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**Permissive roles of cytokines Interleukin-7 and Flt3-ligand in mouse B cell  
lineage commitment**

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## ABSTRACT

Hematopoietic cells are continuously generated throughout life from hematopoietic stem cells, thus making hematopoiesis a favorable system to study developmental cell lineage commitment. The main factors incorporating environmental signals to developing hematopoietic cells are cytokines, which regulate commitment of hematopoietic progenitors to the different blood lineages by acting either in an instructive or a permissive manner. Flt3-ligand (FL) and Interleukin-7 (IL7) are cytokines pivotal for B cell development, as manifested by the severely compromised B cell development in their absence. However, their precise role in regulating B cell commitment has been the subject of debate. In the present study we assessed the rescue of B cell commitment in mice lacking IL7 but simultaneously over-expressing FL. Results obtained demonstrate that FL over-expression in IL7 deficient mice rescues B cell commitment, resulting in significant Ebf1 and Pax5 expression in Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> precursors and subsequent generation of normal numbers of CD19<sup>+</sup> B cell progenitors, therefore indicating that IL7 can be dispensable for commitment to the B cell lineage. Further analysis of Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors in IL7- or FL-deficient mice over-expressing *Bcl2*, as well as in IL7-transgenic mice suggests that both FL and IL7 regulate B cell commitment in a permissive manner; FL by inducing proliferation of CD135<sup>+</sup>CD127<sup>+</sup>Ly6D<sup>+</sup>CD19<sup>-</sup> progenitors and IL7 by providing survival signals to these progenitors.

## **SIGNIFICANCE STATEMENT**

The generation of different blood lineages is regulated by hematopoietic cytokines, either in an instructive or in a permissive manner. The cytokines Interleukin-7 and Flt3-ligand are required for B cell development but their precise mode of action remains controversial. Our study has addressed the role of these cytokines in B cell commitment by analyzing the progenitor stage where B cell commitment occurs in mice over-expressing one of the two cytokines in the absence of the other. Our results demonstrate a permissive role for both cytokines in B cell commitment. Interleukin-7 promotes survival of progenitors instead of up-regulation of B cell commitment factors Ebf1 and Pax5, as previously hypothesized, whereas Flt3-ligand facilitates progenitor expansion by inducing their proliferation.

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## **INTRODUCTION**

Hematopoiesis, the generation of all blood cells from hematopoietic stem cells (HSC), takes place continuously in the adult bone marrow. Accumulating evidence suggests that HSC generate the different hematopoietic lineages via oligo-potent progenitors having limited self-renewal capacity and restricted developmental potentials. Activation of lineage-specific gene transcription in these progenitors eventually leads to their commitment to a particular lineage. Cytokines are the most prominent environmental factors regulating hematopoietic lineage commitment, doing so by acting either in an instructive or a permissive manner (1). In the instructive model, cytokines induce a signaling cascade in progenitors leading 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, with cytokines acting as a selection rather than a commitment factor, 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 roles of cytokines is hotly debated (2-4). Although the permissive model was favored in the past, recent data provide solid evidence for the instructive action of several cytokines including M-CSF, G-CSF, EPO and Flt3-ligand (5-8). 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 (9). Moreover, most

studies to date have addressed cytokine regulated myeloid differentiation with relatively little information on lymphoid lineage commitment.

That Interleukin-7 (IL7) is a crucial cytokine for B cell generation is demonstrated by the dramatic defect in B cell generation in mice lacking either the cytokine (10) or its receptor (11). Interestingly, while human B cell progenitors are also responsive to IL7 (12), disruption of IL7 signaling caused by mutations does not ablate B cell development in man (13, 14). IL7 was initially identified as a growth factor for B cell progenitors (15) and early studies demonstrated that *in vivo* over-expression of the pro-survival gene *Bcl2* did not rescue B cell development in the absence of IL7 signaling suggesting that IL7 acts in an instructive manner in B cell commitment (16, 17). The subsequent findings that uncommitted Common Lymphoid Progenitors (CLP) from *Il7*<sup>-/-</sup> mice lacked expression of the transcription factor Early B-cell Factor 1 (Ebf1) (18) and that Ebf1 over-expression partially restored B cell generation from these CLP (19), led to the hypothesis that IL7, through Stat5 activation, instructs commitment to the B cell lineage by initiating Ebf1 expression in uncommitted progenitors. Supporting this hypothesis, a putative Stat5 binding site was later identified in one of the *Ebf1* promoters (20). However, a more recent study has shown that *Bcl2* can rescue B cell generation in a *Stat5* conditional knock-out mouse (21). Furthermore, the Ebf1-expressing fraction of CLP (Ly6D<sup>+</sup> CLP) is dramatically reduced in *Il7*<sup>-/-</sup> mice (22), therefore providing an alternative possibility for the reduced Ebf1 expression observed in *Il7*<sup>-/-</sup> CLP. Interestingly, B cell lineage commitment is initiated at the molecular level in Ly6D<sup>+</sup>CD19<sup>-</sup> progenitors (23). 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 the 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 Leukemias (AML) (24). Committed B cell progenitors do not express CD135 since expression of the B cell commitment factor Pax5 leads to its down-regulation (25). However, upon transplantation, bone marrow progenitors from *Flt3<sup>-/-</sup>* and *Flt3l<sup>-/-</sup>* mice reconstitute the B cell compartment poorly (26, 27), and FL was found to be essential for maintaining normal numbers of uncommitted B cell progenitors (28).

Recently, we described a FL-transgenic mouse model (hereafter *Flt3ltg*) expressing high levels of FL *in vivo*, which has enabled us to suggest an instructive role for FL in early stages of hematopoiesis (8). By breeding these mice with *Il7<sup>-/-</sup>* mice we herein show that increased FL levels can rescue B cell commitment in CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors and restore early CD19<sup>+</sup> B cell progenitor numbers 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 *Bcl-2*, have enabled us to identify a permissive role for both IL7 and FL in B cell commitment.

## RESULTS

### Increased *in vivo* levels of FL rescue B cell commitment in *Il7*<sup>-/-</sup> Ly6D<sup>+</sup>CD19<sup>-</sup> progenitors.

We have previously characterized an uncommitted B cell progenitor with combined lymphoid and myeloid potential (Early Progenitor with Lymphoid and Myeloid potential – EPLM) (29). EPLM can be further subdivided by SiglecH, CD11c, CD115 and Ly6D expression enabling us to identify the Ly6D<sup>+</sup>SiglecH<sup>-</sup>CD11c<sup>-</sup>CD115<sup>-</sup> fraction of EPLM (hereafter Ly6D<sup>+</sup> EPLM) as the population containing most B cell potential, while being devoid of myeloid potential (Fig. S1B). This EPLM subpopulation is identified as Lin<sup>-</sup>CD19<sup>-</sup>CD117<sup>int</sup>B220<sup>int</sup>Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup> (Figure 1A) therefore partially overlapping phenotypically with Ly6D<sup>+</sup> CLP (Fig. S1A) and pre-pro-B cells (30, 31). Ly6D<sup>+</sup> EPLM numbers in *Il7*<sup>-/-</sup> and *Flt3l*<sup>-/-</sup> mice are significantly decreased compared to WT; 7-fold for *Il7*<sup>-/-</sup> and 13-fold for *Flt3l*<sup>-/-</sup> respectively and a similar dramatic decrease was observed in Ly6D<sup>+</sup> CLP from both mutant mice (Figure 1B-C). FL deficiency also affected the numbers of Ly6D<sup>-</sup> EPLM and CLP, while IL7 did not (Fig. S1C-D). Therefore, Ly6D<sup>+</sup> EPLM/CLP represent the earliest developmental stage of the B cell pathway affected by the absence of IL7.

We have recently generated a mouse model expressing high *in vivo* levels of FL (8). The progenitor compartment of these mice showed a dramatic increase in EPLM and CLP numbers, with their Ly6D<sup>+</sup> fractions increased 90-fold and 28-fold respectively relative to WT (Figure 1D-E). We crossed *Flt3ltg* with *Il7*<sup>-/-</sup> mice to assess the extent to which increased FL levels could potentially rescue the loss of Ly6D<sup>+</sup>CD19<sup>-</sup> progenitors in *Il7*<sup>-/-</sup> mice. As shown in Figure 2 A-B, *in vivo* over-expression of FL leads to a significant increase in *Flt3ltg-Il7*<sup>-/-</sup> EPLM and CLP

numbers, reaching levels of those in *Flt3ltg* mice. Crucially, a full rescue of Ly6D<sup>+</sup> EPLM and CLP can be seen in these mice, with a striking 470-fold and 31-fold increase in numbers compared to their *Il7*<sup>-/-</sup> counterparts (Figure 2A-B). Furthermore, the numbers of the earliest committed CD19<sup>+</sup>CD117<sup>+</sup> pro-B cells were fully restored in *Flt3ltg-Il7*<sup>-/-</sup> mice, showing a 251-fold increase compared to *Il7*<sup>-/-</sup> (Figure 2C and Fig. S2). However, this rescue was less pronounced in downstream CD19<sup>+</sup>CD117<sup>-</sup> IgM<sup>-</sup> and CD19<sup>+</sup>IgM<sup>+</sup> B cell stages, since these cells require IL7 to expand. As a consequence of this rescue in bone marrow B cell development, numbers of splenic marginal zone and follicular B cells were significantly increased in *Flt3ltg-Il7*<sup>-/-</sup> mice compared to *Il7*<sup>-/-</sup> (Figure 2D). While thymic T cell development was not rescued in *Flt3ltg-Il7*<sup>-/-</sup> mice (Fig S3), a significant increase in splenic T cell numbers was observed (Fig. S4) as a result of their expansion upon FL over-expression (32).

To assess whether these rescued *Flt3ltg-Il7*<sup>-/-</sup> Ly6D<sup>+</sup>CD19<sup>-</sup> progenitors could give rise to B cells *in vitro*, we sorted *Flt3ltg-Il7*<sup>-/-</sup> Ly6D<sup>+</sup> EPLM and plated them at limiting dilution on OP9 stromal cells in the presence of IL7. As shown in Figure 3A, *Flt3ltg-Il7*<sup>-/-</sup> Ly6D<sup>+</sup> EPLM could generate B cells at similar frequencies to their WT and *Flt3ltg* counterparts, whereas the few *Il7*<sup>-/-</sup> Ly6D<sup>+</sup> EPLM isolated could not. A rescue in Ly6D<sup>+</sup> EPLM was also observed when *Il7*<sup>-/-</sup> mice were injected with FL (Figure 3E) and when plated under the same conditions these rescued Ly6D<sup>+</sup> EPLM also showed a restored *in vitro* B cell potential (Fig. S5A). Further, when transplanted into irradiated *Rag2*<sup>-/-</sup> mice they were able to generate IgM<sup>+</sup> B cells (Fig S5B-C). Thus, increased FL levels restore the generation of Ly6D<sup>+</sup> progenitors, rather than merely expanding the few Ly6D<sup>+</sup> EPLM/CLP found in *Il7*<sup>-/-</sup> mice. Real-time quantitative PCR analysis of Ly6D<sup>+</sup> EPLM from *Flt3ltg-Il7*<sup>-/-</sup> mice revealed significant expression of *Ebf1*, *Pax5* and *Foxo1* transcription factors' mRNA in the

absence of IL7 (Figure 3B). Ebf1 expression at the protein level was confirmed by intracellular FACS staining (Figure 3C-D). Even though the percentage of *Flt3ltg-Il7*<sup>-/-</sup> Ebf1<sup>+</sup>Ly6D<sup>+</sup> EPLM did not reach WT levels, it was similar to the one found in *Flt3ltg* mice, which produce IL7. Therefore, *Ebf1/Pax5* expression and subsequent commitment to the B cell fate can occur in the absence of IL7 signaling, arguing against an instructive role of this cytokine in B cell commitment.

CD127 (IL7R $\alpha$ ) is a receptor shared between IL7 and thymic stromal lymphopoietin (TSLP), a cytokine capable of rescuing B cell development when over-expressed in the absence of IL7 (33). Since TSLP is produced by dendritic cells (34), which are dramatically expanded in *Flt3ltg* mice (8), *in vivo* FL over-expression could lead to increased levels of TSLP thereby rescuing B cell development in *Flt3ltg-Il7*<sup>-/-</sup> mice. To investigate this possibility we injected *Il7*<sup>-/-</sup> or *Il7ra*<sup>-/-</sup> mice with FL **as described above** and assessed the rescue of Ly6D<sup>+</sup> EPLM and downstream CD19<sup>+</sup> progenitors. FL injections into *Il7*<sup>-/-</sup> mice resulted in a significant increase in Ly6D<sup>+</sup> EPLM and CD19<sup>+</sup>CD117<sup>+</sup> B cell progenitors, comparable to the rescue observed in *Flt3ltg-Il7*<sup>-/-</sup> mice (Figure 3E). FL injected *Il7ra*<sup>-/-</sup> mice also demonstrated a significant rescue of Ly6D<sup>+</sup> EPLM and CD19<sup>+</sup>CD117<sup>+</sup> pro-B cells, indicating that the observed rescue of B cell commitment in *Flt3ltg-Il7*<sup>-/-</sup> mice is not mediated through the action of TSLP.

### **IL7 promotes survival, but not proliferation, of Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors.**

Even though our *Flt3ltg-Il7*<sup>-/-</sup> mouse model suggests that IL7 is dispensable for B cell commitment, the dramatic decrease in *Il7*<sup>-/-</sup> Ly6D<sup>+</sup> EPLM/CLP argues for a role of IL7 in the maintenance of this population when FL levels are limiting, by promoting

either their survival or their proliferation. To investigate the potential role of IL7 as a survival factor for Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors, we crossed *Il7*<sup>-/-</sup> mice with mice expressing the pro-survival gene *Bcl2* (35). *Bcl2*tg-*Il7*<sup>-/-</sup> mice demonstrated a minor but statistically significant 2.6-fold increase in Ly6D<sup>+</sup> EPLM and 2.2-fold increase in Ly6D<sup>+</sup> CLP numbers compared to *Il7*<sup>-/-</sup> mice (Figure 4A-B). Cell cycle stage analysis of Ly6D<sup>+</sup> EPLM of these mice indicated that **most of the cells rescued by *Bcl2* are in a quiescent state (Fig. S6) and do not proliferate in response to cytokines**, thereby compromising to some extent the rescue of these progenitors' numbers. Importantly, when plated on OP9 stromal cells plus IL7, *Bcl2*tg-*Il7*<sup>-/-</sup> Ly6D<sup>+</sup> EPLM generated B cells at frequencies similar to WT mice (Figure 4C), indicating that these rescued Ly6D<sup>+</sup> cells had B cell potential. Indeed, when analyzing bone marrow CD19<sup>+</sup> committed progenitors, we could see a significant 68-fold increase in the earliest CD19<sup>+</sup>CD117<sup>+</sup> pro-B cell compartment, compared to *Il7*<sup>-/-</sup> (Figure 4D). Due to the **their quiescent state** {Fig. S6 and (36)} and the IL7 dependence of their proliferation, *Bcl2*tg-*Il7*<sup>-/-</sup> CD19<sup>+</sup>CD117<sup>+</sup> numbers did not reach WT levels, whereas downstream CD19<sup>+</sup> immature B cells showed a less pronounced, but significant rescue (Figure 4D). In the periphery of these mice, marginal zone and follicular B cell numbers were increased, whereas as previously reported (16), T cell numbers were rescued (Fig. S7). Therefore, providing an extra *Bcl2*-mediated survival signal *in vivo* partially rescues *Il7*<sup>-/-</sup> Ly6D<sup>+</sup>CD19<sup>-</sup> progenitors with B cell potential and restores significantly the generation of CD19<sup>+</sup> progenitors. This suggests a role for IL7 in facilitating the survival of Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors.

To evaluate the potential proliferative effect of IL7 on Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors, we analyzed a transgenic mouse model in which *Il7* expression is driven by an MHC Class II promoter, resulting in increased *in*

*in vivo* levels of IL7 (37). These mice exhibit a lymphoproliferative phenotype with increased numbers of CD19<sup>+</sup> B cells (38). In contrast to bone marrow CD19<sup>+</sup> cells, Ly6D<sup>+</sup> EPLM numbers did not increase in response to elevated IL7 (Figure 5A-C). In addition, the cell cycle profile of Ly6D<sup>+</sup> EPLM remained unaltered in *Il7tg* mice compared to WT (Figure 5D), arguing against a proliferative action of IL7 on these progenitors. To exclude the possibility that a proliferative signal by FL present in these mice compromised the effect of increased IL7 on the cell cycle status of Ly6D<sup>+</sup> EPLM, we crossed *Il7tg* with *Flt3l*<sup>-/-</sup> mice. Over-expression of IL7 *in vivo* did not result in a significant increase in Ly6D<sup>+</sup> EPLM or CLP numbers in the absence of FL (Figure 5E and FigS8). In contrast, a 3-fold increase in CD19<sup>+</sup>CD117<sup>+</sup> numbers was observed (Figure 5F), in agreement with the proliferative effect of IL7 on CD19<sup>+</sup> B cells. This resulted in a small, but significant, increase in splenic follicular B cells (Fig. S9). Moreover, cell cycle analysis of *Il7tg-Flt3l*<sup>-/-</sup> Ly6D<sup>+</sup> EPLM showed no significant change in their cycling profile compared to their *Flt3l*<sup>-/-</sup> counterparts (Figure 5G). Therefore, we conclude that while IL7 acts as a proliferative factor for CD19<sup>+</sup> committed B cells, it does not do so for their Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> precursors.

### **FL induces proliferation of Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors.**

As evident in Figure 5G, loss of *in vivo* FL signaling affected the proliferative status of Ly6D<sup>+</sup> EPLM. Comparison of Ly6D<sup>+</sup> EPLM numbers in mice either lacking or over-expressing FL showed a 14-fold reduction in *Flt3l*<sup>-/-</sup> Ly6D<sup>+</sup> EPLM numbers compared to WT, while *Flt3l*tg Ly6D<sup>+</sup> EPLM increased 105-fold (Figure 6A). A similar response to FL levels was observed for Ly6D<sup>+</sup> CLP (Figure 6A). Cell cycle analysis of Ly6D<sup>+</sup> EPLM from these mice showed a significant increase in the

percentage of Ki67<sup>+</sup>DAPI<sup>-</sup> cells and a decrease in the percentage of Ki67<sup>+</sup> cells when FL signaling was absent, while *Flt3ltg* Ly6D<sup>+</sup> EPLM showed the reverse (Figure 6B and Fig. S10). Thus, our data indicate that FL promotes the proliferation of Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors.

To evaluate if FL also regulates the survival of Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors, we crossed *Flt3l*<sup>-/-</sup> mice with *Bcl2tg* mice. Thus, *Bcl2tg-Flt3l*<sup>-/-</sup> mice showed a minor 2-fold increase in Ly6D<sup>+</sup> EPLM numbers compared to their *Flt3l*<sup>-/-</sup> counterparts (1.8-fold for Ly6D<sup>+</sup> CLP) (Figure 6C). Nevertheless, the *in vitro* B cell potential of *Flt3l*<sup>-/-</sup> Ly6D<sup>+</sup> EPLM progenitors was not improved by *Bcl2* over-expression (Figure 6D). Downstream CD19<sup>+</sup> progenitors also demonstrated a partial, but significant, rescue (Figure 6E). Our analysis of *Bcl2tg-Flt3l*<sup>-/-</sup> mice suggests that the reduction in Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors observed in *Flt3l*<sup>-/-</sup> mice can only be partially explained by a survival role of FL. In contrast, the clear change in the numbers and cycling profile of these progenitors in response to the absence or 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 FL at this developmental stage.

### **FL does not instruct commitment to the B cell lineage.**

The striking rescue in B cell commitment observed in our *Flt3ltg-Il7*<sup>-/-</sup> mice could be explained by a potential instructive role of FL when present at high levels *in vivo*. However, increased FL did not result in *Ebfl* or *Pax5* up-regulation (Figure 3B-D). Moreover, analysis of *Flt3l*<sup>-/-</sup> Ly6D<sup>+</sup> EPLM showed that while absence of FL *in vivo* leads to a reduction in the numbers of Ly6D<sup>+</sup> EPLM (Figure 1C), it does not significantly reduce the percentage of *Ebfl*<sup>+</sup> cells within the population (Figure 7A-

B), consistent with a permissive rather than instructive role of FL. Finally, the decrease in the Ebf1<sup>+</sup> fraction of Ly6D<sup>+</sup> 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 *Flt3ltg* Ly6D<sup>+</sup> 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 in CD135<sup>+</sup>CD127<sup>+</sup>Ly6D<sup>+</sup> progenitors prior to CD19 expression (30, 31). In *Il7*<sup>-/-</sup> mice, this Ly6D<sup>+</sup> CLP compartment is significantly reduced (22), a finding confirmed in the present study for both CLP and EPLM, a B220<sup>int/+</sup> population partly overlapping with CLP and pre-pro B cells (Figure 1B-C). The proliferative effect of IL7 on committed CD19<sup>+</sup> B cell progenitors (38) makes the investigation of its role in B cell commitment challenging when using CD19<sup>+</sup> cells as readout. Hence, we assessed the role of IL7 in B cell commitment by analyzing the Ly6D<sup>+</sup> CLP/EPLM compartment in different mouse models. Our analysis of *Flt3ltg-Il7*<sup>-/-</sup> mice showed a complete rescue of Ly6D<sup>+</sup> CLP/EPLM numbers *in vivo* and their B cell potential *in vitro* and *in vivo*, while Ebf1 and Pax5 were expressed at similar levels to *Flt3ltg* mice, thereby indicating that IL7 signaling is not required for their up-regulation at the Ly6D<sup>+</sup>CD19<sup>-</sup> stage (Figures 2 and 3). 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<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> stage, as previously hypothesized (18-20), but rather as a permissive one.

Early investigations had shown that *Bcl2* over-expression in the absence of IL7 signaling could rescue T cell (39, 40) but not B cell development (16, 17). However, a more recent study demonstrated a *Bcl2*-mediated rescue of CD19<sup>+</sup> progenitors in conditional *Stat5*<sup>-/-</sup> mice, as well as a strong activation of the pro-survival gene *Mcl1* expression by Stat5 (21), therefore suggesting a survival role for IL7 in B cell development. Our use of *Il7*<sup>-/-</sup> mice instead of *Il7ra*<sup>-/-</sup>, which allows the assessment of progenitor *in vitro* B cell potential, and our focus on Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors, has enabled us to confirm the latter findings and extend them to the CD19<sup>-</sup> stage where B cell commitment events are initiated at the molecular level. Interestingly, *Il7*tg mice analysis showed that IL7 indeed acts as a proliferative factor for committed CD19<sup>+</sup> cells, but not for their CD19<sup>-</sup> precursors. Even in the absence of FL, excess IL7 was unable to significantly increase Ly6D<sup>+</sup> CLP/EPLM numbers, while it did so for CD19<sup>+</sup> B cell progenitors (Figure 5). Hence, we propose that the main role of IL7 at the CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> 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 additionally induces their proliferation (Figure 7D). This survival role becomes particularly critical when FL levels are limiting, thereby explaining the reduction in Ly6D<sup>+</sup> CLP/EPLM seen in *Il7*<sup>-/-</sup> mice. Our study, in agreement with previous data (21), identifies a common, permissive rather than instructive role for IL7 in both B and T cell development (39, 40).

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<sup>+</sup>CD127<sup>+</sup>Ly6D<sup>+</sup>CD19<sup>-</sup> 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<sup>-</sup>CD117<sup>+</sup>Sca1<sup>+</sup> cells (LSK) (8), thereby increasing the developmental input into the CD135<sup>+</sup>CD127<sup>+</sup>Ly6D<sup>+</sup>CD19<sup>-</sup> 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 resulted in a proportional reduction of Ebf- and Pax5-expressing Ly6D<sup>+</sup>CD19<sup>-</sup> progenitors (Figures 3 and 7). One explanation for this reduction could be the increased percentage of cycling *Flt3ltg* Ly6D<sup>+</sup>CD19<sup>-</sup> progenitors, resulting in a decreased fraction 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 *Flt3ltg* mice, thus leading to a smaller fraction of the expanded Ly6D<sup>+</sup>CD19<sup>-</sup> compartment entering the B cell pathway. Our conclusion is that FL is mainly responsible for generating enough CD135<sup>+</sup>CD127<sup>+</sup>Ly6D<sup>+</sup>CD19<sup>-</sup> progenitors, both by inducing their proliferation and by increasing their developmental input from the LSK compartment (Figure 7D) (41, 42). As a result, increased levels of FL in *Flt3ltg-Il7<sup>-/-</sup>* mice lead to a dramatic increase in CD135<sup>+</sup>CD127<sup>+</sup>Ly6D<sup>+</sup>CD19<sup>-</sup> progenitor numbers, therefore surpassing the need for the survival role of IL7 at this stage and resulting in a sufficient fraction of them committing to the B cell lineage.

The generation of B cell progenitors in *Flt3ltg-Il7<sup>-/-</sup>* mice is reminiscent of the apparent IL7 independency of human B lymphopoiesis, where relatively normal numbers of B cells are seen in patients with mutations in components of the IL7 signaling pathway (13, 14). However, all patients with such mutations are neonates and in neonatal *Il7<sup>-/-</sup>* mice, B cell development also takes place (43). Therefore, the apparent difference in the IL7 dependency of B cell development between man and mouse could actually reflect the corresponding difference between fetal/neonatal and adult lymphopoiesis. Our data showing that increased FL signaling can rescue B cell commitment in the absence of IL7 could provide a potential explanation for this difference. Fetal/neonatal CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors might be exposed to higher levels of FL and/or show higher sensitivity to FL signaling than adult CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors. Indeed, previous studies showed that despite a preferable response of fetal B cell progenitors to TSLP, FL signaling remains an absolute requirement for fetal B lymphopoiesis (44, 45).

The instructive or permissive progenitor regulation of lineage commitment by cytokines is a complex process, **in which cytokines can initiate developmental transcription programs in progenitors. However, the reverse is also true, since the particular epigenetic, transcriptional and signaling landscape of a cell can affect its response to a cytokine (9).** Indeed, while previous analysis of *Flt3ltg* mice indicated an instructive role for FL in promoting differentiation of multi-potent progenitors towards lympho-myeloid and away from erythroid fate (8), our present data show that FL acts in a permissive manner for B cell commitment of CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors. In addition, whereas IL7 induces proliferation of committed CD19<sup>+</sup> B cell progenitors, it does not do so on CD127<sup>+</sup>CD19<sup>-</sup> progenitors, suggesting that upon commitment to the B cell lineage, changes in the transcription factor and intracellular

signaling landscape influence the effector function of IL7. Therefore, our present data further support the notion of a cell-context dependent cytokine action.

The *Ebf1/Pax5* up-regulation and subsequent B cell commitment in *Flt3ltg-Il7<sup>-/-</sup>* mice shown herein raises the issue of the potential extra-cellular 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<sup>+</sup>CD127<sup>+</sup>Ly6D<sup>+</sup>CD19<sup>-</sup> progenitors resulting in Pax5/CD19 expression and B cell commitment. Alternatively, as yet uncommitted CD135<sup>+</sup>CD127<sup>+</sup>Ly6D<sup>+</sup>CD19<sup>-</sup> progenitors could express *Ebf1* in a cell-autonomous, stochastic, manner with some obtaining sufficient *Ebf1* to initiate the B cell gene program and eventually commit to the B cell lineage. The intricate transcription factor network sustaining B cell commitment through a series of positive feedback regulatory loops (46) provides conceptual support for the latter hypothesis.

## MATERIALS AND METHODS

### Mice.

For breeding and analysis, age- and sex-matched C57BL/6 *Flt3l<sup>-/-</sup>* (27), *Flt3ltg* (8), *Il7<sup>-/-</sup>* (10), *Il7ra<sup>-/-</sup>* (11), *Il7tg* (38), and (C57BL/6 x C3H) *Bcl2tg* (35) mice backcrossed with C57BL/6 for at least 5 generations 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. 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 (47). Briefly, OP9 or OP9DL1 stromal cells were plated on flat-bottom 96-well plates one day before the initiation of co-cultures, at a concentration of 3000 cells per well. The following day stromal cells were  $\gamma$ -irradiated (3000 rad) and the sorted progenitor cells were added at different concentrations. Cultures were maintained in IMDM medium supplemented with  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol, 1 mM glutamine, 0.03% (wt/vol) primatone, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 5% FBS and 10% IL7-conditioned medium. After 14 days in culture all wells were inspected under an inverted microscope and wells containing colonies of more than 50 cells were scored as positive.

### **Quantitative real-time PCR.**

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).

### **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  $\pm$  SD or SEM. n.s. not significant or  $P > 0.05$ , \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ .

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### **AUTHOR CONTRIBUTIONS**

L.vM. performed experiments, analyzed data and revised the manuscript; L.A-S., F.K. and G.C. performed experiments; D.F. provided *Il7<sup>-/-</sup>* and *Il7ra<sup>-/-</sup>* mice and revised the manuscript; R.C provided *Il7tg* mice and revised the manuscript; A.R. designed

experiments, analyzed data, revised the manuscript and supervised the project; P.T. designed and performed experiments, analyzed data, wrote the manuscript and supervised the project.

## COMPETING FINANCIAL INTERESTS

The authors declare no commercial or financial conflict of interest.

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## FIGURE LEGENDS

**Figure 1. IL7 and FL are necessary for the generation of a normal Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> compartment.** **A.** FACS plots showing the gating strategy used for identification of Ly6D<sup>+</sup> 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*<sup>-/-</sup> and *Flt3l*<sup>-/-</sup> mice. **C.** Absolute numbers of Ly6D<sup>+</sup> EPLM (upper graph) and CLP (lower graph) from the bone marrow of WT (n=13), *Il7*<sup>-/-</sup> (n=5) and *Flt3l*<sup>-/-</sup> (n=10) mice. **D.** Representative FACS plots of EPLM and CLP from WT and *Flt3l*<sup>tg</sup> mice. **E.** Absolute numbers of total EPLM and CLP (left graphs) and Ly6D<sup>+</sup> EPLM and CLP (right graphs) from WT and *Flt3l*<sup>tg</sup> mice.

**Figure 2. Increased *in vivo* FL levels rescue B cell generation in *Il7*<sup>-/-</sup> mice.** **A.** Representative FACS plots of EPLM (upper panel) and CLP (lower panel) from WT, *Il7*<sup>-/-</sup>, *Flt3ltg* and *Flt3ltg-Il7*<sup>-/-</sup> mice. **B.** Numbers of EPLM (top left), CLP (bottom left), Ly6D<sup>+</sup> EPLM (top right) and Ly6D<sup>+</sup> CLP (bottom right) from the mouse genotypes indicated on the x-axes. For each mouse genotype mean±SEM is shown. **C.** Numbers of CD19<sup>+</sup>CD117<sup>+</sup> (top left), CD19<sup>+</sup>CD117<sup>-</sup>IgM<sup>-</sup> (top right) and CD19<sup>+</sup>IgM<sup>+</sup> (bottom) bone marrow cells from the mice indicated on the x-axes. For each mouse genotype mean±SEM is shown. **D.** Numbers of CD19<sup>+</sup>CD21<sup>high</sup>CD23<sup>low</sup> marginal zone (left) and CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup> 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<sup>+</sup> EPLM B cell potential. Ly6D<sup>+</sup> EPLM were sorted from WT, *Il7*<sup>-/-</sup>, *Flt3ltg* and *Flt3ltg-Il7*<sup>-/-</sup> 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<sup>+</sup> 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<sup>+</sup> EPLM of the indicated WT or mutant mice. **D.** Percentages of Ebf1-expressing Ly6D<sup>+</sup> EPLM from WT (n=7), *Il7*<sup>-/-</sup> (n=3), *Flt3ltg* (n=11) and *Flt3ltg-Il7*<sup>-/-</sup> (n=6) mice. Bars show mean±SEM. **E.** Ly6D<sup>+</sup> EPLM (left) and CD19<sup>+</sup>CD117<sup>+</sup> (right) numbers from WT (n=5), *Il7*<sup>-/-</sup> (n=5), *Flt3ltg* (n=3), *Flt3ltg-Il7*

<sup>-/-</sup> (n=5) mice, as well as from *Il7*<sup>-/-</sup> (n=5) and *Il7ra*<sup>-/-</sup> (n=6) mice injected intraperitoneally with 10 daily doses of 10 µg FL each (indicated as +FL) or PBS (+PBS, n=4). Shown is the mean±SEM.

**Figure 4. *Bcl2* over-expression partially rescues B cell commitment in *Il7*<sup>-/-</sup> mice.**

**A.** Representative FACS plots of EPLM (upper panel) and CLP (lower panel) from WT, *Il7*<sup>-/-</sup>, *Bcl2*tg and *Bcl2*tg-*Il7*<sup>-/-</sup> mice. **B.** Numbers of EPLM (top left), CLP (bottom left), Ly6D<sup>+</sup> EPLM (top right) and Ly6D<sup>+</sup> 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<sup>+</sup> EPLM B cell potential. Ly6D<sup>+</sup> EPLM were sorted from WT, *Il7*<sup>-/-</sup>, *Bcl2*tg and *Bcl2*tg-*Il7*<sup>-/-</sup> mice and plated at the indicated concentrations on OP9 stromal cells together with IL7. One representative out of three independent experiments is shown. **D.** Numbers of CD19<sup>+</sup>CD117<sup>+</sup> (top), CD19<sup>+</sup>CD117<sup>+</sup>IgM<sup>-</sup> (middle) and CD19<sup>+</sup>IgM<sup>+</sup> (bottom) 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<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors.**

**A.** CD19<sup>+</sup>CD117<sup>+</sup> numbers in bone marrow of WT (n=10), *Il7*<sup>-/-</sup> (n=5) and *Il7*tg (n=8) mice. **B.** EPLM numbers in bone marrow of WT (n=14), *Il7*<sup>-/-</sup> (n=7) and *Il7*tg (n=5) mice. **C.** Ly6D<sup>+</sup> EPLM numbers in bone marrow of WT (n=14), *Il7*<sup>-/-</sup> (n=7) and *Il7*tg (n=5) mice. **D.** Cell cycle analysis of Ly6D<sup>+</sup> EPLM from WT (n=5) and *Il7*tg (n=2) mice. Graph shows percentages of Ki67<sup>-</sup>DAPI<sup>-</sup>, Ki67<sup>+</sup>DAPI<sup>-</sup> and Ki67<sup>+</sup>DAPI<sup>+</sup> Ly6D<sup>+</sup> EPLM. Bars in **A**, **B**, **C** and **D** show mean±SEM. **E.** Numbers of EPLM (top left), CLP (bottom left), Ly6D<sup>+</sup> EPLM (top right) and Ly6D<sup>+</sup> CLP

(bottom right) from WT and mutant mice, as indicated on the x-axes. For each mouse genotype mean $\pm$ SEM is shown. **F.** Numbers of CD19<sup>+</sup>CD117<sup>+</sup> bone marrow cells from WT and mutant mice, as indicated on the x-axis. For each mouse genotype mean $\pm$ SEM is shown. **G.** Cell cycle analysis of Ly6D<sup>+</sup> EPLM from WT (n=5), *Flt3l*<sup>-/-</sup> (n=3), *Il7tg* (n=2) and *Il7tg-Flt3l*<sup>-/-</sup> (n=3) mice. Graph shows percentages of Ki67<sup>-</sup>DAPI<sup>-</sup>, Ki67<sup>+</sup>DAPI<sup>-</sup> and Ki67<sup>+</sup>DAPI<sup>+</sup> Ly6D<sup>+</sup> EPLM. Bars show mean $\pm$ SEM.

**Figure 6. FL promotes proliferation but not survival of Ly6D<sup>+</sup>CD135<sup>+</sup>CD127<sup>+</sup>CD19<sup>-</sup> progenitors.** **A.** Numbers of EPLM (top left), CLP (bottom left), Ly6D<sup>+</sup> EPLM (top right) and Ly6D<sup>+</sup> CLP (bottom right) from WT (n=14), *Flt3l*<sup>-/-</sup> (n=10) and *Flt3ltg* (n=9) mice. Bars show mean $\pm$ SEM. **B.** Cell cycle analysis of Ly6D<sup>+</sup> EPLM from WT (n=5), *Flt3l*<sup>-/-</sup> (n=3) and *Flt3ltg* (n=9) mice. Graph shows percentages of Ki67<sup>-</sup>DAPI<sup>-</sup>, Ki67<sup>+</sup>DAPI<sup>-</sup> and Ki67<sup>+</sup>DAPI<sup>+</sup> Ly6D<sup>+</sup> EPLM. Bars show mean $\pm$ SEM. **C.** Numbers of EPLM (top left), CLP (bottom left), Ly6D<sup>+</sup> EPLM (top right) and Ly6D<sup>+</sup> CLP (bottom right) from WT and mutant mice, as indicated on the x-axes. For each mouse genotype mean $\pm$ SEM is shown. **D.** *In vitro* limiting dilution analysis of Ly6D<sup>+</sup> EPLM B cell potential. Ly6D<sup>+</sup> EPLM were sorted from WT, *Flt3l*<sup>-/-</sup> and *Bcl2tg-Flt3l*<sup>-/-</sup> mice and plated at the indicated concentrations on OP9 stromal cells together with IL7. **E.** Numbers of CD19<sup>+</sup>CD117<sup>+</sup> (left), CD19<sup>+</sup>CD117<sup>-</sup>IgM<sup>-</sup> (middle) and CD19<sup>+</sup>IgM<sup>+</sup> (right) bone marrow cells from WT and mutant mice, as indicated on the x-axes. For each mouse genotype mean $\pm$ 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<sup>+</sup> EPLM of WT, *Flt3l*<sup>-/-</sup> and *Flt3ltg* mice. **B.** Percentages of *Ebf1*-expressing Ly6D<sup>+</sup>

EPLM from WT (n=7), *Flt3l*<sup>-/-</sup> (n=5) and *Flt3ltg* (n=12) mice. Bars show mean±SEM.

**C.** *In vitro* limiting dilution analysis of Ly6D<sup>+</sup> EPLM T cell potential. Ly6D<sup>+</sup> EPLM were sorted from WT and *Flt3ltg* 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<sup>+</sup> committed B-cell precursors. HSC: Hematopoietic Stem Cell; LMPP: Lymphoid-primed Multi-Potent Progenitor; CLP: Common Lymphoid Progenitor; EPLM: Early Progenitor with Lymphoid and Myeloid potential.