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Threshold levels of Flt3-ligand are required for the generation and survival of lymphoid progenitors and B cell precursors

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Summary

The generation of B cell precursors (BCP) from lymphohematopoietic progenitors (LHP) in bone marrow is dependent on signals provided by the receptor tyrosine kinase Flt3 and its ligand, Flt3-ligand (FL). Mice deficient in *FL* exhibit striking reductions in LHP and BCP. Currently the mechanism by which Flt3 regulates lymphoid lineage/B cell development is unknown. Here we show that haploinsufficiency of FL (*FL*+/−) reduced LHP, CLP and Pro-B cells, suggesting that FL levels set a threshold for B lymphopoiesis. Limiting dilution analysis confirmed reduced BCP frequency in *FL*+/− mice. Real-time PCR of LHP from *FL*+/− animals showed increased transcripts of the B lineage inhibitor *id1*. However, targeted deletion of *id1* did not restore the lymphoid/B lineage deficiencies in *FL*−/− mice, supporting Id1-independent mechanisms. BrdU incorporation studies established that FL is not essential for the proliferation of Flt3+ multipotential progenitors. Analysis of *FL*−/− progenitors expressing low levels of Flt3 revealed decreased levels of the pro-survival factor Mcl1. Consequently, the Flt3+ LHP progeny of $Flt3^{low}$ LSK+ cells exhibited increased Annexin V staining. Together, these data suggest that Flt3 signaling initiates a cascade of events in $Flt3^{low}$ precursors that promote the survival of LHP from which BCP are derived.

Keywords

Flt3-ligand; cell differentiation; B cell development; apoptosis

Introduction

Lymphopoiesis is a stepwise process dependent on signals from the microenvironment. In bone marrow (BM), differentiating multipotential progenitors (MPP) integrate microenvironmental signals to generate lymphoid progenitors from which BCP are derived. Fms-like tyrosine kinase (Flt3) and its ligand, Flt3-ligand (FL), are critical regulators of lymphoid progenitors and their B lineage progeny. However, the mechanism by which Flt3 signaling regulates the generation of BCP from LHP *in vivo* is not well understood.

Hematopoietic stem cells (HSC) and MPP can be identified in BM by a lack of lineage markers (Lin-), expression of stem cell antigen-1 (Sca-1), and high levels of the receptor tyrosine kinase, c-kit [1]. These cells are collectively termed LSK+. Lymphohematopoietic progenitors (LHP) are Flt3+ MPP that are functionally distinct from $Flt3^{-/low}$ MPP in having lost megakaryocyte/erythroid differentiation potential [2]. LHP can be distinguished

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Conflict of Interest
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within MPP by a variety of criteria including green fluorescent protein (GFP) knocked into the recombinase-activating gene one (RAG1) coding region [3,4]. LHP defined by these criteria lack surface expression of the IL-7R. Another way to distinguish LHP *in vivo* is differential expression of Flt3 and VCAM-1 [5]. The ability to distinguish LHP within MPP provides a means to identify and characterize cellular and molecular circuits that regulate lymphoid lineage development.

Abundant experimental evidence suggests that the molecular circuitry initiating lymphoid lineage specification within MPP correlates with expression of Flt3. Targeted-deletion of Flt3 or Flt3-ligand resulted in profound deficiencies in LHP and/or BCP [6–9]. Molecular analysis of the residual $Flt3^+$ MPP in *FL*−/− mice revealed severe reductions in lymphoid transcripts [9]. These data were interpreted to suggest that Flt3 signaling regulates lymphoid priming in MPP. However, upregulation of Flt3 could be concomitant with lymphoid priming and not directly regulate this process. Currently, the role of Flt3/FL in regulation of lymphopoiesis, or a molecular connection between Flt3 signaling and the induction of any B lineage regulatory factor or differentiation-related event, remains to be established.

RAG1 locus activation is a hallmark of lymphoid specification [10]. Through the establishment and analysis of RAG1-GFP/+ reporter mice expressing wildtype, heterozygous, and knockout FL alleles, we show that threshold levels of FL are required for RAG1 locus activation in LHP. Limiting dilution analysis confirmed reduced BCP frequency under B cell promoting conditions *in vitro*. E2A activation is critical for the generation of LHP [11]. Real-time PCR analysis of Flt3+ LHP from *FL*+/− mice revealed reductions in transcripts for the E2A targets *ebf1* and *rag1*, consistent with the B cell deficiency in these animals. Id family members and Scl/Tal1 regulate E2A [12]. Real-time PCR analysis of Flt3+ LHP from *FL*+/− mice revealed increased *id1* transcripts. However, deletion of *id1* in *FL*−/− mice did not rescue LHP or BCP, indicating that Flt3 does not regulate lymphopoiesis solely by modulating Id1. FL synergizes with other cytokines to promote the proliferation of MPPs *in vitro* [13]. BrdU incorporation studies revealed that FL is not essential for the proliferation of Flt3+ MPPs *in vivo*. However, in the absence of FL, there is preferential expansion of Flt3- MPPs to fill a niche that would be competitively filled by Flt3+ MPPs. We show that Flt3 is critical for the survival of LHP *in vivo*, as abrogated FL production leads to reductions in Mcl-1 protein and increased evidence of apoptosis.

Results

Haploinsufficiency of FL reduces LHP and BCP

Exogenous administration of FL *in vivo* increases the frequency of LHP [14,15]. However, it has not been determined if threshold levels of FL are required for normal lymphocyte production. A *RAG1-GFP* reporter mouse expressing varying physiological levels of FL provides an *in vivo* model to determine if haploinsufficiency of FL alters the numbers of LHP and BCP in BM. *RAG1-GFP* x *FL*+/+, *RAG1-GFP* x *FL*+/−, and *RAG1-GFP* x *FL*−/− mice were established. BM from the three genotypes was analyzed for FL transcript abundance. Fig. 1A and 1B illustrates that haploinsufficiency of FL reduced FL transcripts by 50%. ELISA confirmed a similar reduction in FL serum levels in *FL*+/− mice (Fig. 1C). These data establish that heterozygosity of *flt3-l* reduces FL production.

The majority of Flt3+/FL-responsive BM cells are within the LSK+ subset [16,17]. Percentages of LSK+ cells were not altered by *FL*-haploinsufficiency in contrast to *FL*-deficiency (Fig. 2A). Haploinsufficiency of FL had no effect on BM cellularity. In agreement with recent findings [18], percentages and absolute numbers of LSK+ cells were significantly reduced in *FL*−/− mice (Fig. 2A and absolute numbers (mean±s.d./hind leg

bone): *FL*+/+ (n=10): $1.8\times10^4\pm8.9\times10^3$; *FL*+/− (n=15): $1.9\times10^4\pm7.9\times10^3$; *FL*−/− (n=11): $9.3\times10^3\pm4.8\times10^3$, p≤0.01). The reductions in LSK+ cells in *FL*−/− mice was not a consequence of crossing these animals to *RAG1-GFP*/+ mice, as we observed statistically significant reductions in LSK+ cells in *FL*−/− mice not bred to *RAG1-GFP*/+ (data not shown).

Next, we used differential cell surface expression of Flt3 and VCAM1 to determine the sensitivities of LSK+ fractions to threshold levels of FL [5]. HSC/MPP were identified as LSK+ VCAM1+ $Flt3^{-/lo}$ (Fig. 2A, *upper left quadrant*), GMLP (granulocyte-macrophage-lymphoid progenitor) as LSK+ VCAM1+ Flt3+ (Fig. 2A, *upper right quadrant*), and LMPP (lymphoid-biased multipotent progenitor) as LSK+ VCAM1- $Flt3^{+hi}$ (Fig. 2A, *lower right quadrant*). Fig. 2A shows that $Flt3^{-lo}$ HSC/MPP were significantly increased in *FL*−/− mice (*FL*+/+ (n=6): 34.6±4.8%; *FL*+/− (n=5): 50.5±7.9%, p=0.0026; *FL*−/− (n=6): 66.5±7.1%, p<0.0001). In contrast, percentages (*FL*+/+ (n=6): 54.5±2.6%; *FL*+/− (n=5): 41.3±9.4%, p=0.009; *FL*−/− (n=6): 29.0±8.4%, p<0.0001) and absolute numbers (data not shown) of GMLP were significantly reduced in *FL*+/− and *FL*−/− mice with dramatic reductions in GMLP expressing the highest levels of Flt3 (Fig. 2A, *bottom panel*, *boxed region* and 2B). Importantly, *FL*+/− and *FL*−/− mice exhibited a dose-dependent reduction in LMPP (Fig. 2A), confirming that threshold levels of FL are required for lymphopoiesis.

An alternative immunophenotyping schema used to fractionate HSC, MPP, and LHP is differential expression of CD27 and GFP within LSK+ of *RAG1-GFP* reporter mice expressing various levels of FL [10]. The LSK+ CD27- subset is enriched for HSC and we observed a consistent increase in percentages of LSK+ CD27- HSC in *FL*-deficient animals (Fig. 2C)[19]. The LSK+ CD27+ subset includes $Flt3^{lo}$ MPP and Flt3+ GMLP and no significant difference in frequencies of these subsets was found. However, consistent with the dose dependent reduction in LSK+ $Flt3^{+hi}$ cells we showed above, percentages and absolute numbers (mean±s.d./hind leg bone) of LHP defined as LSK+ CD27+ GFP+ were significantly reduced with FL haploinsufficiency (Fig. 2C and *FL*+/+ (n=9): $1.2\times10^3\pm5.1\times10^2$; *FL*+/− (n=13): $6.5\times10^2\pm3.7\times10^2$, p≤0.01; *FL*−/− (n=8): $1.2\times10^2\pm1.0\times10^2$, p<0.0001). Thus, RAG1 locus activation, a hallmark of lymphoid differentiation, is dependent on threshold levels of FL.

BCP are enriched in common lymphoid progenitors (CLP) [20]. Sixty percent of CLP express Flt3, half express the RAG1-GFP reporter, and *ebf1* transcripts are upregulated [10,21,22]. FL is required for the regulation of CLP [6]. However, CLP also express the IL-7R, which could functionally substitute for FL [20]. Therefore, we asked if haploinsufficiency of FL affected CLP. Indeed, *FL*+/− mice exhibited a 40% reduction in CLP and percentages of Flt3+ CLP expressing GFP were reduced (Fig. 2D). Thus, the regulation of CLP, as well as RAG1 locus activation within CLP, requires threshold levels of FL.

Downregulation of Flt3 accompanies B lineage commitment [23]. Therefore, BCP subsets, or RAG1 expression within BCP, may not be sensitive to reductions in FL. Shown in Fig. 2E, B220+ CD43+ Pre-Pro-B/Pro-B and B220+ CD43- Pre-B cells were reduced by FL haploinsufficiency. *FL*+/− mice had significant reductions in percentages of Pre-Pro-B/Pro-B cells and Pre-B cells. In contrast, naïve and mature B cell frequency was not significantly affected by loss of FL. Analysis of GFP within the Pre-Pro B/Pro-B and Pre-B compartments revealed no significant difference in percentages of GFP+ cells (data not shown) indicating that RAG1 activation in BCP is not sensitive to reduction of FL.

Threshold levels of FL are required to establish the B cell developmental potential in MPP

We next asked if reductions in FL altered the B lineage differentiation potential of MPP. GMLP were sorted from WT and *FL*+/− mice and limiting dilution assays (LDA) were performed under B lineage promoting conditions. Fig. 3 shows that *FL*+/− GMLP have reduced B cell developmental potential compared to WT, with 30% more input GMLP required to generate one B220+ cell (Fig. 3, *top panel*). Addition of 100ng/ml of FL did not further increase the B cell precursor frequency beyond that obtained with 50ng/ml, suggesting that 50ng/ml of FL is saturating for B lymphopoiesis in this assay. In contrast, reductions in FL amplified the effect as 60% more input FL+/− GMLP were necessary to generate one B220+ cell (Fig. 3, *bottom panel*). These experimental data reinforce the importance of threshold levels of FL for the generation of BCP.

Loss of Id1 does not rescue LHP in *FL −/−* mice

Lymphoid priming is critically dependent on the transcription factor E2A [11]. E2A activity is negatively controlled via dimerization with Id1 and Scl/Tal1 [12]. Quantitative comparison of *id1* and *scl/tal1* transcripts could be informative with regard to mechanisms by which FL promotes lymphoid specification. GMLP and LMPP were sorted from WT and *FL*+/− mice. Real-time PCR showed equivalent levels of transcripts corresponding to *flt3*, variable levels of *tcfe2a*, and decreased transcripts for *rag1* and *ebf1* (Fig. 4A) in *FL*+/− mice. *Rag1* transcripts were reduced in GMLP and LMPP, which further validates the *RAG1-GFP* reporter flow cytometry data (Fig. 4A and 2C). Reductions in *ebf1* transcripts in LMPP are consistent with the B lineage developmental defect in *FL*+/− mice and the LDA results (Fig. 2 and 3). Next, we compared transcript abundance for *id1* and *scl/tal1*. In two independent experiments we found increased transcripts corresponding to *id1* in GMLP and LMPP from *FL*+/− mice (Fig. 4B). In contrast, transcripts for *scl/tal1* were unaffected by *FL* haploinsufficiency (Fig. 4B). In preliminary studies, we observed increases in *id2* transcripts in LMPP, but not GMLP, in *FL*+/− mice (data not shown). *Id3* levels were unaffected in either GMLP or LMPP by *FL* haploinsufficiency (data not shown).

The increase in *id1* transcripts in GMLP and LMPP from *FL*+/− mice suggested that FL might regulate lymphopoiesis by repressing *id1* transcription. To test this hypothesis, *FL*−/− mice were bred to *Id1*−/− mice and backcrossed to generate *FL*−/− x *Id1*−/− animals. BM cells were harvested and stained with combinations of antibodies to resolve HSC/MPP, GMLP, and LMPP. Loss of *id1* did not rescue the deficiencies in GMLP, LMPP, CLP or BCP in *FL*−/− mice or *FL*+/− animals (Fig. 4C and data not shown). These data indicate that FL regulates lymphopoiesis through mechanisms independent of suppression of *id1* transcription.

FL is not essential for the proliferation of Flt3+ LHP

Cytokines regulate hematopoiesis, in part, by controlling the proliferation of hematopoietic progenitors [24]. Numerous studies showed administration of FL increased numbers of hematopoietic progenitors *in vivo* [25,26]. Therefore, we determined if FL was required for the proliferation of LHP by evaluating BrdU incorporation. Percentages of BrdU+ cells within the SK+ and SK+ Flt3+ were compared between the *FL* genotypes. Percentages of BrdU+ cells within the SK+ compartment were not reduced as a consequence of FL deficiency (Fig. 5A). Relatively equivalent ratios of SK+ Flt3+ cells incorporating BrdU+ (an average of 1 in 6 cells) were detected in *FL*+/+, *FL*+/−, and *FL*−/− bone marrow (Fig. 5A and data not shown). We note that percentages of Flt3+ cells were significantly reduced in *FL*−/− mice, consistent with the data presented above (Fig. 2) and previous findings by others [9,18]. However, the ratio of BrdU+ to BrdU− cells within the Flt3+fraction was not diminished as a consequence of FL deficiency. Alternatively, we consistently observed an increase in the frequency of SK+ Flt3- cells that incorporated BrdU, suggesting that FL

deficiency allows preferential expansion of Flt3- MPP (Fig. 5A, *bottom panel, lower right quadrant*). Analysis of subsets enriched for myeloid progenitors, c-kit+ Sca-1-and c-kit+ Sca-1- Flt3+, revealed identical results across the three genotypes (Fig. 5B and data not shown). These results indicate that the deficiency in Flt3+ LHP cells in *FL*−/− mice is not due to defects in proliferation.

Flt3 signaling is required for the survival of LHP

A recent study showed Flt3 signaling prevented spontaneous apoptosis of human stem and progenitor cells *in vitro* [27]. However, similar studies have not been performed *in vivo* in the mouse. Therefore, we determined if Flt3 signaling is required for the survival of murine hematopoietic progenitors *in vivo*. Pre-apoptotic cells can be visualized in BM with Annexin V. Percentages of Annexin V+ cells within HSC/MPP and LHP were compared in WT and *FL*−/− BM (Fig. 5C). No change in percentages of Annexin V+ cells were observed in HSC/MPP (Fig. 5C). However, *FL*−/− BM had significantly increased percentages of Annexin V + LHP suggesting that FL is critical for the survival of LHP (Fig. 5C). We did not observe differences in Annexin V staining in *FL*+/− LHP (data not shown). Interestingly, analysis of Annexin V+ staining in *FL*−/− CLP revealed no significant differences compared to WT CLP (Fig. 5D). CLPs express Flt3 and IL-7R. The lack of Annexin V+ cells within the residual *FL*−/− CLP likely reflects survival signals provided by IL-7R. Consistent with that hypothesis, no differences in Annexin V staining were found in CLP in *FL*+/− animals (data not shown).

Next we sought to determine the mechanisms by which Flt3 contributes to the survival of LHP. Mcl-1 is a critical survival factor for HSC [28]. Therefore, we determined if Mcl-1 expression was altered in *FL*-deficient mice. Flow cytometry revealed reduced Mcl-1 expression in *FL*−/− LSK+ Flt3^{lo} cells (Fig. 5C). *FL*−/− LSK+ Flt3^{hi} cells also exhibited reduced Mcl-1 expression (Fig. 5C). CLP showed no differences in Mcl-1 expression (Fig. 5D). These data suggest that Flt3 signaling in LSK+ Flt3^{lo} cells provides survival signals through induction of Mcl-1. Intracellular staining showed no change in Bcl-2 expression in LHP or CLP (data not shown), indicating that Flt3 signaling likely does not work through Bcl-2 to promote LHP survival *in vivo*.

Discussion

The goal of this study was to determine how Flt3 regulates lymphopoiesis and B cell development. Comparison of mice producing varying levels of FL revealed that LHP genesis is exquisitely sensitive to FL availability. Threshold levels of FL are necessary for the initiation and maintenance of RAG1 locus activation in lymphoid progenitors (LHP, CLP), but not in later stage BCP. LDA confirmed *FL*+/− GMLP had diminished ability to become B220+ BCP. qRT-PCR analysis of sorted GMLP and LMPP from *FL*+/− mice showed increased transcripts for *id1*, an established inhibitor of B lymphopoiesis. However, loss of Id1 did not rescue the lymphoid progenitor deficiency in *FL*−/− mice, suggesting Id1 independent differentiation mechanisms. Previous studies have shown that FL is important for the proliferation of MPPs and CLP *in vitro*. Here we show that FL is dispensable for the proliferation of Flt3+ MPPs *in vivo*. We showed increased Flt3^{+} LHP initiating apoptosis, suggesting that FL is critical for the survival of LHP. Consistent with that finding, Mcl-1 expression was reduced in LSK+ Flt3^{lo} cells, suggesting that signals from Flt3 are important for expression of this critical pro-survival factor. Together, these findings provide mechanistic insight into the role of Flt3 in regulating lymphopoiesis.

Limiting expression of Flt3 has been suggested to control the threshold of Flt3 signaling [25]. Albeit scarce, previous studies have shown that alterations in cytokine levels have consequences, especially those that impact hematopoiesis and/or immune cell function. For

example, haploinsufficiency of B-cell-activating-factor (BAFF) decreased serum immunoglobulin levels, consistent with a critical role for BAFF in B cell homeostasis [29]. Similarly, reductions in IL-7 responding cells in BM of pregnant mice was restored by exogenous administration of IL-7 [30]. Exogenous administration of FL has been shown to increase numbers of LSK+ Flt3+ and B220+ ckit^{lo} Flt3+ BCP in BM *in vivo* [14,15]. Mice heterozygous for *Flk2/Flt3* do not have deficiencies in BCP [7]. However, *flk2/flt3*−/− mice do not exhibit as severe a deficiency in LHP or BCP as *FL*−/−. These data lend credence to our findings that FL availability is the limiting factor. To our knowledge, the current study is the first to demonstrate that alteration in systemic levels of FL alters steady-state lymphopoiesis.

We showed that threshold levels of FL are required for expression of the E2A target genes *ebf1* and *rag1*. A regulatory connection between Flt3 signaling and E2A function has not been reported. It is interesting to note that *Tcfe2a*+/− mice have a similar phenotype to *FL*+/− mice [11]. These parallel findings support a regulatory connection between Flt3 signaling and E2A function and provide an explanation for the deficiency in LHP in *FL*+/− mice. Although we found that *FL* deficiency resulted in elevated *id1* transcripts, loss of *id1* did not restore GMLP or LMPP in *FL*−/− mice, suggesting alternate molecular mechanisms. Loss of multiple *id* family members might be necessary to provide rescue given their functional redundancy. We did document increased *id2* transcripts in *FL*+/− LMPP in preliminary studies. *Id2* is required for NK cell development [31]. The increase in *id2* transcripts we observed *in vivo* is more variable and could reflect increased frequencies of NK progenitors and not increased *id2* transcription upon *FL*-deficiency. Future studies will address if combined loss of *id1* and *id2* alters the lymphoid defect in *FL*−/− mice.

It is well established that FL functions synergistically with various hematopoietic cytokines to promote the expansion of MPPs and CLPs *in vitro* [32–34]. Thus, FL deficiency might be predicted to alter the proliferation of Flt3+ MPPs *in vivo*. However, BrdU analysis of residual Flt3+ MPPs in *FL*−/− mice did not reveal a proliferation defect. This is not necessarily an unexpected finding, given that Flt3+ MPPs are responsive to a number of hematopoietic growth factors that can provide proliferative signals [32,34]. Unexpectedly, we saw increased percentages of Flt3- MPPs incorporating BrdU in *FL*−/− mice. This observation suggests that the absence of FL allows a proliferation advantage to Flt3- MPPs to fill a niche that in *FL* sufficient BM would be occupied by Flt3+ MPPs. Interestingly, close scrutiny of LSK+ cells from *tcfe2a*−/− mice revealed increased BrdU incorporation in Flt3- LSK+, compared to Flt3+ LSK+ cells [11]. These similar findings provide additional support that deficiency in lymphoid progenitors results in preferential expansion of Flt3- MPPs.

Mcl-1 is a critical regulator of murine lymphopoiesis [28,35]. Mcl-1 is highly expressed in LSK+ cells and downregulated in CLP [28]. Conditional-deletion of floxed Mcl-1 established the importance of Mcl-1 in the survival of HSC, CLP, CD19+ BCP [28,35]. Deficiency in *FL* resulted in decreased Mcl-1 in Flt3^{lo} MPP accompanied by increased Annexin V+ Flt3^{hi} MPP. Our findings suggest that Flt3 signaling initiates a cascade of events in Flt3^{lo} MPP that selectively promotes the survival of cells primed to enter the lymphoid pathway. Failure to signal via Flt3 leads to diminished Mcl-1 expression in Flt3^{lo} MPPs and increased propensity for apoptosis in Flt3^{hi} MPP, resulting in selective deficiencies in lymphoid progenitors. Diminished numbers of Flt3+ MPPs allows expansion of Flt3- MPPs to fill an unoccupied niche. Taken together, these studies provide new insight in to the role of Flt3/FL signaling in regulating the survival of lymphoid progenitors by controlling the size of the niche they occupy. Based on our findings that FL haploinsufficiency impacts the numbers of LHP and BCP in BM, it will be interesting to

extend these studies to examine the consequences of FL haploinsufficiency on immune function.

Materials and Methods

Mice

C57Bl/6 (*FL*+/+) and *FL*−/− mice were obtained from Taconic Farms (Germantown, NY). RAG1-GFP/+ and *Id1*−/− mice have been described [36,37]. *FL*−/− and *Id1*−/− mice were bred to generate *FL*−/− x *Id1*−/− mice. *RAG1-GFP*/+ and *FL*−/− were bred to create *RAG1-GFP*/+ x *FL*+/+, *RAG1-GFP*/+ x *FL*+/−, and *RAG1-GFP*/+ x *FL*−/− mice. C57Bl/6 mice were bred to *FL*−/− mice to generate *FL*+/− mice. Age-matched or littermate controls were used for individual experiments, and mice ranged from 4–12 weeks of age. All animals were bred and maintained at the Mayo Clinic animal facility and experiments carried out according to the Institutional Animal Care and Use Committee guidelines.

Genotyping

RAG1-GFP/+ x *FL*+/+, *RAG1-GFP*/+ x *FL*+/−, and *RAG1-GFP*/+ x *FL*−/− mice were identified by separate genotyping reactions with three primer sets: *RAG1*, *GFP*, and *FL*. Genotyping was performed on tail DNA using the following primer combinations: forward *FL* WT, 5′-AGGAACACTGATAGC ATGAG-3′; reverse *FL* WT, 5′-GAAAGCCAAAGCTGGATGAC-3′; forward *FL* mutant, 5′-CAGCTCTATGTGGAGGGGTGAGGT-3′; reverse *FL* mutant, 5′-CAGAAAGCGAAGGAGCAAAG CTG-3′. The WT primers generate a 910 bp product and the mutant primers a 1073 bp product. The wildtype forward primer binds 5′ of the *FL* coding region and the 3′ primer within the *FL* coding region. The mutant forward primer binds within the *FL* genomic locus and the mutant reverse primer binds within the inserted PGK-neo cassette [38]. Tail DNA was amplified using *FL* WT primers as follows: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 61 °C for 45 sec, and 72 °C for 1 min. Using the *FL* mutant primers, the PCR parameters are: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 58 °C for 45 sec, and 72 °C for 1.5 min. Published *RAG1* and *GFP* primers were used to confirm the *RAG1* and *GFP* genotypes [36]. *Id1* primer sequences were provided by R. Benezra.

RT-PCR

RNA was isolated from BM using TRIzol and cDNA synthesized using the SuperScript III Reverse Transcriptase kit and random primers. Serial dilutions of cDNA were amplified by semi-quantitative PCR to measure *FL* transcript levels compared to *β-actin*. The following parameters were used for amplification: 95°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec for 30 cycles. *FL* RT-PCR primers are published [39]. The Gel Doc XR system (Bio Rad, Hercules, CA) and Quantity One software (Bio Rad) was used to analyze and quantify bands. For quantitative PCR analysis, RNA was isolated from sorted GMLP (LSK+ Flt3+ VCAM-1+) and LMPP (LSK+ Flt3+ VCAM-1-) from B6 and *FL*+/− mice using Qiagen RNeasy Micro kit. RNA was treated with DNase I kit to remove gDNA and cDNA synthesized. Quantitative real time RT-PCR (qRT-PCR) was performed using GMLP and LMPP cDNA and gene-specific primers (*Flt3*, *tcfe2a*, *Rag1*, *ebf*, *Scl*, *Id1*, and *GAPDH*). The primers used are: *Flt3* (F) 5′-CATCCAAGACAACATCTCCT-3′, (R) 5′-CCCTGAAGTCAACGTAGAAG-3′; *tcfe2a* (F) 5′-CCAGTCTTTTGCATAACCAT-3′, (R) 5′-AGGTCCTTCTTGTCCTCTTC-3′; *rag1* (F) 5′-CTGCAGACATTCTAGCACTC-3′, (R) 5′-AACTGAAGCTCAGGGTAGAC-3′; *id1* (F) 5′-CTGAAC GGCGAGATCAGT-3′, (R) 5′-GCCTCAGCGACACAAGAT-3′; *scl* (F) 5′-ATGTTCACCAACAACAA CC-3′, (R) 5′-GTGTGAGGACCATCAGAAAT-3′. *Ebf1* and *GAPDH* are published [5,39]. *GAPDH* was

the reference for all qRT-PCR experiments. Comparisons were made using the $2^{-\Delta\Delta CT}$ or ΔCT method [40].

Flow cytometry

BM was harvested and stained with combinations of the following mAbs: B220 (APC, PE), Flt3 PE, CD27 PE, bio IL-7R, bio VCAM-1, Sca-1 (bio, PeCy5.5, FITC, PerCp-Cy5.5), CD43 PE, c-kit (APC, PeCy7), and Mac-1 PE. Incubation with streptavidin conjugates (SA-APC, SA-PerCP, SA-780) was used to visualize biotinylated antibodies. All antibodies were obtained from eBioSciences or BD Pharmingen. In experiments where lineage-positive cells were excluded, a PeCy7 antibody cocktail was used: B220, CD3ε, Mac-1, Gr-1, and Ter119. For Annexin V staining, BM stained with primary antibodies was incubated with Annexin V FITC per manufacturers' instructions (eBioSciences). For intracellular Mcl-1 staining, BM cells stained with primary antibodies were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences), then incubated with rabbit anti-mouse Mcl-1 (Rockland Immunochemicals), followed by incubation with goat anti-rabbit IgG FITC (Southern Biotech). For cell sorting, positive selection was used to enrich c-kit+ cells from BM. BM was incubated with CD117 MACS microbeads and separated using VarioMACS or OctoMACS cell separator. CD117+ cells were stained with mAbs distinguish GMLP (LSK+ Flt3+ VCAM-1+) and LMPP (LSK+ Flt3+ VCAM-1−) and sorted on the FACS Aria (Becton Dickinson) for limiting dilution analysis and qRT-PCR. Surface marker expression was visualized with the FACS Canto, LSRII, or Calibur cytometers (Becton Dickinson). Data was analyzed with FloJo software (Treestar, Inc., San Carlos, CA), and mononuclear and doublet exclusion gates were used to initially enrich for cells of interest.

Limiting dilution analysis

Varying numbers of GMLP from WT and *FL*+/− mice were sorted directly into 96-well plates in X-VIVO 15 media (Biowhittaker/Lonza, Walkersville, MD) with 10% BSA (Stem Cell Technologies), standard supplements, and the following cytokines: 10 ng/mL IL-7 + 10 ng/mL SCF, +/− 100, 50, or 10 ng/mL of FL (Peprotech). On day 2, 100 uL fresh media was added, and every 3 days onward (days 5, 8, and 11) 100 uL of media was removed and 100 uL fresh media added. On day 14, cells were harvested and stained with antibodies to B220 and MAC1 to determine B220+ progeny. The number of input cells at 37% negative line was used to determine precursor frequency [41].

BrdU analysis

FL+/+, *FL*+/−, and *FL*−/− mice were injected intraperitoneally with 2 mg BrdU twelve hours before BM harvest. Following positive selection using CD117 microbeads, CD117+ enriched cells were stained with c-kit APC, bio Sca-1, and Flt3 PE followed by incubation with SA-780 to visualize biotinylated antibodies. Following surface staining, BrdU intracellular staining was performed using FITC BrdU Flow Kit (BD Biosciences). Surface and intracellular marker expression was visualized with the Canto (Becton Dickinson) and analyzed with FloJo software (Treestar, Inc., San Carlos, CA).

ELISA

The serum concentration of FL was calculated by ELISA on serum harvested from *FL*+/+, *FL*+/−, and *FL*−/− mice using mouse Flt3 Ligand Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) per kit instructions. The sensitivity for FL was 5pg/mL.

Statistical analysis

Statistics were performed using the Students T-test. Differences were significant at $p \leq 0.05$. All data reflect mean +/− standard deviation (SD) with the exception of Fig. 4 where the error bars represent mean +/− standard error of the mean (SEM).

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Abbreviations

FL	Flt3 ligand
MPP	multipotential progenitor
LHP	lymphohematopoietic progenitor
GMLP	granulocyte-macrophage-lymphoid progenitor
LMPP	lymphoid-biased multipotent progenitor
CLP	common lymphoid progenitor
BCP	B cell precursor
BrdU	bromodeoxyuridine

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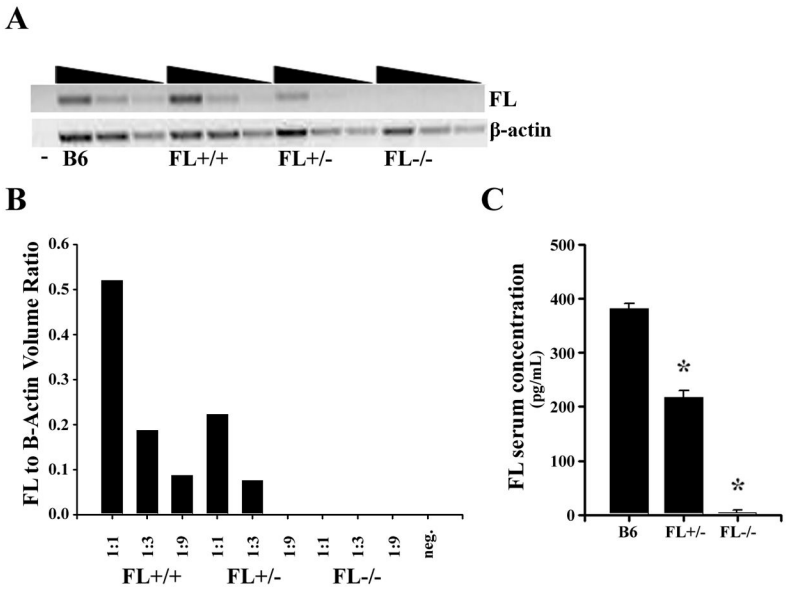

Figure 1.

Monoallelic expression of *FL* reduces FL production. (A) Semi-quantitative RT-PCR of FL transcripts in BM cells from C57BL/6 (B6), *FL*+/+ x *RAG1-GFP*/+, *FL*+/− x *RAG1-GFP*/+, or *FL*−/− x *RAG1-GFP*/+ mice. The cDNA was serially diluted 1:1, 1:3, and 1:9 for semi-quantitative analysis. Beta-actin was used as a loading control. Data are representative of two BM samples for each genotype. (B) Quantification of FL transcripts. Intensity data are the average of two BM samples for each genotype. (C) Concentration of FL (pg/mL) in the serum of *C57Bl/6* (B6), *FL*+/−, and *FL*−/− mice as determined by ELISA. Data represents the mean ± S.D. (*$p \leq 0.0001$) FL concentration in serum from ≥ 5 mice/genotype and 2 independent experiments. *P*-values were determined using the Students T-test.

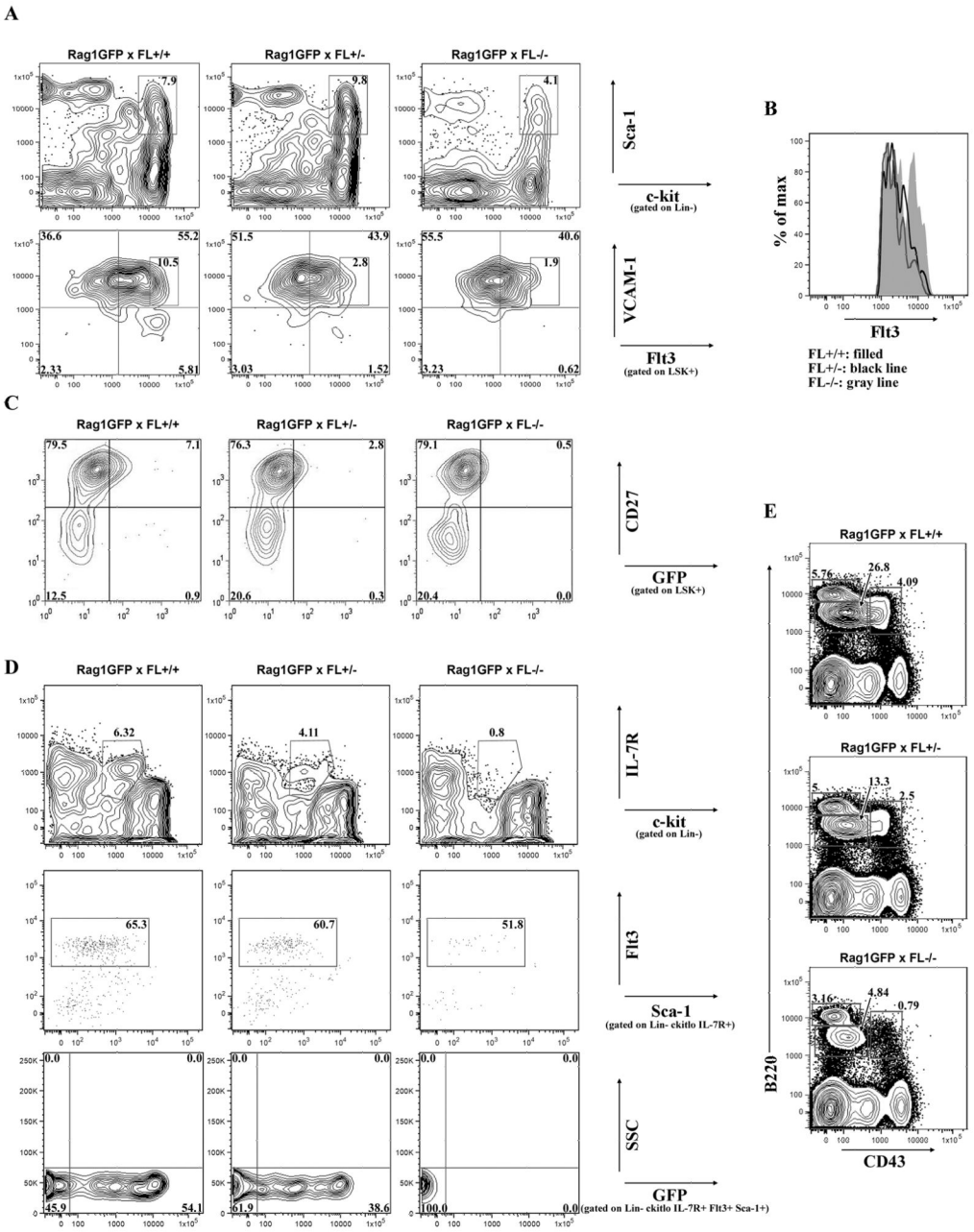

Figure 2.

FL haploinsufficiency reduces LHP and RAG1 locus activation. (A) Flow cytometric analysis of LSK+ BM cells from *RAG1-GFP* x *FL+/+*, *RAG1-GFP* x *FL+/−*, and *RAG1-GFP* x *FL−/−* mice (*top panels*). Differential expression of Flt3 and VCAM-1 in LSK+ BM cells to discriminate HSC/MPP, GMLP, and LMPP (*bottom panels*). Boxed regions indicate Flt3^{hi} GMLP. (B) Histogram of boxed region from (A) *bottom panels*, indicative of Flt3 expression in Flt3^{hi} GMLP in different FL genotypes. (C) Flow cytometric analysis of LSK+ BM cells from *RAG1-GFP+* x *FL* mice to distinguish HSC, MPP, and LHP. A littermate GFP- control was analyzed in each experiment to determine GFP+ gates (data not shown). (D) Flow cytometric analysis of Lin- BM cells to distinguish CLP: Lin- c-kit^{lo} IL-7R+ (*Top panels*). CLP were further discriminated by Flt3 and Sca-1 expression (*middle panels*). GFP

expression within Lin- c-kit^{lo} IL-7R+ Flt3+ Sca-1+ CLP (*bottom panels*). GFP+ gates were determined by analysis of Lin- c-kitlo IL-7R- Flt3+ cells which do not express GFP (data not shown). (E) Flow cytometric analysis of BCP. Pre-Pro-B/Pro-B cells are B220+ CD43+, Pre-B cells are B220+ CD43-, and naïve/mature B cells are B220^{hi} CD43-. Data are representative of ≥ 5 mice/genotype and ≥ 3 independent experiments.

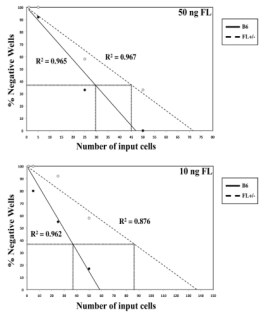

Figure 3.

Reduced BCP frequency in *FL+/−* GMLP. GMLP from *FL+/−* and *C57Bl/6* mice were FACS sorted and cultured in IL-7, SCF, and high (50ng) (*top panel*) or low (10ng) amount of FL (*bottom panel*). The solid (*C57Bl/6*) and dashed (*FL+/−*) lines represent the % negative wells per number of input cells. The line at 37% negative wells indicates precursor frequency, or the number of input *C57Bl/6* or *FL+/−* cells necessary to generate one BCP. Data are representative of cells pooled from ≥ 5 mice/genotype and 2 independent experiments.

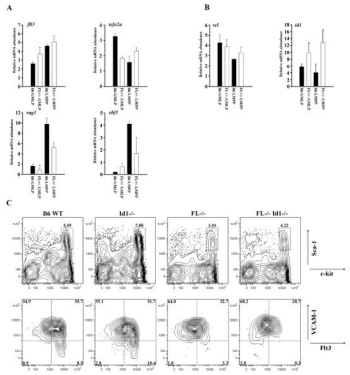

Figure 4.

Id1 does not rescue the lymphoid defect in *FL−/−* mice. (A) *Flt3*, *tcfe2a*, *rag1*, and *ebf1* and (B) *Scl* and *id1* were analyzed from FACS sorted GMLP and LMPP from *C57Bl/6* (B6) and *FL+/−* mice by quantitative PCR. (A+B) Data are representative of 2 independent experiments with BM pooled from ≥ 5 mice/genotype. Error bars represent the mean ± SEM. (C) Flow cytometric analysis of BM from *C57Bl/6* (B6), *Id1−/−*, *FL−/−*, and *FL−/− Id1−/−* mice stained with antibodies for lineage markers, c-kit, and Sca-1 to determine LSK + cells (*top panels*). LSK+ cells stained with antibodies to Flt3 and VCAM-1 to distinguish HSC/MPP, GMLP, and LMPP (*bottom panels*). Data are representative of ≥ 3 mice/genotype and ≥ 3 independent experiments.

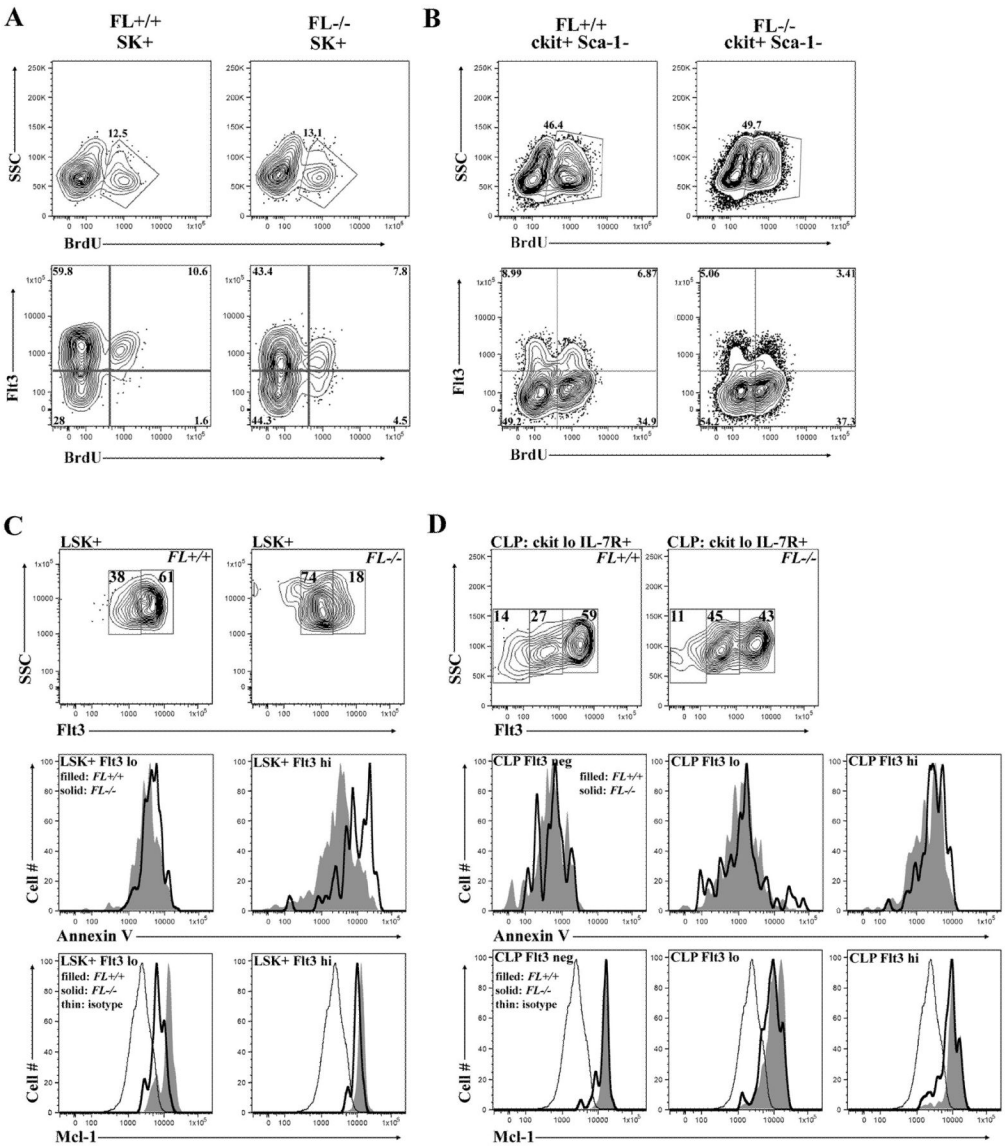

Figure 5.

Flt3 regulates the survival, but not the proliferation of LHP. (A-B) Flow cytometric analysis of bone marrow to examine BrdU incorporation 12 hours after 2 mg were injected *i.p.* into *FL*+/+ and *FL*−/− mice in c-kit+ Sca-1+ (SK+) (A) and c-kit+ Sca-1- (B). (A) Total BrdU incorporation in SK+ cells (*top panels*). BrdU incorporation in SK+ cells using Flt3 as additional criteria (*bottom panels*). (B) Total BrdU incorporation in c-kit+ Sca-1- cells (*top panels*). BrdU incorporation in c-kit+ Sca-1- cells using Flt3 as additional criteria (*bottom panels*). BrdU quadrants are set on the IgG isotype control for each genotype (data not shown). Data are representative of ≥ 3 mice/genotype and ≥ 3 independent experiments. (C-D) Annexin V staining and intracellular Mcl-1 expression in LSK+ cells (C) and CLP (D). *FL*+/+ represented by filled histogram, *FL*−/− indicated by solid line, and thin line depicts isotype control. (C) LSK+ cells were fractionated into $Flt3^{lo}$ and $Flt3^{+hi}$ subsets (*top panels*). Overlaid histograms depicting Annexin V staining in LSK+ $Flt3^{lo}$ and LSK+ $Flt3^{+hi}$ cells (*middle panels*). Overlaid histograms depicting intracellular Mcl-1 staining in LSK+ $Flt3^{lo}$ and LSK+ $Flt3^{+hi}$ cells (*bottom panels*). (D) CLP fractionated into three subsets: $Flt3^{neg}$,

Flt3^{lo}, and Flt3^{hi} (*top panels*). Overlaid histograms depicting Annexin V staining in CLP subsets (*middle panels*). Overlaid histograms depicting intracellular Mcl-1 staining in CLP subsets (*bottom panels*). Data are representative of 3 independent experiments (C+D).