral blood was analyzed 10 weeks after transfer (Fig. 4A). CD45.1$^+$ CD4$^+$ (Treg-derived) cells (identified as CD45.2-negative, left population) were still present alongside CD45.2$^+$ donor progenitor-derived cells comprising both CD4$^+$ and CD4$^-$ (presumably CD8$^+$), lymphocytes (right population). Thus, provision of a small number of Treg cells protected adult mice from the development of wasting following transplantation of progenitor T cells. In fact, in the peripheral CD4$^+$ compartment of transplanted mice, not only progeny of the injected (CD45.1$^+$) Treg cells could be found, but also Foxp3$^+$ cells from the injected CD45.2$^+$pro-T cells (Fig. 4B).

To test whether these peripheral CD4$^+$ T cells were functional, mice were immunized with the T-dependent Ag NIP-OVA (NIP is 4-hydroxy-5-iodo-3-nitrophenyl) in CFA. After 14 days, the IgG anti-NIP Ab titer in CD3ε-deficient mice injected with in vitro propagated pro-T cells together with Treg cells was practically indistinguishable from that in immunized normal C57BL/6 mice (Fig. 4C). In marked contrast, in immunized CD3ε-deficient mice that had received only Treg cells, the anti-NIP Ab titer was very low. Thus, CD4$^+$ T cells derived from the in vitro propagated pro-T cells are functional in that they can help the Ab response to a classical T-dependent Ag. Moreover, we also addressed the functionality of the CD8 T cells derived from the in vitro propagated pro-T cells. Therefore, these cells derived from a B6 pro-T cell were in vitro stimulated with irradiated BALB/c spleen cells. At day 5 of culture, the capacity of these cells to lyse BALB/c target cells was tested. As shown in Supporting Information Figure 2, stimulated pro-T-cell-derived CD8 T cells were as efficient as stimulated CD8 T cells derived from B6 mice in lysing BALB/c targets. Thus, CD8$^+$ T cells derived from the in vitro propagated pro-T cells seem to be functional.

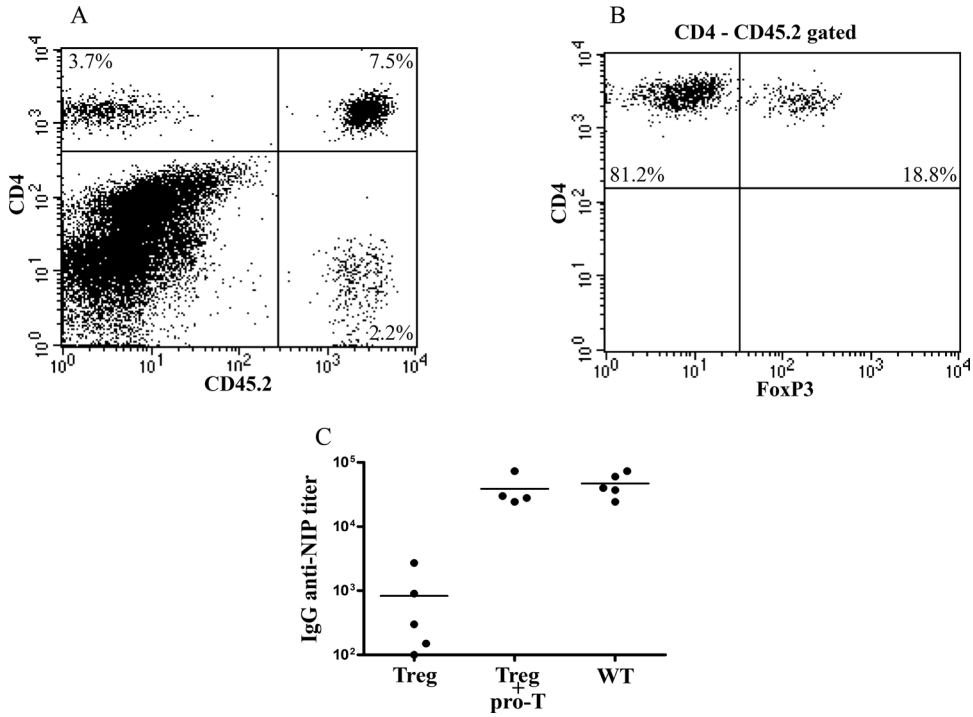

Figure 4. Prevention of wasting disease by cotransfer of mature Treg cells. Sublethally irradiated CD45.1 CDε$^{-/-}$ mice were reconstituted with 5 × 10^6 CD45.2 pro-T cells and 10^5 CD45.1$^+$ CD4$^+$ CD25$^+$ T cells. (A) CD4 and CD45.2 expression in peripheral blood was analyzed 70 days after injection by flow cytometry. (B) CD4 and Foxp3 expression in peripheral blood gated on CD4–CD45.2 cells was analyzed 70 days after injection.(A, B) One representative of over 20 reconstituted mice is shown. (C) Sublethally irradiated CD45.1 CDε$^{-/-}$ mice were reconstituted with 5 × 10^6 CD45.2 pro-T cells and 10^5 CD45.1$^+$ CD4$^+$ CD25$^+$ T cells and immunized at day 50 with NIP-OVA. IgG anti-NIP titer was determined 10 days after immunization. Each symbol represents an individual mouse and the horizontal line represents the mean titer. The NIP-OVA immunization was performed twice with five mice each.

Long-term in vitro propagated pro-T cells can improve the T-cell compartments in T-lymphopenic mice

Pre-Tα deficient mice are highly T-cell lymphopenic [9, 30]. To test whether our pro-T cells could correct the T-lymphopenia of such mice, we transplanted CD45.1$^+$ pro-T cells into sublethally irradiated CD45.2$^+$ pre-Tα$^{-/-}$ mice. In the LNs 6 weeks after transplantation, 15.0 ± 5.3% (n = 6) of lymphoid cells were derived from the injected CD45.1$^+$ cells and these contained 66.9 ± 2.5% CD4 and 29.7 ± 1.5% CD8 single-positive cells. Most of these donor CD45.1$^+$ cells expressed the TCRβ chain and 24.4 ± 5.6% of the CD4$^+$ cells also expressed Foxp3. A representative FACS analysis is shown in Figure 5A–D. Comparison of the donor- and host-derived T-cell compartments revealed that 9.2 ± 3.8% of the LN cells were donor CD4 and 5.2 ± 1.5% host CD4 cells.

Among LN CD4 T cells from pre-Tα$^{-/-}$ mice, the percentage of Foxp3-expressing cells is highly increased (around 30% in pre-Tα$^{-/-}$ mice and 10% in WT mice). Surprisingly, only 13.7 ± 1.7% of the host CD4 cells expressed Foxp3, indicating a normalization of this compartment (data not shown). Moreover, 4.0 ± 1.4% of the LN cells were found to be donor-derived CD8 cells whereas 5.3 ± 0.5% were of host origin. Thus, the transfer of pro-T cells to pre-Tα$^{-/-}$ mice resulted in at least a twofold increase of peripheral T-cell numbers. In the spleens, the number of pro-T-cell-derived CD4 cells was 3.7 × 10^6 ± 0.5 × 10^6 (n = 6), whereas that of CD8 T cells was 0.9 × 10^6 ± 0.2 × 10^6 (n = 6). Moreover, these findings also demonstrate that the inefficient generation of Treg cells from transplanted pro-T cells observed in CD3ε-deficient recipients is not due to an intrinsic pro-T-cell defect but rather due to an environmental effect within recipients.

In vitro propagated pro-T cells can reconstitute all T-cell compartments of lethally irradiated mice

In men, HSC transplantation is an effective treatment for various hematological disorders [31–34]. However, due to prolonged lymphopenia, T-cell reconstitution and T-cell immunity in these patients are impaired for prolonged periods of time [32–34]. Therefore, methods that would accelerate T-cell reconstitution might further improve the utility of HSC transplantation treatment. Here we tested whether our pro-T cells could provide such a tool. Thus, lethally irradiated CD45.1$^+$ B6 mice were reconstituted with a mixture of unfractionated BM cells from CD45.1$^+$ CD3ε$^{-/-}$ and pro-T cells from CD45.2$^+$ mice.

FACS analyses of thymi performed 3–4 weeks after reconstitution (Fig. 6A) showed that virtually all thymocytes were CD45.2$^+$ (Fig. 6A) with the expected CD4- and CD8-defined subpopulations (Fig. 6B), and TCRβ brightly expressed on the expected proportions of SP thymocytes (Fig. 6C). Analysis of LNs after 6–10 weeks

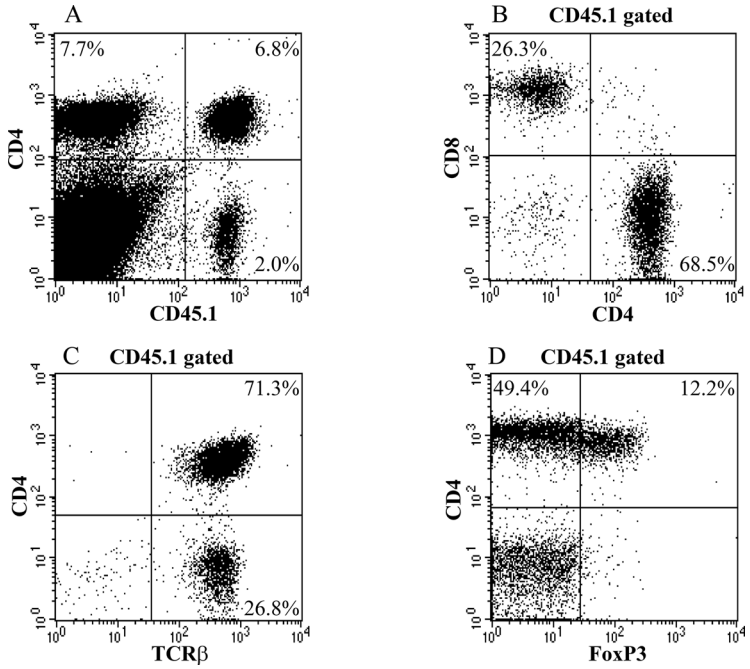

Figure 5. Improvement of T-cell compartments by in vitro propagated pro-T cells. Sublethally irradiated pre-Tα-deficient mice (CD45.2) were injected with 10^7 CD45.1 pro-T cells. LNs were analyzed 30 days after injection by flow cytometry for pro-T (A) CD4 and CD45.1 expression, (B) CD8 and CD4 expression on CD45.1-gated cells, (C) CD4 and TCRβ expression on CD45.1-gated cells, and (D) CD4 and Foxp3 expression on CD45.1-gated cells. One representative of ten reconstituted pre-Tα-deficient mice is shown.

revealed that 12.2 ± 1.8% of lymphoid cells were of donor origin ($n = 8$) and 66.2 ± 7.2% of these were CD4-positive and 23.2 ± 5.8% CD8-positive. Moreover, 13.6 ± 4.8% of the donor-derived CD4 cells expressed Foxp3. Also, small number of donor-derived γδT cells as well as NK1.1-positive cells were detectable. A representative FACS analysis is shown in Figure 6D–H. Thus, our in vitro propagated pro-T cells can indeed take part in the reconstitution of the T-cell compartments of lethally irradiated BM-reconstituted WT mice. Up to now, the latest time point after transfer we analyzed these mice was 3 months. By this time pro-T-cell-derived T cells were still readily detectable.

Discussion

Herein we describe a stromal cell free culture method for the expansion of mouse progenitor T cells for therapeutic purposes. For this, the Notch ligand DL4 was prepared as a fusion protein with the Fc fragment of human IgG_1 and used to coat tissue culture plastic plates. To avoid the release of soluble DL4/Fc fusion protein from the plates that could inhibit T-cell development, the fusion protein was fixed to the plates using the Huf5.4 mouse anti-human Fc mAb.

Using sorted BM multipotent LSK progenitors incubated with IL-7 and SCF, a continuous expansion of cells was seen for up to 6 months. During this time, cells divided every 30 h and retained a DN2–DN3 phenotype, i.e. cells were $CD25^+$, $CD117^+$, $CD44^{+/-}$, $CD3\varepsilon^+$, and $TCR\beta^-$ and had their TCRβ locus partially D–J rearranged. Furthermore, CD27, Gata3, Notch1, and Bcl11b were expressed. Thus, this culture system allows the generation of very large numbers of early T-lineage cells and as we have recently shown [35] is an ideal tool to study the early molecular and epigenetic changes accompanying early T-cell development.

The long-term proliferation without changes in phenotype shows that the DN2–DN3 like cells generated in this in vitro culture system, unlike the equivalent cells in the in vivo thymus, possess a very robust self-renewal capacity. Thus, the total pool of thymocytes is normally replaced every 4 weeks approximately by a new cohort of BM-derived progenitors recently immigrated to the thymus [4]. Moreover, transplantation of WT thymus grafts into SCID or Rag-deficient mice only results in one wave of T-cell production; thereafter the thymus gets colonized by host BM-derived cells [36, 37]. Based on these findings, it was concluded that the thymus lacks self-renewing progenitors. However, in mice unable to generate thymus-settling progenitors in their BM, early thymocytes can show self-renewing capacity [38, 39]. As shown herein, when grown in vitro, early thymocytes can have self-renewing capacity. However, under physiological conditions, newly immigrated BM progenitors seem to be very efficient at replacing the endogenous early thymocyte progenitors from the niches that provide the environment for this self-renewal.

Recently, Ikawa et al. [27] also described a stromal cell free culture system allowing fetal-derived LSKs to differentiate into pro-T cells and in which pro-T cells differentiated into DP thymocytes upon IL-7 withdrawal. We have also found (data not shown) that upon IL-7 withdrawal, fetal liver but not adult BM-derived LSKs differentiated to CD4/CD8-expressing DP cells. Currently we are trying to identify the genes that could account for this obvious difference in differentiation capacity between fetal and adult pro-T cells.

Lymphopenia following BM transplantation or diseases such as AIDS result in immunodeficiency and a compromised immune system [32–34, 40, 41]. Novel approaches to ameliorate this situation are required. Some time ago, Zakrzewski et al. [28] showed that T-cell reconstitution after allogeneic HSC

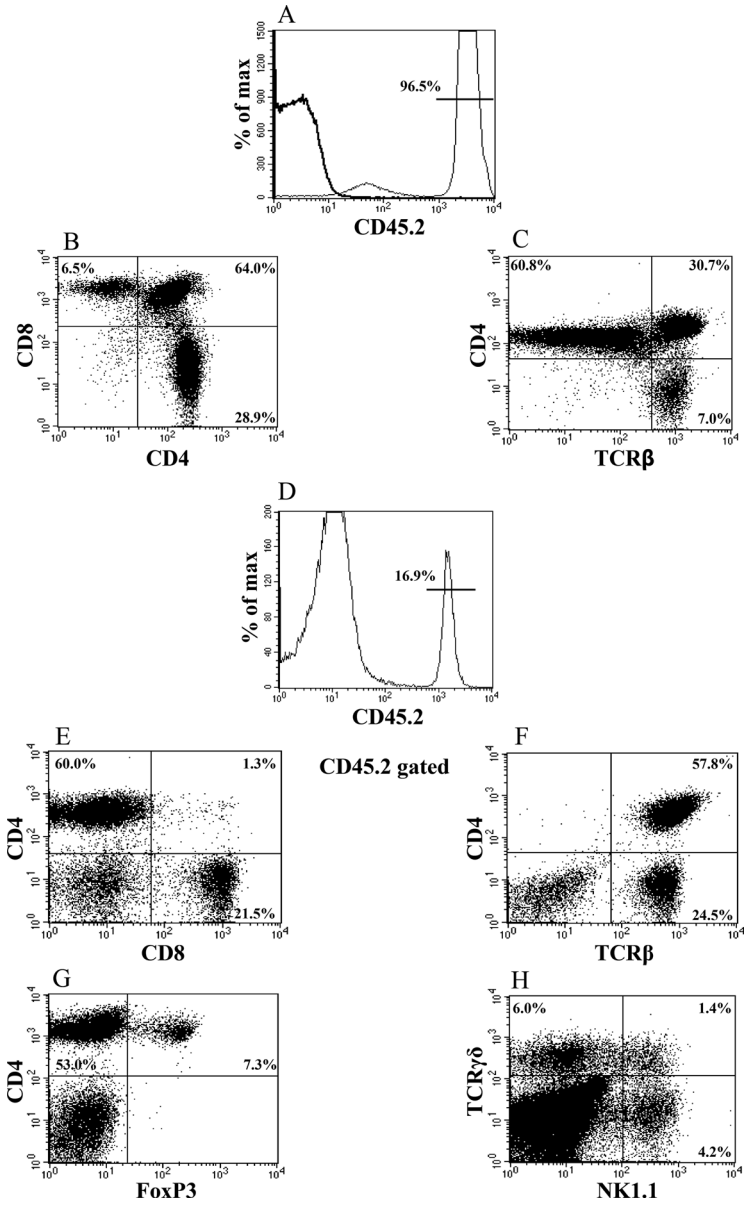

Figure 6. Different T-cell compartments are reconstituted by in vitro propagated pro-T cells. Lethally irradiated CD45.1 B6 mice were reconstituted with 3×10^6 CD45.1$^+$ CD3ε-deficient BM cells and 10^7 CD45.2$^+$ pro-T cells. (A–C) Thymi were analyzed 25 days after reconstitution by flow cytometry for (A) CD45.2 expression, (B) CD4 and CD8 expression, and (C) CD4 and TCRβ expression. One representative of ten reconstituted mice is shown. (D–H) LNs were analyzed 60 days after reconstitution by flow cytometry for pro-T (D) CD45.2 expression, (E) CD4 and CD8 expression, (F) CD4 and TCRβ expression, (G) CD4 and Foxp3 expression, and (H) TCRγδ and NK1.1 expression. (E–H) Dot plots were gated on CD45.2$^+$ cells. One representative of seven reconstituted mice is shown.

transplantation in mice could be improved by the adoptive transfer of in vitro generated T-cell progenitors. However, a major drawback of this study was that progenitor T cells were generated by coculture on Notch ligand DL1 expressing OP9 stromal cells, i.e. a method unlikely to be approved for use in patients. Pro-T cells generated by the stromal cell free culture system described herein would be more likely approved for therapeutic purposes in men. Therefore, T-cell reconstitution using pro-T cells generated in a stromal cell free system is, in our opinion, of great relevance.

Previously we reported that sublethally irradiated T-cell-deficient mice reconstituted with WT BM cells eventually suffer from a lethal wasting disease due to a paucity of Treg cells [29]. Disease onset could be prevented by the cotransplantation of mature Treg cells. Herein we have shown similar results using transplanted in vitro grown pro-T cells. The inability of injected pro-T cells to generate Treg cells does not seem to be due to an intrinsic defect since when they are used to reconstitute T-cell-proficient mice, about 20% of donor pro-T-cell-derived CD4 cells expressed Foxp3. Therefore, the inefficiency of the pro-T cells to generate Treg cells in T-cell-deficient recipient mice is most probably due to a defective thymus anlage. Medullary thymic epithelial cells play an important role in the generation of Treg cells [42] and it is known that the medullary thymic architecture in T-cell-deficient mice is particularly defective [43, 44], thereby resulting in poor Treg generation from transplanted in vitro generated pro-T cells.

In men, various immunodeficiency diseases are caused by the complete or partial absence of T cells [40, 41, 45] (and references herein). Therapeutic approaches that would improve the T-cell compartments in these patients should result in an amelioration of the disease. That the lymphopenic T-cell compartment of pre-Tα-deficient mice can be greatly improved by the transfer of in

vitro derived pro-T cells as described herein suggests that a similar treatment might be efficacious in patients.

The T-cell compartments of patients receiving hematopoietic stem cell transplants are frequently abnormal and not functional [32–34]. That reconstitution of the T-cell compartments in lethally irradiated WT mice transplanted with BM from donors incapable of generating T cells can in part be rescued by the cotransfer of in vitro derived pro-T cells strongly suggests that such type of cells would greatly improve the reconstitution of the T-cell compartments in patients after HSC transplantation.

Materials and methods

Mice

Female C57BL/6 CD45.1, C57BL/6 CD45.2, BALB/c, and CD3$\varepsilon^{-/-}$ [46] as well as pre-Tα-deficient [9] mice of 5–8 weeks of age were used. All mice were bred and maintained in our animal facility under specific pathogen-free conditions. All animal experiments were carried out within institutional guidelines (authorization numbers 1886 and 1888 from Kantonales Veterinäramt, Basel).

Recombinant DL4-Fc fusion protein

The production and purification of a fusion protein consisting of the extracellular portion of mouse DL4 (amino acids 1–521) and the Fc portion of human IgG1 have been described before [47].

mAb against human IgG1 Fc

A mouse monoclonal IgG Ab against the Fc part of human IgG_1 (clone Huf5.4) was produced by conventional techniques using the fusion partner Sp2/0.

Cell lines and cell culture

Pax-5$^{-/-}$ pro-B cells were generated and maintained on OP9 stromal cells in IMDM supplemented with 2% FBS, 5×10^{-5}M β-mercaptoethanol, 1 mM glutamine, 0.03% w/v Primatone (Quest, Naarden, The Netherlands), 100 U/mL penicillin, and in the presence of IL-7 (100 U/mL) as previously described [48].

To establish pro-T cells, 48- or 24-well tissue culture plates from Nunc (Nunc A/S, Roskilde, Denmark) were initially used. Once established, cells were maintained in 6-well bacterial-grade plates (Greiner Bio-One, Kremsmünster, Austria), because of their higher coating capacity. Wells were pre-coated overnight or longer with 10 μg/mL mAb anti-human IgG_1-Fc in PBS (0.25 mL per well for 48-well plates, 0.5 mL per well for 24-well plates, or 2 mL per well for 6-well plates) at 4°C. Thereafter, wells were washed twice with IMDM (see above) and then used for coating with DL4-Fc at 2 μg/mL (or as indicated) in IMDM overnight, washed, and used for cell culture.

Flow cytometry and cell sorting

FITC-, PE-, allophycocyanin-, or biotin-labeled mAbs specific for CD3ε, CD4, CD8α, CD25, CD44, CD45.1, CD45.2, CD93, CD117, Sca-1, TCRβ, TCR$\gamma\delta$, Foxp3, and NK1.1 were either purchased from BD Biosciences (Franklin Lakes, NJ, USA) or eBiosciences (San Diego, CA, USA), or purified from hybridoma culture supernatants. Staining of the cells was performed as described before [24]. Flow cytometry was performed using a FACS Calibur (BD Biosciences) and data were analyzed using the Cell Quest Pro Software (BD Biosciences). For cell sorting, a FACS Aria (BD Biosciences) was used. Reanalysis of sorted cells revealed >98% purity in all instances. LSKs were defined and sorted as previously described [49–51].

Histological analysis

Frozen sections were stained with H&E according to standard procedures.

PCR analyses of *TCR*β gene rearrangements

DNA was isolated from indicated cells and analyzed by PCR for Dβ1 to Jβ1 rearrangements as described before [51].

Transfers of cultured progenitor cells

Recipient mice were irradiated as indicated and injected with the pro-T cells intravenously.

NIP-specific IgG responses

Mice were immunized subcutaneously at day 0 with 100 μg NIP-OVA in a 1:1 CFA emulsion. Serum IgG against NIP was analyzed at day 14 using ELISA as described in [52].

Gene expression analysis using Affymetrix chip array

For detection of mRNA quantity, an Affymetrix Gene Chip Mouse Gene 1.0 ST Array was used. Mean RIN (RNA Integrity Number) values ($\pm$ SD) were 9.7 $\pm$ 0.4. All steps were carried out according to the GeneChip Whole Transcript Sense Target Labeling Assay Manual, Version 4 provided by Affymetrix. Raw data were analyzed with Agilent GeneSpringGX 9.0 or Partek genomics suite.

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Abbreviations: **DL**: Delta-like · **DN**: double negative · **DP**: double positive · **NIP**: 4-hydroxy-5-iodo-3-nitrophenyl

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Supporting information table 1: Genes more than 3 fold up- or down-regulated in Pax5 deficient pro-B cells cultured for 5 days on plate bound DL4-Fc in the presence SCF and IL-7. As controls, Pax5$^{-/-}$ pro-B cells cultured in the presence of IL-7 and SCF only (no stromal cells) were used.

Fold Change	Regulation	Transcript ID	Gene Title
187,6	up	NM_009850	CD3 antigen, gamma (**Cd3g**), mRNA
156,7	up	NM_010370	Granzyme A (**Gzma**), mRNA
70,4	up	NM_008220	Haemoglobin, beta adult major chain (**Hbb-b1**), mRNA
67,9	up	NM_001033904	Nur77 downstream gene 1 (**Ndg1**), mRNA
44,4	up	NM_008809	Platelet derived growth factor receptor, beta polypeptide (**Pdgfrb**), mRNA
39,4	up	NM_008091	GATA binding protein 3 (**Gata3**), mRNA
34,3	up	NM_013542	Granzyme B (**Gzmb**), mRNA
33,5	up	NM_008354	Interleukin 12 receptor, beta 2 (**Il12rb2**), mRNA
31,1	up	NM_011594	Tissue inhibitor of metalloproteinase 2 (**Timp2**), mRNA
30,7	up	NM_009854	CD7 antigen (**Cd7**), mRNA
22,3	up	NM_009430	Protease, serine, 2 (**Prss2**), mRNA
20,5	up	NM_013566	Integrin beta 7 (**Itgb7**), mRNA
16,6	up	NM_009864	Cadherin 1 (**Cdh1**), mRNA
15,1	up	NM_008052	Deltex 1 homolog (Drosophila) (**Dtx1**), mRNA
15	up	NM_029529	Solute carrier family 35, member D3 (**Slc35d3**), mRNA
13,9	up	NM_009331	Transcription factor 7, T-cell specific (**Tcf7**), mRNA
13,5	up	NM_183322	Nur77 downstream gene 1 (**Ndg1**), mRNA
13,1	up	NM_008399	Integrin, alpha E, epithelial-associated (**Itgae**), transcript variant 2, mRNA
12,6	up	NM_177204	RIKEN cDNA D330017J20 gene
12,5	up	NM_009640	Angiopoietin 1 (**Angpt1**), mRNA
12,4	up	NM_024253	Natural killer cell group 7 sequence (**Nkg7**)**,** mRNA
11,9	up	ENSMUST00000 103558	---
11,4	up	NM_011815	FYN binding protein (**Fyb**), mRNA
10,8	up	NM_017379	Tubulin, alpha 8 (**Tuba8**), mRNA
10,8	up	GENSCAN00000 009248	---
10,7	up	NM_007753	Carboxypeptidase A3, mast cell (**Cpa3**), mRNA
10,5	up	NM_134133	RIKEN cDNA 2010002N04 gene
10,4	up	BC038285	RIKEN cDNA A630038E17 gene
10,3	up	NM_008709	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian) (**Mycn**), mRNA
9,2	up	AK168113	interleukin 2 receptor, alpha chain
9	up	XR_031927	peptidase M20 domain containing 2
8,8	up	NM_001033126	CD antigen 27 (**Cd27**), transcript variant 2, mRNA
8,7	up	NM_001033468	G protein-coupled receptor 114 (**Gpr114**), mRNA
8,5	up	BC034889	T-cell receptor gamma, variable 1-3
8,2	up	NM_198034	SID1 transmembrane family, member 1 (**Sidt1**), mRNA
8	up	NM_021334	Integrin alpha X (**Itgax**), mRNA
8	up	NM_175367	Stonin 2 (**Ston2**), mRNA
7,5	up	NM_009994	Cytochrome P450, family 1, subfamily b, polypeptide 1 (**Cyp1b1**), mRNA
7,2	up	NM_013698	TXK tyrosine kinase (**Txk**), mRNA
6,9	up	ENSMUST00000 111960	---
6,8	up	NM_013487	CD3 antigen, delta polypeptide
6,7	up	NM_008007	Fibroblast growth factor 3
6,7	up	M18858	---
6,5	up	NM_011408	Schlafen 2 (**Slfn2**), mRNA
6,3	up	NM_201639	Desmuslin
6,1	up	NM_008859	Protein kinase C, theta (**Prkcq**), mRNA

6,1	up	NM_145149	RAS guanyl releasing protein 4 (**Rasgrp4**), mRNA
6,1	up	NM_010696	Lymphocyte cytosolic protein 2 (**Lcp2**), mRNA
6,1	up	NM_145581	Sialic acid binding Ig-like lectin 5 (**Siglec5**), mRNA
6	up	NM_010658	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian) (**Mafb**), mRNA
6	up	NM_008036	FBJ osteosarcoma oncogene B (**Fosb**), mRNA
6	up	NM_013464	Aryl-hydrocarbon receptor (**Ahr**), mRNA
5,9	up	NM_175414	Tetraspanin 9 (**Tspan9**), mRNA
5,9	up	NM_010583	IL2-inducible T-cell kinase (**Itk**), mRNA
5,5	up	NM_008048	Insulin-like growth factor binding protein 7 (**Igfbp7**), mRNA
5,5	up	NM_009217	Somatostatin receptor 2 (**Sstr2**), mRNA
5,4	up	NM_011854	2'-5' oligoadenylate synthetase-like 2 (**Oasl2**), mRNA
5,1	up	NM_198297	T cell receptor associated transmembrane adaptor 1 (**Trat1**), mRNA
5,1	up	NM_008706	NAD(P)H dehydrogenase, quinone 1 (**Nqo1**), mRNA
5	up	NM_009928	Procollagen, type XV (**Col15a1**), mRNA
5	up	NM_007913	Early growth response 1 (**Egr1**), mRNA
4,9	up	NM_011346	Selectin, lymphocyte (**Sell**), mRNA
4,9	up	NM_025980	Notch-regulated ankyrin repeat protein (**Nrarp**), mRNA
4,9	up	NM_001013384	Podocan-like 1 (**Podnl1**), mRNA
4,8	up	AF491829	Leukocyte-associated Ig-like receptor 1
4,7	up	NM_010220	FK506 binding protein 5 (**Fkbp5**), mRNA
4,6	up	NM_011673	UDP-glucose ceramide glucosyltransferase (**Ugcg**), mRNA
4,6	up	NM_008452	Kruppel-like factor 2 (lung) (**Klf2**), mRNA
4,5	up	NM_008152	G-protein coupled receptor 65 (**Gpr65**), mRNA
4,5	up	NM_031395 /// NM_183370	synaptotagmin-like 3 (**Sytl3**), mRNA
4,5	up	NM_008741	Neuron specific gene family member 2 (**Nsg2**), mRNA
4,5	up	NM_029094	Phosphatidylinositol 3-kinase, catalytic, beta polypeptide (**Pik3cb**), mRNA
4,4	up	NM_009985	Cathepsin W (**Ctsw**), mRNA
4,4	up	NM_027979	Chitinase 1 (chitotriosidase) (**Chit1**), mRNA
4,4	up	NM_008353	Interleukin 12 receptor, beta 1 (**Il12rb1**), mRNA
4,4	up	NM_172743	Pleckstrin homology domain containing, family A member 7 (**Plekha7**), mRNA
4,3	up	NM_144839	Ubiquitin-conjugating enzyme E2E 2 (UBC4/5 homolog, yeast) (**Ube2e2**), mRNA
4,3	up	NM_001005421	Adhesion molecule, interacts with CXADR antigen 1 (**Amica1**), mRNA
4,3	up	NM_001033399	Glucose-fructose oxidoreductase domain containing 1 (**Gfod1**), mRNA
4,3	up	NM_001033780	RIKEN cDNA I830077J02 gene
4,3	up	NM_178743	Solute carrier family 26, member 11 (**Slc26a11**), mRNA
4,3	up	NM_022032	PERP, TP53 apoptosis effector (**Perp**), mRNA
4,2	up	NM_133662	Immediate early response 3 (**Ier3**), mRNA
4,1	up	NM_029983	Src-like-adaptor 2 (**Sla2**), mRNA
4,1	up	NM_008479	Lymphocyte-activation gene 3 (**Lag3**), mRNA
4,1	up	NM_172264 /// NM_175343	Choline dehydrogenase (**Chdh**), mRNA
4,1	up	NM_008965	Prostaglandin E receptor 4 (subtype EP4) (**Ptger4**), mRNA
4,1	up	NM_026056	CAP, adenylate cyclase-associated protein, 2 (yeast) (**Cap2**), mRNA
4,1	up	NM_011391	Solute carrier family 16 (monocarboxylic acid transporters), member 7 (**Slc16a7**), mRNA
4	up	XR_031416	Similar to ornithine decarboxylase

4	up	BC141885	---
3,9	up	NM_025779	Coiled-coil domain containing 109B (**Ccdc109b**), mRNA
3,9	up	NM_030165	Chondroitin sulfate GalNAcT-2 (**Galnact2**), mRNA
3,8	up	NM_008512	Low density lipoprotein receptor-related protein 1 (**Lrp1**), mRNA
3,7	up	NM_001099624	Rap guanine nucleotide exchange factor (GEF) 2 (**Rapgef2**), mRNA
3,7	up	NM_021099	Kit oncogene (**Kit**), mRNA
3,7	up	NM_008869	Phospholipase A2, group IVA (cytosolic, calcium-dependent) (**Pla2g4a**), mRNA
3,7	up	NM_172637	HECT domain containing 2 (**Hectd2**), mRNA
3,7	up	NM_009370	Transforming growth factor, beta receptor I (**Tgfbr1**), mRNA
3,6	up	NM_023543	Chimerin (chimaerin) 2 (**Chn2**), mRNA
3,6	up	NM_010738	Lymphocyte antigen 6 complex, locus A (**Ly6a**), mRNA
3,5	up	AF012139	T-cell receptor beta, joining region /// T-cell receptor beta, variable 8.2
3,5	up	NM_009631	Adenosine A3 receptor (**Adora3**), transcript variant 1, mRNA
3,5	up	NM_010234	FBJ osteosarcoma oncogene (**Fos**), mRNA
3,5	up	NM_016740	S100 calcium binding protein A11 (**calgizzarin**)
3,5	up	NM_177823	Ubiquitin associated and SH3 domain containing, A (**Ubash3a**), mRNA
3,5	up	NM_016740	S100 calcium binding protein A11 (**calgizzarin**)
3,4	up	NM_173014	Acyltransferase like 1A (**Aytl1a**), mRNA
3,4	up	NM_007725	Calponin 2 (**Cnn2**), mRNA
3,4	up	NM_011662	TYRO protein tyrosine kinase binding protein (**Tyrobp**), mRNA
3,4	up	NM_008638	Methylenetetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase (**Mthfd2**), mRNA
3,4	up	NM_021435	Solute carrier family 35, member B4 (**Slc35b4**), mRNA
3,4	up	XR_002131	predicted gene, EG639787
3,4	up	XR_034503	similar to Odc1 protein
3,3	up	NM_008719	Neuronal PAS domain protein 2 (**Npas2**), mRNA
3,3	up	XM_001476259	DENN/MADD domain containing 4A
3,3	up	NM_010575	Integrin alpha 2b (**Itga2b**), mRNA
3,3	up	NM_008716	Notch gene homolog 3 (Drosophila) (**Notch3**), mRNA
3,3	up	NM_009616	A disintegrin and metallopeptidase domain 19 (meltrin beta) (**Adam19**), mRNA
3,3	up	XM_001476259	DENN/MADD domain containing 4A
3,3	up	NM_053149	Hemogen (**Hemgn**), mRNA
3,2	up	NM_177420	Phosphoserine aminotransferase 1 (**Psat1**), mRNA
3,2	up	NM_009916	Chemokine (C-C motif) receptor 4 (**Ccr4**), mRNA
3,2	up	NM_007656	CD82 antigen (**Cd82**), mRNA
3,2	up	NM_011125	Phospholipid transfer protein (**Pltp**), mRNA
3,1	up	NM_023525	Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (**Cad**), mRNA
3,1	up	XM_001476259	DENN/MADD domain containing 4A
3,1	up	NM_172436	Solute carrier family 25 (mitochondrial carrier, Aralar), member 12 (**Slc25a12**), mRNA
3,1	up	NM_011459	Serine (or cysteine) peptdiase inhibitor, clade B, member 8 (**Serpinb8**), mRNA
3,1	up	ENSMUST00000026496	proline-serine-threonine phosphatase-interacting protein 2 (**Pstpip2**), mRNA
3,1	up	NM_013778	Aldo-keto reductase family 1, member C13 (**Akr1c13**), mRNA

3,1	up	NM_011256	Phosphatidylinositol transfer protein, membrane-associated 2 (**Pitpnm2**), mRNA
3,1	up	NM_011777	zyxin (**Zyx**), mRNA
3,1	up	XM_001476259	DENN/MADD domain containing 4A
3	up	NM_178754	Rho GTPase activating protein 6 (**Arhgap6**), transcript variant 1, mRNA
3	up	NM_008685	Nuclear factor, erythroid derived 2 (**Nfe2**), mRNA
3	up	XM_001476259	DENN/MADD domain containing 4A
3	up	NM_134080	Filamin, beta (**Flnb**), mRNA
3	up	NM_007633	Cyclin E1 (**Ccne1**), mRNA

Fold Change	Regulation	Transcript ID	Gene Title
41,5	down	NM_001043228	Deoxynucleotidyltransferase, terminal (**Dntt**), mRNA
31,7	down	NM_025956	RIKEN cDNA 1700011H14 gene
28,8	down	NM_009263	Secreted phosphoprotein 1 (**Spp1**), mRNA
25,5	down	NM_007897	Early B-cell factor 1 (**Ebf1**), mRNA
23	down	BC062250	Pre-B lymphocyte gene 3 (**Vpreb3**), mRNA
22,8	down	NM_001081385	Protocadherin 11 X-linked (**Pcdh11x**), mRNA
21,8	down	NM_019511	Receptor (calcitonin) activity modifying protein 3 (**Ramp3**), mRNA
21,7	down	NM_009019	Recombination activating gene 1 (**Rag1**), mRNA
21,6	down	NM_007932	Endoglin (**Eng**), mRNA
18,9	down	NM_016803	Carbohydrate (chondroitin 6/keratan) sulfotransferase 3 (**Chst3**), mRNA
17,8	down	NM_029338	RIKEN cDNA 1700027N10 gene
17,1	down	NM_011521	Syndecan 4 (**Sdc4**), mRNA
17	down	NM_024204	Ankyrin repeat domain 22 (**Ankrd22**), mRNA
16,1	down	NM_001081227	RIKEN cDNA 6330403A02 gene
15,9	down	NM_133775	Interleukin 33 (**Il33**), mRNA
15,3	down	NM_001081377	Protocadherin 9 (**Pcdh9**), mRNA
14,9	down	NM_010703	lymphoid enhancer binding factor 1 (**Lef1**), mRNA
14,7	down	NM_011580	Thrombospondin 1 (**Thbs1**), mRNA
14,7	down	NM_013540	Glutamate receptor, ionotropic, AMPA2 (alpha 2), mRNA
13,3	down	NM_021406	Triggering receptor expressed on myeloid cells 1 (**Trem1**), mRNA
13,3	down	NM_008115	Glial cell line derived neurotrophic factor family receptor alpha 2 (**Gfra2**), mRNA
12,5	down	NM_016982	pre-B lymphocyte gene 1 (**Vpreb1**), mRNA
12,4	down	NM_174850	MICAL-like 2 (**Micall2**), mRNA
12,4	down	NM_001081377	Protocadherin 9 (**Pcdh9**), mRNA
12	down	NM_024007.3	early B-cell factor 1 (**Ebf1**), mRNA
11,7	down	NM_008829	Progesterone receptor (**Pgr**), mRNA
11,6	down	NM_152804	Polo-like kinase 2 (Drosophila) (**Plk2**), mRNA
11,3	down	NM_007960	Ets variant gene 1 (**Etv1**), mRNA
11,2	down	NM_175461	RIKEN cDNA C030014K22 gene
10,9	down	NM_172715	RIKEN cDNA A230097K15 gene
10,8	down	NM_015730	Cholinergic receptor, nicotinic, alpha polypeptide 4 (**Chrna4**), mRNA
10,7	down	XM_484312	predicted gene, EG432800
10,4	down	NM_013838	Transient receptor potential cation channel, subfamily C, member 6 (**Trpc6**), mRNA
10,2	down	NM_024124	Histone deacetylase 9 (**Hdac9**), mRNA
9,5	down	NM_016983	pre-B lymphocyte gene 2 (**Vpreb2**), mRNA
9,3	down	NM_207231	ADP-ribosylation factor-like 5C (**Arl5c**), mRNA

8,7	down	NM_001033409	Leucine-rich repeat-containing G protein-coupled receptor 6 (**Lgr6**), mRNA
8,7	down	NM_001081039	Dedicator of cytokinesis 9 (**Dock9**), mRNA
8,5	down	NM_001008548	Phosphodiesterase 2A, cGMP-stimulated (**Pde2a**), mRNA
8,5	down	NM_009917	Chemokine (C-C motif) receptor 5 (**Ccr5**), mRNA
8,5	down	NM_009380	Thyroid hormone receptor beta (**Thrb**), mRNA
8,4	down	NM_024444	Cytochrome P450, family 4, subfamily f, polypeptide 18 (**Cyp4f18**), mRNA
8,3	down	NM_033567	Cat eye syndrome chromosome region, candidate 6 homolog (human), mRNA
7,9	down	NM_028078	Immunoglobulin superfamily, member 5 (**Igsf5**), mRNA
7,7	down	NM_008351	Interleukin 12a (**Il12a**), mRNA
7,6	down	NM_027864	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 14 (**Galnt14**), mRNA
7,4	down	NM_183221	FAT tumor suppressor homolog 4 (Drosophila) (**Fat4**), mRNA
7,2	down	NM_010954	Neural cell adhesion molecule 2 (**Ncam2**), mRNA
7,1	down	NM_153095	MAS-related GPR, member A1 (**Mrgpra1**), mRNA
6,8	down	NM_138751	Transmembrane protein 47 (**Tmem47**), mRNA
6,7	down	NM_008626	Mannose receptor, C type 2 (**Mrc2**), mRNA
6,7	down	NM_010867	Myomesin 1 (**Myom1**), mRNA
6,6	down	NM_010610	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1 (**Kcnma1**), mRNA
6,6	down	NM_144850	Rap guanine nucleotide exchange factor (GEF) 3 (**Rapgef3**), mRNA
6,6	down	NM_011595	Tissue inhibitor of metalloproteinase 3 (**Timp3**), mRNA
6,5	down	NM_001004174	Expressed sequence AA467197 (AA467197), mRNA
6,5	down	NM_029879	Regulator of G-protein signalling 7 binding protein (**Rgs7bp**), mRNA
6,3	down	NM_008505	LIM domain only 2 (**Lmo2**), mRNA
6,2	down	NM_011858	odd Oz/ten-m homolog 4 (Drosophila) (**Odz4**), mRNA
6,1	down	NM_172750	ADP-ribosylhydrolase like 1 (**Adprhl1**), mRNA
6,1	down	NM_001042528	Calcium channel, voltage-dependent, N type, alpha 1B subunit (**Cacna1b**), transcript vari
6	down	NM_001033437	Gene model 889, (NCBI) (**Gm889**), mRNA
6	down	NM_019668	Ubiquitin-conjugating enzyme E2A, RAD6 homolog (S. cerevisiae) (**Ube2a**), mRNA
6	down	NM_017372	Lysozyme (**Lyzs**), mRNA
6	down	AF028616	immunoglobulin heavy variable V1-72
5,8	down	NM_011224	Muscle glycogen phosphorylase (**Pygm**), mRNA
5,7	down	NM_001033289	Solute carrier family 9 (sodium/hydrogen exchanger), member 2 (**Slc9a2**), mRNA
5,7	down	NM_172621	Chloride intracellular channel 5 (**Clic5**), mRNA
5,6	down	NM_145933	Beta galactoside alpha 2,6 sialyltransferase 1 (**St6gal1**), mRNA
5,6	down	NM_029682	Stam binding protein like 1 (**Stambpl1**), mRNA
5,5	down	NM_009685	Amyloid beta (A4) precursor protein-binding, family B, member 1 (**Apbb1**), mRNA
5,4	down	NM_011809	E26 avian leukemia oncogene 2, 3' domain (**Ets2**), mRNA
5,4	down	NM_027491	Ras-related GTP binding D (**Rragd**), mRNA
5,4	down	NM_010229	FMS-like tyrosine kinase 3 (**Flt3**), mRNA
5,3	down	NM_146162	Transmembrane protein 119 (**Tmem119**), mRNA
5,3	down	NM_146167	GTPase, IMAP family member 7 (**Gimap7**), mRNA
5,3	down	NM_016846	Ral guanine nucleotide dissociation stimulator,-like 1 (**Rgl1**), mRNA
5,3	down	NM_010570	Insulin receptor substrate 1 (**Irs1**), mRNA
5,2	down	NM_008372	Interleukin 7 receptor (**Il7r**), mRNA

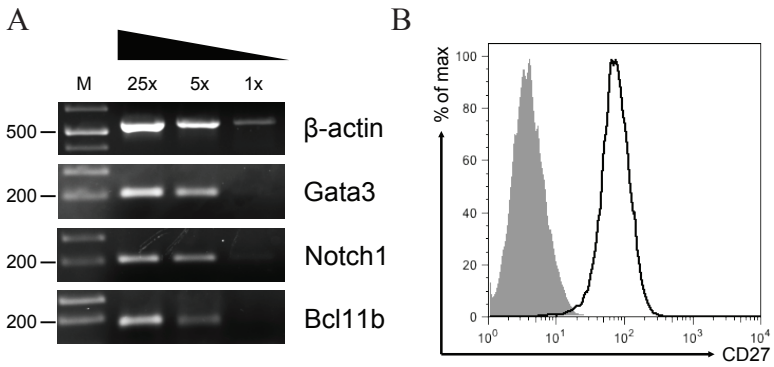
5,2	down	AB061277	cytidine monophospho-N-acetylneuraminic acid hydroxylase, mRNA
5,2	down	NM_007549	B lymphoid kinase (**Blk**), mRNA
5,2	down	NM_008320	Interferon regulatory factor 8 (**Irf8**), mRNA
5,2	down	NM_001102615	Kinesin family member 19A (**Kif19a**), mRNA
5,2	down	NM_023794	Ets variant gene 5 (**Etv5**), mRNA
5,1	down	NM_007521	BTB and CNC homology 2 (**Bach2**), transcript variant 2, mRNA
5,1	down	NM_009904	Calmegin (**Clgn**), mRNA
4,9	down	NM_009235	SRY-box containing gene 15 (**Sox15**), mRNA
4,9	down	XM_001480949	Similar to natural killer cell receptor-P1, mRNA
4,9	down	NM_175329	Nur77 downstream gene 2 (**Ndg2**), mRNA
4,9	down	NM_013674	Interferon regulatory factor 4 (**Irf4**), mRNA
4,8	down	NM_172588	Serine incorporator 5 (**Serinc5**), mRNA
4,8	down	NM_018784	ST3 beta-galactoside alpha-2,3-sialyltransferase 6 (**St3gal6**), mRNA
4,7	down	AF005353	immunoglobulin kappa chain variable 1-110, mRNA
4,7	down	NM_007595	Calcium/calmodulin-dependent protein kinase II, beta (**Camk2b**), mRNA
4,6	down	NM_007956	Estrogen receptor 1 (alpha) (**Esr1**), mRNA
4,6	down	NM_010739	Mucin 13, epithelial transmembrane (**Muc13**), mRNA
4,5	down	NM_172469	Chloride intracellular channel 6 (**Clic6**), mRNA
4,5	down	NM_011303	Dehydrogenase/reductase (SDR family) member 3 (**Dhrs3**), mRNA
4,5	down	NM_008126	Gap junction membrane channel protein beta 3 (**Gjb3**), mRNA
4,5	down	NM_177192	RIKEN cDNA D030011O10 gene, mRNA
4,5	down	NM_008608	matrix metallopeptidase 14 (membrane-inserted), mRNA
4,5	down	NM_007901	Endothelial differentiation sphingolipid G-protein-coupled receptor 1 (**Edg1**), mRNA
4,5	down	NM_008548	Mannosidase 1, alpha (**Man1a**), mRNA
4,5	down	NM_146008	T-complex 11 (mouse) like 2 (**Tcp11l2**), mRNA
4,4	down	NM_019739	Forkhead box O1 (**Foxo1**), mRNA
4,4	down	NM_027496	RIKEN cDNA 5730557B15 gene, mRNA
4,4	down	AK082395	RIKEN cDNA 4930486G11 gene, mRNA
4,4	down	NM_207237	Mannosidase, alpha, class 1C, member 1 (**Man1c1**), mRNA
4,4	down	NM_145133	Traf2 binding protein (**T2bp**), mRNA
4,4	down	NM_020286	Tetraspanin 32 (**Tspan32**), mRNA
4,4	down	NM_011405	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 7 (**Slc7a7**), mRNA
4,4	down	NM_011104	Protein kinase C, epsilon (**Prkce**), mRNA
4,4	down	NM_001033228	Integrin alpha 1 (**Itga1**), mRNA
4,3	down	NM_031185	A kinase (PRKA) anchor protein (gravin) 12 (**Akap12**), mRNA
4,3	down	NM_145827	NLR family, pyrin domain containing 3 (**Nlrp3**), mRNA
4,3	down	NM_201519	Mitogen-activated protein kinase kinase kinase kinase 5 (**Map4k5**), mRNA
4,2	down	NM_001081134	Potassium voltage-gated channel, subfamily G, member 1 (**Kcng1**), mRNA
4,2	down	NM_021415	Calcium channel, voltage-dependent, T type, alpha 1H subunit (**Cacna1h**), mRNA
4,2	down	NM_030017	Retinol dehydrogenase 12 (**Rdh12**), mRNA
4,2	down	NM_198294	Tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1 (**Tanc1**), mRNA
4,2	down	NM_172734	Serine/threonine kinase 38 like (**Stk38l**), mRNA
4,2	down	NM_009330	HNF1 homeobox B (**Hnf1b**), mRNA

4,2	down	NM_001005423	Melanoregulin (**Mreg**), mRNA
4,1	down	NM_007564	Zinc finger protein 36, C3H type-like 1 (**Zfp36l1**), mRNA
4,1	down	NM_019840	Phosphodiesterase 4B, cAMP specific (**Pde4b**), mRNA
4,1	down	NM_008088	Growth arrest specific 7 (**Gas7**), mRNA
4,1	down	NM_016920	ATPase, H+ transporting, lysosomal V0 subunit A1 (**Atp6v0a1**), mRNA
4,1	down	BC026950	Kynureninase (L-kynurenine hydrolase), mRNA
4,1	down	NM_153584	cDNA sequence BC031353, mRNA
4,1	down	NM_010740	CD93 antigen (**Cd93**), mRNA
4,1	down	NM_015786	Histone cluster 1, H1c (**Hist1h1c**), mRNA
4,1	down	NM_010128	Epithelial membrane protein 1 (**Emp1**), mRNA
4,1	down	NM_213616	ATPase, Ca++ transporting, plasma membrane 4 (**Atp2b4**), mRNA
4	down	NM_177303	RIKEN cDNA B430119L13 gene, mRNA
4	down	NM_177645	RIKEN cDNA 1110028C15 gene
4	down	AF329833	GH regulated TBC protein 1 (**Grtp1**), mRNA
4	down	NM_133791	WW, C2 and coiled-coil domain containing 2 (**Wwc2**), mRNA
4	down	NM_008606	matrix metallopeptidase 11, mRNA
4	down	NM_172842	lymphocyte transmembrane adaptor 1, mRNA
4	down	NM_027410	RIKEN cDNA 2210010N04 gene
3,9	down	NM_011925	CD97 antigen (**Cd97**), mRNA
3,9	down	NM_008339	CD79B antigen (**Cd79b**), mRNA
3,9	down	NM_019949	ubiquitin-conjugating enzyme E2L 6, mRNA
3,9	down	NM_172753	RIKEN cDNA 4732435N03 gene
3,9	down	AK171913	macrophage activation 2 like, mRNA
3,9	down	NM_001079686	Synaptic nuclear envelope 1 (**Syne1**), mRNA
3,9	down	AY344585	predicted gene, ENSMUSG00000074792
3,9	down	NM_015753	Zinc finger E-box binding homeobox 2 (**Zeb2**), mRNA
3,9	down	NM_133655	Cd81 antigen (**Cd81**), mRNA
3,8	down	NM_174851	Interleukin 28 receptor alpha (**Il28ra**), mRNA
3,8	down	NM_198724	EGF-like domain 7 (**Egfl7**), mRNA
3,8	down	NM_011175	Legumain (**Lgmn**), mRNA
3,8	down	NM_138309	Cd99 antigen-like 2 (**Cd99l2**), mRNA
3,8	down	AY512938	predicted gene, ENSMUSG00000069163
3,8	down	NM_144805	Transmembrane protein 40 (**Tmem40**), mRNA
3,8	down	NM_134068	Dual specificity phosphatase 22 (**Dusp22**), transcript variant 1, mRNA
3,8	down	NM_011206	Protein tyrosine phosphatase, non-receptor type 18 (**Ptpn18**), mRNA
3,7	down	NM_177192	RIKEN cDNA D030011O10 gene
3,7	down	NM_019976	Proline/serine-rich coiled-coil 1 (**Psrc1**), mRNA
3,7	down	NM_007426	Angiopoietin 2 (**Angpt2**), mRNA
3,7	down	NM_028994	Phosphoenolpyruvate carboxykinase 2 (mitochondrial) (**Pck2**), mRNA
3,7	down	NM_029629	Fumarylacetoacetate hydrolase domain containing 2A (**Fahd2a**), mRNA
3,6	down	BC106179	cDNA sequence BC106179
3,6	down	NM_019823	Cytochrome P450, family 2, subfamily d, polypeptide 22 (**Cyp2d22**), mRNA
3,6	down	NM_008801	Phosphodiesterase 6D, cGMP-specific, rod, delta (**Pde6d**), mRNA
3,6	down	NM_007420	Adrenergic receptor, beta 2 (**Adrb2**), mRNA
3,6	down	NM_009367	Transforming growth factor, beta 2 (**Tgfb2**), mRNA
3,6	down	NM_016764	Peroxiredoxin 4 (**Prdx4**), mRNA
3,6	down	NM_013739	Docking protein 3 (**Dok3**), mRNA

3,6	down	NM_172728	cAMP responsive element binding protein 5 (**Creb5**), mRNA
3,5	down	NM_011491	Stanniocalcin 2 (**Stc2**), mRNA
3,5	down	NM_173731	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase-like 1 (**Hmgcll1**), mRNA
3,5	down	BC058790	major facilitator superfamily domain containing 4, mRNA
3,5	down	AF045503	predicted gene, EG434609
3,5	down	NM_145973	Elongation factor RNA polymerase II-like 3 (**Ell3**), mRNA
3,5	down	NM_010800	Basic helix-loop-helix domain containing, class B, 8 (**Bhlhb8**), mRNA
3,5	down	NM_177768	cDNA sequence BC038156
3,5	down	NM_009511	Vasoactive intestinal peptide receptor 2 (**Vipr2**), mRNA
3,5	down	NM_011977	Solute carrier family 27 (fatty acid transporter), member 1 (**Slc27a1**), mRNA
3,5	down	NM_008902	Placental protein 11 related (**Pp11r**), mRNA
3,4	down	NM_009194	Solute carrier family 12, member 2 (**Slc12a2**), mRNA
3,4	down	NM_025282	Myocyte enhancer factor 2C (**Mef2c**), mRNA
3,4	down	NM_178045	Ras association (RalGDS/AF-6) domain family 4 (**Rassf4**), mRNA
3,4	down	NM_001033210	Expressed sequence AI427122 (**AI427122**), mRNA
3,4	down	NM_080575	Acyl-CoA synthetase short-chain family member 1 (**Acss1**), mRNA
3,4	down	AK156585	RIKEN cDNA 9530009G21 gene
3,4	down	NM_153175	GTPase, IMAP family member 6 (**Gimap6**), mRNA
3,4	down	NM_133697	RIKEN cDNA 1110003E01 gene
3,4	down	NM_010165	Eyes absent 2 homolog (Drosophila) (**Eya2**), mRNA
3,4	down	NM_176930	Neuron-glia-CAM-related cell adhesion molecule (**Nrcam**), mRNA
3,4	down	NM_008099	Germinal center expressed transcript 2 (**Gcet2**), mRNA
3,3	down	NM_010137	Endothelial PAS domain protein 1 (**Epas1**), mRNA
3,3	down	NM_007655	CD79A antigen (immunoglobulin-associated alpha) (**Cd79a**), mRNA
3,3	down	NM_010623	Kinesin family member 17 (**Kif17**), mRNA
3,3	down	NM_145554	Low density lipoprotein receptor adaptor protein 1 (**Ldlrap1**), mRNA
3,3	down	NM_019563	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4 (**Cited4**), mRNA
3,3	down	NM_027401	RIKEN cDNA 1700010C24 gene, mRNA
3,3	down	NM_011074	PFTAIRE protein kinase 1 (**Pftk1**), mRNA
3,3	down	NM_174857	MAM domain containing 2 (**Mamdc2**), mRNA
3,3	down	NM_010158	KH domain containing, RNA binding, signal transduction associated 3 (**Khdrbs3**), mRNA
3,3	down	NM_012054	Acyloxyacyl hydrolase (**Aoah**), mRNA
3,3	down	NM_018732	Sodium channel, voltage-gated, type III, alpha (**Scn3a**), mRNA
3,3	down	NM_029870	RIKEN cDNA A930001N09 gene, mRNA
3,3	down	NM_008349	Interleukin 10 receptor, beta (**Il10rb**), mRNA
3,3	down	NM_026622	RIKEN cDNA 3110057O12 gene, mRNA
3,3	down	NM_172442	Deltex 4 homolog (Drosophila) (**Dtx4**), mRNA
3,3	down	NM_016773	Nucleobindin 2 (**Nucb2**), mRNA
3,3	down	NM_015767	Tocopherol (alpha) transfer protein (**Ttpa**), mRNA
3,2	down	AF057537	immunoglobulin kappa chain variable 1-135, mRNA
3,2	down	NM_011309	S100 calcium binding protein A1, mRNA
3,2	down	NM_145376	Acyltransferase like 2 (**Aytl2**), mRNA
3,2	down	NM_009250	Serine (or cysteine) peptidase inhibitor, clade I, member 1 (**Serpini1**), mRNA

3,2	down	NM_153582	CKLF-like MARVEL transmembrane domain containing 4 (**Cmtm4**), mRNA
3,2	down	NM_008803	Phosphodiesterase 8A (**Pde8a**), mRNA
3,2	down	NM_009368	Transforming growth factor, beta 3 (**Tgfb3**)
3,2	down	NM_145976	cDNA sequence BC027057, mRNA
3,2	down	NM_015749	Transcobalamin 2 (**Tcn2**), mRNA
3,2	down	BC115567	RIKEN cDNA 4930485B16 gene, mRNA
3,2	down	NM_134042	Aldehyde dehydrogenase family 6, subfamily A1 (**Aldh6a1**), mRNA
3,2	down	NM_007415	Poly (ADP-ribose) polymerase family, member 1 (**Parp1**), mRNA
3,2	down	AK084447	RIKEN cDNA C330007P06 gene, mRNA
3,2	down	NM_001013390	Sodium channel, type IV, beta (**Scn4b**), mRNA
3,2	down	NM_010585	Inositol 1,4,5-triphosphate receptor 1 (**Itpr1**), mRNA
3,1	down	NM_010358	Glutathione S-transferase, mu 1 (**Gstm1**), mRNA
3,1	down	NM_019969	Pleiomorphic adenoma gene 1 (**Plag1**), mRNA
3,1	down	NM_145506	Erythrocyte protein band 4.1-like 5 (**Epb4.1l5**), mRNA
3,1	down	NM_013750	Pleckstrin homology-like domain, family A, member 3 (**Phlda3**), mRNA
3,1	down	NM_001039394	RAB43, member RAS oncogene family (**Rab43**), mRNA
3,1	down	NM_001003955	RAB11 family interacting protein 5 (class I), mRNA
3,1	down	NM_173006	Paraoxonase 3 (**Pon3**), mRNA
3,1	down	NM_001025250	Vascular endothelial growth factor A (**Vegfa**), mRNA
3,1	down	AK034303	RIKEN cDNA 9330175E14 gene, mRNA
3,1	down	NM_133667	Pyruvate dehydrogenase kinase, isoenzyme 2 (**Pdk2**), mRNA
3,1	down	NM_001001986	RIKEN cDNA 8430427H17 gene
3,1	down	NM_029100	Selenoprotein N, 1 (**Sepn1**), mRNA
3,1	down	NM_146096	RIKEN cDNA 5730446C15 gene
3,1	down	NM_198642	RIKEN cDNA 5031414D18 gene
3,1	down	NM_175484	Coronin, actin binding protein, 2B (**Coro2b**), mRNA
3	down	NM_027222	RIKEN cDNA 2010001M09 gene, mRNA
3	down	NM_009255	Serine (or cysteine) peptidase inhibitor, clade E, member 2 (**Serpine2**), mRNA
3	down	NM_015767	Tocopherol (alpha) transfer protein (**Ttpa**), mRNA
3	down	XM_887671	Transmembrane protein 200B, mRNA
3	down	NM_025273	Pterin 4 alpha carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1) 1 (**Pcbd1**), mRNA
3	down	NM_023341	Chaperone, ABC1 activity of bc1 complex like (S. pombe) (**Cabc1**), mRNA
3	down	NM_172731	FYVE, RhoGEF and PH domain containing 5 (**Fgd5**), mRNA
3	down	NM_027496	RIKEN cDNA 5730557B15 gene
3	down	NM_020332	Progressive ankylosis (**Ank**), mRNA
3	down	AK173319	Ring finger protein 169, mRNA
3	down	NM_010794	Mannoside acetylglucosaminyltransferase 1 (**Mgat1**), mRNA
3	down	NM_025730	Leucine-rich repeat kinase 2 (**Lrrk2**), mRNA
3	down	NM_029001	ELOVL family member 7, elongation of long chain fatty acids (yeast) (**Elovl7**)
3	down	NM_028724	Ras and Rab interactor 2, mRNA

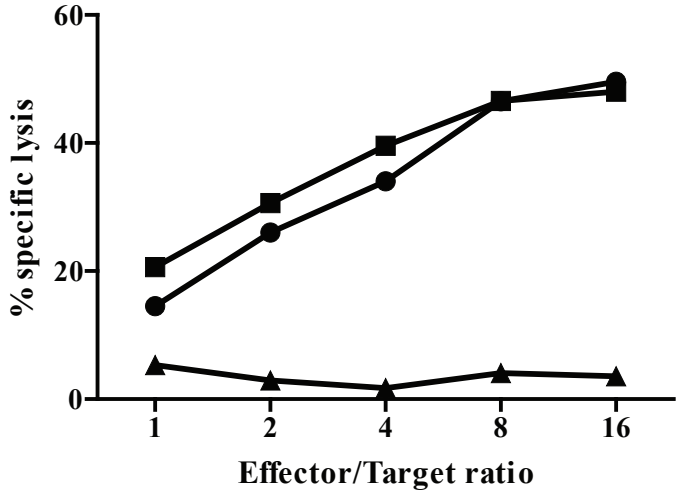
Supporting Information Figure 1



Gata3, Notch1, Bcl11b and CD27 expression by pro-T cells cultured on DL4-Fc coated plates in the presence of IL-7 and SCF for more than 5 weeks. (A) Semiquantitative RT-PCR analysis. (B) FACS analysis. Gray histogram represents the unstained control.

These experiments were performed with three individually established pro-T cell lines The results obtained with one representative line are shown.

Supporting Information Figure 2



Cytotoxic activity of pro T cell derived B6 T cells (■) wild type B6 T cells (●) and Balb/c T cells (▲) stimulated with irradiated Balb/c spleen cells. ^{3}H-thymidine labeled Balb/c LPS blast were used as targets.

Supporting information materials and methods

Semiquantitative RT-PCR

Total RNA from pro T cells was reverse transcribed and PCR amplified. The following primers were used: *β-actin*, 5'-GAAGTCTAGAGCAACATAGCACAGCTTCTC-3' and 5'-GTGGGAATTCGTCAGAAGGACTCCTATGTG-3', *GATA3*, 5'-GTCATCCCTGAGCCACATCT-3' and 5'-TAGAAGGGGTCGGAGGAACT-3', *Notch1*, 5'-AACTGCTCCGAGGAGATCAA-3' and 5'-ACACAGGTGCCATTGTTGAA-3', *Bcl11b*, 5'-TGCCTTCCCATCTATGTTCC-3' and 5'-GAAGAGGAGGAGGAGGAGGA-3'.

Cytotoxic T-cell assay

Total spleen cells from B6 Rag2 deficient mice at 6 weeks after transplantation of *in vitro* propagated B6 pro T cells were co-cultured at a density of 2.5 x 10^6/ml with irradiated Balb/c spleen cells (2.5 x 10^6/ml) for 5 days. At day 5 the capacity to lyse Balb/c LPS blasts was tested. Normal B6 spleen cells stimulated with irradiated Balb/c spleen cells and Balb/c spleen cells co-cultured with irradiated Balb/c spleen cells were used as controls.

III Reconstitution of a functional B-cell compartment in immunodeficient mice with pro-B cells propagated with or without stromal cells

Reconstitution of a functional B-cell compartment in immunodeficient mice with pro-B cells propagated with or without stromal cells

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Conflict of interest

The authors declare no commercial or financial conflict of interest.

Abstract

Fetal liver (FL) and bone marrow (BM) derived pro-B cells were propagated long-term in stromal cell free cultures supplemented with interleukin-7 (IL-7), stem cell factor (SCF) and FLT3 ligand. Within a week, most cells expressed surface CD19, CD79a and CD79b, λ5 and VpreB antigens and had rearranged immunoglobulin D-J heavy chain genes. Cells efficiently differentiated into IgM^+ B cells upon IL-7 withdrawal. Both FL and BM pro-B cells reconstituted the B-cell compartments of immuno-incompetent Rag2-deficient mice with FL pro-B generating follicular, marginal zone (MZB) and B-1a B cells, but BM pro-B cells mainly MZB. Reconstituted mice generated significant IgM and IgG antibodies to a type II T-independent antigen with FL pro-B cell reconstituted mice generating surprisingly high IgG_1 titers. Finally, we show for the first time that *in vitro* propagated pro-B and pro-T cells reconstituted both the B- and T-cell compartments of Rag2-deficient mice and mounted a T-cell dependent antibody response. Thus it is possible to reconstitute a functional adaptive immune system with *in vitro* propagated, stromal cell free, lymphocyte progenitors. This novel stromal cell free culture system facilitates our understanding of B-cell development and might be applied clinically.

Introduction

Like all other hematopoietic cells, B cells are derived from hematopoietic stem cells (HSCs). In mice, most B-cell development takes place in the FL before birth whereas in adults, the BM is the major site. The various stages of B-cell development can be distinguished by combinations of cell surface and intracellular markers, cell cycle profile and rearrangement status of IgH and IgL chain loci [1-4]. B-cell commitment is determined by the transcription factor Pax5 [5-7]. One of the target genes of Pax5 is CD19 [8] and the earliest B-cell committed precursor, also called a pro-B cell, is $CD19^+$ $CD117^+$ [1, 9]. These cells have their IgH chains in D – J rearranged configuration [4] and a large fraction is proliferating [1]. Such cells are absent in Pax5-deficient [10] and *Il7* or *Il7R* gene deleted mice [11, 12] indicating the importance of these molecules in early B-cell development. Also the receptor tyrosine kinases CD117 and CD135 and their corresponding ligands, SCF and Flt3L, play an important role in early B-cell development [13-17]. Blocking SCF binding with an anti-CD117 antibody inhibits pro-B cell proliferation in IL-7-containing stromal cell cultures [18] and CD135 or Flt3L-deficient mice have a dramatically reduced BM pro-B-cell compartment [19]. Taken together, these results show the importance of Pax5 and cytokine-related molecules in early B-cell development.

Over 20 years ago, we showed that FL-derived pro-B cells could be grown long-term on stromal cells and IL-7 [20]. Moreover, using Bcl2 transgenic pro-B cells IL-7 withdrawal resulted in an efficient differentiation into surface IgM positive B cells [20, 21]. However, until now, it has not been possible to grow BM-derived pro-B cells long-term using stromal cell based culture systems. In the present report we now show for the first time that it is also possible to grow BM-derived pro-B cells long-term, with and even without stromal cell support. We have described an early (E) progenitor (P) with lymphoid (L) and myeloid (M) developmental potential, called EPLM, in the BM [22]. EPLM express B220 and CD117 but not CD19 or NK1.1. When

cultured either on OP9 stromal cells plus IL-7 or with IL-7, SCF and FLT3L but without stromal cells, EPLM from the FL or BM can be propagated long-term and differentiate into $CD19^{+}$ pro-B cells. Moreover, upon *in vivo* transplantation into immunodeficient *Rag2* gene deficient hosts, these pro-B cells can reconstitute a functional B-cell compartment. Finally, we also show that when these mice are reconstituted with a mixture of *in vitro* propagated pro-B and stromal cell free propagated pro-T cells [23], a small, but functional adaptive immune system is generated.

Results

Establishment of a culture system that allows the long-term in vitro-propagation of BM pro-B cells

More than 20 years ago we described a stromal cell based culture system permitting the long-term *in vitro* growth of FL-derived pro-B cells [20]. However, until now, we were unable to grow BM pro-B cells long-term. More recently we identified a $B220^{+}CD117^{+}CD19^{-}NK1.1^{-}$ progenitor in the BM that showed a strong lymphoid and myeloid developmental potential and that we called EPLM [22]. Now we tested the capacity of BM-derived EPLM to generate long-term growing pro-B cells. Therefore $B220^{+}CD117^{+}CD19^{-}NK1.1^{-}$ ($CD19^{-}$) cells and $B220^{+}CD117^{+}CD19^{+}$ $NK1.1^{-}$ ($CD19^{+}$) cells from FL or BM were sorted (Figure 1a) and plated on OP9 stromal cells plus IL-7. After an initial culture period of 6 days, cells were harvested every 3 - 4 days and re-plated on fresh stromal cells and IL-7. As shown in figure 1b, $CD19^{-}$ (EPLM) cells showed continuous growth with doubling times of about 30 hours, whereas $CD19^{+}$ cells proliferated for the first 7 days and then their numbers plateaued. In order to test whether differential gene expression was underlying this different *in vitro* expansion capacity we performed RNAseq on $CD19^{+}$ cells derived from $CD19^{-}$ EPLM grown for 12 days on OP9 plus IL-7 and freshly-isolated $CD19^{+}CD117^{+}$ cells similarly grown for 5 days. This analysis revealed that only 83 genes, none of which were B-cell related, were twofold or more differentially expressed (summary table 1). In fact, the 44 B-cell related genes found in this analysis were similarly expressed (summary table 2 and supplementary figure 1). Gene ontology analysis performed on the 83 genes did not identify genes that might explain the growth difference observed.

Comparison of the growth rate of FL and BM-derived EPLM on OP9 stromal cells with IL-7 revealed that FL-derived cells grew marginally better than those from the BM (Figure 1c). Most importantly, FACS analysis showed that over 95% of these cultured FL and BM EPLM gained the expression of CD19 (Figure 1d). Around 25% BM and over 60% FL-derived cells expressed CD117 (Figure 1d). RT-PCR analysis revealed that both expressed CD79a, CD79b, Igll1 (λ5) and Vpreb1 and both had undergone D_H - J_H rearrangements (Figure 1e). Thus, BM and FL EPLM cultured on OP9 plus IL-7 give rise to long-term proliferating pro-B cells.

Establishment of a stromal cell free culture system that allows the long-term propagation of BM and FL pro-B cells

FL pro-B cells propagated on stromal cells plus IL-7 reconstituted the B-cell compartment of immuno-deficient mice [20, 24, 25]. However, it is unlikely that pro-B cells derived from co-culture settings will ever be approved for therapeutic purposes. Therefore, we developed a stromal cell free culture system consisting of soluble IL-7, SCF, and FLT3L. As shown in Figure 2a and b, EPLM derived from FL and BM showed very robust growth under these stromal cell free conditions. In fact, the growth rate of BM-derived EPLM was identical under stromal cell and stromal cell free conditions (Figure 2a). On the other hand the growth of FL EPLM was slightly slower without stromal cell support (Figure 2b). FACS analysis revealed that stromal cell free cultured EPLM acquired CD19 expression and around 35% BM and over 75% FL-derived cells expressed CD117 (Figure 2c and d). Moreover, stromal cell free cultured cells expressed CD79a, CD79b, Igll1 (λ5) and Vpreb1 and had their IgH chains in D_H-J_H rearranged configuration (Figure 2e). Thus, both BM and FL EPLM cultured with IL-7, SCF and FLT3L without stromal cells also give rise to long-term proliferating pro-B cells.

Previously we showed that upon IL-7 removal, *in vitro* propagated pro-B cells underwent massive apoptosis that was prevented by the transgenic over-expression

of the anti-apoptotic Bcl2 gene. IL-7 removal from cultured transgenic Bcl2 expressing pro-B cells resulted in a large fraction differentiating into surface IgM positive B cells [21]. To test whether BM EPLM-derived pro-B cells could differentiate upon IL-7 withdrawal, we sorted such cells from Bcl2 transgenic mice and maintained cultures either on OP9 or in stromal cell free conditions. As shown in figure 2f (upper panels) very few (0.4% on OP9, left panel and 1.2% stromal cell free, right panel) cells grown in the presence of IL-7 expressed a μH (IgM) and Igκ light chain on their surface. However, 6 days after IL-7 withdrawal, 26% of the stromal cell culture derived pro-B cells were IgM^+, Igk^+ (Figure 2f, left upper panel, upper right quadrant) and the remaining 7.1% IgM^+, Igk^- (upper left quadrant) cells being $Ig\lambda^+$ (data not shown). As shown in figure 2f (lower right dot plot), these values were 17.3% and 7.8% respectively for pro-B cells cultured in stromal cell free conditions. Thus BM EPLM-derived pro-B cells propagated either on stromal cells or grown stromal cell free differentiated efficiently into IgM positive B cells upon IL-7 withdrawal.

In vivo reconstitution of the B-cell compartments by pro-B cells derived from BM or FL EPLM

To test the capacity of *in vitro* generated FL or BM-derived pro-B cells to reconstitute mice, 10^7 EPLM-derived pro-B from FL or BM of C57BL/6 CD45.1 mice cultured on OP9 stroma or stromal cell free were transferred into sub-lethally irradiated CD45.2 C57BL/6 Rag2-deficient mice. Five to ten weeks following transfer, reconstitution was assessed by FACS analysis of spleen or peritoneal cavity (PerC) cells. As shown in figure 3a for FL-derived and figure 4a for BM-derived donor cells (upper row dot plots), $CD45.1^+$ expression was restricted to $CD19^+$ cells and all $CD19^+$ cells co-expressed IgM (second row). Thus, all *in vitro* propagated pro-B cells generated B cells but no other lineages. Based on expression of CD5, FL-derived pro-B cells grown either on stroma or stromal cell free, gave rise to a large fraction of B-

1a B cells especially in the PerC (Figure 3a third row cytograms). As expected, BM-derived pro-B cells generated few CD5 expressing B cells (Figure 4a third row cytograms). Spleen marginal zone B (MZB) and follicular B cells (FB) were identified using combined CD21 and CD23 expression on gated $CD19^+$ cells. The CD21 and CD23 expression pattern on splenic B cells derived from FL pro-B cells propagated on either OP9 stromal or stromal cell free were similar (Figure 3a lower row cytograms). Thus 40 – 60% were $CD21^+/CD23^+$ FB and 30 – 40% were $CD21^+/CD23^-$ MZB. The $CD19^+/CD21^-/CD23^-$ cells (lower left quadrant) most likely represent B-1 B cells. In figure 3a a representative immunohistochemical picture of a spleen section of such a reconstituted mouse is shown. Thus like in wild type mice B-cell follicles were formed with IgM^{high} (green) IgD^{low} (blue) MZB cells surrounding metallophilic macrophages (red) and IgM^+IgD^{high} FB inside. Mice reconstituted with BM pro-B cells cultured by the two methods also showed no obvious differences in CD21 and CD23 expression (Figure 4a lower row cytograms). However, in marked contrast to FL-derived pro-B cells, more than 70% of BM pro-B cell-derived B cells were MZB and only 15 – 20% were FB (Figure 4a lower row cytograms).

Spleens of mice reconstituted with FL-derived pro-B cells contained around 5 x 10^6 B cells with no difference between cells cultured with or without stroma (figure 4b). In contrast, spleens of mice reconstituted with BM-derived pro-B cells contained only 0.5 x 10^6 B cells irrespective of whether they had been propagated with or without stroma. Thus FL-derived pro-B cells seem to be much more efficient at reconstitution than BM-derived cells. To test this latter conclusion more stringently, we performed a competitive reconstitution experiment. Sub-lethally irradiated CD45.2 C57BL/6 Rag2-deficient mice were reconstituted with a 1:1 mixture of 5 x 10^6 CD45.2 FL-derived and CD45.1 BM-derived pro-B cells. After 8 weeks, FACS analysis of one representative mouse showed (Figure 4c) that 44% of total splenocytes were $CD19^+$ and of these 97.5% (42.9/44) were FL and only 2.5% (1.1/4.4) were BM-derived. Thus FL-derived pro-B cells are superior to BM-derived ones also in competitive transplantation settings. Similar results were obtained in more than three independent experiments. Taken together, the difference in MZB to FB-cell

ratio between FL and BM-derived pro-B cell-reconstituted mice is most likely the consequence of the different efficiency of B-cell reconstitution.

Some time ago it was shown that adult HSC could be functionally converted into FL HSC upon enforced expression of the *Lin28B* gene [26]. More recently it was also shown that *Lin28B* expressing BM-derived pro-B cells gained properties of FL-derived ones [27, 28]. To test whether *Lin28B* expression could also influence the reconstitution efficiency, we introduced a MigR1 retrovirus encoding *Lin28B* into BM-derived pro-B cells. Then 5 x 10^6 *Lin28B* transduced and 5 x 10^6 non-transduced BM-derived pro-B cells were co-transferred into sub-lethally irradiated Rag2-deficient recipients. Representative FACS blots of spleens of such mice are shown in supplementary figure 2. Only 2% $CD19^+$ cells were found and about half of these expressed *Lin28B* (GFP). Thus the overall reconstitution was very poor and the expression of *Lin28B* did not improve the *in vivo* B-cell generating capacity of BM-derived pro-B cells. However, practically all $CD19^+$ cells were IgM^+ and over 80% $Lin28B^+$ cells expressed CD5, thereby resembling B-1 B cells. Thus with respect to CD5 expression, we also found that *Lin28B* over-expression was able to convert BM pro-B cells to ones phenotypically resembling FL cells.

BM or FL pro-B cell reconstituted Rag2-deficient mice mount antibody response to a T-cell independent type 2 antigen

To test whether the B-cell compartments of Rag2-deficient reconstituted mice were functional, 8 weeks after cell transfer they were immunized with the T-cell independent antigen NIP-Ficoll. Serum anti-NIP titers were determined one week before and two weeks after immunization. All reconstituted mice mounted a good IgM anti-NIP response with IgM titers comparable to those in immunized wild type C57BL/6 mice (Figure 5a). On the other hand, mice reconstituted with BM-derived pro-B cells showed a rather low, but still significant, IgG anti-NIP response (Figure 5b). However, the IgG anti-NIP response induced in mice reconstituted with FL pro-B

cells was as high or even higher than in wild type C57BL/6 mice (Figure 5b). Thus the pro-B cell reconstituted B-cell compartments were functional.

The surprisingly high IgG anti-NIP titers in FL pro-B reconstituted mice prompted us to determine their isotypes. As shown in figure 5c, the IgG2A and IgG2B anti-NIP titers were low and comparable to those in immunized wild type C57BL/6 mice, whereas IgG1 and IgG3 titers were considerably higher. Although high IgG3 titers are observed in other T-cell independent responses, switching to IgG1 was thought to be a highly T-cell dependent phenomenon since it requires IL-4 [29-31]. In addition to T cells, IL-4 was also reported to be produced by mast cells [32, 33], basophils [34], eosinophils [35] and group 2 innate lymphoid cells (ILC2) [36]. The generation of ILCs is largely dependent on IL-7 and consequently Rag2/common gamma chain (Rag2cγ) double-deficient mice are practically devoid of ILCs [37, 38]. Therefore, we reconstituted Rag2 and Rag2cγ double-deficient mice with 10^7 FL-derived pro-B cells and analyzed immune responses as described above. Results are summarized in Figure 6a-d. Both Rag2 and Rag2cγ double-deficient mice showed a very significant IgM and IgG anti-NIP response (Figure 6a and b), similar and/or even higher than wild type C57BL/6 mice. Moreover, both types of reconstituted mice showed a very strong IgG1 and IgG3 anti-NIP response (Figure 6c and d). Thus the IgG class switching observed in immunized reconstituted mice does not seem to be regulated by ILCs.

Reconstitution of the adaptive immune system in Rag2-deficient mice by in vitro-propagated pro-B cells and pro-T cells

Recently, we described a new stromal cell free culture system that allows the long-term propagation of pro-T cells and showed that they could be used to reconstitute the T-cell compartment of T-cell deficient mice [23]. However, the thymus of these reconstituted mice was rather poor at generating regulatory T cells (Tregs) and mice developed a wasting disease preventable by the co-transfer of

mature Treg cells. We have since observed that co-transfer of pro-T cells and pro-T cells transduced with a retrovirus encoding *Foxp3-IRES-GFP* could overcome the need of co-transferring mature Tregs. Therefore, we tested whether the transfer of *in vitro* propagated pro-B cells together with a mixture of non-transduced and *Foxp3* transduced pro-T cells (ratio 4:1) could lead to the formation of an adaptive immune system in Rag2-deficient mice. In initial experiments, where pro-B and pro-T cells were injected simultaneously, resulted in T but no B-cell reconstitution (not shown). We therefore performed sequential cell transfers. Sub-lethally irradiated C57BL/6 Rag2-deficient mice were first reconstituted with 10^7 FL-derived pro-B cells and 4 weeks later injected with 10^7 pro-T cells of which 2.5 x 10^6 were Foxp3$^+$. Six weeks after pro-T cell transfer FACS analysis on peripheral blood revealed a significant B and T-cell reconstitution (data not shown). Because FL pro-B cells partially reconstituted the B-cell compartment of B-cell deficient, T-cell proficient, µMt mice (Supplementary Figure 3a) and mounted a T-cell dependent immune response (Supplementary Figure 3b and c) we tested whether the established B and T cells were functional and could cooperate. Reconstituted mice were therefore immunized with the T-cell dependent antigen NIP-OVA. Rag2-deficient mice that only received pro-B cells were used as controls. The anti-NIP titers were determined in sera taken one week before and two weeks after immunization. No IgM or IgG anti-NIP response was observed in Rag2-deficient mice reconstituted with pro-B cells only (Figure 7a). In marked contrast, mice reconstituted with both pro-B and pro-T cells mounted a strong IgM and relatively weak, but significant, IgG anti-NIP response (Figure 7a). Thus the reconstituted adaptive immune system was functional.

To test the extent of reconstitution, mice were subjected to detailed FACS analysis at 10 – 14 weeks after pro-T cell-transfer. A representative FACS staining of the spleen of one of these mice is shown in figure 7b. Splenocytes comprised 10% B cells, 10% CD8 T cells and 20% CD4 T cells. Moreover, around 10% of the CD4 T cells expressed GFP indicating they were Tregs. To quantitate reconstitution, cell numbers in the spleens of 7 individual mice 14 weeks after pro-T cell transfer were assessed and are shown in figure 7c. Thus around 3.5 x 10^6 CD19$^+$ IgM$^+$ B cells, 1 x 10^6 CD8 T cells and 3 x 10^6 CD4 T cells were found. Of the CD4 T cells 0.5 x 10^6 were GFP

positive indicating that they were derived from the Foxp3 transduced pro-T cells. Thus the transfer of *in vitro* propagated pro-B and pro-T cells into Rag2-deficient mice resulted into reconstitution of a small, but functionally active, adaptive immune compartment.

Discussion

Culture methods allowing the long-term propagation of FL-derived pro-B cells have been available for more than 20 years [20]. However, to the best of our knowledge, it has not yet been possible to grow BM-derived pro-B cells long-term. To our knowledge, this is the first report describing the long-term *in vitro* propagation of BM-derived pro-B cells and how these can be used to reconstitute a functional immune system in immunodeficient recipient mice. Pro-B cells from $Pax5^{-/-}$ mice were previously shown to have multi-lineage developmental potential [39]. We identified an equivalent $B220^{+}CD117^{+}CD19^{-}NK1.1^{-}$ cell with lymphoid and myeloid developmental potential in the BM of wild type mice, which we called EPLM [22]. Herein we show that BM-derived EPLM when cultured on OP9 stromal cells plus IL-7 differentiated into pro-B cells phenotypically expressing CD19, CD79a, CD79b, Igll1 and Vpreb1 and genotypically having their D_H-J_H genes rearranged. In contrast to freshly isolated $CD19^{+}CD117^{+}$ pro-B cells, those derived from EPLM could be cultured long-term *in vitro*. Gene expression profiling did not reveal an obvious explanation for this difference in growth capacity. One possibility is that this is regulated via RNA modification or at the translational level [40-42].

Up to now, the long-term growth of pro-B cells required co-culture on stromal cells, yet the specific role of stromal cells in this culture system was unknown. For mouse B-cell development, IL-7 and its receptor components play a crucial role [11, 12, 43], best illustrated by the finding that mice deficient for IL-7 or its receptor components are practically devoid of pro-B cells. Additionally, pro-B cell growth is inhibited by an anti-CD117 antibody that blocks ligand (SCF) binding [18] and mice deficient for FLT3L or its receptor have a strongly reduced pro-B-cell compartment [15, 16, 19]. Taken together, these results suggest that IL-7, SCF, and FLT3L play a crucial role in early B-cell development. Indeed, we now show that EPLMs efficiently grow and differentiate into pro-B cells when cultured in the combined presence of

IL-7, SCF and FLT3L without contact with stromal cells. Thus pro-B-cell development and proliferation is not absolutely dependent on stromal cells and indirectly suggests that SCF and FLT3L can substitute for stromal cells. Moreover, we showed that following withdrawal of IL-7 from both stromal cell and stromal cell free cultures, propagated pro-B cells efficiently differentiated into IgM^+ B cells. This finding strongly supports our previous conclusion that IL-7 mediated proliferation of pro-B cells blocks their differentiation [44]. Previously it was shown that pro-B cells maintained on stromal cells plus IL-7 could reconstitute the B-cell compartment of immunodeficient mice [20]. Here we confirm and extend these findings. Thus both FL and BM EPLM-derived pro-B cells propagated either on stromal cells or stromal cell free could give rise to a significant B-cell compartment upon transfer into Rag2-deficient mice. However, FL-derived pro-B cells were about 40 fold more efficient at B-cell reconstitution than their BM-derived partners.

Recently it was shown that upon introduction of the *Lin28B* gene BM pro-B cells gained characteristics of FL-derived pro-B cells [27]. Indeed, as we show herein, *in vitro* propagated BM-derived pro-B cells transduced with the *Lin28B* gene could generate CD5 positive B cells *in vivo*. However, expression of the *Lin28B* gene did not improve the *in vivo*-reconstitution capacity of BM pro-B cells.

Previously, a relative enlargement of the MZB compartment was observed in mice with reduced B lymphopoiesis [45-49] and it was hypothesized that in B-lymphopenic mice, newly-formed B cells first fill up the MZB compartment. The preferred reconstitution of the MZB compartment could be due to the specialized environment within the splenic marginal zone. Additionally, the specificity of the BCR might play a role in that only those B cells with a certain specificity home to the marginal zone and once there, expand by proliferation [50].

When immunized with the T-cell independent antigen NIP-Ficoll, all reconstituted mice mounted an anti-NIP response indicating their B-cell compartments were functional. Mice reconstituted with BM-derived pro-B cells had a slightly lower IgM anti-NIP titer than wild type mice and showed a significant IgG anti-NIP response. However, this response was at least 10 fold lower than that

observed in wild type mice. Thus BM pro-B cell derived B cells are functional; the lower anti-NIP titer is most likely due to the relatively poor reconstitution capacity of these pro-B cells. In marked contrast, Rag2-deficient mice reconstituted with FL pro-B cells mounted an IgM and IgG anti-NIP response that was identical or even higher than that observed in wild type mice. This finding might suggest that B cells generated from fetal progenitors are the main anti-NIP responders in a primary immunization. In C57BL/6 mice, the primary anti-NIP antibodies are characterized by the very high usage of λ1 light chains [51]. Similar dominance of λ1 over κ light chain was observed in the anti-NIP response of pro-B cell reconstituted mice (data not shown). This finding might indicate that the B-cell repertoires that mount an anti-NIP response in the pro-B cell reconstituted mice are identical to those responding in wild type mice.

A rather surprising finding were the high IgG1 anti-NIP titers observed in FL pro-B cell reconstituted mice. Since the class switch to IgG1 is thought to be IL-4-dependent and reconstituted mice were devoid of T cells, the question arose which cell type was responsible for IL-4 production [29-31]. Given that pro-B cell reconstituted Rag2cγ double-deficient mice, which are deficient in ILCs, were also able to mount such a high IgG1 response makes it rather unlikely that ILCs were the source of IL-4 [37, 38]. This further suggests that mast cells, basophils and/or eosinophils, which are also able to produce IL-4 [32-35], might be involved in this class switching. FL pro-B cells were also able to reconstitute a functional B-cell compartment in T-cell contatining, B-cell deficient μMt mice (Supplementary Figure 3). This result might seem surprising given that the T-cell compartments in these mice may not be tolerant to mature B cells.

Recently we showed that pro-T cells propagated *in vitro* under stromal free conditions were able to reconstitute the T-cell compartments of T-cell deficient mice [23]. Here we show that the combined transfer of stromal cell free propagated pro-B and pro-T cells into Rag2-deficient mice results in the generation of a functional adaptive immune repertoire capable of mounting a T-cell dependent antibody response. However, it should be noted that the pro-T cell transfer has to be performed 2-3 weeks after the pro-B cell reconstitution. When both populations

were transferred together, mice showed a good T-cell but no B-cell reconstitution. This finding might suggest that under such combined transfer conditions T cells develop that are not tolerant to B cells and therefore eliminate them.

Overall the findings described here show that progenitor lymphocytes can be readily propagated under stromal free conditions *in vitro* and that these cells can be used to reconstitute mice with mature functional lymphocytes. Based on these results the establishment of stromal cell free culture systems for human lymphocyte progenitors might be of great interest since unlike stromal cell propagated lymphocyte progenitors, stromal cell free propagated cells could be potentially used for therapeutic purposes in patients with B and/or T-cell deficiencies.

Materials and Methods

Mice

Female C57BL/6 CD45.1 and CD45.2, C57BL/6 Rag2-deficient [52], and C57BL/6 Bcl2 transgenic mice [53] with 5–8 weeks of age were used. The appearance of vaginal plugs was counted as day 0 of gestation and embryos were taken at day E17.5. All mice were bred and maintained in our animal facility under specific pathogen free conditions. Animal experiments were carried out within institutional guidelines (authorization numbers 1886 and 1888 from Kantonales Veterinäramt, Basel).

Cell lines, cell culture, and supplements

The OP9 stromal cell line [54] was cultured as a monolayer in IMDM supplemented with 2% FBS, 5×10^{-5} M β-mercaptoethanol, 1 mM glutamine, 0.03% w/v Primatone (Quest, Naarden, The Netherlands), and 100 U/mL penicillin. For pro-B cell culture, $CD117^{+}B220^{+}CD19^{-}NK1.1^{-}$ cells were sorted from the FL or the femoral BM of adult mice and cultured at 10^{4}/ml in supplemented IMDM either on a semi-confluent layer of 30 Gy γ-irradiated OP9 stromal cells in the presence of 100U/ml IL-7, or without stromal cell support but in the presence of 100U/ml IL-7, 50ng/ml FLT3L, and 100ng/ml SCF. IL-7 was derived from culture supernatant of J558L cells transfected with murine IL-7 cDNA. Polyhistidin-tagged SCF was purified from transfected Rosetta pLacI bacteria using Ni-NTA-agarose beads (Qiagen, Venlo, NL). A vector expressing a human FLT3L-Fc fusion protein was expressed in Chinese hamster ovary cells. The supernatant was passed over a protein A-Sepharose (GE Healthcare, Chalfont St. Giles, GB) column in order to purify the protein. Pro-T cells were cultured as previously described [23].

Antibodies, flow cytometry, and sorting

FITC-, PE-, allophycocyanin-, or biotin-labeled mAbs specific for CD117, B220, CD19, NK1.1, IgM, Igκ, CD5, CD45.1, CD21, CD23, CD4, CD8α, and TCRβ were either purchased from BD Biosciences (Franklin Lakes, NJ, USA) or eBiosciences (San Diego, CA, USA), or purified from hybridoma culture supernatants according to standard procedures. Staining of the cells was performed as described before [55]. Flow cytometry was done using a FACS Calibur (BD Biosciences) and data were analyzed using the CellQuest Pro (BD Biosciences) or FlowJo Software (Treestar). For cell sorting, a FACSAria IIu (BD Biosciences) was used (>98% purity).

Transfer of cultured progenitor cells

Recipient mice were γ-irradiated using a Cobalt source (Gammacell 40, Atomic Energy of Canada, Ltd) 4 hours prior to reconstitution. The indicated number of pro-B or pro-T cells was then injected into the tail vein.

Immunohistochemical analysis

To analyze pro-B-cell derived B-cell localization in the spleen, the 5µm snap frozen and acetone-fixed sections were incubated with FITC-labelled anti-IgM (clone M41, self-made), APC-labelled anti-IgD (clone 1.19 self-made), and biotinylated anti-MOMA-1 (Vector, Burlingame, CA), which was revealed with PE-conjugated streptavidin (SouthernBiotech, Birmingham, AL). Confocal microscopy images were taken with a LSM 510 Meta (Zeiss, Oberkochen, D) and analyzed using the ImageJ software and the Fiji image-processing package.

NIP-specific antibody responses

Reconstituted mice were immunized subcutaneously with 100µg NIP-Ficoll or NIP-OVA in a 1:1 CFA emulsion. Serum IgM and IgG against NIP was analyzed at day 14 using ELISA as described in [56].

PCR analyses

The PCR conditions for amplifying D_HJ_H rearrangements were described elsewhere [4, 6, 57]. The primers used were D_H 5′-TTCAAAGCACAATGCCTGGCT-3′ and J_H3 5′-GTCTAGATTCTCACAAGAGTCCGATAGACCCTGG-3′. Oligonucleotide primers used for CD79a verification from pro-B cDNA were 5′-TGTTTGGGTCCCGGATGCCA-3′ and 5′-CACGCGGAGGTAAGTACCACA-3′, for CD79b 5′-TCTTCTCAGGTGAGCCGGTA-3′ and 5′- TATGGTTGGCGCTGTCACAT-3′, for Igll1 5′-AGTAGGACAGACTCTGGGCA-3′ and 5′- GGCTGACCTAGGATTGTGAGC-3′, for Vpreb1 5′-CTCCGGGTCCAAAGATACGAC-3′ and 5′-GCTCATAGCAACACCGCAGAA-3′, and for beta-actin 5′-GAAGTCTAGAGCAACATAGCACAGCTTCTC-3′ and 5′-GTGGGAATTCGTCAGAAGGACTCCTATGTG-3′.

Statistical analysis

Statistical analysis was performed with Prism 6.0g software (GraphPad Software, Inc.). Two-tailed unpaired Student t tests were used for statistical comparisons. If not differently indicated, data are presented as mean values ± SEM from three independent experiments (n.s. not significant or $P > 0.05$, * or + $P \leq 0.05$, ** or ++ $P \leq 0.01$, *** or +++ $P \leq 0.001$, **** $P \leq 0.0001$).

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Tables

Summary table 1

Differentially (Up and Down regulated) expressed genes identified from edgeR analysis (FDR, adjusted p-value < 0.05, fold change > 2) in sorted pro-B maintained on OP9 plus IL-7 (n=3) versus pro-B cells derived from sorted EPLM (n=3) cultured in the same way.

Up-regulated genes			
ENTREZID	**SYMBOL**	**Fold change**	**FDR**
69169	Faim3	6.22	9.56E-11
19699	Reln	4.76	0.0001
677296	Fcrl6	4.64	2.99E-18
14368	Fzd6	4.52	4.68E-07
17199	Mc1r	4.39	9.36E-16
77717	6030408B16Rik	4.3	4.59E-10
12290	Cacna1e	4.19	3.18E-06
270893	Tmem132e	3.78	3.13E-14
12797	Cnn1	3.68	7.54E-09
214791	Sertad4	3.14	6.97E-05
217071	Gm525	2.99	0.0007
22152	Tubb3	2.9	2.34E-20
72630	Hspa12b	2.85	2.22E-07
15019	H2-Q8	2.82	0.0037
110557	H2-Q6	2.8	0.004
211949	Spsb4	2.73	2.22E-05
94352	Loxl2	2.69	5.34E-06
14012	Mpzl2	2.67	4.90E-21
102371	Myzap	2.63	0.0023
20497	Slc12a3	2.49	1.87E-07
278795	Lrrc10b	2.42	0.0005
216881	Wscd1	2.42	0.0003
12522	Cd83	2.4	0.0236
56198	Heyl	2.4	3.56E-12
382077	Ccdc33	2.36	2.26E-05
20562	Slit1	2.36	4.28E-11
12931	Crlf1	2.34	6.21E-17

641361	Pinlyp	2.32	5.05E-05
26380	Esrrb	2.31	0.0388
109272	Mybpc1	2.31	7.35E-05
67937	Tmem59l	2.27	5.57E-14
13421	Dnase1l3	2.27	0.0011
12293	Cacna2d1	2.23	6.97E-05
13390	Dlx1	2.22	7.99E-07
12740	Cldn4	2.21	0.0035
209195	Clic6	2.2	0.0001
276829	Smtnl2	2.18	9.36E-16
16493	Kcna5	2.16	0.0002
11941	Atp2b2	2.15	0.0209
74050	4921525O09Rik	2.15	0.0173
104174	Gldc	2.14	9.00E-15
386750	Slitrk3	2.13	0.0025
11433	Acp5	2.08	0.0016
22092	Rsph1	2.08	8.77E-05
12578	Cdkn2a	2.07	0.0038
15375	Foxa1	2.07	0.02
16528	Kcnk4	2.06	0.0045
654812	Angptl7	2.06	0.0054
64011	Nrgn	2.04	0.0022
170757	Eltd1	2.04	0.0099
15016	H2-Q5	2.04	0.0173
14786	Grb7	2.03	9.48E-10
235633	Als2cl	2	0.0005

Down-regulated genes			
ENTREZID	**SYMBOL**	**Fold Change**	**FDR**
381310	6330403A02Rik	-7.8	1.62E-07
20202	S100a9	-3.97	0.0013
226610	Fam78b	-3.86	8.25E-07
12772	Ccr2	-3.73	2.39E-06
20201	S100a8	-3.63	2.48E-05
68713	Ifitm1	-3.55	0.0013
214968	Sema6d	-3.51	0.0055
12655	Chil3	-3.41	4.26E-05
667034	Pnp2	-3.26	0.0066
241633	Atp8b4	-3.21	2.93E-14
17523	Mpo	-2.97	0.0001
11801	Cd5l	-2.72	0.011
72310	Nkg7	-2.62	4.89E-08
70602	5730488B01Rik	-2.61	9.08E-07

20305	Ccl6	-2.53	4.86E-06
52024	Ankrd22	-2.51	0.0055
13733	Emr1	-2.44	0.0002
17105	Lyz2	-2.43	0.0008
12978	Csf1r	-2.37	0.0051
11639	Ak4	-2.37	0.0001
26368	Ceacam9	-2.31	0.0045
17476	Mpeg1	-2.25	0.0184
17381	Mmp12	-2.22	0.0055
13386	Dlk1	-2.21	0.0033
54373	Prss16	-2.09	0.0055
14728	Lilrb4	-2.08	0.0037
14425	Galnt3	-2.08	1.31E-06
330671	B4galnt4	-2.08	0.0141
18022	Nfe2	-2.05	0.001
17474	Clec4d	-2	0.0007

Summary table 2

Gene expression level (log2 FPKM) of 44 B-cell related genes in sorted pro-B maintained on OP9 plus IL-7 (n=3) and pro-B cells derived from sorted EPLM (n=3) cultured in the same way.

ENTREZID	SYMBOL	ProB.1	ProB.2	ProB.3	EPLM.1	EPLM.2	EPLM.3
108655	Foxp1	12.17	12.19	12.19	12.13	12.15	12.22
12043	Bcl2	8.40	8.53	8.54	9.43	9.32	9.29
12145	Cxcr5	13.15	13.15	13.26	12.81	12.66	12.61
12445	Ccnd3	15.96	15.93	15.84	16.07	16.03	16.01
12478	Cd19	15.00	14.95	15.03	14.80	14.88	14.95
12483	Cd22	11.68	11.57	11.44	11.31	11.42	11.62
12484	Cd24a	16.72	16.87	16.79	17.03	17.12	17.09
12494	Cd38	10.80	10.86	10.66	9.95	10.04	10.57
12505	Cd44	11.70	12.07	11.82	11.76	11.86	12.02
12515	Cd69	10.65	10.62	10.75	10.89	11.01	11.16
12517	Cd72	13.13	13.10	13.06	12.76	12.93	12.81
12518	Cd79a	15.65	15.61	15.62	15.53	15.48	15.46
12520	Cd81	14.62	14.87	14.57	14.37	14.33	14.52
13591	Ebf1	13.75	13.86	13.80	13.74	13.71	13.73
14678	Gnai2	15.23	15.24	15.15	15.15	15.12	15.13
15184	Hdac5	11.77	11.67	11.55	11.59	11.64	11.64
15985	Cd79b	15.69	15.61	15.67	15.55	15.60	15.66
16136	Igll1	17.04	17.04	17.14	17.09	17.06	17.06
16197	Il7r	11.56	11.73	11.72	11.42	11.71	11.70

16364	Irf4	12.37	12.43	12.39	11.85	11.92	11.89
16842	Lef1	13.93	13.78	13.90	13.86	13.80	13.80
17060	Blnk	14.34	14.27	14.38	14.43	14.46	14.42
17068	Ly6d	13.60	13.18	13.25	13.26	13.34	13.44
17210	Mcl1	13.43	13.45	13.48	13.27	13.39	13.48
17869	Myc	13.64	13.48	13.55	13.76	13.78	13.75
18507	Pax5	14.11	14.04	13.96	14.08	14.04	14.04
18707	Pik3cd	13.96	13.90	13.94	13.93	13.93	13.92
18751	Prkcb	12.84	12.89	12.95	12.91	12.94	12.99
18753	Prkcd	12.78	12.84	12.79	12.82	12.91	12.90
18985	Pou2af1	15.55	15.57	15.52	15.38	15.41	15.43
19265	Ptprcap	15.54	15.36	15.30	15.40	15.38	15.35
19373	Rag1	11.92	11.65	11.89	12.14	12.13	12.28
19374	Rag2	10.39	10.40	10.51	11.03	11.03	10.93
20677	Sox4	13.50	13.43	13.24	13.19	13.19	13.21
20877	Aurkb	13.46	13.47	13.43	13.56	13.59	13.58
20963	Syk	13.71	13.67	13.67	13.67	13.69	13.72
20969	Sdc1	12.18	12.54	12.11	11.92	12.04	12.20
21423	Tcf3	14.72	14.54	14.49	14.51	14.47	14.49
21673	Dntt	14.80	14.79	15.07	15.20	15.04	14.98
22362	Vpreb1	16.55	16.61	16.64	16.57	16.52	16.44
22778	Ikzf1	13.30	13.35	13.31	13.17	13.21	13.24
240754	Lax1	11.67	11.69	11.82	11.31	11.30	11.48
56458	Foxo1	12.38	12.31	12.34	12.26	12.24	12.31
72049	Tnfrsf13c	11.83	11.91	11.80	11.46	11.51	11.55

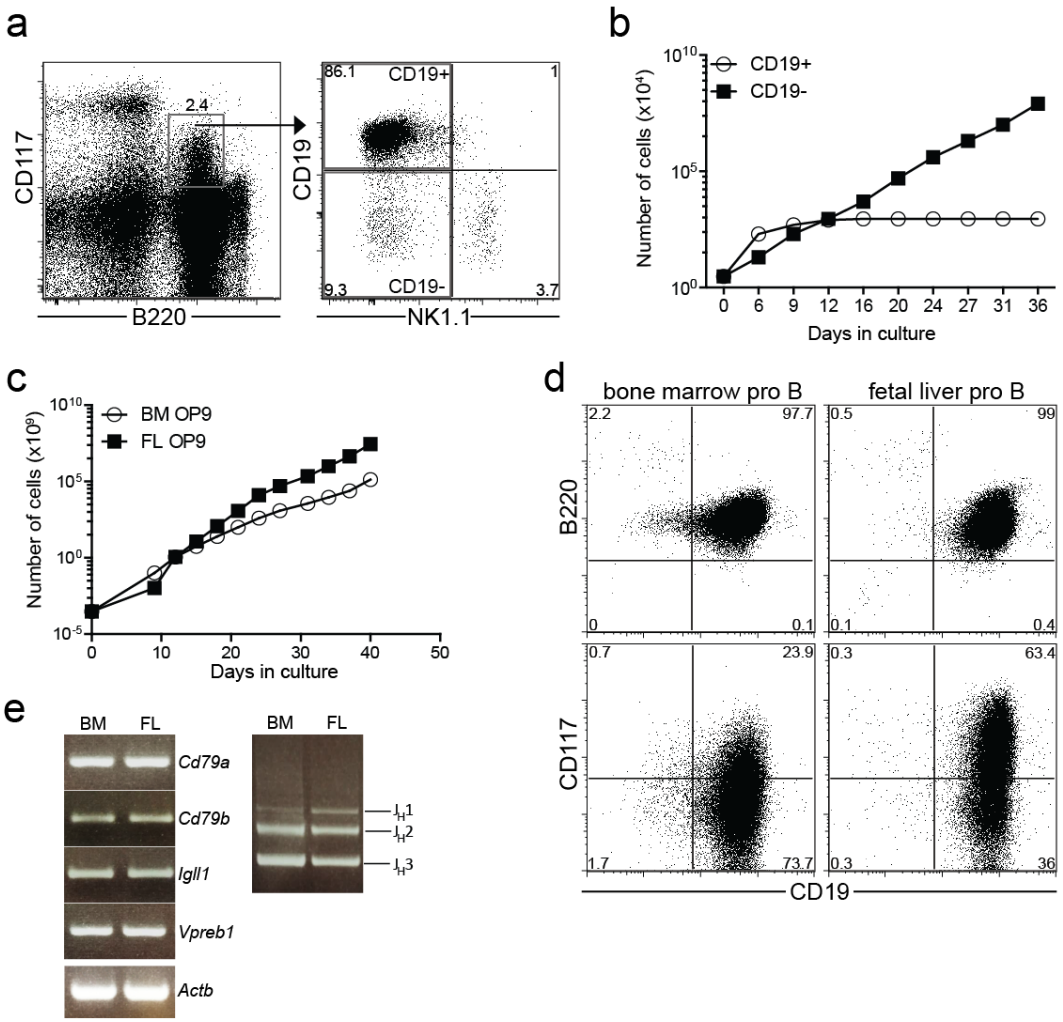


Figure 1: Establishment of EPLM derived long-term proliferating pro-B cell lines from BM and FL. (a) CD19 and NK1.1 expression by $B220^{+}CD117^{+}$ C57BL/6 BM cells. **(b)** Growth of sorted BM-derived $CD19^{+}B220^{+}CD117^{+}NK1.1^{-}$ cells and $CD19^{-}B220^{+}CD117^{+}NK1.1^{-}$ cells on OP9 stromal cells plus IL-7. **(c)** Comparison of the growth of FL and BM-derived $CD19^{-}B220^{+}CD117^{+}NK1.1^{-}$ cells on OP9 stromal cells plus IL-7. **(d)** B220, CD19 and CD117 expression by FL and BM-derived CD19-B220+CD117+NK1.1- cells cultured for 12 days on OP9 stromal cells plus IL-7. **(e)** RT-PCR expression analysis of Cd79a, Cd79b, Igll1, Vpreb1, and Actb by FL and BM-derived $CD19^{-}B220^{+}CD117^{+}NK1.1^{-}$ cells cultured for 12 days on OP9 stromal cells plus IL-7 and D_H-J_H rearrangement analysis of the same cells.

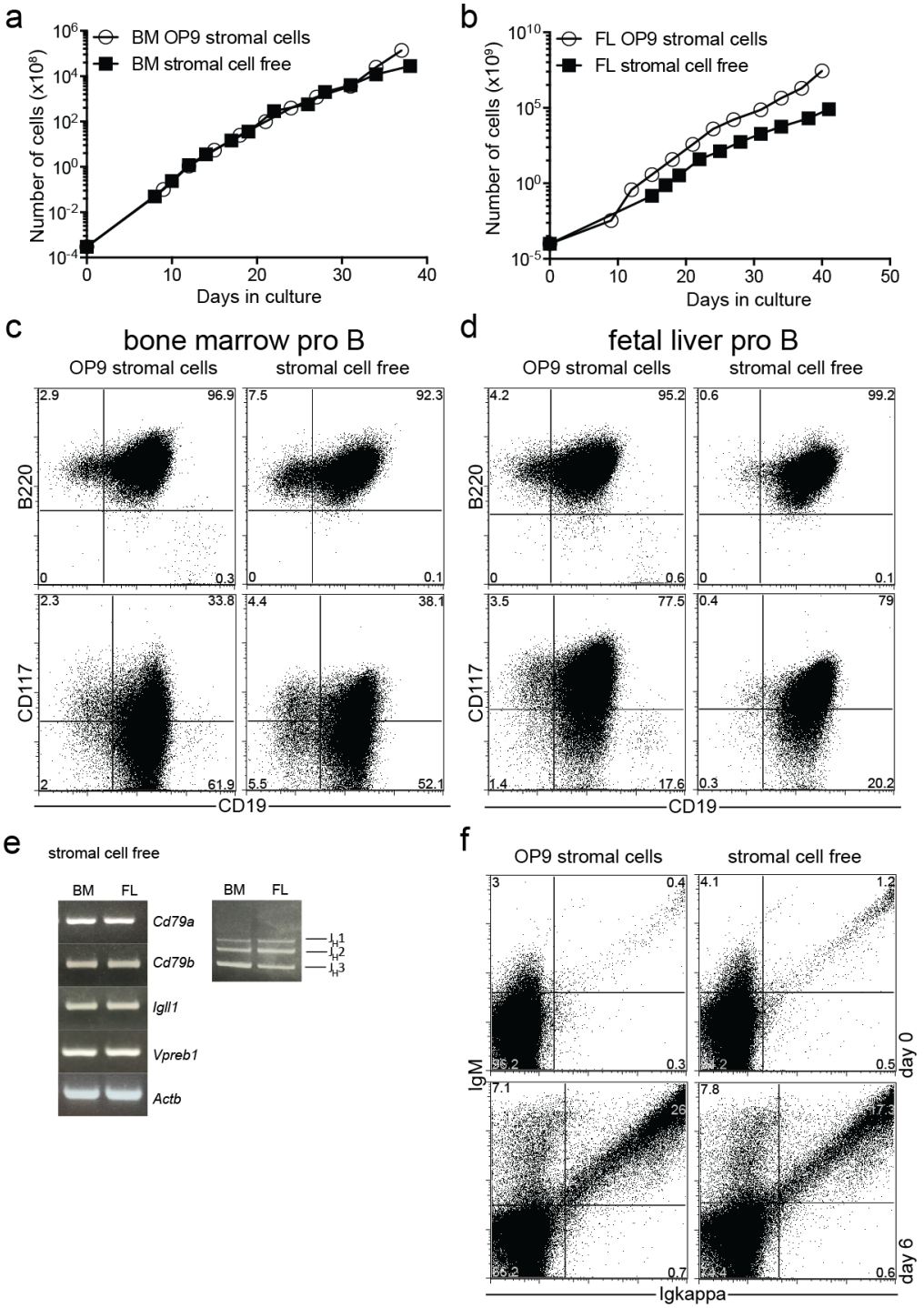


Figure 2: Establishment of EPLM-derived long-term proliferating pro-B cell lines from BM and FL under stromal cell free conditions. (a) Growth of BM-derived $CD19^{-}B220^{+}CD117^{+}NK1.1^{-}$ cells on OP9 stromal cells plus IL-7 or in the presence of IL-7, SCF and FLT3L in absence of stromal cells. **(b)** Growth of FL-derived $CD19^{-}B220^{+}CD117^{+}NK1.1^{-}$ cells on OP9 stromal cells plus IL-7 or in the presence of IL-7, SCF and FLT3L in absence of stromal cells. **(c)** B220, CD19 and CD117 expression by BM-

derived $CD19^{-}B220^{+}CD117^{+}NK1.1^{-}$ cells cultured on OP9 stromal cells plus IL-7 or cultured in the presence of IL-7, SCF and FLT3L in absence of stromal cells for 14 days. **(d)** B220, CD19 and CD117 expression by FL-derived $CD19^{-}B220^{+}CD117^{+}NK1.1^{-}$ cells cultured on OP9 stromal cells plus IL-7 or cultured in the presence of IL-7, SCF and FLT3L in absence of stromal cells for 14 days. **(e)** RT-PCR expression analysis of *Cd79a*, *Cd79b*, *Igll1*, *Vpreb1* and *Actb* by FL and BM-derived $CD19^{-}B220^{+}CD117^{+}NK1.1^{-}$ cells cultured for 14 days in the presence of IL-7, SCF and FLT3L and the absence of stromal cells and D_H-J_H rearrangement analysis of the same cells. **(f)** µH and Igκ expression by BM-derived $CD19^{-}B220^{+}CD117^{+}NK1.1^{-}$ cells from Bcl2 transgenic mice cultured on OP9 stromal cells plus IL-7 or cultured in the presence of IL-7, SCF and FLT3L (upper dot blots) and by the same cells cultured for 3 weeks as described above and then for 6 days in plain medium.

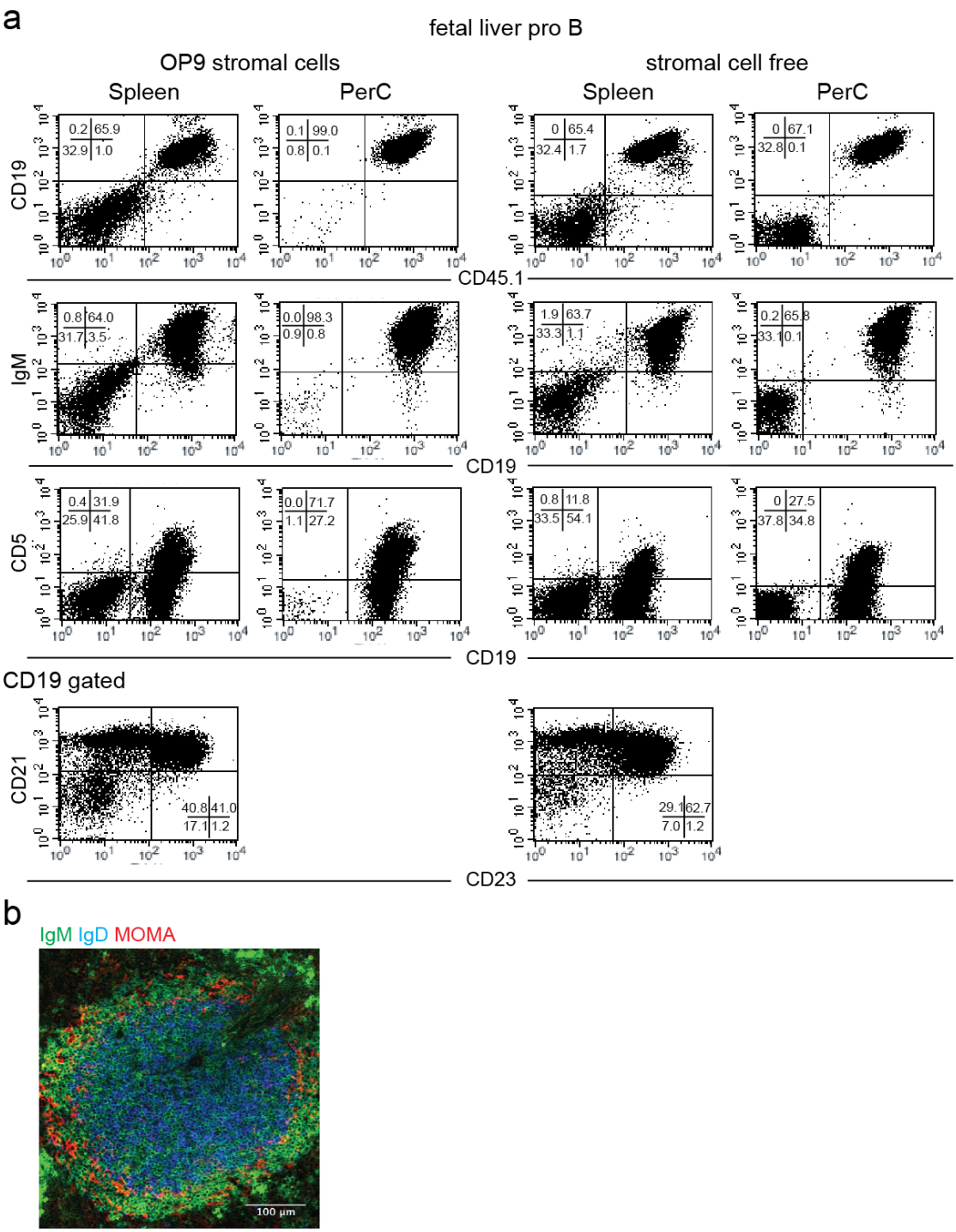


Figure 3: Reconstitution of sub-lethally irradiated CD45.2 C57BL/6 Rag2-deficient mice with CD45.1 FL-derived pro-B cells propagated on stromal cells or stromal cell free. (a) CD45.1, CD19, IgM and CD5 expression on spleen cells and PerC cells from CD45.2 C57BL/6 Rag2-deficient mice 8 weeks after transfer of FL-derived pro-B cells *in vitro* propagated on OP9 stromal cells (left panel of dot blots) or stromal cell free (right panel of dot plots). Also CD21 and CD23 expression on CD19

gated spleen cells are shown. **(b)** Representative picture of a staining for IgM (green), IgD (blue), and metallophilic macrophages (MOMA-1 in red) in spleens of C57BL/6 Rag2-deficient mice 8 weeks after transfer of FL-derived pro-B cells *in vitro*-propagated on OP9 stromal cells in the presence of IL-7.

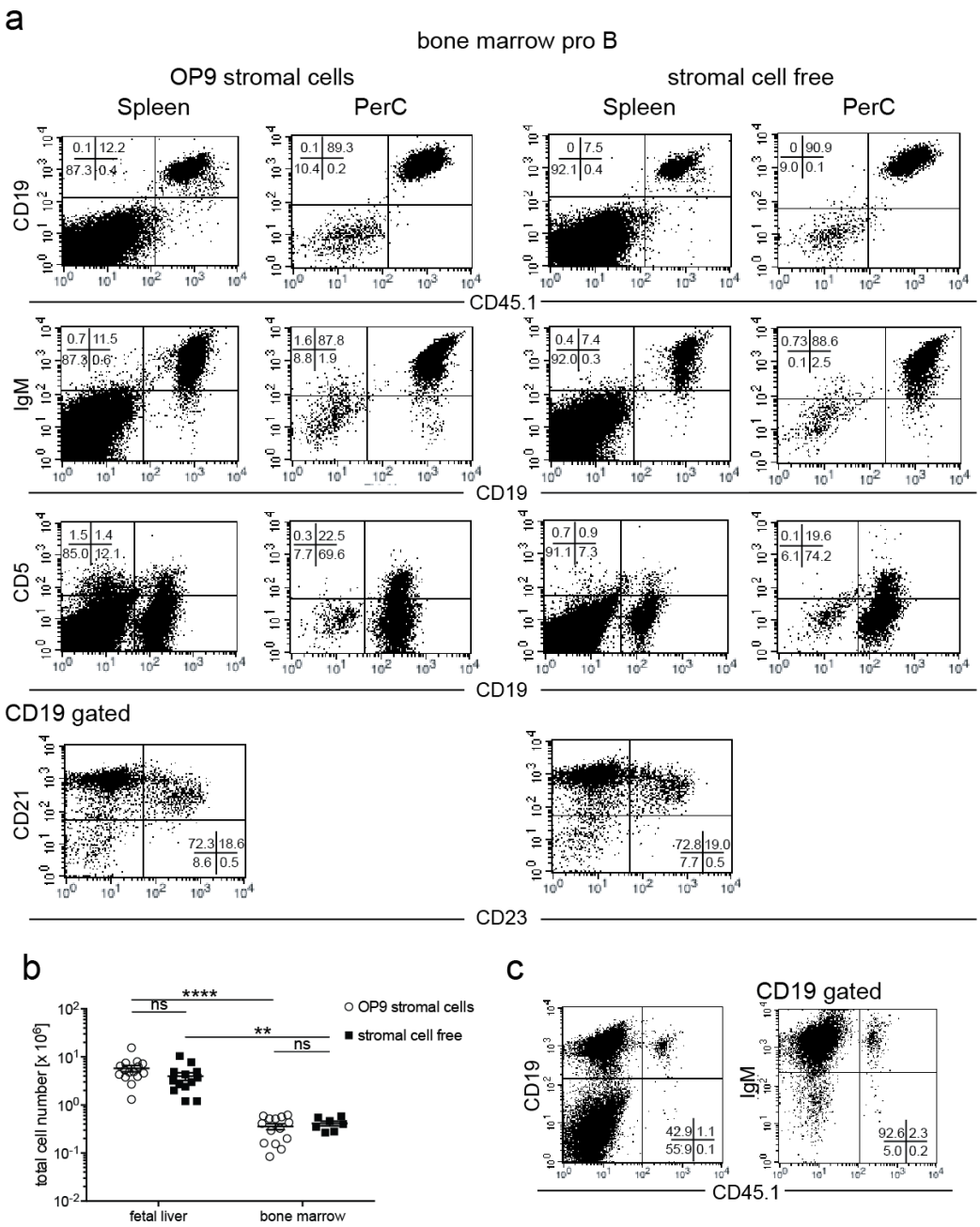


Figure 4: Reconstitution of sub-lethally irradiated CD45.2 C57BL/6 Rag2-deficient mice with CD45.1 BM-derived pro-B cells propagated on stromal cells or stromal cell free. (a) CD45.1, CD19, IgM and CD5 expression on spleen cells and PerC cells from CD45.2 C57BL/6 Rag2-deficient mice 8 weeks after transfer of BM-derived pro-B cells *in vitro* propagated on OP9 stromal cells (left panel of dot blots) or stromal cell free (right panel of dot plots). Also CD21 and CD23 expression on CD19 gated spleen cells are shown. **(b)** Absolute numbers of $CD19^+$ IgM^+ cells found in the spleens of Rag2-deficient mice reconstituted with FL pro-B propagated on OP9 stromal cells (n = 15), FL pro-B propagated stromal cell free (n = 13), BM pro-B

propagated on OP9 stromal cells (n = 14), BM pro-B propagated stromal cell free (n = 7). Mice were analyzed 8 – 12 weeks after pro-B cell transfer. Student's t test. Data shown above are mean ± SEM. ns: not significant or P> 0.05, **P ≤ 0.01 and ****P ≤ 0.0001. **(c)** CD19, IgM, and CD45.1 expression from a spleen of a CD45.2 C57BL/6 Rag2-deficient mouse 8 weeks after transfer of 5 x 10^6 CD45.2 FL-derived pro-B cells and 5 x 10^6 CD45.1 BM-derived pro-B cells. Both pro-B cells were propagated stromal cell free.

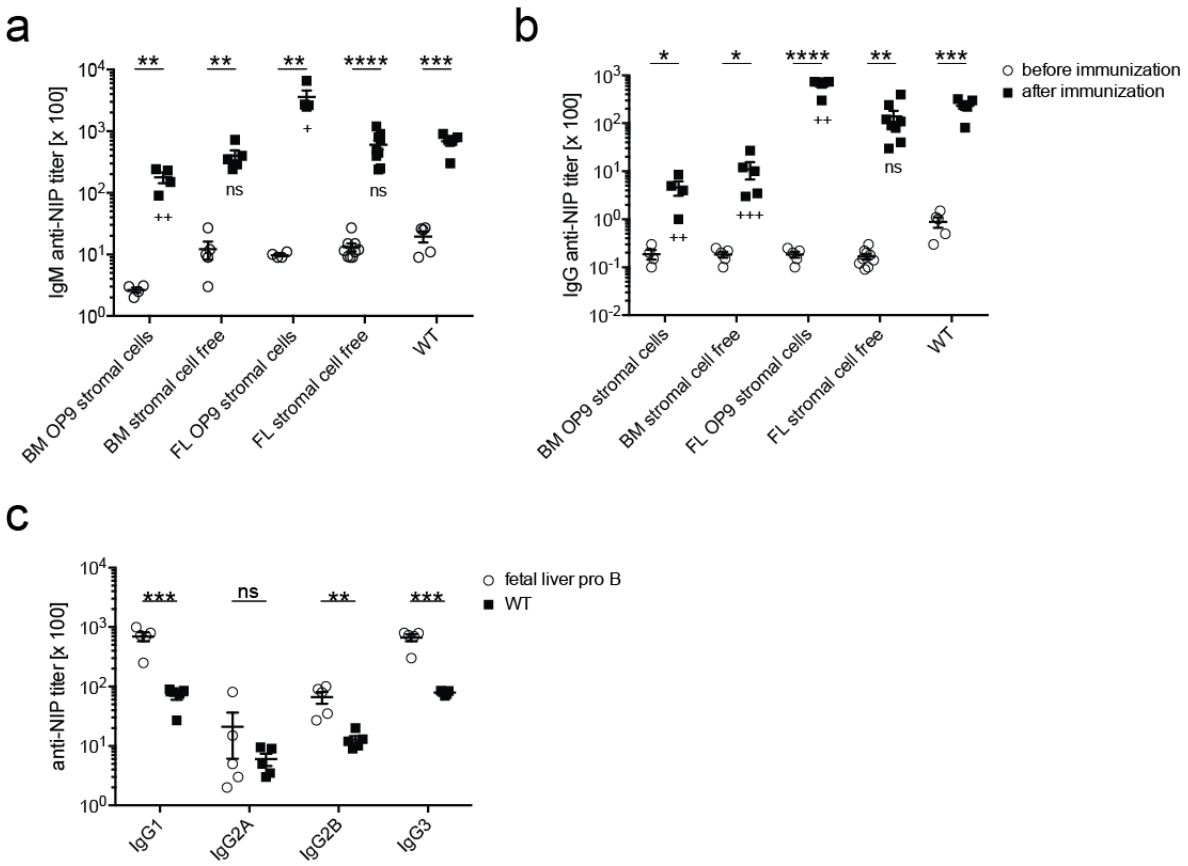


Figure 5: Anti-NIP response of C57BL/6 Rag2-deficient mice reconstituted with BM or FL pro-B cells propagated either on stromal cells or stromal cell free and immunized with NIP-Ficoll at 8 weeks after cell transfer. Normal C57BL/6 wild type mice were used as controls and statistical significance compared to wild type is indicated below after immunization. Titers were defined as the serum dilutions that gave 2 times background OD values in the ELISA. Titers were determined in sera taken 1 week before immunization (a and b open symbols) and 2 weeks after immunization (a and b closed symbols) **(a)** IgM anti-NIP titers. **(b)** IgG anti-NIP titers. **(c)** Anti-NIP IgG subclass titers in the serum of mice reconstituted with FL-derived pro-B cells and wild type mice. ns: not significant or $P > 0.05$, * or + $P \leq 0.05$, ** or ++ $P \leq 0.01$, *** or +++ $P \leq 0.001$, ****$P \leq 0.0001$. * represents significance compared to pre-bleed. + represents significance compared to titer of wild type mice. Student's t test. n = 5-8. Data shown above are mean ± SEM.

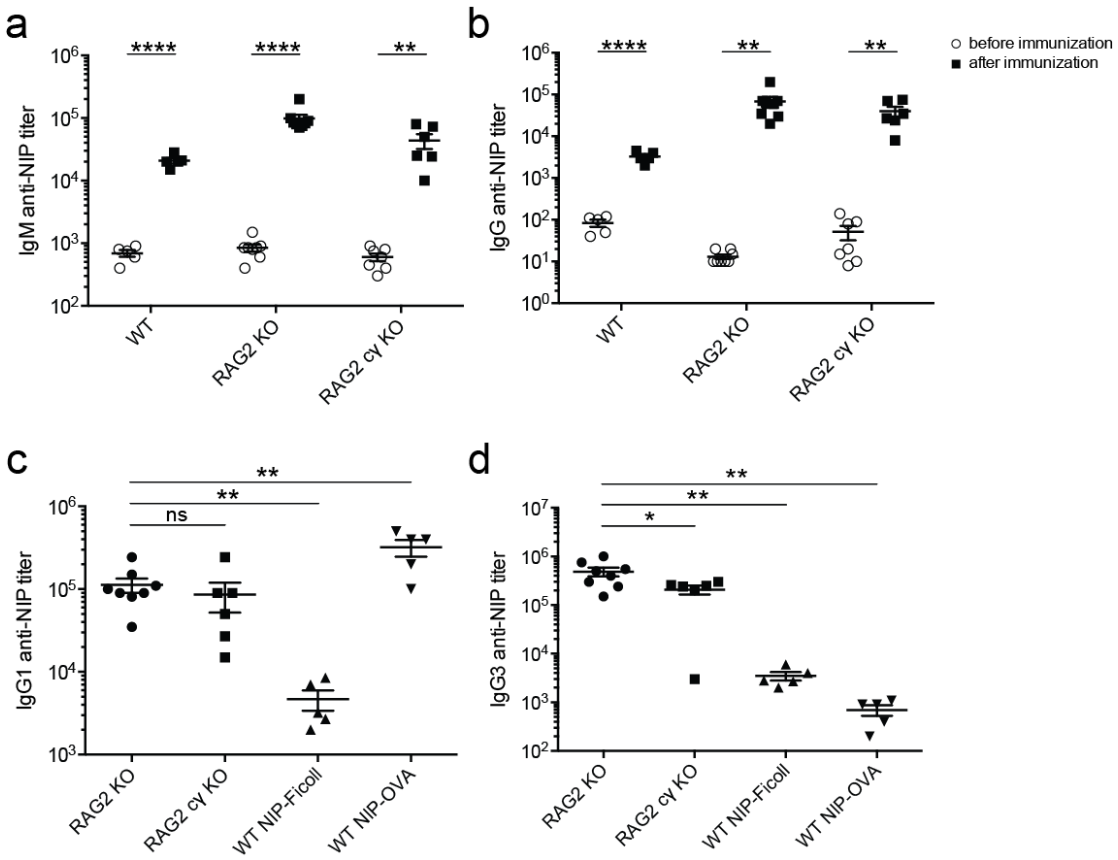


Figure 6: Anti-NIP response of C57BL/6 wild type mice, Rag2-deficient and Rag2cγ double-deficient mice reconstituted with FL pro-B cells propagated on stromal cells and immunized with NIP-Ficoll 8 weeks after cell transfer. Titers were defined as the serum dilutions that gave 2 times background OD values in the ELISA. Titers were determined in sera taken 1 week before immunization (a and b open symbols) and 2 weeks after immunization (a and b closed symbols) **(a)** IgM anti-NIP titers. **(b)** IgG anti-NIP titers. **(c)** IgG1 anti-NIP titers. Sera from C57BL/6 mice immunized with NIP-Ficoll or NIP-OVA were used as positive controls. **(d)** IgG3 anti-NIP titers. Sera from C57BL/6 mice immunized with NIP-Ficoll or NIP- OVA were used as positive controls. ns: not significant or $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$. Student's t test. n = 5-8. Data shown above are mean ± SEM.

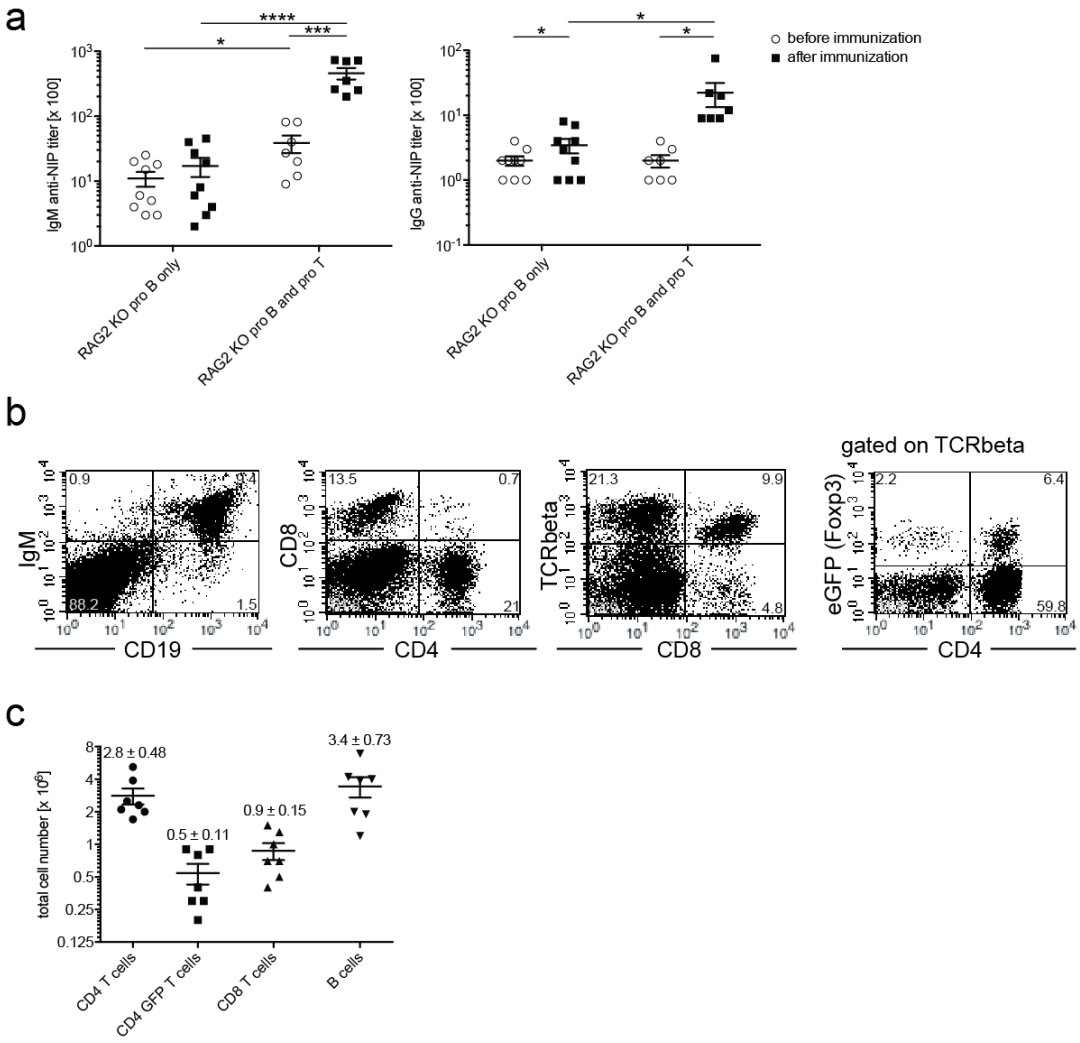
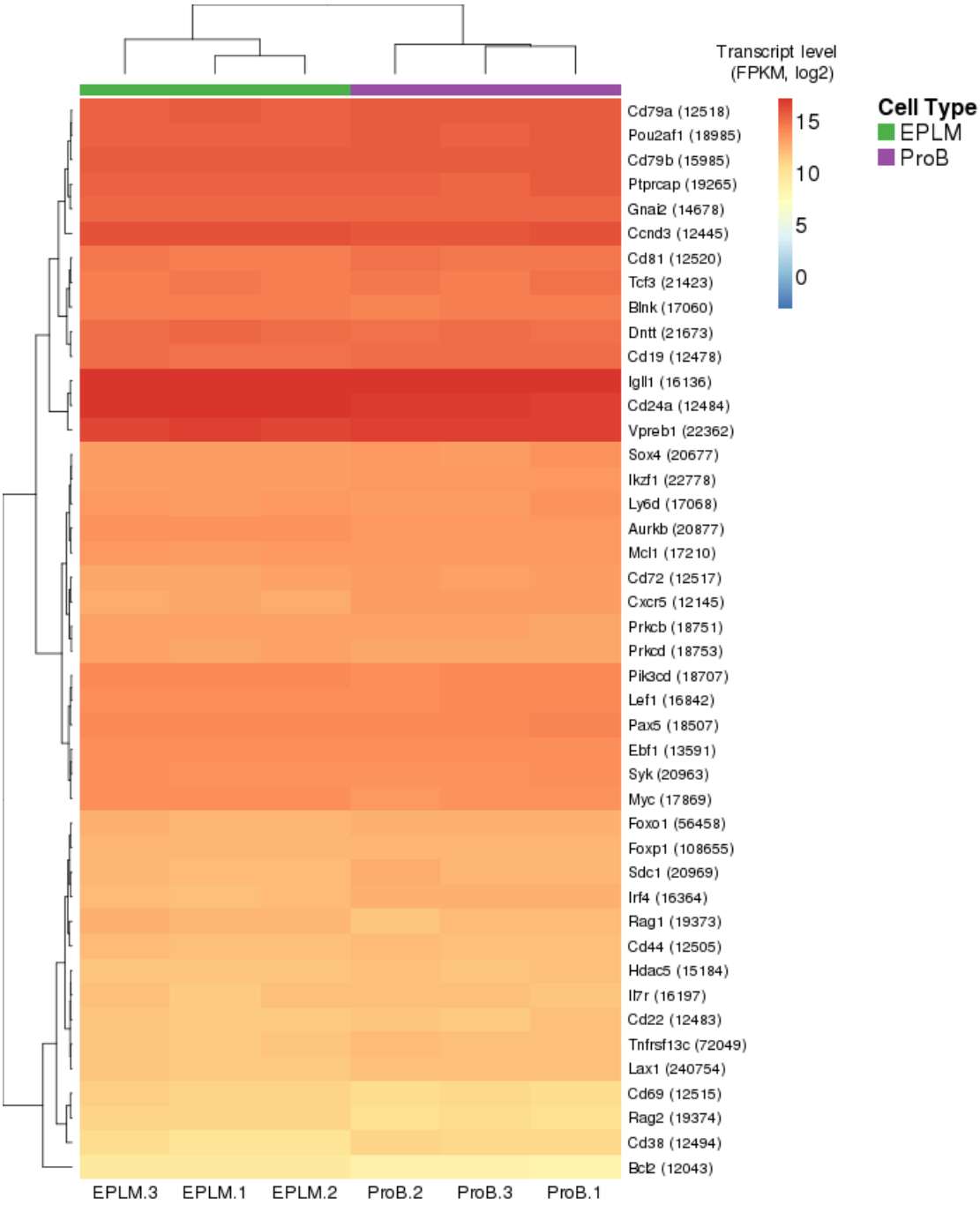
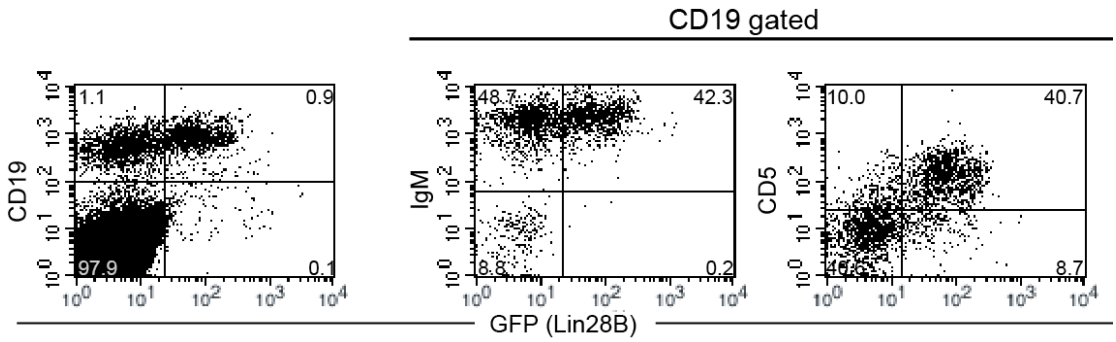


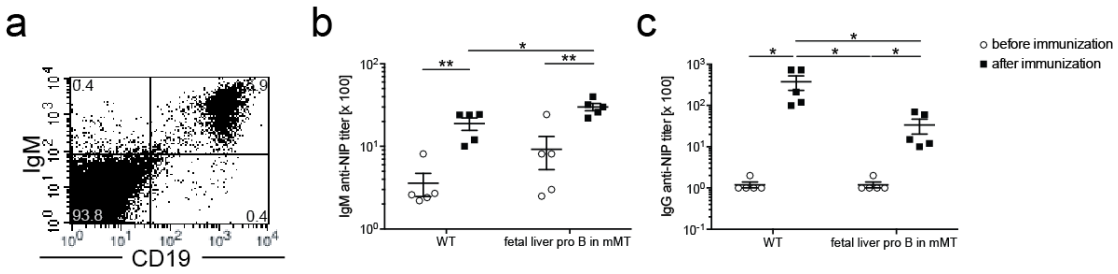
Figure 7: Reconstitution of C57BL/6 Rag2-deficient mice with *in vitro* propagated pro-B plus pro-T cells. (a) IgM and IgG anti NIP response of C57BL/6 Rag2-deficient mice reconstituted with pro-B cells or pro-B cells and pro-T cells and immunized with NIP-OVA 11 weeks after pro-B cell transfer (7 weeks after pro-T cell transfer). Anti-NIP titer was determined 2 weeks after immunization. Sera taken 1 week before immunization were used as negative controls. **(b)** IgM, CD19, CD4, CD8, TCRβ, and GFP expression by splenic cells derived from C57BL/6 Rag2-deficient mice reconstituted with pro-B cells and pro-T cells. Analysis was performed 12 weeks after pro-T cell transfer. **(c)** Absolute numbers of CD4, CD4-GFP and CD8 T cells and $CD19^+$ IgM^+ B cells found in the spleen of C57BL/6 Rag2-deficient mice reconstituted with pro-B and pro-T cells. Analysis was performed at 10-14 weeks after pro-T cell transfer. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Student's t test. n = 7-9. Data shown above are mean ± SEM.



Supplementary figure 1: B-cell related genes are equally expressed. Heatmap illustrating gene expression level of B-cell related genes (log2 FPKM) in sorted pro-B maintained on OP9 plus IL-7 (n=3) versus pro-B cells derived from sorted EPLM (n=3) cultured in the same way.



Supplementary Figure 2: *In vitro*-propagated BM-derived pro-B cells transduced with the *Lin28B* gene could generate CD5 positive B cells *in vivo.* CD19 and GFP expression and IgM, GFP and CD5 expression on CD19-gated cells recovered from C57BL/6 Rag2-deficient mice reconstituted with 5 x 10^6 BM-derived pro-B cells and 5 x 10^6 BM-derived *Lin28B-IRES-GFP* transduced pro-B cells. Analysis was performed 8 weeks after cell transfer. This has been observed in more than three independent experiments.



Supplementary Figure 3: Reconstitution of the B-cell compartment in µMt mice with FL-derived pro-B cells. (a) CD19 and IgM expression by µMT spleen cells 8 weeks after transfer of FL-derived pro-B cells. **(b)** IgM anti-NIP titer of µMT mice immunized with NIP-OVA at 8-10 weeks after pro-B cell transfer. Anti-NIP titers were determined in the sera taken 2 weeks after immunization. Sera taken 1 week before immunization were used as negative controls. Sera from wild type C57BL/6 mice immunized with NIP-OVA were used as positive controls. **(c)** IgG anti-NIP titer of µMT mice immunized with NIP-OVA at 8-10 weeks after pro-B cell transfer. Anti-NIP titers were determined in the sera taken 2 weeks after immunization. Sera taken 1 week before immunization were used as negative controls. Sera from wild type C57BL/6 mice immunized with NIP-OVA were used as positive controls. *P ≤ 0.05, **P ≤ 0.01. Student's t test. n = 5. Data shown above are mean ± SEM.

Supplemental Materials and Methods

Mice

Female C57BL/6 µMT mice [1] with 5–8 weeks of age were used. All mice were bred and maintained in our animal facility under specific pathogen free conditions. Animal experiments were carried out within institutional guidelines (authorization numbers 1886 and 1888 from Kantonales Veterinäramt, Basel).

Plasmids

Complete murine *Lin28b* cDNA amplified from mouse FL cDNA was cloned into the MigR1 retroviral expression vector. MigR1 was a gift from Warren Pear (Addgene plasmid # 27490) [2].

Supplemental references

1. Kitamura, D., et al., *A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene.* Nature, 1991. 350(6317): p. 423-6.
2. Pear, W.S., et al., *Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow.* Blood, 1998. 92(10): p. 3780-92.

IV The selection of mature B cells is critically dependent on the expression level of the co-receptor CD19

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The selection of mature B cells is critically dependent on the expression level of the co-receptor CD19

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ABSTRACT

CD19 plays a crucial role in mature B cell development as best exemplified by the finding that CD19 deficient mice have severely reduced mature B cell compartments (Engel et al., 1995; Rickert et al., 1995). In the present study we show that the transition into the mature B cell compartments is heavily dependent on the correct amount of CD19 expression. Thus, Nup-98–HoxB4 immortalized hematopoietic stem cells (HSCs) over-expressing CD19 show upon transplantation an impaired pro/pre B to immature B cell transition in the bone marrow, whereas Nup-98–HoxB4 HSCs expressing a shRNA that down-modulates CD19 expression show upon transplantation a strongly reduced mature B cell compartment. Overall our findings indicate that too high CD19 expression might result into too strong BCR signaling in the bone marrow and therefore causing negative selection. Too low CD19 expression might result into too little BCR signaling and thereby preventing the B cells to enter the mature pool (absence of positive selection).

1. Introduction

Early stages of B cell development take place in the bone marrow (BM), whereas final maturation takes place in the spleen. In order to get selected into the long-lived mature compartment B cells have to pass several crucial selection steps. Signal strength delivered from the B cell receptor (BCR) seems to be a key determination factor. This is best exemplified by the large number of BCR signaling mutants which show a developmental arrest [1]. Among the different modulators of BCR signaling, CD19 is known as positive regulator. Together with CD21, CD81, and CD225 it builds the co-receptor of the BCR [2,3]. CD19 has been shown to lower the threshold for B cell activation and decreases the amount of anti-IgM that is needed to induce DNA synthesis [4]. Upon ligation the conserved tyrosine residues of the cytoplasmic domain become phosphorylated and serve as binding sites for SH2 domains of regulatory proteins, such as PI3K and Lyn, thereby acting on BCR signaling [5,6]. However, also ligand-independent "tonic" signaling activity has been described for CD19, which is possibly involved in generating a basal signaling level essential for positive selection [7,8]. CD19 is a 95 kDa transmembrane protein which is first detectable on the surface of D_H–J_H rearranged proB cells and remains expressed until terminal differentiation into memory B cells. However, antibody secreting plasma cells are CD19 negative [9,10]. CD19 deficient mice have been described previously [11,12]. In these mice early stages of B cell development do not seem to be affected. However, they have reduced numbers of mature B cells suggesting that the transition from immature to mature B cells is impaired. Also mice over-expressing human CD19 have been generated [13]. The earliest stages of B cell development were not affected. However, beginning with IgM^+ immature cells in the bone marrow total numbers were reduced. Taken together these findings strongly suggest that CD19 plays a crucial role in the transition of immature to mature B cells.

In the present study we analyzed this phenomenon in more detail. In order to do this, we employed the recently described method of immortalizing multipotent hematopoietic cells with a retrovirus encoding a Nup98–HoxB4 fusion protein [14]. It was shown that upon injection into irradiated recipients these cells are able to give rise to all hematopoietic lineages [14]. We introduced in these cells a retrovirus encoding CD19 and a retrovirus containing a shRNA directed against CD19. Our *in vivo* findings with these cells show that early stages of B cell development are independent of the CD19 expression level, whereas only those immature B cells that express amounts of CD19 comparable to non-transduced B cells can be selected into the long-lived mature compartment.

Abbreviations: BCR, B cell receptor; FoB, follicular B cell; HSC, Hematopoietic stem cell; Ig, Immunoglobulin; MZB, marginal zone B cell; OV.EX. CD19, over-expression of murine CD19; shCD19 shRNA, directed against murine CD19 mRNA.

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2. Materials and methods

2.1. Mice

RAG-2 deficient mice were first described by Shinkai et al. and CD3ε deficient mice by Malissen et al. [15,16]. Animals were bred under pathogen-free conditions at the Center for Biomedicine at the University of Basel. Experiments were carried out within institutional guidelines with the permission of national or local authorities (permission numbers 1886 and 1888).

2.2. Generation of Nup98–HoxB4 transduced multipotent hematopoietic progenitor cells from CD3ε$^{-/-}$ mice

In order to "immortalize" hematopoietic progenitor cells of CD3ε$^{-/-}$ mice using the Nup98–HoxB4 construct we employed a method, that was first described by Sauvageau et al., and then further improved by Ruedl et al. [14,16,17]. In short, bone marrow of mice, treated with 5-Fluorouracil (5-FU), was transduced with the pMYc-IP vector containing a Nup98–HoxB4 fusion protein. After selection for transduced cells using the puromycin-resistance (1 μg/ml), cells were kept in culture by addition of 3% (v/v) IL-6 supernatant and 100 ng/ml stem cell factor (SCF).

2.3. Cell lines and cell culture

The CD19 positive Abelson virus-transformed preB cell line 40E1 was grown in IMDM supplemented with 5×10^{-5} M β-mercaptoethanol, 1 mM glutamine, 0.03% (w/v) Primatone (Quest; Naarden, NL), 100 U/ml penicillin, 100 μg/ml streptomycin and 5% (v/v) fetal bovine serum [18]. Once established, Nup98–HoxB4 transduced hematopoietic progenitor cells were maintained in the same IMDM, additionally supplemented with 3% (v/v) IL-6 supernatant and 100 ng/ml SCF.

2.4. Plasmids

Complete murine CD19 cDNA was cloned into the pMigR1-IRES-GFP expression vector. To decrease CD19 protein levels a shRNA (5′-CTCGAGAAGGTATATTGCTGTTGACAGT GAGCGCTCTGAGAAGCTGGCTTGGTATTAGTGAAGCCACAGATGTAA TACCAAGCCAGCTTCTCAGAATGCCTACTGCCTCGGAATTC-3′) targeting the beginning of exon 2 of murine CD19 mRNA was introduced into the MSCV-LTRmiR30-PIG (LMP) vector. The pMYc-NUP98–HOXB4-IP retroviral vector was a kind gift from Christiane Ruedl and Klaus Karjalainen. Its preparation was described before [14].

2.5. Transfection of phoenix-eco cells, retroviral transduction and sorting

Phoenix-eco cells were seeded in a 6-well plate at a density of 2×10^5 cells/well one day before transfection [19]. Medium was removed and transfection conducted using FuGENE HD (Promega; Fitchburg, WI) according to the manufacturer's protocol. Two days after transfection virus supernatant was collected and used immediately or stored at −80 °C. For transduction 10^6 target cells were resuspended in virus supernatant and spun 3 h at 1157 rcf and 33 °C. Successfully transduced cells were sorted using a FACSAria (BD Biosciences; Franklin Lakes, NJ) to >98% purity.

2.6. Transplantation

Mice were sublethally irradiated (4 Gy). For reconstitution 10×10^6 cells were injected intravenously. At around 5 weeks after transfer, mice were sacrificed and organ cell suspensions were prepared by mechanical disruption. Bone marrow was aspirated from the femur.

2.7. Antibodies and flow cytometry

Anti-CD19-PE (6D5) was purchased from BioLegend (San Diego, CA). Anti-B220-PE (RA3-6B2), anti-CD23-PE (B3B4), anti-CD19-PECy7 (1D3) and anti-Ter119-PE (TER-119) were purchased from BD Bioscience (Franklin Lakes, NJ). Anti-B220 (RA3 6B2), anti-IgM (M41) and anti-CD21 (7G6) were purified from hybridoma culture supernatants on protein G-Sepharose columns (Pharmacia) as recommended by the supplier. Purified monoclonal antibodies were conjugated with Alexa647 according to standard protocols. Staining of cells was performed as previously described [20]. Propidium iodide (Sigma–Aldrich; St. Louis, MO) was used at 0.5 μg/ml. Flow cytometry was performed using a FACSCalibur (BD Biosciences) and data were analyzed using the FlowJo (Tree Star; Ashland, OR) software.

3. Results

3.1. Generation of retroviruses encoding murine CD19 and containing shRNA directed against CD19

To analyze the effect of the CD19 "dose" on various B subpopulations, plasmids containing either complete murine CD19 cDNA or a shRNA directed against CD19 mRNA were generated. In both the sequence of interest was linked to a GFP cassette via an IRES sequence. In this way a broad spectrum of CD19 protein expression could be covered and easily monitored by GFP expression levels. Functionality of the various retroviruses was tested *in vitro* through transduction of the CD19$^+$ Abelson virus-transformed preB cell line 40E1 [18]. Non-transduced cells expressed identical levels of CD19 compared to the ones infected with the empty pMigR1 vector. In marked contrast, shRNA transduced GFP positive cells expressed about an order of magnitude less CD19 on the surface than non-transduced controls. On the other hand, GFP positive cells transduced with CD19 encoding retrovirus expressed about a 10 fold higher level of CD19 than non-transduced cells (Fig. 1A).

3.2. Generation of multipotent progenitors expressing CD19 or containing shRNA for down-modulation of CD19 expression

Recently it was shown that BM cells with hematopoietic stem cell properties (hereafter called HSCs) can be immortalized by expression of a Nup98–HoxB4 fusion protein [14]. Here we employed these cells in order to analyze the effect of over and/or under expression of CD19 on B cell development. Therefore, HSCs from CD3ε deficient mice were generated [16]. In brief, BM cells were collected from 5-Fluorouracil treated mice and cultured for 3 days in medium containing IL-6 and SCF. Thereafter, the cells were infected with a retrovirus encoding a Nup98–HoxB4 fusion protein and containing a puromycin resistance cassette. By day 3 of culture puromycin was added (1 μg/ml). After 3 days of culture with puromycin cells were harvested and infected with retroviruses containing the empty pMigR1-IRES-GFP expression vector, the CD19 encoding pMigR1-IRES-GFP expression vector and the MSCV-LMP vector containing the CD19 shRNA and GFP. After 4 days of reculture GFP expressing cells were FACS sorted and further propagated. As shown in Fig. 1B all transduced HSCs expressed robust levels of GFP. Moreover, HSCs transduced with the CD19 encoding pMigR1-IRES-GFP expression vector expressed homogenous levels of CD19 on their surface (Fig. 1B).

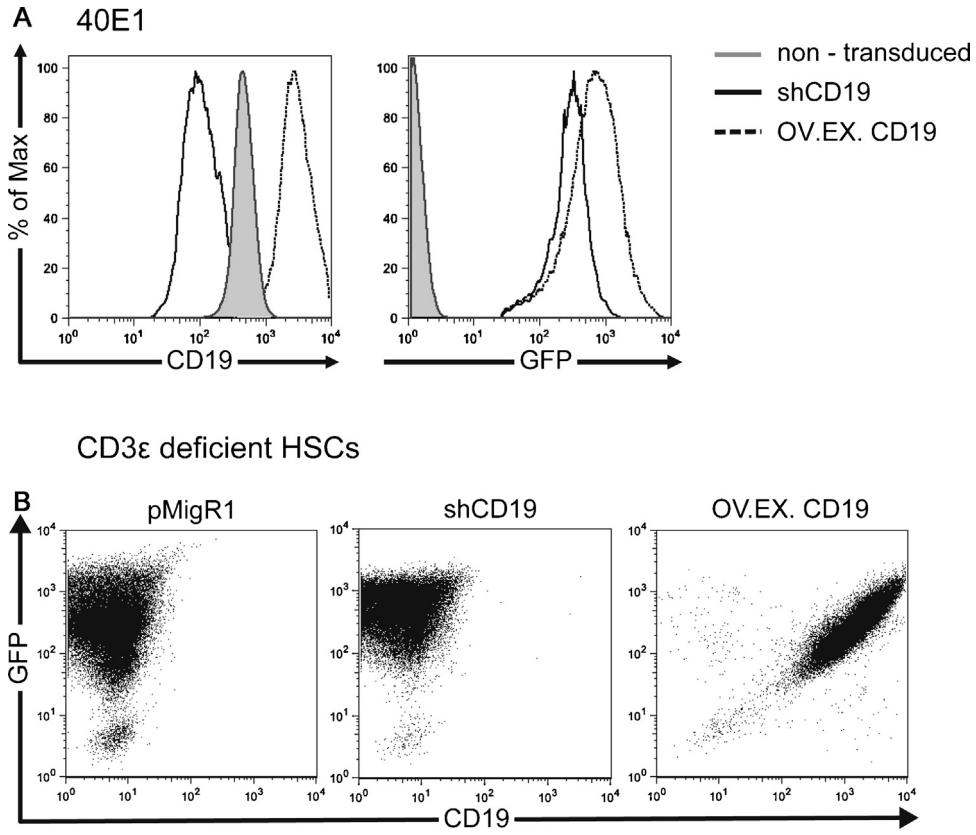

Fig. 1. *In vitro* testing of the various retroviral constructs. (A) Left histograms: CD19 expression on non-transduced 40E1 cells (gray histogram), CD19 expression on shCD19 transduced 40E1 cells (solid line histogram) and CD19 expression on OV.EX. CD19 transduced 40E1 cells (broken line histogram). Right histogram: GFP expression by non-transduced 40E1 cells (gray histograms), GFP expression by shCD19 transduced 40E1 cells (solid line histogram) and GFP expression by OV.EX. CD19 transduced 40E1 cells (broken line histogram). (B) GFP and CD19 expression by Nup98–HoxB4 HSCs from CD3ε deficient mice transduced with pMigR1 (control) retrovirus (left dot plot) shCD19 encoding retrovirus (middle dot plot) and OV.EX. CD19 encoding retrovirus (right dot plot).

3.3. Decreased CD19 expression does not influence bone marrow B cell development, whereas increased CD19 expression inhibits the formation of IgM⁺ bone marrow cells

In order to test the potential influence of increased or decreased CD19 expression on B cell development in the BM, the various transduced HSCs were intravenously injected into sublethally irradiated B6 RAG-2 deficient mice [15]. We used B6 CD3ε deficient HSCs, since we showed previously that RAG deficient mice reconstituted with wild type HSCs develop a fatal "syngeneic graft versus host disease" due to an impaired regulatory T cell development [21].

At around 5 weeks after transfer B cell development in these mice was analyzed. In the BM of all 3 groups over 90% of the cells expressed GFP indicating a very robust reconstitution (data not shown). CD19 expression analysis on B220 positive cells revealed that expression was practically identical to wild type cells on those derived from pMigR1 transduced HSCs (Fig. 2A and B). However, B220⁺ cells derived from CD19 shRNA transduced HSCs expressed about 4 fold less CD19 whereas those derived from CD19 over-expressing HSCs expressed about 4 fold more CD19 than wild type cells (Fig. 2A and B). The percentage of B220⁺ IgM⁻ cells found in the BM of pMigR1 and shCD19 transduced HSCs was practically identical, whereas this percentage was slightly lower in the group that received the CD19 over-expressing HSCs (Fig. 2C). Moreover, also the percentage of B220⁺ IgM⁺ cells found in the groups transplanted with pMiR1 and shCD19 HSCs was practically identical (Fig. 2D). However, in the mice that received the CD19 over-expressing HSCs the percentage of B220⁺ IgM⁺ cells was found to be 3–4 fold lower (Fig. 2D), indicating that too high expression of CD19 inhibits the transition from pro/pre to immature B cells in the BM. We also determined GFP expression levels by the various B cell subpopulations in the BM and used Ter119 positive cells as controls. As shown in Fig. 2E, Ter119⁺ cells (gray histogram), pro/pre B cells (dotted histogram) and immature B cells (solid line histogram) obtained from the BM of recipients of shCD19 transduced HSCs, all expressed the same level of GFP and thus strengthen the conclusion that down-modulation of CD19 does not influence B cell development in the BM. However, BM immature B (solid line histogram) cells from mice that received CD19 over-expressing HSCs seemed to express lower amounts of GFP than pro/pre B (dotted line histogram) and Ter119⁺ cells (gray histogram) (Fig. 2-F). This finding strengthens the conclusion that CD19 over-expression inhibits the transition from pro/pre to immature B cells in the BM.

3.4. CD19 over-expression and CD19 down-modulation severely reduce the splenic B cell compartments

In the spleens of the mice reconstituted with the 3 differently transduced HSCs over 70% of the cells expressed GFP indicating that our HSCs also gave a very robust peripheral reconstitution (data not shown). In marked contrast to the BM, CD19 expression by B220⁺ splenic cells derived from CD19 over-expressing HSCs was practically identical to CD19 expression by wild type B cells (gray histogram) (Fig. 3-A and B). Moreover, also the majority (over 60%) of B220⁺ splenic cells derived from the shCD19 HSCs expressed CD19 levels comparable to their wild type controls and only 40% expressed lower levels of CD19 (Fig. 3A and B). The latter cells presumably represent immature (transitional) B cells. Likely as a consequence of the counter selection of CD19 over- and under-expressing cells, IgM⁺ IgD⁺ B cell numbers found in the spleen of

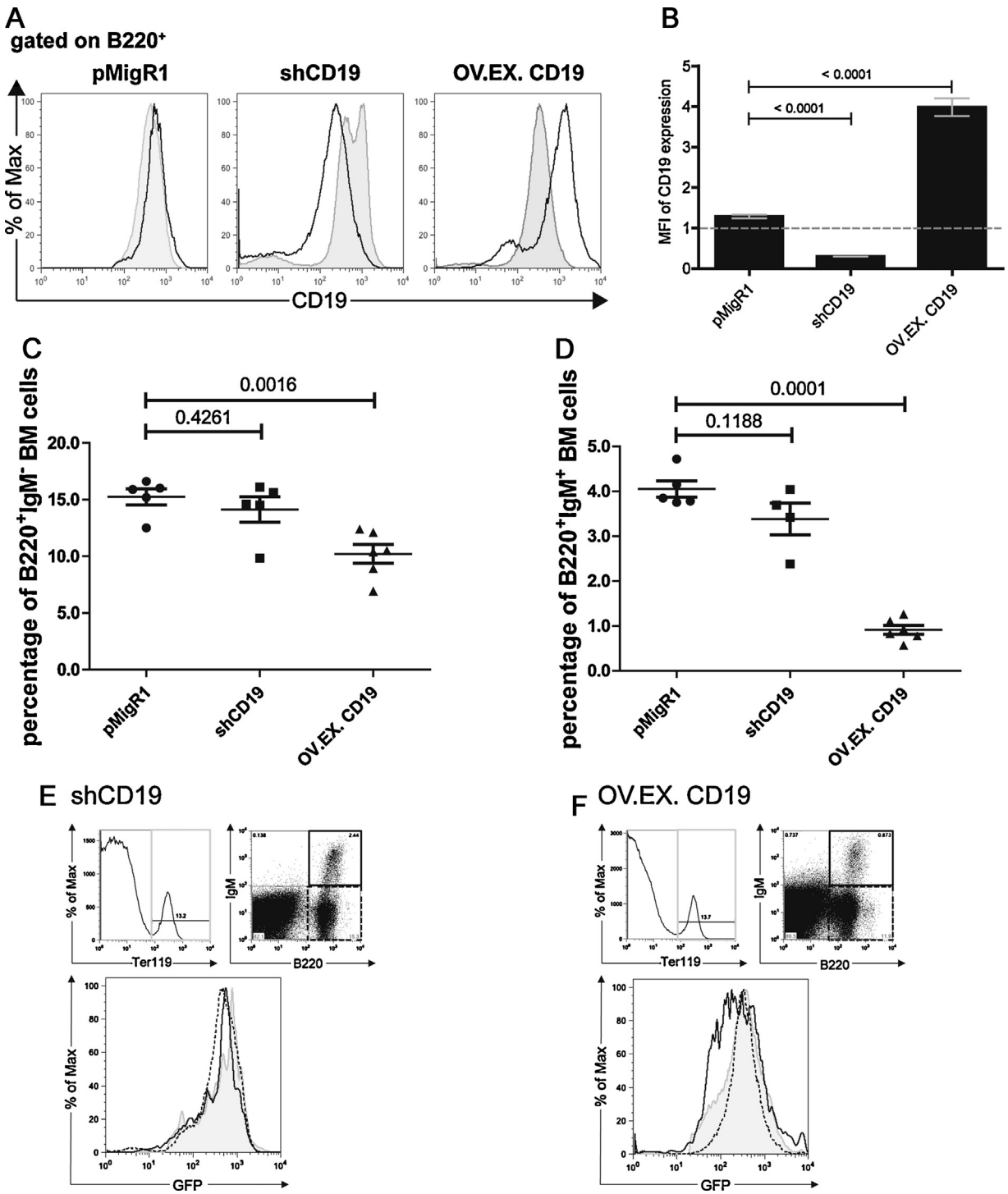

Fig. 2. B cell development in the BM of sublethally irradiated B6 RAG-2 deficient mice at 5 weeks after reconstitution with Nup98–HoxB4 HSCs transduced pMiR1 (control), shCD19 and OV.EX. CD19 expressing retroviruses. (A) CD19 expression on B220$^+$ BM cells derived from mice that received: left histogram pMigR1 transduced HSCs (gray histogram WT control and solid line histogram pMigR1 derived), middle histogram shCD19 transduced HSCs (gray histogram WT control, solid line histogram shCD19 HSCs) and right histogram OV.EX. CD19 HSCs (gray histogram WT control, solid line histogram OV.EX.CD19 HSCs). (B) Mean fluorescence index (MFI) of CD19 expression on B220$^+$ BM cells derived from the recipients of the various transduced HSCs. The MFI of wild type controls is defined as 1. $N = 5–6$. (C) Percentage of B220$^+$ IgM$^-$ cells in the BM of the recipients that received the various HSCs. (D) Percentage of B220$^+$ IgM$^+$ cells in the BM of the recipients that received the various HSCs. (E) GFP expression by Ter119$^+$ erythrocyte precursors (gray histogram), B220$^+$ IgM$^-$ cells (dotted line histogram) and B220$^+$ IgM$^+$ BM cells (solid line histogram) derived from recipients transplanted with shCD19 HSCs. Upper histogram and dot plot show the gating strategy. (F) GFP expression by Ter119$^+$ erythrocyte precursors (gray histogram), B220$^+$ IgM$^-$ cells (broken line histogram) and B220$^+$ IgM$^+$ BM cells (solid line histogram) derived from recipients transplanted with OV.EX. CD19 HSCs. Upper histogram and dot plot show the gating strategy.

mice reconstituted with shCD19 HSCs and CD19 over-expressing HSCs were severely reduced in comparison to those that received pMigR1 transduced control HSCs (Fig. 3C). The observed reduction in B cells affected the follicular (FoB) and marginal zone B (MZB) cell compartments in a similar way (Fig. 3D and E). Moreover, MZB and follicular B cells found in the spleens of mice reconstituted with shCD19 HSCs or CD19 over-expressing HSCs expressed similar levels of CD19 than the corresponding cells derived from wild type

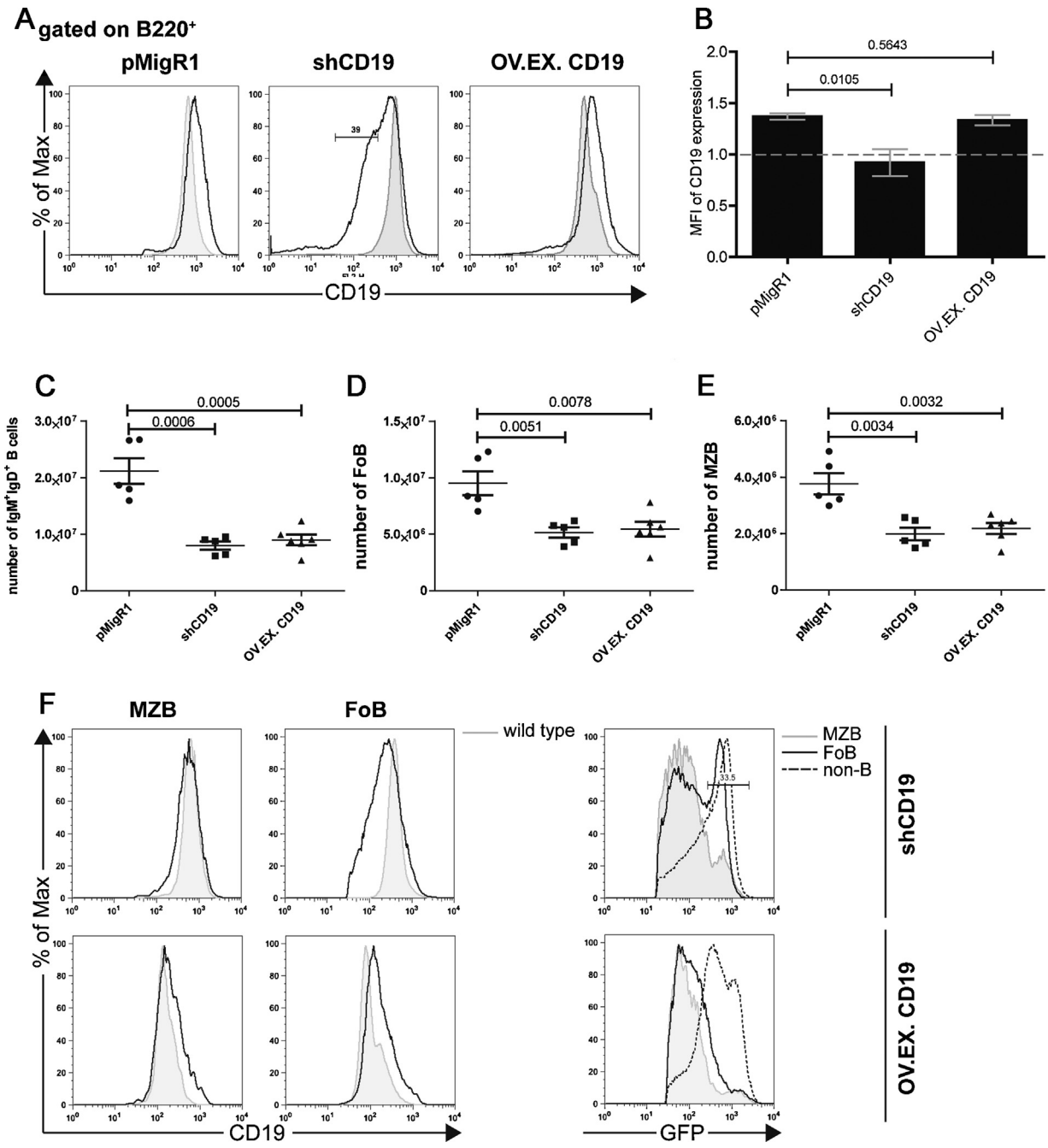

Fig. 3. Splenic B cell compartments of sublethally irradiated B6 RAG-2 deficient mice at 5 weeks after reconstitution with Nup98–HoxB4 HSCs transduced with pMiR1 (control), shCD19 and OV.EX. CD19 expressing retroviruses. (A) CD19 expression on B220$^+$ spleen cells derived from mice that received: left histogram pMigR1 transduced HSCs (gray histogram WT control and solid line histogram pMigR1 derived), middle histogram shCD19 transduced HSCs (gray histogram wild type control, solid line histogram shCD19 HSCs) and right histogram OV.EX. CD19 HSCs (gray histogram WT control, solid line histogram OV.EX. CD19 HSCs). (B) Mean fluorescence index (MFI) of CD19 on B220$^+$ splenocytes derived from the recipients of the various transduced HSCs. The MFI of wild type controls is defined as 1. $N = 5$–6. (C) Total number of IgM$^+$ IgD$^+$ splenic B cells found in the recipients of the various transduced HSCs. (D) The total number of follicular B cells (FoB) is defined as CD21^{intermediate} CD23^{high} found in the spleen of the recipients of the various transduced HSCs. (E) The total number of marginal zone B cells (MZB) is defined as CD21^{high} CD23^{low} found in the spleen of the recipients of the various transduced HSCs. (F) CD19 and GFP expression by MZB cells (defined as CD21^{high} CD23^{low}) and by follicular B cells (FoB) (defined as CD21^{intermediate} CD23^{high}) found in the spleens of recipients reconstituted with shCD19 transduced HSCs (upper histograms) and recipients reconstituted with CD19 over-expressing HSCs (lower histograms). Left hand histograms: CD19 expression by MZB cells and follicular B cells (gray histograms represent CD19 expression on corresponding WT control B cells). Right hand histograms: GFP expression of MZB cells (gray histograms), follicular B cells (solid line histogram) and non B cells (broken line histogram).

(gray histogram) mice (Fig. 3F). Furthermore, GFP expression levels were found to be strongly down-modulated in MZB and in 70% of the follicular B cells derived from shCD19 HSCs (Fig. 3D) and in all mature B cells derived from CD19 over-expressing HSCs (Fig. 3E) in comparison to non-B cells derived from the same HSCs (Fig. 3F). Thus, only B cells that express CD19 levels similar to wild type B cells were selected into mature B cell compartments.

4. Discussion

CD19 is known to be a specific co-receptor of the BCR, but its exact mode of action is not yet completely understood. Co-ligation of CD19 and IgM was shown to lower the threshold for B cell activation and proliferation in response to IgM cross-linking [4]. Therefore, CD19 is thought to act as a response regulator of

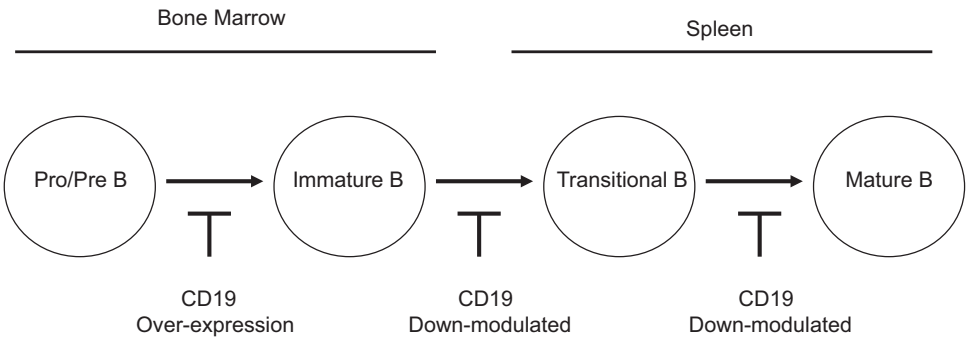

Fig. 4. Schematic representation of B cell development and the stages in which CD19 over-expression and CD19 under-expression can result in an impairment of this developmental process.

BCR signaling. Additionally, CD19 has also been implicated in a BCR-independent tonic signaling activity via the CD19/Lyn/Vav autonomous amplification loop [7,22]. The very important role of CD19 in B cell development was best revealed by the generation of a CD19 deficient mouse [11,12]. In these mice early stages of B cell development are non-altered however, mature B cell compartments are severely reduced [11,12].

In the present study we analyzed if B cell development is also influenced by lower or higher amounts of CD19 expression. In order to do so we employed the recently by Ruedl et al. developed Nup-98–HoxB4 immortalized HSC system [14]. These cells can be easily propagated *in vitro* and very efficiently can give rise to all hematopoietic lineages upon *in vivo* transplantation [14]. Moreover genes can be relatively easy introduced in these HSCs by retroviral infection [this manuscript and unpublished data]. However, the retroviral transduction efficiency is only around 5% as determined by GFP expressing retroviral vectors [unpublished observation]. An advantage of this low efficiency is that most likely only a single retroviral copy will be integrated per cell. Moreover, the expression of the introduced gene rather broad and can vary by two orders of magnitude as determined by FACS (Fig. 1B). Furthermore, the expression level of the gene of interest correlates very well with IRES GFP expression (Fig. 1B) i.e. low GFP expression–low expression of the gene of interest and high GFP expression–high expression of the gene of interest. Therefore the Nup-98–HoxB4 HSCs form to our opinion a unique experimental system to address the over-expression and silencing of genes in a quantitative way.

Here we used this system to analyze the effect of CD19 over-expression and CD19 silencing on B cell development. With respect to CD19 over-expression we observed an inhibition of B cell development already at the transition of pro/pre B to immature B cells in the BM. Thus, only the cells that expressed CD19 comparable to wild type cells were able to enter the immature pool. This finding suggests that CD19 over-expression results into strong BCR signaling leading to deletion and/or receptor editing [23,24]. Our finding that the pro/pre B cell compartment in the BM of the mice that received CD19 over-expressing HSCs was already significantly reduced (Fig. 2C) might indicate that immature B cells over-expressing CD19 cannot or only poorly be rescued by receptor editing.

CD19 silencing does not seem to influence B cell development in the BM. However, CD19 silencing results in severely impaired mature B cell formation. Therefore, CD19 down-modulation inhibits the migration of immature B cells from the BM into the spleen and/or due to too little BCR signaling might prevent the positive selection of splenic transitional B cells into the mature compartments. Currently we favor the second explanation and the finding that a significant fraction of $CD21^+$ $CD23^+$ cells express relatively high levels of GFP and could be transitional B cells might support this hypothesis. However, definite experimental proof for this is still missing. In Fig. 4 a schematic view of how CD19 over-expression and silencing can influence B cell development is shown.

Taken together the data presented here show that not only the mere expression of CD19 determines efficient B cell development, but that also the amount of CD19 expression plays a critical role in this developmental process. Therefore, under physiological conditions the regulation of CD19 expression is a crucial process. Since it was shown that CD19 expression is under control of the transcription factor Pax5, it might well be Pax5 activity regulates CD19 expression [25].

Finally, our findings that deregulated CD19 expression impairs B cell development might suggest that B cell deficiencies in patients could also be due to deregulated CD19 expression. The finding by van Zelm et al., that null-mutations in the *CD19* gene lead to an antibody-deficiency syndrome in humans, supports this hypothesis [26].

Conflict of interest

The authors have no financial conflict of interest.

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6. Conclusions and Perspectives

In man, hematopoiesis generates approximately 10^{11} to 10^{12} new blood cells daily in a healthy adult person [290]. Between HSCs and mature blood cells, progenitors undergo proliferation and progressive loss of developmental potentials. The process is governed by the combined use of soluble and membrane-bound factors. Thereby a stable gene expression profile is established in progenitors, which ultimately generates the unique phenotype of differentiated cells. The cells of the adaptive immune system undergo substantial somatic rearrangements of gene segments encoding their specific antigen-recognizing receptors. Thereby, double-strand breaks are introduced in the DNA, parts are excised, and eventually the ligated positions are complemented with non-templated nucleotide insertions. The enormous variety of receptor specificities emerging from the random recombination of gene segments can obviously generate cells recognizing self-structures. Sophisticated selection processes together with peripheral tolerance mechanisms ensure that the immune system fends off only foreign structures and intruders and is tolerant to self. At the same time a sufficient number of lymphocytes bearing differing antigen specific receptors needs to be maintained in order to ensure adequate protection. Despite the incredible number of differentiation, maturation, recombination, selection, and proliferation events occurring every day in the hematopoietic system, there are relatively few mistakes made, at least if failure is defined as its translation into an observable phenotype or disease setting. However, due to its complexity the process needs tight control mechanisms. In our laboratory we try to understand the molecular and cellular mechanisms and requirements guiding mouse lymphocyte development.

About a decade ago, our laboratory identified a new bone marrow progenitor cell population with lymphoid and myeloid potential. We called this cell the EPLM. Now we have found subpopulations within EPLM and that the multipotentiality of this population is reflected in a $Ly6D^{+}$ subpopulation, which is likely to contain the

direct progenitor of $CD19^+$ committed pre-BI B cells. IL-7 deficient mice display a severe B-cell defect and in these mutant mice, the $Ly6D^+$ subpopulation is absent. We observed a complete rescue of early B-cell populations in these mice when excess Flt3L was provided. It is known that fetal B-cell development is less dependent on IL-7 [211] than that of adults and that the Flt3L cytokine is able to drive IL-7-independent B lymphopoiesis [225]. Therefore excess Flt3L might mediate fetal-like hematopoiesis in the absence of IL-7. The fact that commitment to the B-cell lineage is possible without the assistance of IL-7 is in contrast to reports claiming that IL-7 is a non-redundant cytokine [2]. We could also show that besides Flt3L, Bcl2 rescued to some extent the B-cell defect of IL-7-deficient mice. Initial reports describing this phenomenon might have missed the effect due to the omitted IL-7 mediated proliferation [133, 134]. However, the recently described rescue of Stat5-deficient pro-B cells by transgenic Bcl2 expression agrees with our data [139]. The different mutant and transgenic mice we generated reveal that IL-7 acts as a pro-survival factor before commitment to the B-cell lineage and following commitment as a proliferative factor. In contrast, IL-7 is not an absolute requirement for B-cell commitment. Therefore we propose that no instructive signal is necessary for commitment of progenitor cells to the B-cell lineage. The B-cell fate might be the "default" course that is adopted if no other lineage option is assigned by commitment signals for example via Notch1 [104]. Thereby, in IL-7KO x Flt3L transgenic mice, the immense number of lymphoid progenitor cells is sufficient to generate detectable numbers of committed B cells.

We also developed *in vitro* cell culture systems for the long-term growth of functional mouse progenitor T as well as B lymphocytes. We were able to overcome the need for supporting stromal cell layers by adding soluble or plate-bound factors, which we think are indispensable for the propagation of early lymphocyte progenitors. In this way we circumvent co-culture systems where the strength and duration of signal delivery is difficult to control. We already used the stromal cell free pro-T cell culture system successfully and could show that the transition from DN3 to DP thymocytes is strictly dependent on Delta-like 4 induced Notch signaling [291]. Thereby we could confirm previously generated results, which were obtained

in a stromal cell based culture system [292]. Moreover using stromal cell free grown pro-T cells, we were able to investigate the epigenetic profile of early mouse T-cell development without contamination of transcripts from stromal cells [293]. This system mimics the bone marrow or thymus environment, respectively, in a way making the co-culture with stromal cells redundant. The successful propagation of progenitor cells without stromal cell support promotes the concept that lymphoid development is not strictly dependent on stromal cells. It all depends on the factors provided by the stromal cells and not on close proximity to the stromal cell body itself. One further indication for this is the presence of all normal stages of B-cell development in the secondary lymphoid organs of IL-7 transgenic mice [294]. This suggests that when IL-7 is readily available and no longer a limiting factor for the expansion of progenitor B cells, B-cell development can happen everywhere.

We aim to use these stromal cell free culture systems in the future to address the requirements for human lymphocyte development as well as to expand human T- and B-cell progenitors. Potentially, these cells could be used to treat lymphocytopenias caused for example by HIV infection or after chemo- or radiotherapy. Since these progenitors would originate from expansion cultures without stromal cell support, they are much more likely to be approved for human therapy. However, first attempts using $CD34^+$ cord blood derived progenitor cells were not successful. The differential growth capacities of human and mouse lymphocyte precursors might be attributed to the different life spans of mouse and man. In mice, development as well as aging is faster compared to the human system. For example the temporal maturation of the MZB cells in humans is only completed after 1-2 years, whereas in rodents it takes only 2-3 weeks [295]. Also the turnover rate of naïve T cells is much faster in mice than in man [296]. It might also be true that hematopoietic progenitor cells from humans grow much slower in tissue culture. Also in a recent publication by Kraus et al. the authors had to pass the human progenitor cells through different cytokine cocktails to get them to proliferate and to differentiate [297].

The striking difference in reconstitution capacity of bone marrow versus fetal liver derived pro-B cells, irrespective of whether they were grown with or without

stromal cell support, is still an unresolved phenomenon. Only few differentially expressed genes have been identified that characterize fetal versus bone marrow-derived precursors. One gene product we found differentially expressed amongst fetal liver and bone marrow derived pro-B as well as pro-T cells was the transcription factor Sox13. *Sox13* is a very interesting gene, because there are some clues that link it to fetal hematopoiesis. Sox13 has been shown to mediate the αβ-γδ lineage decision in the thymus [298] and γδ T cells are one of the innate-like lymphocyte subsets generated in distinct waves mainly during embryogenesis. When *Sox13* was first cloned and it's expression tracked in the mouse embryo it was found to be expressed in the arterial walls of E13.5 embryos [299]. From E11.5 onward the AGM region, in particular the ventral wall of the dorsal aorta, is the first site where definitive HSCs autonomously arise [300]. Therefore a closer investigation of the AGM region for *Sox13* expression might be worthwhile, even though *Sox13* remains expressed in arterial walls even after birth indicating a more general requirement for Sox13 in angiogenesis [299]. Unfortunately, a later study investigating the spatiotemporal expression pattern of Sox13 protein during mouse embryonic development did not describe expression in the arteriovascular system [301]. Also early fetal thymocytes expressed high levels of Sox13 and it was also found to be expressed in a day 18 thymocyte cell line [299].

More recently, Sox13 has been implicated in fine-tuning of the Wnt/Tcf pathway by directly interacting with the transcription factor Hhex (hematopoietically expressed homeobox) in the early embryo [302]. Hhex, in turn, is an important modulator of the earliest stages of definitive hematopoiesis [303]. Hhex itself has already been shown to be a downstream target of Sox17, which is a close relative of Sox13 and an important regulator of fetal liver derived HSCs [173, 304]. In addition to that, Hhex determined cKit expression in an immature T-cell leukemia mouse model [305]. In line with this observation, ectopic expression of *Sox13* in bone marrow derived pro-B cells results in increased expression of cKit *in vitro*. Yet *in vivo* reconstitution capability was still poor (unpublished observation). Nevertheless, it might be interesting to investigate a potential downstream effect of Sox13, acting on cKit expression via Hhex.

At least in our hands, none of the genes we found differentially expressed in fetal liver and bone marrow derived precursors could confer the quantitative reconstitution competence of fetal liver derived pro-B cells if they were expressed in bone marrow derived pro-B cells. For instance, when we transduced bone marrow derived pro-B cells with *Lin28b* we did not see increased reconstitution. However, the B-1 B cell compartment was overly represented suggesting therefore, that *Lin28b* expression in bone marrow derived pro-B cells conferred on them some qualities of fetal progenitors. It might well be that the superior reconstitution capacity of fetal liver derived pro-B cells, as well as their biased lineage fate, are consequences of independent transcriptional programs. Clearly the exceptional features of fetal liver derived progenitors are the consequence of a complex network of exclusively expressed or regulated factors, which largely remain to be elucidated.

Our stromal cell free cell culture systems for progenitor B and T cells are an ideal tool to closely investigate the cues mediating the divergent features of adult bone marrow and fetal liver derived lymphocyte development. Next we are planning to analyze the V_H repertoires of fetal liver and bone marrow-derived progenitors. Due to the absence of *Dntt* gene expression, encoding for the TdT enzyme, fetal liver derived lymphocytes lack non-germline nucleotide additions at the D_H-J_H and V_H-D_H junctions of the IgH chain [196, 306-308]. This strongly restricts CDR3 diversity and consequently the B-cell repertoire. It is possible, that the divergent repertoire in B cells is one of the main determinants instructing a different developmental fate characteristic of fetal progenitors. An indication for this is the elegant experiment performed by Lam et al., where they showed that replacing an “adult” with a “fetal” BCR conferred fetal properties to adult cells [202]. This supports our finding that the fetal and adult properties of progenitors are cell intrinsic.

Besides the above experiments, we also successfully employed the culture system recently developed by Ruedl et al. [3]. Bone marrow cells with hematopoietic stem cell properties can be immortalized by expression of a Nup98-HoxB4 fusion protein. Under appropriate culture conditions, they can be expanded and readily manipulated *in vitro*. At the same time they retain complete hematopoietic reconstitution capability upon injection into myelo-ablated recipient mice. Indeed

we used these stem cells to address the effect of varying CD19 expression levels on the development of B cells [309]. The CD19 molecule is part of the BCR co-receptor complex and is CD19 known to act as a positive regulator of BCR signaling activity [11]. From several mutants affecting BCR signaling it is known that B cells depend on a certain signal strength range for developmental progression into the mature B-cell compartment [4, 100, 310-312]. This is a possible explanation for the developmental block observed when either higher or lower than normal CD19 levels were expressed [309]. These might mimic an autoreactive or not fully functional BCR, respectively, resulting in failure of positive selection. That CD19 is able to modulate the responsiveness of cells to mitogenic stimuli has already been shown in CD19 transgenic and CD19 deficient mice [272, 279]. Wild type B-1 B cells express around 50% more CD19 molecules on their cell surface compared to B-2 B cells [95] and their development depends on the amount of CD19. This is best illustrated by the dramatic loss of B-1 B cells in CD19-deficient mice and their increase in hCD19tg mice [95, 272, 280]. In our system, we could not specifically address the role of CD19 in B-1 B-cell development because the establishment of Nup98-HoxB4 HSCs was so far only successful from adult bone marrow derived cells and B-1 B cells are generated most efficiently from fetal progenitors [206]. In its role as response regulator of BCR-mediated signals [285] aberrant expression of CD19 can influence B-1 B-cell generation [272, 279, 280]. This is in accordance with other mutations affecting BCR signaling, which have the same effect [96, 313-317]. A similar kind of positive selection acts in the splenic MZB-FoB lineage decision [201, 318]. With the Nup98-HoxB4 HSC reconstitution system we observed that the reduced B-cell output caused by aberrant CD19 expression preferentially generated MZB [309]. This has also been observed in other B lymphopenic mice [201, 211, 295, 319] and even when we reconstituted Rag2-deficient mice with bone marrow derived pro-B cells or µMt mice with fetal liver derived pro-B cells. By generating MZB the most rapidly responding B-cell population located at the interface with the environment becomes established first. This might reflect the evolutionary pressure acting on the immune system to provide a first-line of defense even if the slower T-cell dependent immunity is not fully established. This is in contrast to what is happening during ontogeny. In mice, the marginal zone is only fully established two to three weeks

after birth [320]. In this special neonatal setting, maternal antibodies as well as fetally generated B-1 B cells serve as an immediate line of defense prior to the emergence of a functional T-cell dependent immune system. The differential appearance of MZB versus FoB cell compartments has implications for neonatal vaccinology in man.

Mutations in the *CD19* gene were also associated with an antibody-deficiency syndrome in humans [321-323]. The disruption of the CD19 complex caused impaired BCR signaling and thereby impaired humoral immune responses. Since in more than 90% of patients diagnosed with primary immunodeficiencies no associated genetic defect has yet been found it might be worth comparing CD19 levels expressed by the remaining B cells. It is very likely that complex genetic networks and not a single gene mutation are responsible for disease manifestations. When these genetic defects impair CD19 expression, this might cause the disease.

In summary, the results presented in this thesis show:

1. We could identify the direct progenitor of committed $CD19^+$ B cells within the EPLM $Ly6D^+$ subpopulation.
2. We showed that IL-7 is not absolutely required for commitment to the B-cell lineage and most likely acts as a permissive factor.
3. We developed stromal cell free *in vitro* culture systems for the long-term propagation of functional bone marrow and fetal liver derived progenitor T and B cells.
4. We could show the importance of fine-tuning CD19 expression levels on B-cell selection.

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8. Appendix

Further publication

Versatility of stem and progenitor cells and the instructive actions of cytokines shape haematopoiesis

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REVIEW ARTICLE

Versatility of stem and progenitor cells and the instructive actions of cytokines on hematopoiesis

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Abstract

For many years, developing hematopoietic cells have been strictly compartmentalized into a rare population of multi-potent self-renewing hematopoietic stem cells (HSC), multi-potent hematopoietic progenitor cells (MPP) that are undergoing commitment to particular lineage fates, and recognizable precursor cells that mature towards functional blood and immune cells. A single route to each end-cell type is prescribed in the "classical" model for the architecture of hematopoiesis. Recent findings have led to the viewpoint that HSCs and MPPs are more versatile than previously thought. Underlying this are multiple routes to a particular fate and cells having clandestine fate options even when they have progressed some way along a pathway. The primary role of cytokines during hematopoiesis has long been seen to be regulation of the survival and proliferation of developing hematopoietic cells. Some cytokines now clearly have instructive actions on cell-fate decisions. All this leads to a new way of viewing hematopoiesis whereby versatile HSC and MPP are directed towards lineage outcomes via cytokine regulated cell-fate decisions. This means greater flexibility to the shaping of hematopoiesis.

Abbreviations: **ALL**: acute lymphoblastic leukemia; **AML**: acute myeloid leukemia; **CIC**: cancer-initiating cells; **CLP**: common lymphoid progenitor; **CMP**: common myeloid progenitor; **CSC**: cancer stem cell; **DC**: dendritic cell; **DN**: double-negative; **DP**: double-positive; **EBF**: early B cell factor; **Epo**; erythropoietin; **EPLM**: early progenitors with lymphoid and myeloid potential; **ETP**: early thymocyte progenitors; **Flt3**: Fms-like tyrosine kinase 3 receptor; **Flt3L**: Flt3 ligand; **G-CSF**: granulocyte colony-stimulating factor; **GM-CFU**: granulocyte/macrophage colony-forming unit; **GM-CSF**: granulocyte/macrophage colony-stimulating factor; **GMP**: granulocyte and monocyte progenitor; **HPC**: hematopoietic progenitor cells; **HSC**: hematopoietic stem cell; **IL**: interleukin; **IL-7R**: interleukin-7 receptor; **LIC**: leukemia-initiating cell; **LMPP**: lymphoid-primed multi-potent progenitors; **LSC**: leukemia stem cells; **LSK**: lineage markers , Sca-1$^+$, c-Kit$^+$ population of bone marrow cells; **LT-HSC**: long-term reconstituting hematopoietic stem cell; **M-CSF**: macrophage colony-stimulating factor; **MegE**; megakaryocyte/erythroid; **MEP**: megakaryocyte and erythroid progenitor; **MPP**: multi-potent hematopoietic progenitor cells; **NK**: natural killer cell; **SCF**: stem cell factor; **SLAM**: signaling lymphocyte activation molecule; **SOCS**: suppressor of cytokine signaling; **ST-HSC**: short-term reconstituting hematopoietic stem cell; **TCR**: T cell receptor; **TGF-β1**: transforming growth factor-β1; **Tpo**: thrombopoietin; **TSP**: thymus-settling progenitor; **vWF**: von-Willebrand factor

Keywords

Cell differentiation, cell-fate decisions, cytokines, hematopoiesis, leukemia

History

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Introduction

The hematopoietic stem cell (HSC) gives rise to a wide range of blood and immune cell types. The generation of large numbers of each cell type is a complex and tightly regulated process that is ultimately governed by commitment of rare, and generally quiescent, HSCs to pathways of cell differentiation. These cells, which reside within the bone marrow in adult mammals, are the apex of the hematopoietic hierarchy. HSCs, which can self-renew, give rise to multi-potent

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progenitor cells (MPPs) that undergo decision-making, expansion, and differentiation, via recognizable lineage precursors, to give rise to the final compartment of functional cells. Since the early 1980s, the end-cell types have been viewed as two families: lymphoid that includes B and T lymphocytes and natural killer (NK) cells, and myeloid consisting of the rest of the blood and immune cells. Commensurate with this has been the identification of progenitors of each family; namely the common lymphoid progenitor (CLP)[1] and common myeloid progenitor (CMP)[2]. This lymphoid/myeloid dichotomy is the basis of a long-standing model for the architecture of hematopoiesis which also encompasses single routes of differentiation towards individual fates[3]. Over the past 15 years, the above strict architecture has become less black and white. HSCs and hematopoietic progenitor cells (HPCs) are now viewed as more versatile and developing HSCs/HPCs as less rigorously compartmentalized.

Cell heterogeneity and versatility during hematopoiesis

Heterogeneity of hematopoietic stem cells

HSCs are better described in mouse than in human, and mouse HSCs can be purified to homogeneity to a greater extent[4]. There are differences between mouse and human HSCs, and between fetal and adult HSCs. Human HSCs are isolated for transplantation on the basis of expression of the cell surface molecule CD34. In contrast, a single mouse $CD34^{low/-}$ HSC reconstitutes hematopoiesis long-term in a lethally irradiated mouse[5,6]. Matsuoka et al. observed that HSCs of bone marrow, liver, and spleen from fetal and neonatal mice express CD34, whereas HSCs are enriched in the $CD34^-$ fraction and MPPs are $CD34^+$ in mice older than 10 weeks[7]. Albeit, the principles of hematopoiesis derived from human and mouse studies, including of fetal and adult cells, are substantially similar.

The description of HSCs, and isolation by fluorescence activated cell sorting, relies on the presence and absence of a range of cell surface molecules. Studies of HSCs in the mouse have the advantage that HSCs can be rigorously defined during purification as cells that repopulate long-term (LT-HSC) and short-term (ST-HSC) the entire hematopoietic system. A cardinal aspect of HSCs is that they do not express markers that are associated with the various hematopoietic cell lineages (Lin^-), including, for example, CD3 (T lymphocytes), B220 (B lymphocytes), CD11b (monocytes/macrophages), Ly-6G (neutrophils), and TER-119 (erythroid cells). HSCs and MPPs express the two molecules, c-Kit, a mast/stem cell growth factor receptor and tyrosine kinase, and Sca-1, a phosphatidylinositol-anchored membrane protein. LT-HSC and ST-HSC reside in the Lin^-, $Sca\text{-}1^+$, $c\text{-}Kit^+$ population of bone marrow cells, termed LSK.

Originally the Weissman group sub-divided the LSK compartment on the basis of expression of Thy-1.1 and the Flt3 (fms-like) tyrosine kinase, a type 3 receptor kinase[3,8]. The loss of a low level of expression of Thy-1.1 and gain of expression of Flt3 was observed to correlate with a loss of the capacity of HSCs to self-renew (Figure 1). LT-HSCs were isolated from adult bone marrow as LSK $Flt3^-$, and

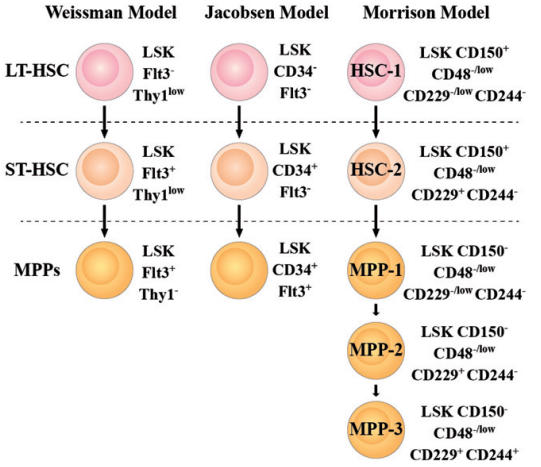

Figure 1. Identification of hematopoietic stem and progenitor cells in adult bone marrow. The loss and gain of cell surface molecules is used to delineate sub-populations of hematopoietic stem cells (HSCs) and multi-potent progenitors (MPPs). Weissman subdivides the lineage markers , $Sca\text{-}1^+$, $c\text{-}Kit^+$ (LSK) population of bone marrow cells on the basis of expression of Flt3 and Thy1.1. Jacobsen makes use of CD34 and Flt3 expression. Signaling lymphocyte activation molecule (CD150, CD48, CD229, and CD244) markers are used to subdivide LSK cells in the model proposed by Morrison. These markers are representative for hematopoiesis in mice. Flt3, fms-like tyrosine kinase; LT-HSC, long-term reconstituting hematopoietic stem cells; ST-HSC, short-term reconstituting hematopoietic stem cells.

transplantation of LSK $Flt3^+$ cells resulted in short-term multi-lineage reconstitution. However, fetal liver HSCs are contained within the $Flt3^+$ and $Flt3^-$ LSK cells. MPPs are $Thy1^-Flt3^+$. In 2005, Yang et al. combined LSK markers with CD34 and Flt3. The definitions provided by the Jacobsen group, which are commonly used, are LT-HSC as LSK $CD34^-Flt3^-$, ST-HSC as LSK $CD34^+$ $Flt3^-$, and MPPs as LSK $CD34^+$ $Flt3^+$ (Figure 1)[9].

Recently, the signaling lymphocyte activation molecule (SLAM) family of membrane receptors (CD150, CD48, CD229, and CD244) has been used to describe subpopulations of HSCs and MPPs (Figure 1). Kiel et al. purified HSCs and MPPs as $CD150^+CD48^-CD244^-$ and $CD150^{-/+}CD48^-CD244^+$, respectively. Lineage-restricted progenitors can be identified by acquired expression of CD48 and are mostly $CD150^-CD48^+CD244^{+}$[10]. Adding CD229 to the panel of SLAM, CD34 and LSK markers led Oguro et al. to propose HSC-1 and HSC-2 populations, which have different lineage biases, and three populations of MPPs (Figure 1). $CD229^-$ HSC-1 are cells that rarely divide and are myeloid-biased as revealed by transplantation studies. $CD229^+$ HSC-2 cells divide more frequently and are lymphoid biased[10]. Contrary to the findings of the Morrison group, the level of expression of CD150 has also been used to distinguish myeloid- and lymphoid-biased HSCs. Ema et al. have observed that myeloid-biased HSCs are enriched in the $CD150^{high/med}CD34^-LSK$ population and lymphoid-biased HSCs are enriched in $CD150^{low/-}CD34^-LSK$ population. These workers have proposed that these two populations of cells overlap with LT-HSCs and ST-HSCs[11].

As mentioned above and for many years, the two compartmentalizing properties of HSCs are their capacities to reconstitute the entire hematopoietic system and to self-renew. Oguro, in summarizing the sub-populations of hematopoietic stem and progenitor cells distinguished using SLAM markers, provides a scheme whereby the self-renewal potential of HSC-1 is long-term, of HSC-2 is long to intermediate, of MPP-1 is intermediate to transient, and of MPP2 and MPP3 is transient[10]. Oguro also described HPCs (HPC-1 and 2) which do not self-renew. In essence, the boundary between HSCs and MPPs as to the property of self-renewal is blurred, and a distinction between HSCs and MPPs is perhaps somewhat redundant. Both these cell types might be best viewed, and classified as a continuum of HPCs that reduce their capacity to self-renew as they mature.

Strict multi-potency does not always go hand in hand with self-renewal as to the identification of HSCs with lineage biases. As mentioned above, the $CD34^{-}$LSK population of bone marrow cells, which engraft mice including secondary hosts, has been divided into myeloid-biased and lymphoid-biased HSCs. In addition to expressing a higher level of CD150, myeloid-biased HSCs exclude Hoechst 33342 more effectively than lymphoid-biased HSC, and these two HSC sub-types are differentially regulated by transforming growth factor-β1 (TGF-β1)[12]. There is additional heterogeneity within the $CD150^{high}CD34^{-}$LSK population of cells, as revealed by engraftment of single cells in irradiated mice. In primary hosts, some single cells readily gave rise to myeloid cells and cells that were able to engraft a secondary host. Some single cells produced few myeloid cells in primary hosts and cells which when transferred to secondary hosts gave rise progressively to multiple lineages[13]. Furthermore, CD41 and CD86 expression on HSCs has been reported to distinguish myeloid-biased and lymphoid-biased cells, respectively[14,15]. As mice age, there are quantitative differences in lineage biases in the HSC population. The data support a model whereby myeloid-biased HSCs have clonally expanded, while lymphoid-biased HSCs exhaust themselves due to their more extensive proliferative nature[16].

Recently Jacobsen's group have described a platelet-biased LT-HSC that expresses von-Willebrand factor (vWF)[17]. Transplantation of single vWF^{+} HSCs into irradiated hosts resulted in reconstitution biased towards platelets and myeloid cells. vWF^{-} HSCs gave rise to a lymphoid-biased reconstitution. vWF^{+} HSCs require thrombopoietin (Tpo) for their maintenance as these cells were significantly reduced in number in $Tpo^{-/-}$ mice. vWF^{+} HSCs gave rise to vWF^{-} HSCs, and vWF^{-} HSCs were not able to give rise to vWF^{+} HSCs. This led the Jacobsen group to propose that platelet-biased HSCs are the apex of the hematopoietic hierarchy.

Heterogeneity of hematopoietic progenitor cells

In the ''classical'' lymphoid/myeloid dichotomy model of hematopoiesis, the sets of potentials observed for different types of progenitors align themselves to developmental progression along each arm of the dichotomy. However, this is not the case for all the progenitors that have been described to date. At odds with an irrevocable commitment of HSCs to either a lymphoid or myeloid pathway is the early description

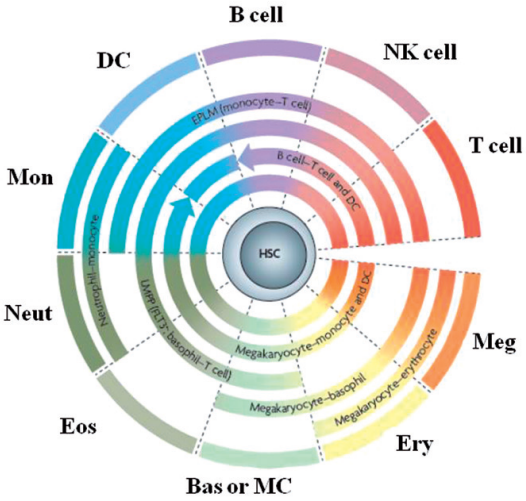

Figure 2. A pair-wise model of hematopoiesis. A fate choice continuum with an invariant series of pair-wise developmental relationships between hematopoietic cells fates is derived from the nature of the sets of potentials of various hematopoietic progenitor cells[22–25]. These are show as segments of the continuum. Dendritic cells (DC) can be derived from common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), as shown by arrowheads on two of the arcs[26]. The figure, modified and with permission is from Ref. (21) © Macmillan Magazines Ltd. Bas, basophil; Eos, eosinophil; EPLM, early progenitor with lymphoid and myeloid potential; Ery, erythroid; MC, mast cell; Meg, megakaryocyte; Mon, monocyte; Neut, neutrophil; NK, natural killer cell.

of a progenitor in mouse fetal liver with just the potentials for B lymphoid and macrophage differentiation[18]. This cell was later shown to be present in adult bone marrow[19]. A further finding that contradicts the notion that HSCs make an immediate and irrevocable decision to commit to either the lymphoid or myeloid pathways of differentiation is the identification of cells with lymphoid potentials and an incomplete set of myeloid potentials. These cells are early progenitors with lymphoid and myeloid potential (EPLM), that can give rise to T and B lymphocytes, NK cells, dendritic cells (DCs), and macrophages[20], and lymphoid-primed multipotent progenitors (LMPP), that have little potential for megakaryocyte or erythroid development while retaining other potentials[21].

Progenitor cells that contradict a lymphoid/myeloid dichotomy led us to propose the pair-wise model of hematopoiesis[22–24]. This model does not assume lineage branching patterns or prescribe a single preferred route to a particular end-cell fate. Instead mature cell fates are shown to be near-neighbors within a continuum of lineage fates (Figure 2). As HSCs mature towards a specific dominant cell fate, fates that are distantly related to this fate are lost first and more closely, related fates remain possible as latent fates. The model envisages versatility of HSCs and MPPs, as to allowing an end-cell type to be reached by more than one route (see also below). Mapping of transcription factor usage and the responsiveness of progenitors to growth factors support the proposed close relationships between cell lineages[22–24].

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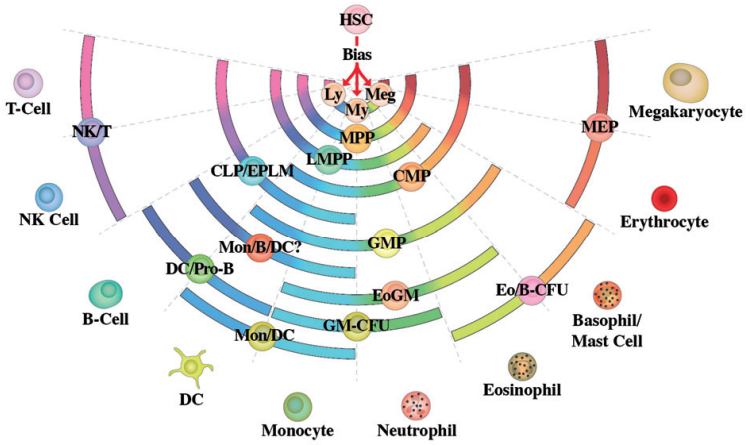

Figure 3. Mapping of hematopoietic progenitor cells to the pair-wise model. The set of fates available to various progenitors underlie near-neighbor placement of lineages in the pair-wise model. Progenitors with commensurate and contiguous lineage potentials are CLP, common lymphoid progenitor[1]; CMP, common myeloid progenitor[2]; DC/Pro-B, dendritic cell and B lymphocyte progenitor[26]; Eo/B-CFU, eosinophil and basophil progenitor[28]; EPLM, early progenitor with lymphoid and myeloid potential[20]; GMP, granulocyte and macrophage progenitor[27]; LMPP, lymphoid-primed multi-potent progenitor[21]; MEP, megakaryocyte and erythrocyte progenitor[29]; Mon/B/DC?, monocyte, B lymphocyte and dendritic cell? progenitor[18,19]; Mon/DC, monocyte and dendritic cell progenitor[30]; NK/T, natural killer cell and T lymphocyte progenitor[31]; HSC, hematopoietic stem cell; Ly, lymphoid bias; Meg, megakaryocyte bias; My, myeloid bias.

A number of progenitor cells that have different sets of potentials have been described. The various combinations of lineage potentials that exist within progenitors are also reflected in a number of different cell lines[25]. Figure 3 shows that the different combinations of differentiation capabilities described for normal progenitors can be mapped to the pair-wise model. Of particular importance to placing cell lineages close to one another in the continuum are progenitor cells that have just two differentiation capacities. In other words, the continuum infers that only certain bi-potentialities are permissible. For example, a cell with the potential for megakaryocyte and T cell differentiation should not exist, and has not been described to date. Bi-potent progenitor cells can be placed within the model with the exception of a bi-potent B lymphocyte/macrophage cell[18,19]. Whether this cell can give rise to DCs has not been studied.

Already there is a considerable variety of stem cells, with differing biases, and progenitor cells with differing sets of potentials. The latter in turn give rise to end-cell types that can be divided into numerous sub-types, for example, as is the case for T helper cells and DCs. The full extent of the heterogeneity of progenitor cell populations, and their mature progeny, is very much contingent on the extent to which new and existing cell surface markers can be used to define new sub-populations. It is highly likely that progenitor cells with multiple lineage options and that we presently view as a homogeneous population of cells will be divided into cells with lineage biases in various directions.

Versatility of hematopoietic progenitor cells

The pair-wise model allows developmental pathways to be flexible. One aspect of this is HSCs and their progeny using more than one route to a particular end-cell type. This principle was demonstrated by deriving DCs *ex vivo* from cells purified as CLPs and CMPs. When the transcription profiles of the two DC populations were compared they were found to be the same[26]. Ishikawa et al. concluded that the developmental program of human DCs operates independently of the pathways for myeloid and lymphoid cells.

Alongside new findings there has been a plethora of new models for the architecture of hematopoiesis. Some of the models depict multiple routes towards granulocytes and monocytes. For example, a model provided by Jacobsen considers the possibilities of these myeloid cells arising from ST-HSC/MPP via (i) CMP and GMP (granulocyte and monocyte progenitor); (ii) LMPP giving rise to GMP; and (iii) LMPP giving rise to a granulocyte/monocyte/T lymphocyte progenitor[32] which in turn gives rise to GMP. In a model proposed by Katsura, the two routes towards myeloid cells are HSCs veering towards: (i) a cell with the potentials for myeloid, erythroid, and megakaryocyte development; and (ii) a cell with the potentials for myeloid and lymphoid development[33]. Ye and Graf compared the production of mature cell types to flows along branches of a tree with a major branch giving rise to platelets, erythroid cells, granulocytes, and monocytes in equal measure and a separate branch giving rise largely to granulocytes and monocytes[34]. Of course, it is difficult to exclude the possibility that all the above routes to myeloid cells occur to some degree.

Precise tracking of a progenitor, as defined by a set of markers, giving rise to the next progenitor, also verified by markers, and so on to an end-cell type(s) is an impossible task. However, a novel approach has been recently described whereby cells can be uniquely marked *in situ*, by transposon tagging, allowing longitudinal analysis of clones in mice and revealing that long-lived progenitors are a main driver of steady-state hematopoiesis in adulthood[35]. This approach will also impact on our view of cell lineages and provide information on the origin of hematopoietic malignancies. Aside from this experimental model, examination of the sets of options available to various progenitors, as defined by appropriate markers, and which progenitors are or are not able via loss of a fate option(s) to give rise to one another, allows configuration of routes to end-cell types. For certain cell types, it is clear that there can be multiple routes. As described by Jacobsen et al., HSCs can give rise to neutrophils and monocytes through LMPP and CMP intermediates. Figure 4 shows different possible routes downstream of LMPP and CMP towards neutrophils. These include

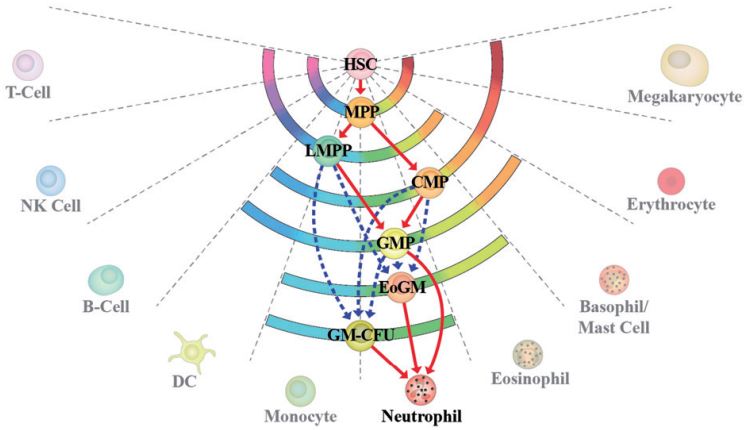

Figure 4. Alternative developmental routes towards neutrophils. The sets of potentials available to known oligopotent progenitors are used to construct possible routes. The red solid arrows are routes delineated from studies of the progeny of progenitors and the blue dash arrows are putative routes. For abbreviations, see the legend to Figure 3.

potential pathways through an eosinophil/granulocyte/monocyte progenitor[36] and/or GM-CFU (granulocyte/macrophage colony-forming unit). The routes are in keeping with the pairwise model as to their close proximity. Similarly, there are multiple potential pathways towards monocytes via CMP, an eosinophil/granulocyte/monocyte progenitor and GM-CFU, and *via* LMPP, EPLM, and a monocyte/dendritic cell progenitor[30]. In fetal liver, a myeloid/B-biased progenitor is a further intermediary to myeloid cells[37]. A caveat to all this is the extent to which routes are used *in vivo*. However, the point of interest is the inherent flexibility of pathways available to progenitors. It is noteworthy that the ability of precursor cells to follow alternative developmental pathways to give rise to the same cell phenotype was described for cell lineages in the embryo of the leech as early as 1987[38].

One aspect underpinning versatility is that progenitor cells retain fate options even after they have progressed some way along a pathway. Thymus-settling progenitors (TSP) have the potential to give rise to myeloid cells, dendritic cells, NK cells, and B lymphocytes in addition to T lymphocytes. TSP give rise to double-negative (DN) 1 early thymocyte progenitors (ETP), which give rise to DN2 cells. DN2 cells have lost the potential for differentiation towards B lymphocytes, but when cultured in the right environment generate myeloid, dendritic cells, and NK cells[39–41]. As to driving pro-T cells to other fates, culture of DN2 cells in IL3 (interleukin) and stem cell factor (SCF) in the absence of Notch signaling revealed mast cell potential, indicating a close unexpected relationship between the pro-T cell and mast cell fates[42]. These clandestine potentials are lost as DN2 cells progress to the DN3 stage of development.

Some cytokines have instructive actions on cell-fate decisions

Early glimpses to the instructive action of cytokines

A long-standing debate is whether the commitment of HSCs to fate options occurs in a cell-autonomous and stochastic way, or is driven (in an ordered way) by instructive signals from the local environment[43–46]. Cytokines are the pivotal external factors that impart environmental signals to control hematopoietic cell development. They have multiple actions that can be viewed as either instructive, by directing HSCs/MPPs towards a specific lineage, or permissive, by selectively allowing cells committed to a particular lineage to survive and/or proliferate[47,48]. For many years, a permissive role of cytokines has been favored. A very recent and complete turnabout in our understanding of the control of hematopoiesis is the notion that cytokines instruct decision-making[49,50].

Information to support an instructive role for cytokines has been available for quite some time. In 1982, Metcalf and Burgess concluded that granulocyte/macrophage colony-stimulating (GM-CSF) factor and macrophage colony-stimulating factor (M-CSF) can ''*irreversibly commit the progeny of GM-CFC respectively to granulocyte and macrophage production*''[51]. When paired daughter cells of GM-CFU were split and one cultured in GM-CSF and the other in M-CSF, some of the cells underwent irreversible commitment to the granulocyte and macrophage pathways, respectively. This occurred during completion of the first cell division and within 24 h. Later in 1991, Metcalf again concluded that colony-stimulating factors have the ability to influence lineage commitment[52]. Metcalf examined the relative frequencies of lineage committed progenitors when blast cell colonies were established from normal bone marrow cells in combinations with granulocyte colony-stimulating factor (G-CSF), GM-CSF, and multi-CSF. The relative frequency of granulocyte progenitors was increased when cultures were established in the combination with GM-CSF or multi-CSF with SCF.

In 2000, Kondo et al. provided more evidence to support the notion that cytokines can convert the fate of lymphoid-committed progenitors[49]. The IL-2 and GM-CSF receptors were exogenously expressed in CLPs, which normally gives rise exclusively to T lymphocytes, B lymphocytes, and NK cells. This resulted in cell-fate conversion to the myeloid lineage. The use of mutants of the beta-chain of the IL-2 receptor revealed that signals for the granulocyte and monocyte differentiation pathways are provoked by different cytoplasmic domains of the IL-2 receptor. Kondo et al. also showed that primitive HSCs express low-to-moderate levels

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of the receptors for GM-CSF and M-CSF. Hence, there is a possibility that HSCs are receptive to the instructive actions of these growth factors. Kondo et al. concluded from all the above that down-regulation of cytokine receptors that drive myeloid cell development is a critical step in commitment of cells to lymphoid development.

Recent studies confirm an instructive action of cytokines

A number of studies have now shown that cytokines have an instructive action on cell-fate decisions. M-CSF, G-CSF, and erythropoietin (Epo) instruct monocytic, neutrophilic and erythroid fates, respectively. In 2009, Rieger et al., by monitoring individual hematopoietic progenitors in culture, confirmed that M-CSF and G-CSF provided instructive cues. Using bioimaging techniques and a LysM-GFP reporter system to detect differentiation and cell death, the group observed that individual GMPs adopted monocytic or neutrophilic fates in the presence of M-CSF or G-CSF, respectively[50]. More recently, Mossadegh-Keller et al. made use of PU.1-GFP reporter mice to show that M-CSF drives expression of myeloid-associated genes in some LT-HSCs. *In vivo*, M-CSF stimulated expression of PU.1 HSCs generated increased numbers of GMPs in the spleen and peripheral myeloid cells at the expense of cells undergoing megakaryocyte, erythroid, and lymphoid development when compared with non-primed PU.1^{-} HSCs[53]. Epo induces priming of erythroid lineage-associated genes in LT-HSCs and *in vivo* skews the potential of these cells towards an erythroid fate[54]. An increase in serum Epo levels in mice led to the expansion of committed erythroid and megakayocyte and erythroid progenitors (MEP) in the bone marrow, whereas megakaryocyte progenitors, pre-GMP, and LMPP populations were decreased.

An instructive action of Flt3 ligand in determining cell fate

One of the cytokines that is essential to cell survival and proliferation during early hematopoiesis is the ligand for Flt3[55,56]. Flt3 ligand (Flt3L) was described two decades ago[55] and is the only known ligand for Flt3. Upon ligand binding, the Flt3 receptor dimerizes and initiates signaling that involve STAT5a, ERK1/2, and PI3K[57]. Flt3 is an important area of research since mutations in Flt3 were among the first ones discovered in acute myeloid leukemia (AML)[58]. Presently, there is substantial interest in elucidating the instructive role of Flt3L.

Flt3 expression occurs during hematopoiesis at the non-self-renewing ST-HSC stage of development[59]. In fact, Flt3 up-regulation relates to the loss of self-renewal capacity[60]. This may just be a coincidence, or Flt3/Flt3L provokes the loss of self-renewal capacity by an as-yet-unknown mechanism. Thereafter, MPP express Flt3 as do several downstream progenitors with myeloid and/or lymphoid potential, while the MEP is Flt3^{-}[59]. As lineage options become more restricted, Flt3 expression is down-regulated with the exception of DCs[61].

Importantly, Flt3L exerts a role by interacting with other cytokines such as IL-7 or SCF[62,63]. For example, IL-7 and

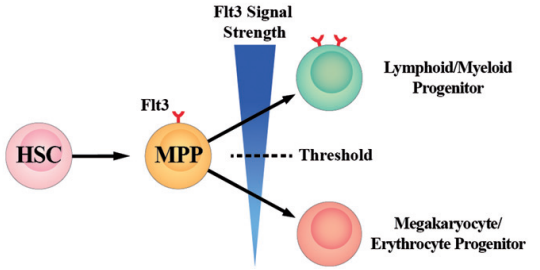

Figure 5. Instructive action of Flt3 ligand in determining lymphoid/myeloid *versus* MegE lineage development. If the Flt3 ligand signal strength exceeds a certain threshold level, cells enter the lymphoid/myeloid branch at the expense of the MegE lineage. HSC, hematopoietic stem cell; MPP, multi-potent progenitor.

Flt3L stimulate lymphoid development in a coordinated manner that occurs in a narrow window during which cells express both receptors. In the case of *in vitro* cultures of hematopoietic progenitor cells, such as ETP, CLP, or EPLM, and in the presence of IL-7, Flt3L provides an additive anti-apoptotic effect while stimulating proliferation[64,65] (and our observations). This additive effect is reported to result from parallel activation of the IL-7 receptor (IL-7R) and Flt3 via separate signaling pathways converging to activate Stat5[63].

Mice with targeted gene disruption of Flt3[66] or its ligand[67] have provided precise information about the actions of Flt3/Flt3L. These mice have defects in the developmental potential of myeloid/lymphoid progenitors as well as reduced numbers of B cells, DCs, and NK cells[66,67]. Tsapogas et al. have provided evidence to support the notion that Flt3L is instructive to cell decision-making. These workers generated a Flt3L transgenic (Flt3L-Tg) mouse that expresses human Flt3L[68]. Flt3L-Tg mice have a tremendous expansion of hematopoietic progenitors in the bone marrow. Of all the progenitor populations analyzed, the only progenitor that was decreased was the MEP. As such, the Flt3-Tg mice had decreased platelet counts and developed anemia. Previous studies had reported that the expression of Flt3 after the ST-HSC stage leads to a reduction of megakaryocyte and erythrocyte potentials[21]. Also, a significant reduction in MEP progenitors was observed by day 3 when wild-type mice were injected with recombinant Flt3L[69]. Considering this rapid response, it is likely that the reduction in MEP numbers was a consequence of a Flt3L threshold response in upstream Flt3^{+} progenitors rather than a space restriction within the bone marrow caused by the over-proliferation of other progenitors. It was proposed that upstream Flt3^{+} progenitors develop towards lymphoid/myeloid lineages on receiving Flt3L stimulation above a certain threshold level and develop towards the MegE (megakaryocyte/erythroid) lineage if this threshold level was not reached. In other words, an increased level of Flt3L guides the development of cells towards the lymphoid/myeloid fates at the expense of the MegE fates (Figure 5). This provides an explanation of the MegE developmental defect in the Flt3L-Tg mice. The exact mechanism by which Flt3L exerts an instructive action remains to be elucidated and whether this occurs at the CLP/EPLM level is of considerable interest.

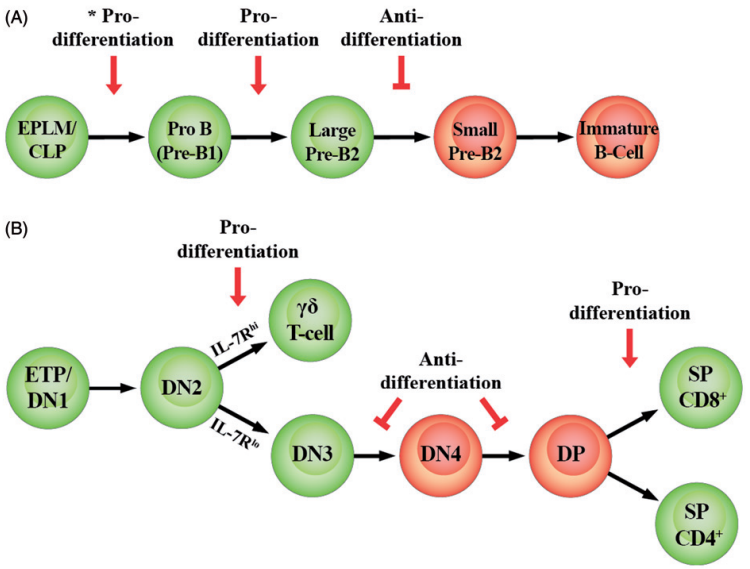

Figure 6. IL-7 action during various stages of murine B and T lymphocyte development. The progressive stages of B lymphocyte development (A) and T lymphocyte development (B) are shown. EPLM/CLP to Large Pre-B2 (green cells in A) and ETP/DN1 to either γδ T-cell or DN3 and SP (green cells in B) represent stages responsive to the survival and proliferative action of IL-7. Pro-differentiation and anti-differentiation actions are also shown.*The IL-7 pro-differentiation action could be instructive. For other stage transitions, more evidence is needed to conclude an instructive action. DN, double-negative; DP, double-positive; EPLM, early progenitor with lymphoid and myeloid potentials; ETP, early thymocyte progenitor.

Cytokines play different roles at various developmental stages

This is best illustrated by consideration of the actions of IL-7 at various stages of B and T lymphocyte development[70–72]. These include promoting cell survival and proliferation and facilitating decision-making during differentiation (Figure 6). IL-7 was described in 1988[73]. In mice, deficiencies in IL-7 or the receptor lead to impairment of B and T lymphopoiesis[74–76]. IL-7 is not required for B lymphopoiesis in humans, as deficiencies in IL-7 result in apparently normal B cell development[72]. However, the B cells could be the result of fetal development since they can be cultured *in vitro* in the absence of IL-7 while postnatal B cells seem to be IL-7 dependent[77].

CLPs express IL-7R, which provides a criterion used to isolate these cells[1]. CLPs are dramatically reduced in IL-7-deficient mice[78], indicating that this cell is critically dependent on IL-7. EPLMs also express IL-7R[20]. A strong proliferative action of IL-7 is seen when CLPs and EPLMs, sorted from mouse bone marrow, are cultured on OP9 stromal cells and treated with IL-7[78] (and our own observations). Furthermore, these cells are able to differentiate to the next B cell developmental stage, the pro-B (pre-B1), indicating that IL-7 acts as a differentiation factor at the CLP/EPLM level. In keeping with this notion is that CLP cells are unable to reach the pro-B (pre-B1) stage in $\gamma c^{-/-}$, IL-7R$\alpha^{-/-}$, or IL-$7^{-/-}$ mice, and instead arrest at an uncommitted level[79]. These data suggest an instructive role for IL-7. This appears to occur via IL-7-induced STAT5 signaling which regulates the expression levels of EBF (early B cell factor)[78,80]. In turn, EBF activates transcription of Pax5[81,82] leading to the expression of CD19. This cell surface marker indicates that cells have gained the pro-B phenotype and commitment to the B cell lineage[83,84].

The pro-B (pre-B1) stage is also sensitive to the action of IL-7. Fetal and bone marrow pro-B (pre-B1) cells can be cultured *in vitro* in the presence of IL-7 and stromal cells for long periods of time (more than 4 months)[85]. The survival action of IL-7 appears to be exerted *via* expression of the gene encoding the anti-apoptotic protein Mcl-1[86]. Moreover, there is evidence to suggest that IL-7 facilitates differentiation of pro-B (pre-B1) cells into cytoplasmic μ immunoglobulin (Ig) heavy chain expressing large pre-B2 cells[87,88] (and our own unpublished observation). IL-7 is not instructive, since cells differentiate irrespective of the presence of IL-7[87,88]. Instead, the data argue for a permissive role of IL-7 in promoting survival and proliferation during the pro-B (pre-B1)/large pre-B2 transition. However, some groups have reported that STAT5-mediated IL-7 signaling controls chromatin accessibility and the rearrangement of distal V_H genes at the *Igh* locus[89–91]. This is exemplified by a significant decrease in distal V_H rearrangements in $B220^+$ IgM^- bone marrow B lymphocytes of $IL7R^{-/-}$ and *Stat5*$^{-/-}$-deficient mice[89,91]. In contrast, Malin has argued that there is no substantial difference in the distal V_H rearrangement genes seen for STAT5 deficient, IL-7R mutant, and control pro-B (pre-B1) cells[86]. Finally, as to the pre-B1/pre-B2 transition, it has been reported that IL-7 acts to prevent premature rearrangements of the Igκ via binding of IL7-mediated STAT5 to the Igκ intronic enhancer (iEκ)[86,92].

The large pre-B2 transition is the last stage in B cell development that is sensitive to the action of IL-7. The proliferation of these cells is improved by the presence of IL-7[93]. However, IL-7 acts as an anti-differentiation factor towards immature B cells by blocking the rearrangement of the light chain loci[86,92,93]. Upon withdrawal of IL-7 from the culture, cells undergo Igκ recombination and differentiation into IgM positive cells[21,86,94,95]. At this stage, as a consequence of the pre-B cell receptor signaling, IL-7R is

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down-regulated and the later stages of B cell development are unresponsive to IL-7[93,96,97].

During T lymphocyte development, the thymus is seeded by ETP[98] and there is a general agreement that these cells can give rise to multiple lineages. In humans, the ETP retain lympho-myeloid potentials and can generate separate lymphoid- and myeloid-primed progenitors. Lymphoid-restricted progenitors seem to be the main thymus colonizers in the mouse[99], although several *in vitro* studies suggest that these cells have myeloid potentials[64,100]. IL-7Rα is a direct target of Notch1[101], the master signaling pathway that regulates thymopoiesis[102–104]. Up-regulation of IL-7R at the ETP stage[105] is a hallmark for lymphoid commitment, and IL-7-mediated signaling triggers the first wave of expansion of lymphoid-primed progenitors[106,107]. IL-7 is essential to survival and proliferation of ETP, and appears to transmit signals for the survival and proliferation of lymphoid-committed cells at the expense of the myeloid branch[72]. Sustained Notch signaling favors cell development along the T cell lineage by inducing the transcription of important T lymphocyte differentiation factors and blocking the development of cells towards other lineages[105,108]. IL-7 is also required for the transition of ETP cells to the DN2 stage *in vitro*[64]. Moreover, an early block is observed in T lymphocyte development in IL-7 or IL-7R-deficient mice[109]. However, Bcl-2 is sufficient to rescue T lymphocyte development (at least the α/β branch of T lymphocytes), thus confirming a permissive role for IL-7 during the early thymopoiesis[110].

IL-7Rα chain expression increases progressively until the DN2 stage, coinciding with the first massive cellular expansion, and then steadily decreases. DN2 cells with high IL-7R expression levels are diverted to the γδ T cell lineage, at least in mice[111], and IL-7R signaling controls accessibility to the T cell receptor (TCR)γ locus and its rearrangement[112]. Thus, IL-7 plays an instructive role in this developmental process and Bcl-2 is unable to rescue the development of γδ T cells[110,113]. The cells with reduced IL-7R expression and/or limited IL-7 availability progress to the DN3 stage. The proliferation of these cells is still IL-7 dependent[110,113]. However, a diminished IL-7R signaling seems necessary for DN2 mouse thymocytes to up-regulate Bcl11b, a transcription factor that is essential to the T cell lineage[114]. Thereafter, on successful rearrangement of the TCRβ chain and subsequent pre-TCR expression, IL7-R is down-regulated, and cells move to the DN4 stage[115,116]. Here, at the β selection checkpoint, there is a second wave of expansion that is controlled by Notch1 signaling and pre-TCR expression[64]. As such, the DN3 to DP (double-positive) transition is IL-7 independent. However, the continued presence of IL-7 in *in vitro* cultures blocks differentiation and DP cells are generated only on IL-7 removal. In this case, IL-7 is acting as an anti-differentiation factor[64,117]. This highlights the importance of the IL-7R signaling suppression during the DP transition, which is guaranteed by SOCS-1 (suppressor of cytokine signaling)[115]. After the DP selection stage, IL-7R surface expression levels are restored and IL-7 signaling seems to be required for the DP to single positive CD8 transition[118]. In keeping with this, Park et al. reported differentiation of single positive CD8 cells ($CD8^+$) and RUNX-3 transcription factor up-regulation in transgenic TCR mice expressing a transgene-derived IL-7Rα chain in an IL-7 over-expressed environment[119]. Finally, IL-7 is required for homeostatic expansion of naïve $CD8^+$ and $CD4^+$ T cells[120,121].

In the context of the role of IL-7 in lymphopoiesis and in particular the flexibility of the hematopoietic process, the effect of pregnancy on hematopoiesis and lymphopoiesis is worthy of mention. Pregnancy is a physiological process in which there is an increase in blood volume and the bone marrow responds by increasing erythropoiesis[121]. Lymphopoiesis is also affected, and two mechanisms account for the reduced B lymphopoiesis observed in pregnancy. First, early B progenitor cells are sensitive to the increased levels of sex steroids in pregnancy[122] and second, in mice, IL-7 production and availability dramatically decrease[123]. Not only is the bone marrow affected during pregnancy but also the maternal thymus undergoes dramatic involution[124]. Both B and T lymphopoieses return to pre-pregnancy levels following parturition and weaning. A full explanation of these associated phenomena is not currently available and is certainly worthy of further investigation. In regard to the effect of cytokines on lymphopoiesis in the developing fetus, an interesting and recent finding is that inflammatory cytokines interferon- (IFN) α and γ positively regulate the number of lymphoid progenitors in mouse embryos, and IFN-γ signaling also affects the number of HSCs[125]. These findings have important implications to enhancement of the formation of hematopoietic stem and progenitor cells in the embryo and the differential regulation of hematopoiesis during fetal and adult life.

Implications of heterogeneity and versatility to leukemia

A Darwinian viewpoint on leukemia

A widely-held viewpoint for a number of years is that the cell population that makes up an overt leukemia and solid tumor is heterogeneous and that there is a hierarchical organization. A small subset of cells, which are at the apex of the hierarchy, is responsible for maintaining the long-term growth of the leukemia and tumor mass. These leukemia- and cancer-maintaining cells, which have an inherent capacity to self-renew, are termed leukemia stem cells (LSC) and cancer stem cells (CSC), respectively[126,127]. There is also the question of the normal cell from which the leukemia/tumor has arisen, which are termed leukemia-initiating cells (LIC) and cancer-initiating cells (CIC), respectively. These are the cells that suffered an oncogenic lesion and that evolve to give rise to the leukemia/tumor. And, many leukemias and cancers arise from a self-renewing stem cell or downstream progenitor if self-renewal is reactivated. The origin and LSC/CSC nature of leukemias/cancers is important to putting into practice a cancer stem cell-based therapeutic to cure patients[128–132].

To add to the difficultly of designing therapies to eliminate LSC/CSC, Greaves has proposed a Darwinian viewpoint on the nature of CSC which pays attention to the dynamics of the cancer[133,134]. Studies of single acute lymphoblastic leukemia (ALL) cells have revealed that the leukemia "stem" cells are genetically diverse. Greaves has likened the complex and branching clonal architecture of ALL "stem" cells to Darwin's evolutionary tree-like divergence diagram which was drawn in 1837. To add to the Darwinian analogy, as the leukemia evolves,

in an almost entirely clinically silent manner, cells acquire gene copy number alterations. The cytokine TGF-β has been proposed to exert a selective Darwinian advantage to expand the cells that are first "hit" as to genomic alteration and at risk of acquiring secondary multiple gene alterations. In essence, this is similar to "natural selection" as described by Darwin.

Often what we discover from studies of cancer cells, and ascribe to these cells, turns out to be a feature of normal cells. A simple example is that the common ALL-associated antigen (CD10) was first described as a candidate leukemia-specific antigen[135]. In fact, CD10 is expressed by rare B cell progenitor cells and its presence on ALL cells was telling us something about the origin of common ALL[136]. So, we might view normal hematopoiesis as a Darwinian process that is driven by selective pressures, namely cytokines, acting on an inherent diversity that is sufficient to generate the various types of blood and immune cells. In essence, inherent cell heterogeneity must have been the template to the evolution of the wide variety of immune cells that exist in higher mammals.

Are leukemia stem cells as versatile as their normal counterpart?

An important question that arises from the above considerations is the following: are LSCs as versatile as normal HSCs in terms of their capacity to access routes to end-cell types? There is evidence to suggest that LSCs are less versatile in this regard[137,138], as exemplified by erythroleukemia [acute myeloid leukemia (AML) FAB-M6] and pre-B/pro-B/common acute lymphoblastic leukemia (ALL). The cells that sustain these leukemias appear to have become directed to generate cells of a particular cell type. An accumulation of erythroid precursors and myeloblasts in AML FAB-M6 reveals a disease origin in a cell with multi-lineage potential[139,140]. However, the partial differentiation of the leukemic blast cells is restricted to certain pathways, and accordingly disease sub-sets are characterized as myeloblast-rich (FAB-M6A), proerythroblast-rich (FAB-M6B) and myeloblast- and proerythroblast-rich (FAB-M6C)[135].

In the case of childhood ALL, including pre-B ALL, pro-B ALL, and common ALL (c-ALL), there are arguments to support a disease origin in either a cell that is committed to B lymphocyte development[141,142] or a cell that is more stem cell like[143]. The former notion is a long-held viewpoint. In favor of the latter cellular origin is that c-ALL-derived cells lacking the B-lineage markers CD10 and CD19 and expressing the stem cell marker CD34 can give rise to c-ALL and pre-B ALL when transplanted into mice[143]. The argument about the precise "target" cell that is transformed in childhood ALL could be set aside as versatility of lineage options extends to progenitor cells, as illustrated by the cytokine-mediated redirection of CLPs to the myeloid lineage[49]. Strikingly, the blast cells that accumulate in the blood and bone marrow in childhood ALL are restricted to B-lineage development.

Conclusions and outlook

There is good evidence to support the notions of developmental plasticity in the hematopoietic system and that growth factors can instruct lineage potentials. This leads to interesting questions as to how HSCs establish and, as required for steady-state hematopoiesis, maintain pluripotency, and how a network of factors might participate to control HSC identity and commitment ability to drive contributions to homeostasis and adaption to inflammatory conditions. Presumably to maintain HSC pluripotency, there is the requirement to control genomic stability, in part via controlling intrinsic DNA repair machinery. As mentioned above vWF^+ HSCs require Tpo for their maintenance. This growth factor also plays a role in regulating HSC genomic stability, since the efficiency of DNA-Protein Kinase-dependent DNA repair, in response to DNA damage, is increased by Tpo[144]. Tpo-induced activation of ERK and NF-κB in HSCs is important to damage repair[145]. As to Tpo playing a role to ensure chromosomal integrity, the plot thickens regarding the roles of growth factors.

Ineffective DNA repair of genomic instability in LIC and/or LSC might curtail the availability of lineage options of these cells. Global genomic instability is unusual for AML[146], and for both AML and pro-B ALL, a very small number of mutations are required to generate the leukemia[147]. Genomic instability is a feature of the chronic phase of chronic myeloid leukemia, resulting in BCR-ABL-1 mutations that encode resistance to the tyrosine kinase inhibitors (for example imatinib) used to treat the disease. Importantly, instability has been postulated to occur in primitive leukemia progenitor cells in patients who have not been treated with tyrosine kinase inhibitors. Also, it has been suggested that the instability is due to high levels of DNA damage by reactive oxygen species and inefficient/unfaithful repair of DNA double-strand breaks that lead to chromosomal aberrations[148].

In summary, for normal stem and progenitor cells, the versatile nature of the pair-wise model provides an appropriate template for the persuasive action of growth factors to shape hematopoiesis. As yet, we do not know whether LIC and/or LSC are as or less versatile in terms of lineage options than their normal counterparts. Selective instability in genomic elements encoding controls on lineage decision-making (for example transcription factors and signaling molecules) might restrict the versatility of LIC/LSC.

Declaration of interest

The authors alone are responsible for the content and writing of the paper.

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