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Flt3 signaling regulates the proliferation, survival, and maintenance of multipotent hematopoietic progenitors that generate B cell precursors

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Abstract

Flt3 signaling plays a crucial role in regulating the survival and differentiation of lymphoid progenitors into B cell precursors (BCPs) in bone marrow. To define further the role of Flt3 signaling in lymphoid progenitor survival, mice deficient in Flt3 ligand that also expressed a Bcl2 transgene (*Eμ-bcl2tg flt3l*$^{-/-}$) were generated. Intracellular flow cytometry established transgene expression in primitive hematopoietic progenitors, including lineage-negative Sca-1^{+} c-kit^{+} (LSK^{+}) CD27^{-} cells enriched for functional hematopoietic stem cells. Compared with *flt3l*$^{-/-}$ mice, *Eμ-bcl2tg flt3l*$^{-/-}$ mice had significantly increased multipotential progenitors (MPPs), IL-7R^{+} common lymphoid progenitors, and B cell precursors. To determine whether forced expression of Bcl2 was sufficient to restore lymphoid priming in the absence of Flt3 signaling *Eμ-bcl2tg flt3l*$^{-/-}$ *rag1-gfp*$^{+}$ mice were generated. Analysis of *Eμ-bcl2tg flt3l*$^{-/-}$ *rag1-gfp*$^{+}$ mice revealed that the Bcl2 transgene had no effect on lymphoid priming before CD19 expression. Thus, forced expression of a survival gene can bypass the requirement for threshold levels of Flt3 signaling requisite for lymphoid priming. Temporal Flt3 ligand (FL) replacement therapy in *flt3l*$^{-/-}$ mice revealed specific requirements for Flt3 signaling in the expansion and maintenance of Flt3^{+hi} MPP and Flt3^{+} all lymphoid progenitors, but not Flt3^{+} B lymphoid progenitors (BLPs), the immediate precursors of BCPs. BCPs were restored after temporal in vivo FL treatment, albeit with delayed kinetics. Together, these results show that Flt3 regulates the proliferation, and survival, and maintenance of developmental stage–specific hematopoietic progenitors that give rise to BCPs.

B cell development from hematopoietic stem cells (HSCs) requires the concerted activities of transcription factors that regulate cell fate decisions and signaling molecules that temporally control the differentiation, proliferation, and survival of select progenitor subsets. Fms-like tyrosine kinase (Flt3) and its ligand, Flt3 ligand (FL), play a critical role in regulation of the multipotential progenitor (MPP) and common lymphoid progenitor (CLP)

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hematopoietic progenitor pools from which B cell precursors are derived [1–3]. While it is widely appreciated that Flt3 signaling is critical for lymphoid lineage and B-cell development, much remains to be learned concerning the roles of this growth factor receptor–ligand pair in their regulation. Indeed, precise cellular targets and mechanisms by which Flt3 signaling regulates the MPP and CLP pools, or their differentiation into B cell precursors (BCPs), remains largely unknown.

The generation of BCPs from HSCs proceeds through a series of developmental intermediates that initiate a series of molecular events that culminate in the activation of a genetic program that selectively orchestrates B cell differentiation. Upregulation of Flt3 receptor density in MPPs correlates with lymphoid-myeloid restriction [4–6]. A small subset of $Flt3^+$ MPPs express high levels of Flt3 ($Flt3^{hi}$ MPPs) and exhibit a lymphoid-biased genetic program and differentiation potential in vitro and in vivo [4–6]. The significance of high Flt3 receptor density on lymphoid-biased progenitors remains to be elucidated, but it is consistent with our previous findings that threshold levels of Flt3 signaling are required for lymphoid development.

$Flt3^{hi}$ MPPs express *il7ra* transcripts and are the presumed immediate precursors of IL-$7R^+$ $Flt3^+$ CLPs [2,6]. A recent study determined that a subset of IL-$7R^+$ $Flt3^+$ CLPs express the surface marker Ly6D [7]. In vitro and in vivo assays revealed that the $Ly6D^-$ fraction of CLPs retained all lymphoid potential, while the $Ly6D^+$ fraction was enriched for B and dendritic cell potentials. Based on these findings, IL-$7R^+$ $Flt3^+$ $Ly6D^-$ CLPs are now referred to as *all lymphoid progenitors* (ALPs), and IL-$7R^+$ $Flt3^+$ $Ly6D^+$ CLPs are known as *B lineage-restricted B lymphoid progenitors* (BLPs). IL-7R signaling in BLPs induces expression of early B cell factor (EBF), a transcription factor critical for specification and commitment to the B cell fate [8–10].

Cytokine signaling also contributes to hematopoiesis by promoting progenitor expansion and survival. Although we did not find a proliferation defect in vivo in mice deficient for FL (*flt3l*$^{-/-}$), in vivo administration of FL expands MPPs with lymphoid–myeloid differentiation potential [11]. It is well established that FL synergizes with other early-acting cytokines in the expansion of MPPs [12–15]. The combination of FL, stem cell factor, and IL-7 is widely used to generate lymphoid–B lineage cells from MPPs [16–18]. In addition to proliferation, Flt3 signaling regulates hematopoietic progenitor survival. *Flt3l*$^{-/-}$ mice have increased percentages of Annexin V^+ lineage-negative Sca-1^+ c-kit^+ (LSK^+) $Flt3^+$ cells and reduced levels of the prosurvival protein Mcl-1, supporting a role for Flt3 signaling in the survival of primitive hematopoietic progenitors [3].

In this study, we sought to pinpoint developmental stage–specific roles for Flt3 signaling in the proliferation, survival, and maintenance of $Flt3^+$ MPPs into BCPs in vivo. We show that enforced expression of the antiapoptotic protein Bcl2 largely restored LSK^+, $Flt3^+$ CLP, and select BCP deficiencies in *flt3l*$^{-/-}$ mice. We crossed our *Eμ-bcl2tg flt3l*$^-$ mice to *rag1-gfp*$^+$ mice to determine whether lymphoid priming was restored in Lin^- progenitor subsets. Strikingly, the frequency of GFP^+ cells within $Flt3^{+hi}$ MPP, ALP, and BLP in *Eμ-bcl2tg flt3l*$^{-/-}$ *rag1-gfp*$^+$ was similar to the *flt3l*$^{-/-}$ *rag1-gfp*$^+$ mice, indicating that restoration was independent of lymphoid priming. Importantly, temporal FL administration in vivo

expanded $Flt3^+$ LSK^+ and $Flt3^+$ ALP subsets enriched for multilymphoid lineage differentiation potential, but only minimally affected $Flt3^+$ BLPs. Regardless of the minor effect of FL administration on BLP, BCP numbers were normalized after temporal FL administration. Together, these experimental findings show that Flt3 signaling regulates the survival and expansion of $Flt3^+$ LSK^+ into BCPs. Furthermore, these results suggest that IL-7R signaling coupled with enforced expression of a survival gene can circumvent the requirement for Flt3 signaling in B cell development.

Methods

Mice

All mice in this study have been maintained on a C57Bl/6 genetic background for greater than 10 generations. WT (C57Bl/6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). *Flt3l*$^{-/-}$ mice were obtained from Taconic Farms (Germantown, NY, USA) and then bred and maintained in our colony. *Eμ-Bcl2-36* transgenic (*Eμ-bcl2tg*) mice have been described previously and were provided by Paul W. Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA) [19]. *Flt3l*$^{-/-}$ and *Eμ-bcl2tg* mice were bred to generate *Eμ-bcl2tg flt3l*$^{-/-}$ mice. *Eμ-bcl2tg flt3l*$^{-/-}$ mice were crossed to *rag1-gfp*$^+$ mice to generate *Eμ-bcl2tg flt3l*$^{-/-}$ *rag1-gfp*$^+$ progeny. *Rag1-gfp*$^+$and *flt3l*$^{-/-}$ *rag1-gfp*$^+$ mice have been described [3,48]. Age-matched or littermate controls were used for individual experiments, and all mice analyzed in this study ranged from 8–12 weeks of age. All animals were bred and maintained at the Mayo Clinic animal facility, and experiments were performed according to the Mayo Clinic Institutional Animal Care and Use Committee guidelines.

Genotyping

Eμ-bcl2tg flt3l$^{-/-}$ mice were identified by separate genotyping reactions with two primer sets: *flt3l* and *bcl2tg*. *Eμ-bcl2tg flt3l*$^{-/-}$ *rag1-gfp*$^+$ were identified by separate genotyping reactions using four primer sets: *rag1*, *gfp*, *flt3l*, and *bcl2tg*. PCR sequences and conditions for genotyping *rag1*, *gfp*, and *flt3l*$^{-/-}$ mice have been described [3]. *Bcl2tg* genotyping was performed using primer combinations and PCR conditions found on The Jackson Laboratory website, mouse stock number 002321.

Flow cytometry

Methods for flow cytometry and progenitor isolation have been described [20,49,50]. BM or spleen was harvested and stained with combinations of the following antibodies: c-kit (APC, APC-eFluor 780), Sca-1 PerCP-Cy5.5, Flt3 PE, CD34 eFluor 450, CD150 PE-Cy7, IL-7Rα (bio or PE-Cy7), Ly6D (APC, eFluor 450), IgM APC-Cy7, CD19 (APC, PE-Cy7), B220 APC, CD43 PerCP-Cy5.5, and a FITC- or biotin-labeled (for *rag1*- *gfp*$^+$ mice) lineage cocktail (B220, CD3ε, CD11b/Mac-1, Gr-1, and Ter119) to exclude Lin^+ cells. Incubation with streptavidin- PE-Cy7 or streptavidin-APC was used to visualize bio-IL-7Rα or bio-lineage markers, respectively. For intracellular staining of the Bcl2 transgene in LSK^+ cells, BM was stained with a biotin-labeled lineage mixture (B220, CD19, Gr-1, Ter119, CD3ε, NK1.1, IgM, and CD8α) and depleted of Lin^+ cells using streptavidin magnetic bead depletion. Lin^{-lo} cells were surface stained with c-kit APC, Sca-1 PE, and CD27 APC-

eFluor 780, fixed, and permeabilized using Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA, USA), followed by intracellular staining with hamster anti-human Bcl2 FITC or PE (for *rag1-gfp*$^{+}$ mice) or isotype control FITC or PE (BD Biosciences, San Jose, CA, USA). All antibodies were obtained from eBioscience (San Diego, CA, USA), BD Biosciences, or BioLegend (San Diego, CA, USA). Flow cytometric analysis was performed on the LSRII or Canto cytometers (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Progenitor frequency and absolute number calculations

Progenitor frequencies were calculated by multiplying percentages of sequential gated populations. Absolute numbers were calculated by multiplying mononuclear cell counts obtained after BM harvest by progenitor frequencies. The frequencies and number calculations reflect mononuclear and doublet exclusion gates.

Flt3 ligand replacement therapy

Flt3 ligand was obtained from PeproTech (Rocky Hill, NJ, USA). Two cohorts of *flt3l*$^{-/-}$ mice were administered Flt3 ligand five times over the course of an 8-day injection schedule. Each injection, spaced 2 days apart, consisted of 10 μg of FL delivered in 200 μL of PBS. For controls, WT and *flt3l*$^{-/-}$ mice were similarly injected with equivalent volumes (200μL) of PBS. Two days following the last injection, one cohort of mice were euthanized, BM harvested, and progenitor populations were analyzed by flow cytometry. The second cohort of mice was euthanized, and BM was harvested 5 days after the last injection for flow cytometric analysis.

Statistical analysis

Statistical analysis was performed using the Student *t* test; $p \leq 0.05$ was significant. All numerical data are presented as mean ± SEM.

Results

Enforced expression of Bcl2 rescues LSK^{+} cells, but not deficiencies in Flt3^{+hi} MPPs in flt3l$^{-/-}$ mice

We previously reported increased percentages of Annexin V^{+} Flt3^{+hi} MPPs and decreased intracellular levels of the prosurvival protein Mcl-1 in *flt3l*$^{-/-}$ mice compared with their wild type (WT) counterparts [3]. To further elucidate the role of Flt3 signaling in hematopoietic progenitor survival in vivo, we generated *Eμ-bcl2tg flt3l*$^{-/-}$ mice. Eμ-Bcl2 line 36 transgenic mice have been described previously [19]. In these mice, the Eμ enhancer drives expression of human Bcl2, primarily in the B lineage. We previously showed that the Eμ enhancer is active in LSK^{+} cells [20]. FL is required from an early stage in hematopoiesis; therefore, it was important to establish that the Bcl2 transgene was expressed early enough to effect the survival of Flt3^{+} MPPs. As shown in Figure 1A, the Bcl2 transgene is expressed in LSK^{+} cells and initiates before upregulation of CD27, a marker acquired as HSC transition into MPPs (Fig. 1A) [21].

Bone marrow (BM) cellularity is reduced in FL-deficient mice, and this was not significantly altered by expression of a Bcl2 transgene (Table 1). Percentages and absolute cell numbers of LSK^+ cells are significantly reduced in *flt3l*$^{-/-}$ mice, and this deficiency was restored by expression of the Bcl2 transgene (Fig. 1B, C, and D). MPPs can be discriminated from HSCs within the LSK^+ compartment by differential expression of CD150 and CD34. HSCs are $LSK^+CD150^+CD34^-$ and MPPs are $LSK^+CD150^-CD34^+$ (Supplementary Figure E1, online only, available at www.exphem.org) [22,23]. Consistent with our previous report, percentages of $LSK^+CD150^+CD34^-$ HSCs are increased in *flt3l*$^{-/-}$ mice, and this is not altered by expression of the Bcl2 transgene (Fig. 1C, D, and Supplementary Figure E1) [24]. Indeed, the most significantly upregulated subset of LSK^+ cells affected by expression of Bcl2 are $LSK^+CD150^-CD34^+$ MPPs (Fig. 1D). Although forced expression of the Bcl2 transgene corrected the *flt3l*$^{-/-}$ deficiency in LSK^+ cells, it did not restore frequencies of $Flt3^{+hi}$ MPPs enriched for lymphoid-biased progenitors (Fig. 1B and C).

Due to the impact of the Bcl2 transgene on the LSK^+ compartment, we observed statistically significant increases in absolute numbers of $LSK^+CD150^-CD34^+$ MPPs, $Flt3^{+hi}$ MPPs, and $LSK^+CD150^+CD34^-$ HSCs, in the *Eμ-bcl2tg flt3l*$^{-/-}$ mice compared with *flt3l*$^{-/-}$ mice ($LSK^+CD150^-CD34^+$ MPPs: $1.24 \times 10^5 \pm 2.60 \times 10^4$ vs. $3.12 \times 10^4 \pm 3.42 \times 10^3$, $p = 0.012$; $Flt3^{+hi}$ MPPs: $2.79 \times 10^4 \pm 4.97 \times 10^3$ vs. $7.29 \times 10^3 \pm 1.04 \times 10^3$, $p = 0.0066$; $LSK^+CD150^+CD34^-$ HSCs: $3.96 \times 10^4 \pm 8.25 \times 10^3$ vs. $1.09 \times 10^4 \pm 9.00 \times 10^2$, $p = 0.013$; Fig. 1D). However, the Bcl2 transgene did not alter Flt3 receptor density on LSK^+ of *Eμ-bcl2tg flt3l*$^{-/-}$ mice (Fig. 1B). Consequently, the $Flt3^{+hi}$ MPP subset was not restored. To determine whether upregulation of Flt3 is important for lymphoid priming, we crossed the *Eμ-bcl2tg flt3l*$^{-/-}$ mice to *rag1-gfp*$^+$ mice. RAG1 expression is a hallmark of lymphoid priming [25]. *rag1-gfp*$^+$ mice have GFP knocked into the RAG1 locus, facilitating tracking of RAG1 induction via GFP expression. In agreement with our previous study, *flt3l*$^{-/-}$ *rag1-gfp*$^+$ mice had a severe reduction in frequencies of GFP^+ cells in the $Flt3^{+hi}$ MPP subset (Table 2) [3]. Strikingly, the percentage of GFP^+ cells in the $Flt3^{+hi}$ MPP subset from *Eμ-bcl2tg flt3l*$^{-/-}$ *rag1-gfp*$^+$ was similar to the *flt3l*$^{-/-}$ *rag1-gfp*$^+$ mice (Table 2). Thus, forced expression of a survival protein rescues the LSK^+ deficiency, but not the immunophenotypic $Flt3^{+hi}$ MPP subset in *flt3l*$^{-/-}$ mice or lymphoid priming in the LSK^+ compartment.

Enforced expression of Bcl2 partially rescues Flt3$^+$ CLPs in flt3l$^{-/-}$ mice

LSK^+ cells that express high levels of Flt3 have increased abundance of lymphoid lineage-associated transcripts, including *il7ra*, compared to LSK^+ cells expressing lower levels of Flt3 [6]. $Flt3^{hi}$ LSK^+ cells are the presumed precursors of $Flt3^+$ CLPs, the lymphoid-restricted progenitor subset that gives rise to BCPs [26]. To determine whether upregulation of Flt3 in LSK^+ cells is an obligate step in the generation of $Flt3^+$ CLPs, we examined the $Flt3^+$ CLP compartment in *Eμ-bcl2tg flt3l*$^{-/-}$ mice. Consistent with previous findings, percentages and numbers of Lin^-IL7R^+ CLPs were significantly reduced in *flt3l*$^{-/-}$ mice (Fig. 2A) [1,3]. In contrast, frequencies and absolute numbers of Lin^- $IL\text{-}7R^+$ CLPs in *Eμ-bcl2tg flt3l*$^{-/-}$ mice were comparable to controls (Fig. 2A, C, and D). Numbers of $Flt3^+$ CLPs increased 2.4-fold in the *Eμ-bcl2tg flt3l*$^{-/-}$ mice compared with *flt3l*$^{-/-}$ mice ($3.74 \times 10^3 \pm 4.69 \times 10^2$ vs. $1.57 \times 10^3 \pm 4.04 \times 10^2$, $p = 0.013$; Fig. 2D). $Flt3^+$ CLPs can be fractionated into $Flt3^+$ $Ly6D^-$ ALPs and $Flt3^+$ $Ly6D^+$ BLPs that we and others have shown

have upregulated expression of combinations of genes that accompany B cell fate specification and commitment [7,27]. Percentages of ALPs and BLPs were both increased approximately twofold to threefold in *Eμ-bcl2tg flt3l*$^{-/-}$ mice, compared with *flt3l*$^{-/-}$ mice (ALP: 0.017 ± 0.006 vs. 0.005 ± 0.002, $p = 0.079$; BLP: 0.010 ± 0.002 vs. 0.004 ± 0.001, $p = 0.029$; Fig. 2C). Similarly, numbers of ALPs and BLPs were elevated twofold to threefold in the *Eμ-bcl2tg flt3l*$^{-/-}$ mice compared with *flt3l*$^{-/-}$ mice (ALP: $2.30 \times 10^3 \pm 6.20 \times 10^2$ vs. $9.48 \times 10^2 \pm 3.44 \times 10^2$, $p = 0.10$; BLP: $1.44 \times 10^3 \pm 2.99 \times 10^2$ vs. $6.18 \times 10^2 \pm 2.04 \times 10^2$, $p = 0.063$; Fig. 2D). Although the increases in ALP and BLP did not reach statistical significance, they do reflect a similar proportional increase (approximately threefold) that we observed in Flt3^{+hi} MPPs in the *Eμ-bcl2tg flt3l*$^{-/-}$ mice. To determine whether Flt3$^+$ CLP subsets in the *Eμ-bcl2tg flt3l*$^{-/-}$ mice exhibited lymphoid priming compared with *flt3l*$^{-/-}$ mice, we compared the frequencies of GFP$^+$ ALPs and GFP$^+$ BLPs in *Eμ-bcl2tg flt3l*$^{-/-}$ *rag1-gfp*$^+$ to *flt3l*$^{-/-}$ *rag1-gfp*$^+$ mice. Similar to findings observed in GFP$^+$ Flt3^{+hi} MPPs, percentages of GFP$^+$ cells in ALPs or BLPs were comparable in these two mice (Table 2). These data show that forced expression of a survival protein restores IL-7R$^+$ CLPs and partially restores Flt3$^+$ CLP subsets in *flt3l*$^{-/-}$ mice, but does not alter lymphoid priming in the ALP or BLP compartments.

Enforced expression of Bcl2 restores BCP in flt3l$^{-/-}$ mice

The B lineage–restricted progeny of Flt3$^+$ BLPs are B220$^+$CD19$^-$Ly6D$^+$PDCA1$^-$ Pre-pro B, CD19$^+$B220$^+$ CD43^{+lo}IgM$^-$ Pro-B, CD19$^+$B220$^+$CD43$^-$IgM$^-$ Pre-B, and CD19$^+$IgM$^+$ B cells. *Flt3l*$^{-/-}$ mice have significant reductions in all B lineage progeny (Fig. 3) [3,27]. Bcl2 can functionally substitute for Mcl-1 deficiency in Pro-B cell lines [28]. Furthermore, the same study showed a Bcl2 transgene partially restored numbers of BCP in *IL-7R*$^{-/-}$ mice. Therefore, we examined whether forced expression of Bcl2 corrected the B lineage deficiencies in *flt3l*$^{-/-}$ mice.

Pre-pro B cells are discriminated from BLP by surface expression of CD45R/B220 [27]. Forced expression of the Bcl2 transgene partially restored percentages and absolute numbers of Pre-pro B cells in *flt3l*$^{-/-}$ mice (Fig. 3C and D). CD19$^+$B220$^+$CD43^{+lo}IgM$^-$ Pro-B cells were also increased, but the increase was not statistically significant (Fig. 3C, D, and Supplementary Figure E2 [online only, available at www.exphem.org]). In contrast to the severe decreases in percentages of GFP$^+$ cells within Flt3^{+hi} MPP, ALP, and BLP in the *flt3l*$^{-/-}$ *rag1-gfp*$^+$ mice, regardless of expression of the Bcl2 transgene, the frequency of Pro B cells from *Eμ-bcl2tg flt3l*$^{-/-}$ *rag1-gfp*$^+$ was similar to that from *rag1-gfp*$^+$ control mice (Table 2). Pre-B defined as CD19$^+$ B220$^+$ CD43$^-$IgM$^-$ were significantly increased in *Eμbcl2tg flt3l*$^{-/-}$ mice (Fig. 3C, D, and Supplementary Figure E2). Interestingly, mature B cells in the marrow, identified as CD19$^+$ IgM$^+$, which includes naive and recirculating B cells, were not substantially altered by expression of the Bcl2 transgene. Naive B cells can be discriminated from mature B cells within the CD19$^+$IgM$^+$ subset by GFP expression. As Table 2 illustrates, the decrease in GFP$^+$ naive B cells in the *flt3l*$^{-/-}$ *rag1-gfp*$^+$ mice was not altered by expression of the Bcl2 transgene.

To determine whether the decrease in IgM$^+$ B cells in the marrow mirrored the peripheral B cell pool, spleens were harvested from WT, *flt3l*$^{-/-}$, *Eμ-bcl2tg*, *Eμ-bcl2tg flt3l*$^{-/-}$, and

numbers of IgM^+ B cells were determined. As we and others reported previously, B cell numbers in the spleen are reduced in *flt3l*$^{-/-}$ mice [24,29,30]. Splenic cellularity was dramatically increased in *Eμ-bcl2tg* mice (data not shown) [31]. Importantly, numbers of IgM^+ splenic B cells were reduced in *Eμ-bcl2tg flt3l*$^{-/-}$ compared with control *Eμ-bcl2tg* mice (Supplementary Figure E3, online only, available at www.exphem.org). These data suggest that FL deficiency does not affect survival pathways in peripheral B cells. Taken together, these experimental findings show that forced expression of a survival gene selectively affects developmental stage$^-$ specific B cell precursor subsets in the bone marrow of *flt3l*$^{-/-}$ mice.

Flt3 signaling is required for the expansion and maintenance of LSK$^+$ Flt3^{+hi} MPPs

Previous studies using *flt3l*-deficient mice implicated Flt3 signaling in the regulation of $Flt3^{+hi}$ MPPs and CLPs [1,2]. In vivo administration of FL expands hematopoietic progenitors, which could reflect roles in proliferation or maintenance. Reductions in $Flt3^{+hi}$ MPPs or CLPs in *flt3l*$^{-/-}$ mice during homeostasis does not distinguish roles for Flt3 signaling in maintenance of $Flt3^+$ progenitor subsets. To make these distinctions in vivo, we performed temporal FL replacement therapy. Ten micrograms of recombinant murine FL was administered to *flt3l*$^{-/-}$ mice over an 8-day period (five injections spaced 2 days apart). WT and *flt3l*$^{-/-}$ mice injected with phosphate-buffered saline (PBS) using the same injection schedule served as a control group. Two cohorts of mice were analyzed. The first set of mice was analyzed 2 days after the last injection to determine the requirement for Flt3 signaling in the expansion of $Flt3^+$ progenitor subsets. The second set was analyzed 5 days after the final injection of FL to determine the requirement for Flt3 signaling in the maintenance of $Flt3^+$ progenitor subsets.

Two days after cessation of FL administration, the LSK^+ compartment in *flt3l*$^{-/-}$ mice remained comparable with PBS-treated WT mice (Fig. 4A, C, and D). FL administration significantly increased percentages and numbers of $Flt3^{+hi}$ MPPs within the LSK^+ subset compared with PBS-injected *flt3l*$^{-/-}$ mice (Fig. 4A, C, and D). We noted that frequencies and numbers of $Flt3^{+hi}$ MPPs increased in FL injected *flt3l*$^{-/-}$ mice, but did not reach WT levels following FL injection. This result likely reflects the in vivo half-life of FL [32]. The increase in LSK^+ $Flt3^{+hi}$ MPPs 2 days after FL administration was significantly decreased 5 days after the cessation of FL administration (Fig. 4B–D). These in vivo results support roles for Flt3 signaling in the expansion and maintenance of LSK^+ $Flt3^{+hi}$ MPPs.

Differential requirements for Flt3 signaling in expansion and maintenance of Flt3$^+$ CLP subsets

Next, we examined whether FL administration affected the expansion and maintenance of $Flt3^+$ CLPs. Similar to the LSK^+ subset, FL administration in *flt3l*$^{-/-}$ mice increased total Lin^- IL-7Rα^+ c-kit^{+lo} CLPs and $Flt3^+$ CLPs comparable to PBS-injected WT mice (Fig. 5A, C, and D). However, 5 days after FL administration, percentages and numbers of $Flt3^+$ CLPs resembled that of *flt3l*$^{-/-}$ mice (Fig. 5B–D). To define FL-sensitive CLP subsets, we fractionated $Flt3^+$ CLPs into $Ly6D^-$ ALPs and $Ly6D^+$ BLPs. ALPs were highly sensitive to FL replacement therapy, whereas BLPs were not (Fig. 5C and D). We observed a sharp increase in ALPs 2 days after FL treatment, coupled with a decline in this population 3 days

later (Fig. 5C and D). In contrast, frequencies and numbers of BLPs were more refractory to FL administration than ALPs in *flt3l*$^{-/-}$ FL-injected mice compared with PBS-injected *flt3l*$^{-/-}$ control mice (% BLP: *flt3l*$^{-/-}$ FL, 1.0 ± 0.1; *flt3l*$^{-/-}$ PBS, 0.4 ± 0.1, $p = 0.0025$ (to *flt3l*$^{-/-}$ PBS); no. of BLPs: *flt3l*$^{-/-}$ FL, $2.7 \times 10^4 \pm 7.0 \times 10^3$; *flt3l*$^{-/-}$ PBS, $4.3 \times 10^3 \pm 8.3 \times 10^2$, $p = 0.01$ (to *flt3l*$^{-/-}$ PBS; Fig. C and D). In addition, we observed that BLPs remain stable between 2 and 5 days after FL treatment (Fig. 5C and D). Overall, these data indicate that FL is critical for the expansion and maintenance of Flt3$^+$ ALPs, but not Flt3$^+$ BLPs.

Delayed recovery of BCPs after temporal FL administration

FL replacement therapy primarily targeted Flt3$^+$ MPPs and ALPs. BLPs, the immediate precursor of BCPs, were less sensitive to FL. This observation suggested that the temporal in vivo administration model could be informative regarding the kinetics of B cell genesis from Flt3$^+$ hematopoietic progenitors. First, we determined whether BCPs were restored within the time frame evaluated. Two days after cessation of FL administration, BCPs were increased approximately twofold (Fig. 6A, C, and D). Interestingly, at this time point, the increase in BCPs was directly proportional to the increase in BLPs (% BCP: *flt3l*$^{-/-}$ FL, 10 ± 2; *flt3l*$^{-/-}$ PBS, 4 ± 1, $p = 0.0075$ (to *flt3l*$^{-/-}$ PBS); no. of BCPs: *flt3l*$^{-/-}$ FL, $9.0 \times 10^6 \pm 1.9 \times 10^6$; *flt3l*$^{-/-}$ PBS, $3.3 \times 10^6 \pm 1.1 \times 10^6$, $p = 0.025$ (to *flt3l*$^{-/-}$ PBS; Fig. 6A, C, and D). These data are consistent with BLPs being the immediate precursor of BCPs. In contrast, 5 days after cessation of FL, the BCP compartment was fully restored in *flt3l*$^{-/-}$ mice (Fig. 6B–D). The complete restoration of BCPs five days after cessation of FL administration suggests that approximately 72 hours is sufficient for ALPs to differentiate into CD19$^+$IgM$^-$ BCPs.

Discussion

In this study, we sought to elucidate the roles of Flt3 signaling in the proliferation, survival, and maintenance of Flt3$^+$ hematopoietic progenitor subsets into BCPs. We generated *Eμ-bcl2tg flt3l*$^{-/-}$ mice to determine whether Flt3 signaling is critical for the survival of Flt3^{+hi} MPPs and Flt3$^+$ CLPs. Forced expression of Bcl2 at the transition of HSC to MPP, the stage where Flt3 signaling initializes, restored the LSK$^+$ and Lin$^-$IL-7R$^+$ CLP compartments [33,34]. Furthermore, CD19$^+$ IgM$^-$ BCPs in *Eμ-bcl2tg flt3l*$^{-/-}$ mice were comparable to WT. Importantly, restoration of the BCP compartment by forced expression of Bcl2 was independent of lymphoid priming at the earliest stages of lymphopoiesis. Cytokines also regulate the proliferation and maintenance of developmental stage–specific hematopoietic progenitor subsets [35]. Temporal FL administration in vivo substantially expanded Flt3^{+hi} MPPs and Flt3$^+$ ALPs in *flt3l*$^{-/-}$ mice. However, these subsets quickly declined, establishing the importance of continuous Flt3 signaling in their maintenance. In contrast, Flt3$^+$ BLPs were more refractory to Flt3 signaling. We conclude from these new in vivo experimental findings that Flt3 signaling is critical for the proliferation, maintenance, and survival of Flt3$^+$ MPPs and ALPs. In contrast, BLPs that are poised to downregulate Flt3 and become BCPs are less dependent on signals from Flt3.

Forced expression of a survival gene has been shown to rescue lineage-specific developmental blocks imposed by cytokine deficiencies [36–40]. High-density Flt3

expression is commonly used to resolve lymphoid-biased MPPs ($Flt3^{hi}$ MPP) from MPPs with combined lymphoid-myeloid potential that express lower levels of Flt3 [2,3,5,6,41]. Interestingly, restoration of the LSK^+ subset was not accompanied by upregulation of Flt3, unlinking Flt3 signaling activated survival pathways to receptor upregulation. We interpret this finding to suggest that in the absence of sufficient levels of Flt3 receptor expression necessary to activate survival pathways, lymphoid-primed MPPs are susceptible to apoptosis. Indeed, analysis of *EuBcl2tg flt3l*$^{-/-}$ *rag1-gfp*$^+$ mice revealed no rescue or increase in GFP reporter expression in the LSK^+ or CLP subsets. Thus, forced expression of a survival gene bypasses the requirement for threshold levels of Flt3 signaling to activate lymphoid lineage-specific survival pathways and lymphoid priming. Importantly, IL-7R, once expressed, can largely compensate for reduced Flt3-regulated survival [28]. B cell differentiation does not proceed past the CLP stage in mice deficient for IL-7R signaling underscoring the importance of this signaling pathway in B lymphopoiesis [9,27,42]. The BCP compartment in *Eμ-bcl2tg flt3l*$^{-/-}$ mice was largely comparable to WT controls; thus, IL-7R signaling, coupled with forced expression of a survival gene, can bypass the requirement for threshold levels of Flt3 signaling in the generation of BCP from residual $Flt3^+$ CLPs.

The restoration of numbers of select hematopoietic progenitor subsets that we document in this study strongly supports a role for Flt3 signaling in the regulation of cell survival pathways. However, the restoration for all lymphoid–B lineage subsets downstream of the LSK^+ compartment was partial. In addition to regulating cell survival, Bcl2 transgene expression reduces the proliferation of B and T lineage lymphocytes [43,44]. Indeed, a previous study showed that overexpression of Bcl2 reduces the proliferation of all cycling B lymphocytes [44]. Furthermore, Bcl2 overexpression retards the transition between quiescent and cycling states regardless of differentiation state. The effect of Bcl2 overexpression on proliferation provides an explanation for why we document only a partial rescue of $Flt3^+$IL-$7R^+$ CLPs and BCP subsets in *Eμ-bcl2tg flt3l*$^{-/-}$ mice.

This study determined that Flt3 signaling is essential for the expansion and maintenance of $Flt3^{+hi}$ MPPs and $Flt3^+$ ALPs. Temporal FL administration to *flt3l*$^{-/-}$ mice selectively expanded $Flt3^{+hi}$ MPPs and $Flt3^+$ ALPs. Three days after restoration, the increases in these two subsets diminished. These data support and extend previous in vitro and in vivo findings by others that Flt3 signaling augments hematopoietic progenitor proliferation [11,12,15,45]; hhowever, to our knowledge they show for the first time, the select sensitivity of $Flt3^{+hi}$ MPPs and $Flt3^+$ ALPs to Flt3 signaling. In stark contrast, $Flt3^+$ BLPs that have initiated B cell fate specification and commitment are more refractory to Flt3 signaling [7]. Indeed, in contrast to ALPs, BLPs have detectable transcripts for the B cell commitment factor Pax5 [7]. Importantly, Pax5 is a negative regulator of *flt3* transcription [46]. Thus, Pax5 modulation of *flt3* in BLPs likely contributes to the differential sensitivity of BLPs to FL administration.

FL administration in *flt3l*$^{-/-}$ mice restored BCP. We note that the kinetics of restoration of the BCP pool was delayed compared with $Flt3^{+hi}$ MPPs and $Flt3^+$ ALPs. We suggest that these data reflect the timing of two critical events in B lymphopoiesis regulated by Flt3 signaling. First, signaling via FL plays an active role in upregulating levels of Flt3 in MPPs.

$Flt3^{+hi}$ MPPs are initiating immunoglobulin gene recombination, and this event is coupled to cell cycle regulation [20,25,47]. Thus, strong survival signals provided by Flt3 at this stage are likely critical to protect lymphoid progenitors until sufficient upregulation of IL-7R. Second, Flt3 signaling augments the proliferation of $Flt3^{+hi}$ MPPs and $Flt3^{+}$ ALPs, expanding the pool of lymphoid progenitors that can generate BCP.

Overall, this study provides new information concerning how Flt3 signaling regulates early steps in lymphoid–B lineage development in BM (Fig. 7). We show in vivo, that Flt3 signaling is critical for the survival, expansion, and maintenance of developmental stage–specific hematopoietic progenitor subsets that give rise to BCPs. Finally, our findings suggest that FL plays an active role in upregulating and maintaining Flt3 expression in $Flt3^{+hi}$ MPPs, establishing an autoregulatory role for FL in regulation of Flt3 density.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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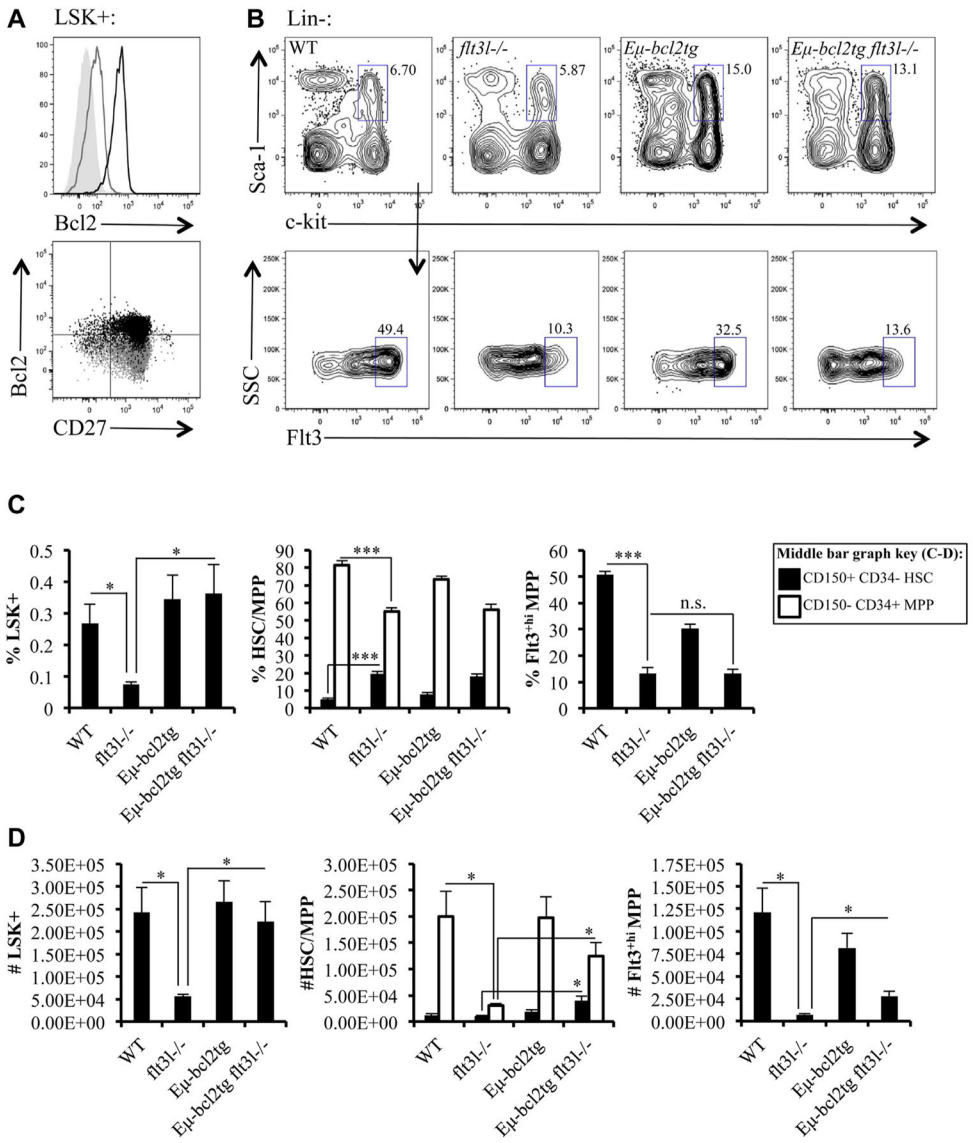

Figure 1.

Enforced expression of Bcl2 rescues LSK^{+} cells, but not deficiencies in Flt3^{+hi} MPPs in *flt3l*$^{-/-}$ mice. **(A)** Intracellular human Bcl2 expression in LSK^{+} cells in *Eμ-bcl2tg* mice. The filled histogram represents the unstained control, the gray line represents the isotype control, and the black line represents Bcl2 staining (top panel). Staining of WT B6 mice with the human Bcl-2 antibody gave identical staining pattern to the isotype control. The bottom panel depicts human Bcl2 versus CD27 staining. The overlaid gray and black dot plots represent isotype control and Bcl2 staining, respectively. Data are representative of three *Eμ-bcl2tg* mice and three independent experiments. **(B)** Flow cytometric analysis of LSK^{+} (pregated on Lin^{-}, top panels) from a representative WT, *flt3l*$^{-/-}$, *Eμ-bcl2tg*, and *Eμ-bcl2tg flt3l*$^{-/-}$ mouse further stained to examine Flt3 expression (bottom panels). **(C, D)** Bar graphs illustrating the frequency (**C**) and numbers (**D**) of LSK^{+}, CD150^{+}CD34^{-} HSCs (black bars) and CD150^{-}CD34^{+} MPPs (white bars) within LSK^{+} (middle bar graphs) and Flt3^{+hi} MPPs within LSK^{+} across the four genotypes. Supplementary Figure E1 (online only, available at

www.exphem.org) displays HSC and MPP gating strategy. Data are representative of four or five mice per genotype and four independent experiments (**B–D**). Error bars represent mean ± SEM. *$p \leq 0.05$, ***$p < 0.0001$, Student *t* test, between the means of different genotypes. n.s. = nonsignificant differences between genotypes measured using Student *t* test at $p > 0.05$.

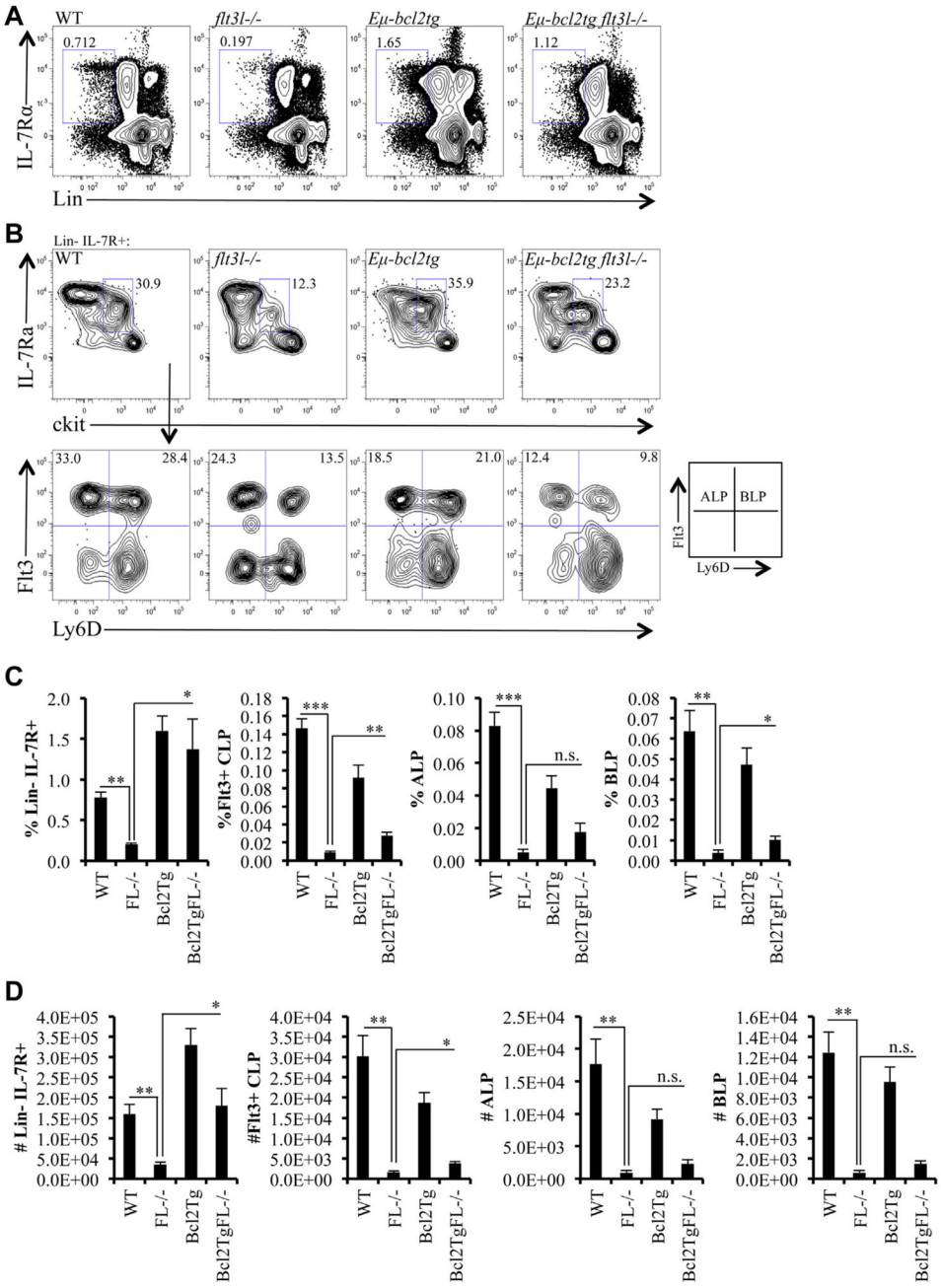

Figure 2.

Enforced expression of Bcl2 rescues CLPs, but not the deficiency in Flt3$^+$ CLPs in *flt3l*$^{-/-}$ mice. **(A)** Flow cytometric analysis of Lin$^-$IL-7Rα$^+$ from a representative WT, *flt3l*$^{-/-}$, *Eμ-bcl2tg*, and *Eμ-bcl2tg flt3l*$^{-/-}$ mouse. **(B)** c-kit^{lo} IL-7Rα$^+$ CLP compartment (pregated on Lin$^-$IL-7Rα$^+$, top panels) across the four genotypes further stained with Ly6D and Flt3 to examine Ly6D$^-$ ALPs and Ly6D$^+$ BLPs (bottom panels). **(C, D)** Bar graphs illustrating the frequency **(C)** and numbers **(D)** of Lin$^-$IL-7Rα$^+$, Flt3$^+$CLPs, ALPs, and BLPs across the four genotypes. Data are representative of four to seven mice per genotype and four to five independent experiments. Error bars represent mean ± SEM. *$p \leq 0.05$, **$p \leq 0.005$, ***$p <$

0.0001, Student's *t*-test at, between the means of different genotypes. n.s. = nonsignificant differences between genotypes measured using Student *t* test at $p > 0.05$.

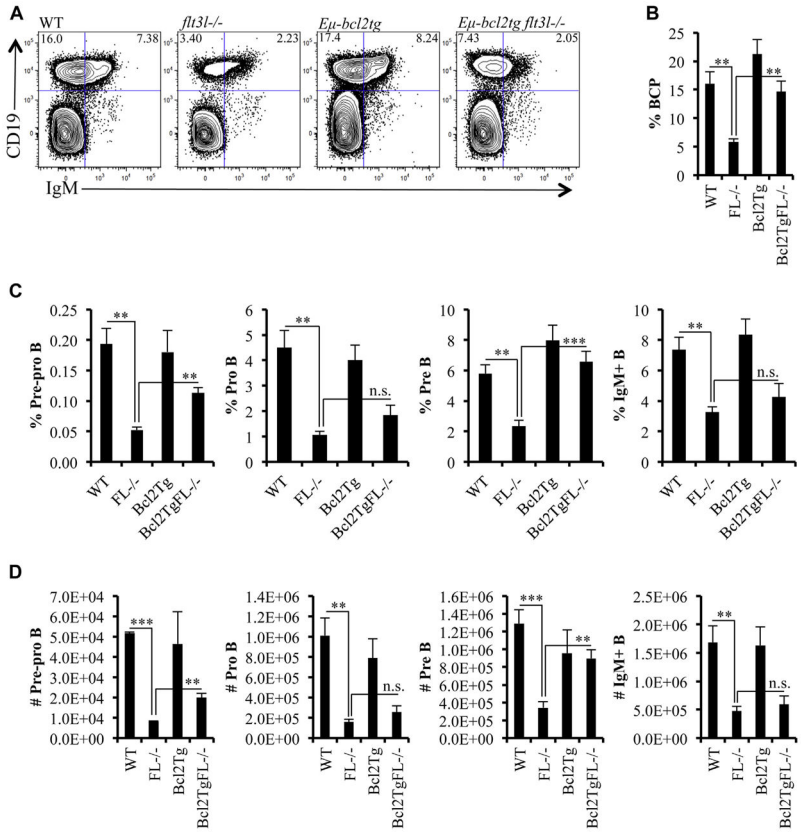

Figure 3.

Enforced expression of Bcl2 restored BCP in *flt3l*$^{-/-}$ mice. **(A)** Flow cytometric analysis of BCPs (CD19^{+}IgM^{-}) and CD19^{+}IgM^{+} B cells from a representative WT, *flt3l*$^{-/-}$, *Eμ-bcl2tg*, and *Eμ-bcl2tg flt3l*$^{-/-}$ mouse. **(B)** Bar graph showing the frequency of BCPs across the four genotypes. **(C, D)** Bar graphs illustrating the frequency **(C)** and numbers **(D)** of pre-pro B (gated on B220^{+}CD19^{-}AA4.1^{+}Ly6D^{+}PDCA-1^{-}), pro B, pre B, and IgM^{+} B cells across the four genotypes. Supplementary Figure E2 (online only, available at www.exphem.org) displays pro B and pre B gating strategy. With the exception of the pre-pro B cell stain (three mice per genotype and two independent experiments), data are representative of six to nine mice per genotype and five independent experiments. Error bars represent mean ± SEM. $^{**}p \leq 0.005$, $^{***}p<0.0001$, Student *t* test, between the means of different genotypes. n.s. = nonsignificant differences between genotypes measured using Student *t* test at $p > 0.05$.

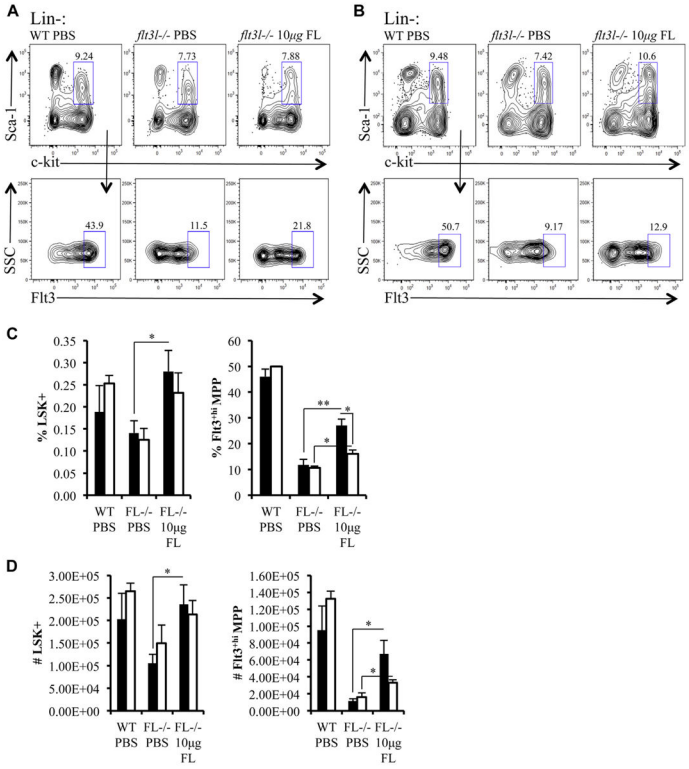

Figure 4.

Flt3 signaling is required for the expansion and maintenance of LSK^{+} Flt3^{+hi} MPPs. **(A, B)** Flow cytometric analysis of BM taken 2 days (**A**) or 5 days (**B**) after either the last injection of PBS (control mice) or FL. LSK^{+} cells (pregated on Lin^{-}, top panels) from PBS-injected WT mice (WT PBS), PBS-injected *flt3l*$^{-/-}$ mice (*flt3l*$^{-/-}$ PBS), and FL-injected *flt3l*$^{-/-}$ mice (*flt3l*$^{-/-}$ 10 μg FL) further stained to examine Flt3^{+hi} MPP (bottom panels). **(C, D)** Bar graphs illustrating the frequency **(C)** and numbers **(D)** of LSK^{+} and Flt3^{+hi} MPPs within LSK^{+} across the three conditions. The bars represent data collected 2 days (black) or 5 days (white) following cessation of PBS or FL injection schedule. Data are representative of four to six mice per genotype and two to three independent experiments. Error bars represent mean ± SEM. *$p \leq 0.05$, **$p \leq 0.005$, Student *t* test, between the means of different conditions.

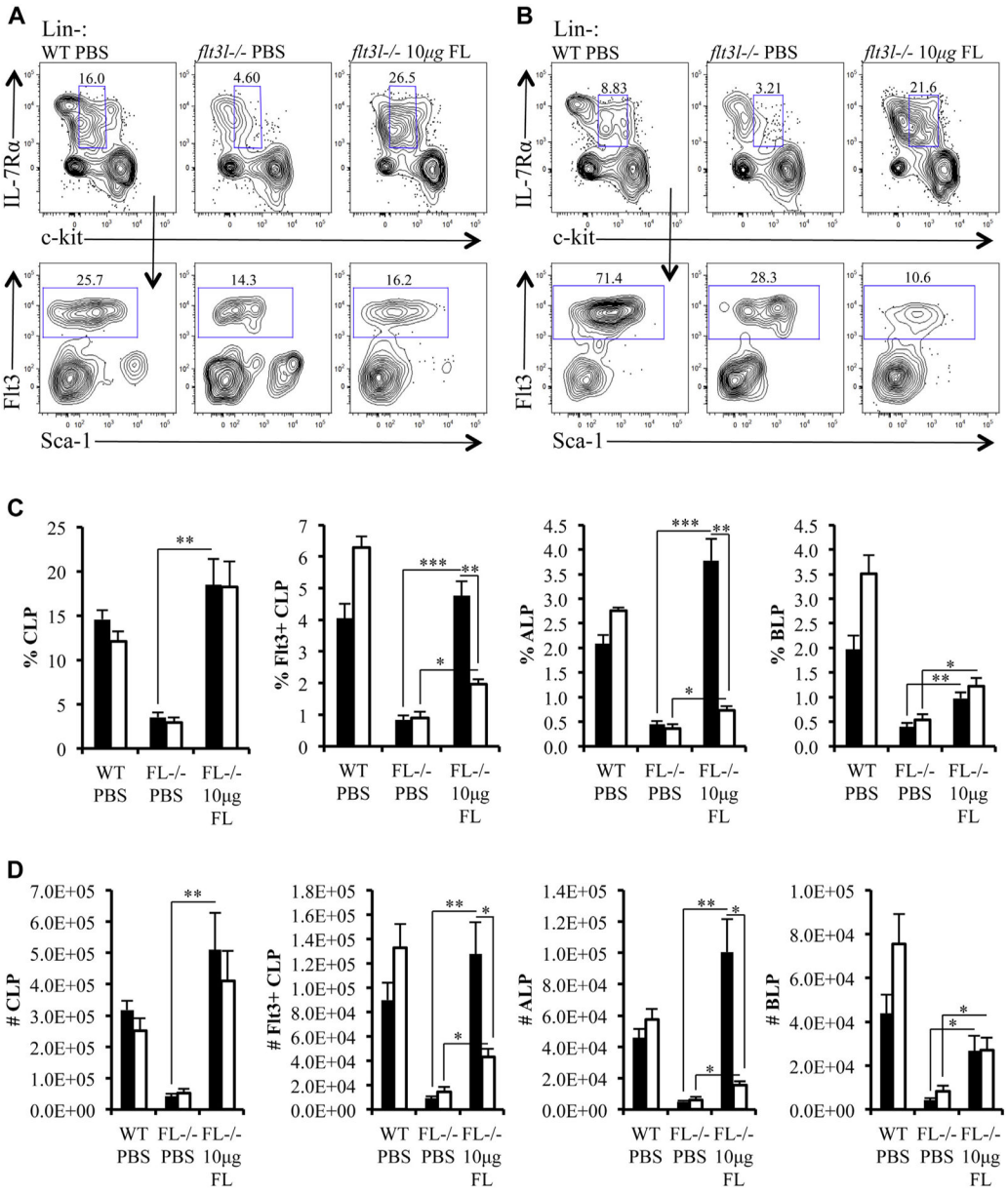

Figure 5.

Differential requirements for Flt3 signaling in expansion and maintenance of Flt3$^+$ CLP subsets. **(A, B)** Flow cytometric analysis of bone marrow taken 2 days **(A)** or 5 days **(B)** after either the last injection of PBS (control mice) or FL. Bone marrow (pregated on Lin$^-$) is stained with antibodies against c-kit and IL-7Rα to visualize CLP compartment in PBS-injected WT mice (WT PBS), PBS-injected *flt3l*$^{-/-}$ mice (*flt3l*$^{-/-}$ PBS), and FL-injected *flt3l*$^{-/-}$ mice (*flt3l*$^{-/-}$ 10 μg FL). Flt3$^+$ CLPs are shown (bottom panels). **(C, D)** Bar graphs illustrating the frequency **(C)** and numbers **(D)** of total Lin$^-$c-kit^{+lo} IL-7Rα$^+$ CLPs, Flt3$^+$ CLPs, Ly6D$^-$ ALPs, and Ly6D$^+$ BLPs across the three conditions. The bars represent data collected 2 days (black) or 5 days (white) after cessation of PBS or FL injection schedule. Data are representative of four to six mice per genotype and two to three independent

experiments. Error bars represent mean ± SEM. *$p \leq 0.05$, **$p \leq 0.005$, and ***$p < 0.0001$ Student *t* test, between the means of different conditions.

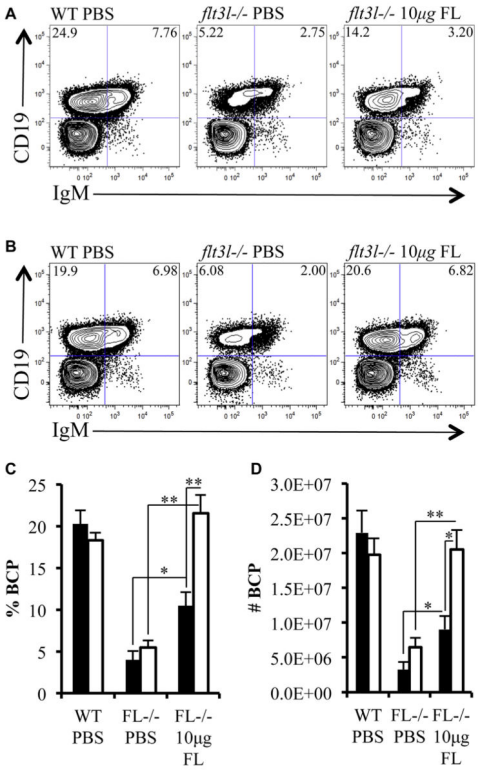

Figure 6.

Delayed recovery of BCPs after temporal FL administration. **(A, B)** Flow cytometric analysis of bone marrow taken 2 days **(A)** or 5 days **(B)** after either the last injection of PBS (control mice) or FL. Bone marrow is stained with antibodies against CD19 and IgM to visualize BCP ($CD19^+IgM^-$) and $CD19^+IgM^+$ B cells in PBS-injected WT mice (WT PBS), PBS-injected *flt3l*$^{-/-}$ mice (*flt3l*$^{-/-}$ PBS), and FL-injected *flt3l*$^{-/-}$ mice (*flt3l*$^{-/-}$ 10μg FL). **(C, D)** Bar graphs illustrating the frequency **(C)** and numbers **(D)** of BCPs across the three conditions. The bars represent data collected 2 days (black) or 5 days (white) following cessation of PBS or FL injection schedule. Data are representative of four to six mice per genotype and two to three independent experiments. Error bars represent mean ± SEM. *$p \leq 0.05$, **$p \leq 0.005$, Student *t* test, between the means of different conditions.

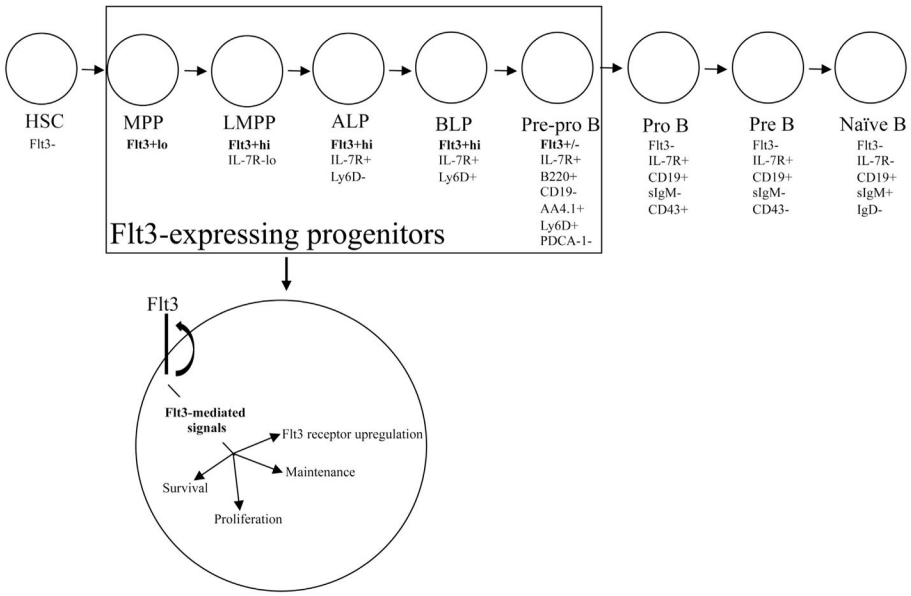

Figure 7.

Model illustrating Flt3 signaling in regulating the proliferation, survival, and maintenance of $Flt3^+$ hematopoietic progenitors. In mice, Flt3 is initially expressed at low levels in MPPs. Flt3 is upregulated in LMPP to high levels, where it remains in ALPs and BLPs until Flt3 is downregulated in pre-pro B cells and silenced at the pro B cell stage of B cell development. Once bound by its ligand, Flt3-ligand, Flt3 sends critical signals necessary for the proliferation, survival, and maintenance of Flt3-expressing hematopoietic progenitors. In addition, Flt3 signaling works in an autoregulatory manner to upregulate and maintain high levels of Flt3 receptor density, ensuring threshold levels of Flt3 signaling are present to maintain the pool of $Flt3^+$ progenitors that give rise to B cell precursors.

Table 1

Bone marrow cellularity[a]

Mice[b]	No. of BM cells ($\times 10^7$)[c]
Wild type	9.5 ± 0.4
ftl3l$^{-/-}$	7.5 ± 0.4
Eμ-bcl2tg	8.1 ± 0.5
Eμ-bcl2tg ftl3l$^{-/-}$	7.3 ± 0.5

[a] Cell counts taken from four hind leg bones per mouse.

[b] n = 7–11 mice per genotype.

[c] Mean ± SEM.

Table 2

Frequencies of RAG1-GFP$^+$ in hematopoietic progenitor populations[a]

Mice[b]	Flt3^{+hi} MPP[c]	ALP	BLP	Pro B	Naïve B[d]	Mature B[d]
WT GFP	14.1 ± 3.2	58.1 ± 7.0	83.0 ± 3.5	94.0 ± 0.5	83.7 ± 0.5	16.3 ± 0.5
flt3l$^{-/-}$ GFP	2.2 ± 0.4	20.1 ± 0.6	40.2 ± 2.7	81.8 ± 0.9	61.9 ± 4.6	38.2 ± 4.6
bcl2tg GFP	7.8 ± 2.8	40.4 ± 4.2	81.6 ± 5.2	94.8 ± 3.5	73.8 ± 2.0	26.2 ± 2.0
bcl2tg flt3l$^{-/-}$ GFP	1.7 ± 0.8	18.5 ± 0.3	38.6 ± 10.0	92.7 ± 1.8	62.7 ± 6.6	37.4 ± 6.6

[a] Frequencies reflect mean ± SEM of two independent experiments.

[b] n = 2 mice per genotype.

[c] Flt3$^+$hi multipotential progenitor defined as LSK$^+$Flt3$^+$hi.

[d] Naive B cells are CD19$^+$IgM$^+$GFP$^+$ whereas mature B cells are CD19$^+$IgM$^+$GFP$^-$.