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Hoxa9 and Flt3 Signaling Synergistically Regulate an Early Checkpoint in Lymphopoiesis

Kimberly A. Gwin, Mariya B. Shapiro, Joseph J. Dolence, Zhixin L. Huang, and Kay L. Medina

Hoxa9 and Flt3 signaling are individually important for the generation of lymphoid lineage precursors from multipotent hematopoietic progenitors (MPP) in bone marrow. Mice deficient for Hoxa9, Flt3, or Flt3 ligand (FL) have reduced numbers of lymphoid-primed multipotential progenitors (LMPP), common lymphoid progenitors (CLP), and B/T cell precursors. Hoxa9 regulates lymphoid development, in part, through transcriptional regulation of Flt3. However, it was unclear whether Hoxa9 has functions in lymphopoiesis independent of, or alternatively, synergistically with Flt3 signaling. In this study, we show that Hoxa9−/−Flt3−/− mice have more severe deficiencies in all B lineage cells, CLP, LMPP, and total Flt3+ MPP in bone marrow than the single knockouts. Although LMPP and Flt3+ CLP contain precursors for NK and dendritic cell lineage cells, no deficiencies in these lineages beyond that in Flt3−/− mice was found. Thymocyte cellularity was significantly reduced in the compound knockout, although peripheral T cell numbers mirrored Flt3−/− mice. Analysis of the hematopoietic progenitor compartment revealed elevated numbers of CD150hiCD34−CD41+ myeloid-biased stem cells in Hoxa9−/−Flt3−/− mice. In contrast, CD150− MPP enriched for lymphoid potential were synergistically reduced, suggesting Hoxa9 and Flt3 signaling function coordinately to regulate lymphopoiesis at a very early stage. Real-time PCR analysis of CD150 Flt3+ cells from wild-type control, Hoxa9−/−, and Flt3−/− single knockouts revealed decreased lymphoid transcripts, corroborating the importance of these regulators in lymphoid development. Taken together, these studies reveal a very early checkpoint in lymphopoiesis dependent on the combinatorial activities of Hoxa9 function and Flt3 signaling. The Journal of Immunology, 2013, 191: 745–754.

Steady-state production of lymphoid–lineage precursors from pluripotent hematopoietic stem cells (HSC) is a stepwise process driven by the concerted activities of transcription factors and signaling molecules (1). For example, at least six developmental intermediates have been characterized between HSC and committed pro-B cells: short-term repopulating cells, Flt3+ MPP, LMPP that are Flt3hi and evidence lymphoid priming, all lymphoid progenitors (ALP), B lineage–restricted progenitors (BLP), and pre-pro-B cells. The ability to resolve these developmental intermediates makes it possible to more accurately define precursor–progeny relationships and assign unique and interconnected gene regulatory modules that instruct the B cell fate decision. Importantly, it also allows more precise identification of developmental stage-specific blocks imposed by gene-targeting strategies. The end result is a better understanding of the roles of individual molecular and cellular determinants in regulation of lymphoid development and B cell differentiation.

In bone marrow (BM), most HSCs are quiescent, and few are actively participating in blood cell genesis at any one time (2). Pluripotent HSC are enriched in the Lineage-negative (Lin−), Sca-1+, c-kithi (collectively referred to as LSK+) fraction of BM cells expressing the signaling lymphocyte activation molecule family marker CD150 (3). Within LSK+CD150+ cells, HSC can be phenotypically distinguished from multipotential progenitors (MPP) by differential expression of a variety of cell surface markers including CD34, CD48, and HSC (3, 4–6). HSC are LSK+CD150−CD48−CD41−, whereas MPPs are CD150+ and express CD34, CD48, CD27, and Flt3. Although most HSC are not actively cycling, the majority of MPP are proliferating to maintain blood cell production.

Surface expression of Flt3 is an early event in HSC differentiation and largely coincides with downregulation of CD150 (7, 8). Acquisition of Flt3 denotes lymphoid–myeloid-biased differentiation potential and reduced capacity to generate erythroid/megakaryocyte progeny (9). A subset of Flt3+ MPPs expressing high levels of Flt3 express the early program of lymphoid–lineage gene expression and have been denoted early lymphoid progenitors or lymphoid-primed multipotential progenitors (LMPP) (10). Flt3 signaling plays a critical role in regulation of lymphoid priming in primitive hematopoietic progenitors (10, 11). However, a direct regulatory connection between Flt3 signaling and the expression and/or activity of any lymphoid–lineage specification factor remains to be established. LMPP can be prospectively identified within the LSK+ fraction using differential expression of VCAM-1 and Flt3 (VCAM−1−Flt3hi−) or by expression of GFP using RAG-1GFP knockin reporter mice (12–14). LMPP are enriched for precursors biased for T, B, NK, and/or dendritic cell (DC) differentiation potential (15, 16). At present, it is unclear whether individual LMPP retain multilineage lymphoid differentiation potential. Regardless, data obtained from numerous gene-targeting strategies have established that failure to generate and/or maintain the LMPP pool has significant consequences on the production of B lineage lymphocytes in BM (11, 17–21).

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Abbreviations used in this article: ALP: all lymphoid progenitor; B6, C57BL/6; BCP, B cell precursor; BLP, B lineage–restricted progenitor; BM, bone marrow; CLP, common lymphoid progenitor; DC, dendritic cell; FL, Flt3 ligand; GMLP, granulocyte–macrophage lymphoid progenitor; HSC, hematopoietic stem cell; LMPP, lymphoid-primed multipotential progenitor; LSK+, Lineage-negative, Sca-1+, c-kithi; MPP, multipotential progenitor; SP, single-positive; WT, wild-type.
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Downregulation of c-kit and surface expression of IL-7R denotes the transition from LMPP to CLP (16). BLP are enriched in a subset of CLPs that express the cell surface marker Ly6D, whereas Ly6D− CLPs exhibit B, T, and NK potentials and are referred to as ALP (22). Resolution of ALP and BLP is an important advance because it enables more accurate determination of the roles of regulatory factors in lymphoid lineage restriction as well as B lineage commitment from lymphoid-restricted progenitors. In that vein, this study seeks to determine individual versus combinatorial roles of two key factors, the homeodomain transcription factor Hoxa9, and the cytokine, Flt3 ligand (FL), in regulation of ALP and BLP.

Hoxa9 is a transcription factor important for the generation of normal numbers of B lineage precursors in BM (17, 19, 23). Hoxa9 is expressed at low levels in HSC, upregulated in MPP and CLP, and then downregulated in B cell precursor (BCP) (17). Hoxa9 regulates B cell development, in part, through transcriptional regulation of Flt3 (17). It is presently unclear whether the B cell deficiency in Hoxa9−/− mice is due solely to impaired Flt3 signaling or whether Hoxa9 and Flt3 signaling provide nonoverlapping functions that guide B cell development. We theorized that if the B lineage deficiency in Hoxa9−/− mice was due to impaired Flt3 alone, then combined loss of Hoxa9 and FL would not exacerbate the B lineage deficiency beyond that observed in the single knockouts. However, if Hoxa9 has additional nonredundant roles or functions cooperatively with Flt3 signaling to promote lymphoid development and B cell differentiation, then combined loss could exacerbate the B lineage deficiency.

In this study, through generation and analysis of Hoxa9−/− Flt3−/− mice, we show that Hoxa9 and Flt3 signaling function cooperatively to establish the lymphoid progenitor pool in BM. Intriguingly, LSK+CD150hiCD34loCD41+ cells that are myeloid-biased HSC are overrepresented in the LSK+ compartment and increased in number in the compound knockout mice. In contrast, LSK+CD150− Flt3+ cells that are enriched for lymphoid progenitors are significantly reduced (5, 24). ALP, BLP, and B lineage lymphocytes are ablated in BM of Hoxa9−/− Flt3−/− mice. NK and DC lineage cells are not impacted beyond what has been documented as a consequence of FL deficiency, suggesting alternative pathways of generation (25). Thymic cellularity is significantly reduced in Hoxa9−/− Flt3−/− mice compared with the single knockouts consistent with the ablation of LMPP in BM that seed the thymus. However, T cell development on the whole appears essentially normal, and numbers of peripheral T cells mirror Flt3−/− mice. These findings establish that there is an extremely early checkpoint in lymphoid lineage development dependent on the combinatorial activities of Hoxa9 and Flt3 signaling.

Materials and Methods

Mice

C57BL/6 (referred to in this paper as wild-type [WT] or B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Hoxa9−/− and Flt3−/− mice have been described previously (11, 17). Hoxa9−/− and Flt3−/− mice were bred, and genotyping was performed to identify compound knockouts (11, 17). All mice in this study were maintained in the Mayo Clinic animal facility and used between 8 and 16 wk of age. Age-matched mice of all four genotypes were used in each experiment, and no differences in phenotype were observed in that range or in male versus female mice. All experiments were carried out in accordance with Mayo Clinic Institutional Animal Care and Use Committee guidelines.

Abs and flow cytometry

Methods for flow cytometry and progenitor isolation have been described previously (11, 14, 17). Flow cytometric analysis was performed on the LSRII cytometer (BD Biosciences, San Jose, CA) and data analysis with FlowJo software (Tree Star, Ashland, OR). All Abs were purchased from eBioscience, BioLegend, or BD Pharmingen. Ab combinations and combinations used to delineate progenitor subsets in BM were the following: LSK+, Lineage mixture (FITC-conjugated CD45R/B220, CD11b/Mac1, Ly6G/Gr1, TER119, CD3ε, CD8α, and NK1.1), c-kit–allophycocyanin–fluor 780, Sca-1-PerCP-Cy5.5, CD150-PE-Cy7, Flt3-PE, CD34-eFluor 450, and CD41-allophycocyanin; CLP, Lineage mixture (FITC-conjugated CD45R/B220, CD11b/Mac1, Ly6G/Gr1, TER119, CD3ε, CD8α, and NK1.1), c-kit–allophycocyanin–fluor 780, Sca-1-PerCP-Cy5.5, CD122-FITC, and NK1.1, c-kit–allophycocyanin–fluor 780, Sca-1-PerCP-Cy5.5, biotin IL-7R (visualized with streptavidin PE-Cy7), Flt3-PE, and Ly6D-allophycocyanin; BCP (CD19-allophycocyanin, B220-PE-Cy7, IgM-FITC, and CD43-PE); myeloid cells, CD11b/Mac-1-allophycocyanin, and Ly6C), c-kit–allophycocyanin–fluor 780, Sca-1-PerCP-Cy5.5, biotin IL-7R (visualized with streptavidin PE-Cy7), Flt3-PE, and Ly6D-allophycocyanin; BCP (CD19-allophycocyanin, B220-PE-Cy7, IgM-FITC, and CD43-PE); myeloid cells, CD11b/Mac-1- allophycocyanin, and Gr-1-FITC; erythroid cells, Ter119-PeCy7; NK subsets, CD3ε-PeCy7, CD122-FTTC, and NK1.1-PeCy5.5; total DCs, CD11c-PE; and thymocyte subsets, CD3ε-allophycocyanin, CD4-FTTC, and CD8α-PE. Gates for discriminating negative and positive subsets were determined based on staining of BM mononuclear cells or previously characterized positive/negative BM subsets. Spleens and thymi were harvested, and single-cell suspensions were made by crushing the organs through a 70-μm nylon mesh. Spleen mononuclear cell counts. (B) B and T cell precursor frequency in spleen. Spleen cell suspensions were incubated with anti–CD19-PE or anti–CD3ε-allophycocyanin, anti–CD4-FITC, and CD8α-PE Abs to resolve B and T lineage lymphocytes, respectively. Percentages reflect cells within the lymphoid light scatter gate. (C) Absolute numbers of B or T cells in spleen. Data in (A)–(C) are representative of the mean ± SD of five to six spleens per genotype.
mesh. Mononuclear cell counts were determined by counting nuclei after lysis in 3% acetic acid/water solution.

Isolation of progenitor subsets for real-time PCR analysis

BM cells were harvested and pooled from six B6 controls, seven Hoxa92/2, and eight Flt3l2/2 mice in each of two independent experiments. Lin− cells were enriched from the three mouse groups by incubating BM cell suspensions with a biotin-labeled Lin− Ab mixture (B220, Gr-1, Ter119, CD3e, CD8a, CD11c, NK1.1, and CD19), followed by incubation with streptavidin-conjugated microbeads and magnetic separation. The Lin− enriched BM fractions were resuspended in 100 µl extraction buffer and then stored at −80°C or processed immediately for RNA isolation using the Arcturus PicoPure RNA isolation kit (Applied Biosystems, Foster City, CA). To remove contaminating genomic DNA, the RNA was treated with DNase I kit (Qiagen, Germantown, MD). cDNA amplification was performed using the Ovation PicoSL WTA System (NuGEN, San Carlos, CA). Amplified cDNA was purified using the MinElute Reaction Cleanup kit (Qiagen). Real-time PCR was performed using TaqMan probes (Applied Biosystems) or gene-specific SYBR Green assays as we described previously. Gene expression was normalized to 18S RNA for TaqMan assays or gapdh for SYBR Green assays. All cDNA samples were assayed in triplicate. Relative transcript abundance was determined using the 2−ΔΔCT method with B6 transcript levels for each sorted subset assigned the comparator.

Statistical analysis

Statistical significance was determined using the Student t test. A p value ≤ 0.05 was deemed significant and is indicated by asterisks.

Results

Synergistic reduction in B lymphopoiesis in Hoxa9−/−Flt3l−/− mice

To determine whether Hoxa9 provides nonredundant roles or functionally coordinates with Flt3 signaling in the generation of lymphoid progenitors and BCPs, Hoxa9−/−Flt3l−/− mice were generated. The compound knockouts were born at normal Mendelian ratios and exhibited no gross abnormalities. Peripheral blood analysis of the double knockouts mirrored Flt3l−/− mice (i.e., reduced numbers and percentages of lymphocytes; data not shown). Consistent with the reduction in blood lymphocytes, spleen cellularity was reduced ~60% in Hoxa9−/−Flt3l−/− mice, compared with 39% in Flt3l−/− mice (Fig. 1A). Flow cytometric analysis of
T cells in the spleen showed no significant differences in percentages or absolute numbers in the compound knockouts beyond that observed in the single knockouts (Fig. 1B, 1C). No alterations in ratios of CD4+ or CD8+ splenic T cells were observed (data not shown). In contrast, percentages of CD19+ splenic B cells were synergistically reduced in the compound knockout mice (splenic CD19+ cells, B6 = 53.4 ± 8.4% [n = 6], Hoxa9−/− = 40.6 ± 9.4% [n = 5], Flt3l−/− = 32.5 ± 11.8% [n = 6], and Hoxa9−/−Flt3l−/− = 23.8 ± 6.6% [n = 6] (Fig. 1B). The reduced percentages together with reduction in cellularity in the compound knockout culminated in a statistically significant reduction in numbers of splenic CD19+ B lymphocytes (12.5 ± 7.4 versus 24.1 ± 10.2 × 106, Hoxa9−/−Flt3l−/− versus Flt3l−/−, respectively; *p = 0.047). These data suggest that combined loss of Hoxa9 and Flt3 signaling has more severe consequences on B lymphopoiesis than deficiency in Hoxa9 or Flt3 alone. Spleenic T cells are reduced but comparable to Flt3 deficiency alone.

**Severe reduction in BM BCP in Hoxa9−/−Flt3l−/− mice**

The spleen results suggested that the compound knockout mice might have selective abnormalities associated with B lymphopoiesis in BM. Hoxa9−/− and Flt3l−/− single knockout mice are known to have reduced BM cellularity, and in this study, we determined that the same was true for Hoxa9−/−Flt3l−/− single knockout mice (Fig. 2A) (23, 25). However, the reduction in BM cellularity in the compound knockouts was not statistically significant when compared with the single knockouts.

To determine the consequence of combined loss of Hoxa9 and Flt3 signaling on B lymphopoiesis, BM cells were harvested from strain-matched B6 controls, Hoxa9−/−, Flt3l−/−, and Hoxa9−/−Flt3l−/− mice and stained with Abs to CD45R/B220, CD19, CD43, and IgM to resolve B lineage subsets. As summarized in Fig. 2B and shown in Fig. 2C and 2D, combined loss of Hoxa9 and FL severely ablated B lymphopoiesis. To document the stages of B lymphopoiesis affected in the compound knockout mice, mature (B220+IgM+) and immature (B220+IgM−) B lineage subsets were examined (Fig. 2C, top panel). Indeed, the prominent subset of BCPs in marrow in the compound knockout mice were IgM− and likely represent recirculating B lymphocytes (26). It is known that naive B cells undergo homeostatic proliferation under conditions of B cell deficiency, and the IgM− cells in the marrow uniformly expressed high levels of B220, characteristic of recirculating B cells (26). In contrast, all IgM− BCP subsets, including B220+CD43− pre-B cells and B220+CD43−CD19+ pro-B cells, were ablated in the compound knockout mice (Fig. 2C). We conclude from these experimental findings that Hoxa9 and Flt3 function in a shared pathway to regulate B lymphopoiesis in BM.

The consequence of combined loss of Hoxa9 and Flt3 signaling was selective for B lineage lymphocytes. We found no significant alterations in frequencies of Mac-1/Gr1+ myeloid or Ter119+ erythroid lineage analysis of Hoxa9−/−Flt3l−/− mice. BM cells were harvested as we previously described and incubated with combinations of Abs to resolve NK or DC differentiation by flow cytometry. The data in (A) represent precursor frequency of NK progenitors defined as CD3−ε−CD122+NK1.1−. The data in (B) represent precursor frequency of mature NK cells defined as CD3ε−CD122+NK1.1+. (C) Percentages of total CD11c+ DC lineage cells in BM. The data are representative of mean frequencies ± SD of three to four BM analyses per genotype. (D) Thymi were harvested from mice of each genotype, and nucleated cell counts were determined. The data are representative of mean frequencies ± SD of four animals per genotype. Statistical significance is compared with B6 controls. (E) Thymic cell suspensions incubated with anti-CD4-FITC and anti-CD8-PE and flow cytometric analysis performed.

Precursor frequencies of the four major T cell developmental subsets were evaluated based on differential expression of CD4 and CD8, Double-negative (DN) = CD4+CD8−, double-positive (DP) = CD4+CD8+, CD4 SP indicates CD4+SP, and CD8 SP indicates CD8+SP. The data are representative of mean frequencies ± SD of four thymic analyses per genotype.

**FIGURE 3.** Alternate lymphoid lineage analysis of Hoxa9−/−Flt3l−/− mice. BM cells were harvested from strain-matched B6 controls, Hoxa9−/−, Flt3l−/−, and Hoxa9−/−Flt3l−/− mice and stained with Abs to CD45R/B220, CD19, CD43, and IgM to resolve B lineage subsets. As summarized in Fig. 2B and shown in Fig. 2C and 2D, combined loss of Hoxa9 and FL severely ablated B lymphopoiesis. To document the stages of B lymphopoiesis affected in the compound knockout mice, mature (B220+IgM+) and immature (B220+IgM−) B lineage subsets were examined (Fig. 2C, top panel). Indeed, the prominent subset of BCPs in marrow in the compound knockout mice were IgM− and likely represent recirculating B lymphocytes (26). It is known that naive B cells undergo homeostatic proliferation under conditions of B cell deficiency, and the IgM− cells in the marrow uniformly expressed high levels of B220, characteristic of recirculating B cells (26). In contrast, all IgM− BCP subsets, including B220+CD43− pre-B cells and B220+CD43−CD19+ pro-B cells, were ablated in the compound knockout mice (Fig. 2C). We conclude from these experimental findings that Hoxa9 and Flt3 function in a shared pathway to regulate B lymphopoiesis in BM.

The consequence of combined loss of Hoxa9 and Flt3 signaling was selective for B lineage lymphocytes. We found no significant alterations in frequencies of Mac-1/Gr1+ myeloid or Ter119+ erythroid lineage analysis of Hoxa9−/−Flt3l−/− mice. BM cells were harvested as we previously described and incubated with combinations of Abs to resolve NK or DC differentiation by flow cytometry. The data in (A) represent precursor frequency of NK progenitors defined as CD3ε−CD122+NK1.1−. The data in (B) represent precursor frequency of mature NK cells defined as CD3ε−CD122+NK1.1+. (C) Percentages of total CD11c+ DC lineage cells in BM. The data are representative of mean frequencies ± SD of three to four BM analyses per genotype. (D) Thymi were harvested from mice of each genotype, and nucleated cell counts were determined. The data are representative of mean frequencies ± SD of four animals per genotype. Statistical significance is compared with B6 controls. (E) Thymic cell suspensions incubated with anti-CD4-FITC and anti-CD8-PE and flow cytometric analysis performed.

Precursor frequencies of the four major T cell developmental subsets were evaluated based on differential expression of CD4 and CD8, Double-negative (DN) = CD4+CD8−, double-positive (DP) = CD4+CD8+, CD4 SP indicates CD4+SP, and CD8 SP indicates CD8+SP. The data are representative of mean frequencies ± SD of four thymic analyses per genotype.
throid cells (data not shown). Thus, the profound deficiency in BCP in the compound knockout mice is not due to a requirement for Hoxa9 and Flt3 signaling in regulating the lymphoid versus myeloid-erythroid fate decision in primitive hematopoietic progenitors.

NK and DC lineage cells in BM share a common progenitor pathway with BCP (15). Flow cytometric analysis of NK precursors defined as CD3e−CD122+CD11c+ or mature NK cells defined as CD3e−CD122+CD11c+ (27) revealed no significant alteration in NK development or maintenance in the compound knockouts beyond that because of Flt3 deficiency (Fig. 3A, 3B). The same was true for dendritic lineage cells defined broadly as CD11c+ (Fig. 3C).

T cells and B cells arise from Flt3+ MPPs in BM (15). Numbers of total thymocytes were similarly reduced in Hoxa9−/− and Flt3−/− mice in accord with previous reports (25, 28, 29). Compound loss of Hoxa9 and Flt resulted in a more severe reduction in total thymocyte cellularity than the single knockout mice (~5.8-fold reduced compared with B6 controls and 3-fold from Hoxa9−/− [p = 0.0058] or Flt3−/− mice [p = 0.0078]) (Fig. 3D). The synergistic reduction in thymocyte cellularity is likely due to the ablation of Flt3+ MPPs in BM that seed the thymus, thus reducing the efficiency of BM progenitor seeding of the thymus (see below) (30). Although total thymocyte numbers are reduced, we found no significant alteration in major hallmarks of T cell development (Fig. 3E). Percentages of CD8+ (double-negative), CD4+ or CD8+ single-positive (SP) thymocyte subsets were comparable in all four mouse models. We previously reported decreased Flt3+LSK+ cells in Flt3−/− and Flt3−/− mice (11, 17). The dramatic reduction in ALP in the compound knockout mice suggested critical roles for Hoxa9 and Flt3 signaling upstream of IL-7R expression. LSK+ are the presumed immediate precursors of ALP as il7rα transcripts are detectable by real-time PCR at this stage (10). LSK+ can be distinguished within LSK+ cells based on differential expression of VCAM-1 and Flt3 (31). As shown in Fig. 5A and summarized in Fig. 5B, LSK+VCAM-1+ Flt3−hi LMP are reduced in Flt3−/− and Hoxa9−/− mice and ablated in Hoxa9−/−Flt3−/− mice.

Severe deficiency in ALP and BLP in Hoxa9−/−Flt3−/− mice

Functional BCP are enriched in Lin−c-kithiIL-7R+Flt3+ CLPs. Hoxa9−/− and Flt3−/− mice each have significantly reduced numbers of Flt3+ CLPs (11, 17, 19). Recently, it was determined that Flt3+ CLPs could be fractionated into Ly6D− ALP and Ly6D+ BLP (22). Both categories of Flt3+ CLPs were depressed in frequencies and numbers in the single knockouts (Fig. 4A and summarized in Fig. 4B, 4C). Combined loss of Hoxa9 and Fl exacerbated the reduction of Flt3+Ly6D− ALP (~70% reduction in Ly6D− compared with Flt3−/− mice) and Flt3+Ly6D+ BLP (~37-fold reduction compared with Flt3−/− mice). We note an increase in abundance of Flt3−lo CLPs in Flt3−/− and Hoxa9−/−Flt3−/− mice (Fig. 4A). This observation suggests that Flt3 signaling might play a role in autoregulation of Flt3 in CLPs or at an earlier stage (see below). The dramatic loss of Flt3+ CLPs in Hoxa9−/−Flt3−/− mice suggests that Hoxa9 and Flt3 signaling are critical components of a genetic network that regulates the generation or maintenance of ALP and BLP.

Reductions in LMPP and granulocyte-macrophage lymphoid progenitor in Hoxa9−/−Flt3−/− mice

We previously reported decreased Flt3+LSK+ cells in Hoxa9−/− and Flt3−/− mice (11, 17). The dramatic reduction in ALP in the compound knockout mice suggested critical roles for Hoxa9 and Flt3 signaling upstream of IL-7R expression. LMPP are the presumed immediate precursors of ALP as il7rα transcripts are detectable by real-time PCR at this stage (10). LMPP can be distinguished within LSK+ cells based on differential expression of VCAM-1 and Flt3 (31). As shown in Fig. 5A and summarized in Fig. 5B, LSK+VCAM-1+ Flt3−hi LMPP are reduced in Flt3−/− and Hoxa9−/− mice and ablated in Hoxa9−/−Flt3−/− mice.

![FIGURE 4](http://www.jimmunol.org/)

**A** Ablation of CLP subsets in Hoxa9−/−Flt3−/− mice. (A) Representative flow cytometry profiles of Lineage (Lin−) IL-7R+ CLP subsets in the four mouse models. Lin− cells (top panels) were discriminated based on differential expression of c-kit and IL-7R to identify CLPs. Lin− c-kithiIL-7R+ cells (middle panels) were further fractionated into ALP and BLP based on differential expression of Flt3 and Ly6D (bottom panels). The plots shown are representative of three to four mice per genotype. (B) Precursor frequency of Flt3+ CLP subsets in the four mouse models. (C) Absolute numbers of ALP (white bar) and BLP (gray bar). Data represent the mean and SD of four mice per genotype. Statistical significance, indicated by the asterisks, is determined relative to B6 WT controls. **p > 0.005. ***p > 0.0001.
In addition to LMPP, Hoxa9−/− and Flt3l−/− mice have statistically significant reductions in granulocyte-macrophage lymphoid progenitor (GMLP), an LSK− subset with combined lymphoid and myeloid differentiation potential (11, 31). Similar to LMPP, combined loss of Hoxa9 and FL results in a more severe reduction in GMLP than observed in the single knockout mice (Fig. 5A and summarized in Fig. 5B). Importantly, the reduction in GMLP had no significant impact on the production of myeloid cells in BM beyond that in Flt3−/− mice (42.3 ± 3.1 versus 32.7 ± 6.0 versus 34.95 ± 5.7% of BM mononuclear cells in B6 versus Hoxa9−/−Flt3−/− versus Flt3−/−, respectively, n = 4 mice/genotype). Thus, the myeloid lineage developmental potential of VCAM-1−1Flt3hi is not compromised.

The reductions in GMLP and LMPP coincided with a significant increase in percentages of LSK−VCAM-1−Flt3−/hi cells enriched for HSC and MPP (Fig. 5A and summarized in Fig. 5B). These observations suggest that Hoxa9 and Flt3 signaling could be important in regulation of the HSC/MPP pool.

**Alterations in HSC and MPP subsets in Hoxa9−/−Flt3l−/− mice**

Flt3 is expressed early during hematopoietic differentiation. Two independent studies showed that all MPP progress through an Flt3+ stage, including erythroid/megakaryocyte progenitors (7, 8). However, because Flt3 expression is sensitive to loss of Hoxa9 and/or Flt3 signaling, evaluation of single or combined requirements for these regulators in subsets defined by Flt3 expression alone is problematic (11, 17). Functional HSC are rare and express the signaling lymphocyte activation molecule family marker CD150 and are CD34− (5, 6). MPP with lymphoid–myeloid-biased differentiation potentials express Flt3 and have downregulated CD150 signaling.

**Summary of HSC/MPP, GMLP, and LMPP subset frequencies as a percentage of LSK+ cells.**

Data represents the mean and SD of four to five mice per genotype. *p > 0.05, **p > 0.005, ***p > 0.0005.

**FIGURE 5.** Reductions in GMLP and LMPP in Hoxa9−/−Flt3l−/− mice. (A) LSK− cells were discriminated as Lin− (data not shown) c-KIThi SCA-1− (boxed region). HSC/MPP, GMLP, and LMPP was resolved within the LSK− gate based on differential expression of VCAM-1 and Flt3. (B) Summary of HSC/MPP, GMLP, and LMPP subset frequencies as a percentage of LSK+ cells. Data represents the mean and SD of four to five mice per genotype. *p > 0.05, **p > 0.005, ***p > 0.0005.

Finally, we consistently observed that the CD150+ cells that increased in the compound knockout mice expressed very high levels of CD150 (Fig. 6). LSK−CD150+ cells expressing high levels of CD150 have been shown to exhibit latent and myeloid-biased reconstitution potential in transplant recipients (3). Myeloid-biased HSC can be further discriminated in adult mice by expression of CD41 (32). As shown in Fig. 6C, there was a significant increase in percentage of LSK−CD150+CD34−lo cells that expressed CD41 in the compound knockout mice (p = 0.034, n = 3 mice/genotype). Thus, combined loss of Hoxa9 and Flt3 signaling results in a disproportionate number of myeloid-biased HSC in BM.

Taken together, these flow cytometry analyses show that combined loss of Hoxa9 and Flt3 signaling significantly alters the HSC/MPP compartment of BM. LSK−CD150+CD34−loCD41+ myeloid-biased HSC are increased, whereas CD150+Flt3− progenitors are severely depleted. We conclude that Hoxa9 and Flt3 signaling regulates a critical early checkpoint in hematopoietic differentiation that restricts B and T lineage developmental potential.

**Differential role of Hoxa9 and/or Flt3 signaling in regulation of the early program of lymphoid gene expression**

Deficiencies in Hoxa9, FL, or both result in significant reduction in lymphoid-biased progenitors and BCP in BM. We and others (10, 11) have shown that the rare MPP expressing high levels of Flt3 in Flt3hi mice have reduced levels of lymphoid–lineage transcripts, suggesting that Flt3 signaling plays a role in lymphoid priming. Hoxa9−/− mice also have reductions in Flt3hi MPP, although a role for Hoxa9 in lymphoid priming has not been reported. Combined roles for Hoxa9 and Flt3 signaling in regulation of lymphoid priming provide an explanation for the severe defect in lymphoid development in the compound knockout mice. To make this de-
termination, LSK⁺CD150⁻ cells from B6 control, \( Flt3^{-/-} \), and \( Hoxa9^{-/-} \) mice were sorted into Flt3⁺ and Flt3⁻ subsets. The paucity of LSK⁺CD150⁻ cells, particularly LSK⁺CD150⁺Flt3⁺ cells that are severely reduced, precluded isolation of these cells from the compound knockout mice. CD150 was included as a gating parameter to exclude alterations to the results contributed by
CD150\(^{+}\)Flt3\(^{lo}\) cells that vary among the three strains. Consistent with previous findings by others, transcripts corresponding to *rag1*, *ccr9*, and *il7ra* are more abundant in Flt3\(^{+}\) than Flt3\(^{lo}\)LSK\(^{+}\) cells (Fig. 7) (10, 33). All three lymphoid–lineage transcripts are reduced in Flt3\(^{+}\) cells in Flt3\(^{-/-}\) mice (Fig. 7) (10). Similarly, *rag1* and *ccr9* transcripts are reduced in *Hoxa9*\(^{-/-}\) mice. However, we did not observe a significant reduction in *il7ra* transcripts in Flt3\(^{+}\) cells in *Hoxa9*\(^{-/-}\) mice. These data suggest that lymphoid priming is more dependent on Flt3 signaling than Hoxa9 function.

**Discussion**

We previously showed that Hoxa9 regulates B cell development, in part, through transcriptional regulation of Flt3. In this study, we sought to determine whether Hoxa9 provides additional functions in BM lymphoid/B cell development, independent of, or coordinately with, Flt3 signaling. We show that Hoxa9\(^{-/-}\)/Flt3\(^{-/-}\) mice have a profound block in the generation of lymphoid progenitors destined to become B or T lineage lymphocytes. Importantly, the ablation of lymphoid progenitors had selective consequences on lymphoid lineage progeny because NK and DC lineage cells were not synergistically reduced. We pinpointed the block in lymphoid development to the LSK\(^{+}\)CD150\(^{+}\)CD150/CD150\(^{+}\)Flt3\(^{+}\) transition. Hematopoietic progenitors deficient in Hoxa9 or Flt3 alone can mediate this developmental transition, albeit inefficiently. However, combined loss of Hoxa9 and Flt3 signaling completely abrogates the generation of LSK\(^{+}\)CD150\(^{+}\)Flt3\(^{+}\) MPPs. The severe lymphoid deficiency was accompanied by an increase in absolute numbers of LSK\(^{+}\)CD150\(^{+}\)CD34\(^{-/-}\)CD41\(^{+}\) phenotypic myeloid-biased HSC in the compound knockout mice. Taken together, these new findings uncover combinatorial and limiting requirements for Hoxa9 and Flt3 signaling in the generation of lymphoid precursors destined to become B or T lineage precursors.

Lymphoid priming is a very early event in hematopoiesis. Transcriptional priming of lymphoid genes has been demonstrated in individual HSC, and one study showed that it is dependent on the Knüppel-type zinc finger transcription factor Ikaros (33, 34). Similar to Hoxa9\(^{-/-}\) and Flt3\(^{-/-}\) mice, Ikaros-null mice have reductions in total Flt3\(^{+}\) MPP, and the residual MPP in these mice are Flt3\(^{lo}\) (35). In WT mice, Flt3\(^{lo}\) MPPs show little evidence of lymphoid priming. Upregulation of Flt3 coincided with increased abundance of lymphoid lineage transcripts, including *ccr9*, *rag1*, and *il7ra*. The Flt3\(^{+}\) MPP pool, which includes LMP, is significantly depleted by Hoxa9 or FL deficiency. Interestingly, *il7ra* transcripts were detectable in the residual Flt3\(^{+}\) MPP in Hoxa9\(^{-/-}\) mice. Given that Lin IL-7R\(^{+}\) cells are detectable by flow cytometry in the single and compound knockout mice, the reduction in *il7ra* transcripts in the Flt3\(^{-/-}\) mice likely reflects the lymphoid-primed progenitor pool simply fails to be established. These results suggest that Flt3 signaling, but not Hoxa9 function, is limiting in establishment of a lymphoid-primed progenitor pool. The contribution of Hoxa9 is likely indirect.

**Table I. Hematopoietic progenitor frequency analysis**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>% Lin(^{+})</th>
<th>% LSK(^{+})</th>
<th>% CD150(^{+})LSK(^{+})</th>
<th>% CD150(^{+})LSK(^{+})Flt3(^{+})</th>
<th>% Flt3(^{-/-})LSK(^{+})</th>
<th>% Flt3(^{lo})LSK(^{+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>2.8 ± 1.0</td>
<td>9.0 ± 2.5</td>
<td>9.8 ± 2.7</td>
<td>86.5 ± 1.2</td>
<td>88.8 ± 2.0</td>
<td>22.2 ± 2.9</td>
</tr>
<tr>
<td>Hoxa9(^{-/-})</td>
<td>3.3 ± 0.7</td>
<td>5.3 ± 1.8</td>
<td>33.2 ± 9.9**</td>
<td>64.5 ± 7.5**</td>
<td>63.3 ± 11.2**</td>
<td>6.6 ± 1.3***</td>
</tr>
<tr>
<td>Flt3(^{-/-})</td>
<td>2.6 ± 0.9</td>
<td>4.7 ± 2.1</td>
<td>33.9 ± 9.5**</td>
<td>62.7 ± 7.8**</td>
<td>76.6 ± 7.7*</td>
<td>5.9 ± 2.9**</td>
</tr>
<tr>
<td>Hoxa9(^{-/-})/Flt3(^{-/-})</td>
<td>2.1 ± 0.6</td>
<td>4.3 ± 0.6</td>
<td>55.1 ± 2.5*</td>
<td>42.3 ± 4.7*</td>
<td>39.9 ± 5.4*</td>
<td>1.3 ± 1.0***</td>
</tr>
</tbody>
</table>

All data represent mean ± SD of three to five mice per genotype. LSK\(^{+}\) frequency reflects % LSK\(^{+}\) cells within the Lin\(^{-}\) gated subset. CD150\(^{+}\), CD150\(^{-}\), Flt3\(^{+}\) (includes Flt3\(^{lo}\) cells), and Flt3\(^{lo}\) indicate percentages of marker positive cells within the LSK\(^{+}\) gated population.

\(^{a}\)Hoxa9\(^{-/-}\)/Flt3\(^{-/-}\) subset statistics compared with B6.

\(^{b}\)Hoxa9\(^{-/-}\)/Flt3\(^{-/-}\) compared with Hoxa9\(^{-/-}\)/ or Flt3\(^{-/-}\).

*p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.

IL-7R expression in Lin\(^{-}\) cells denotes the CLP stage, and IL-7R signaling promotes lymphoid restriction (16, 36). The CLP subset on the whole is heterogeneous, and at present, three distinct subsets have been resolved based on differential expression of Flt3 and Ly6D. Flt3\(^{+}\)Ly6D\(^{-}\) ALP are the presumed immediate progeny of Flt3\(^{+}\)LMP and retain full lymphoid lineage developmental potential (15, 22). Downregulation of Flt3 in CLPs accompanies NK developmental restriction (37, 38). Flt3\(^{lo}\)-CLPs are refractory to treatment with IL-7R signaling and promote lymphoid restriction (16, 36). The CLP subset on the whole is heterogeneous, and at present, three distinct subsets have been resolved based on differential expression of Flt3 and Ly6D. Flt3\(^{+}\)Ly6D\(^{-}\) ALP are the presumed immediate progeny of Flt3\(^{+}\)LMP and retain full lymphoid lineage developmental potential (15, 22). Downregulation of Flt3 in CLPs accompanies NK developmental restriction (37, 38). Flt3\(^{lo}\)-CLPs are refractory to treatment with IL-7R signaling and promote lymphoid restriction (16, 36). The CLP subset on the whole is heterogeneous, and at present, three distinct subsets have been resolved based on differential expression of Flt3 and Ly6D. Flt3\(^{+}\)Ly6D\(^{-}\) ALP are the presumed immediate progeny of Flt3\(^{+}\)LMP and retain full lymphoid lineage developmental potential (15, 22). Downregulation of Flt3 in CLPs accompanies NK developmental restriction (37, 38). Flt3\(^{lo}\)-CLPs are refractory to treatment with IL-7R signaling and promote lymphoid restriction (16, 36). The CLP subset on the whole is heterogeneous, and at present, three distinct subsets have been resolved based on differential expression of Flt3 and Ly6D. Flt3\(^{+}\)Ly6D\(^{-}\) ALP are the presumed immediate progeny of Flt3\(^{+}\)LMP and retain full lymphoid lineage developmental potential (15, 22). Downregulation of Flt3 in CLPs accompanies NK developmental restriction (37, 38). Flt3\(^{lo}\)-CLPs are refractory to treatment with IL-7R signaling and promote lymphoid restriction (16, 36). The CLP subset on the whole is heterogeneous, and at present, three distinct subsets have been resolved based on differential expression of Flt3 and Ly6D. Flt3\(^{+}\)Ly6D\(^{-}\) ALP are the presumed immediate progeny of Flt3\(^{+}\)LMP and retain full lymphoid lineage developmental potential (15, 22). Downregulation of Flt3 in CLPs accompanies NK developmental restriction (37, 38). Flt3\(^{lo}\)-CLPs are refractory to treatment with IL-7R signaling and promote lymphoid restriction (16, 36).

**FIGURE 7.** Requirements for Hoxa9 and Flt3 signaling in lymphoid priming. Real-time PCR of the indicated transcripts in CD150\(^{+}\)LSK\(^{+}\) cells sorted into Flt3\(^{lo}\) and Flt3\(^{+}\) subsets. The panels on the left show transcript abundance in B6 mice CD150\(^{+}\)LSK\(^{+}\)Flt3\(^{lo}\) and CD150\(^{+}\)LSK\(^{+}\)Flt3\(^{+}\) sorted cells. The panels on the right compare the indicated transcripts in CD150\(^{+}\) LSK\(^{+}\)Flt3\(^{+}\) cells isolated from the three indicated genotypes (B6 versus Hoxa9\(^{-/-}\)/Flt3\(^{-/-}\)). The data represent mean values obtained from two independent sorts. Error bars indicated SD.
to loss of Hoxa9 function and Flt3 signaling, and we recently showed that Hoxa9 is not essential for the generation or differentiation of NK lineage cells (39). At present, there is no evidence for an LMPPI/CLP-independent pathway in NK development. IL-7R signaling is not required for NK cell genesis (40). It is possible that NKp can develop directly from LSK/CD150 Flt3<sup>hi</sup> MPP independent of LMPPI/CLP. Future studies using adoptive transfer and in vitro assays will directly test this possibility.

Flt3<sup>hi</sup> CLP also have DC differentiation potential (15). Like NK cells, Hoxa9<sup>+</sup> is dispensable for the generation and differentiation of DC (39). The generation of committed DC progenitors is not dependent on Flt3 signaling although the cytokine is important for DC differentiation and homeostasis (25, 41). An IL-7R Flt3<sup>+</sup> common DC progenitor was described recently (42). Because DC progeny have multiple development origins and the transcription factor PU.1 is a critical regulator of Flt3 in this lineage, it is not unexpected that DC are not perturbed in the compound mice (15, 43–45).

We previously showed that Flt3<sup>lo</sup>LSK<sup>+</sup> cells from Flt3<sup>l</sup>-/mice have reduced expression of prosurvival factors, suggesting that Flt3 signaling plays a critical role in regulation of progenitor survival (11). Hoxa9, in contrast, has been shown to regulate the proliferation of multipotent hematopoietic progenitors in vitro (46). Hoxa9<sup>−/−</sup> mice are deficient in the serine–threonine kinase Pim-1 (47). Flt3 signaling activates Pim-1, and reductions in Pim-1 impact B lymphopoiesis (48, 49). Importantly, Pim-1 has been linked to c-Myb activity, and c-Myb plays a critical role in lymphoid priming and B cell development (21, 50). Hoxa9 has been implicated in regulation of c-Myb in leukemic blasts (51). These experimental findings together with the profound lymphoid/B lineage deficiency manifested by the compound knockout mice suggest that Hoxa9 and Flt3 signaling regulate lymphoid/B cell development through regulatory circuits that impact the survival and proliferation of Flt3<sup>hi</sup> MPP. Preliminary analyses of Flt3<sup>lo</sup> MPP isolated from the single knockout mice did not reveal significant alterations in expression of c-Myb or Pim-1. However, these findings do not preclude that similar analysis of Flt3<sup>lo</sup> MPP from the compound knockout mice might reveal different results. Future studies using adoptive transfer and in vitro assays will directly test this possibility.

We note that the increase in myeloid-biased HSC did not influence the lymphoid/B lineage. Preliminary analyses of Flt3<sup>lo</sup> MPP isolated from the single knockout mice did not reveal significant alterations in expression of c-Myb or Pim-1. However, these findings do not prevent that similar analysis of Flt3<sup>lo</sup> MPP from the compound knockout mice might reveal different results. Future studies using adoptive transfer and in vitro assays will directly test this possibility.
Hoxa9 AND Flt3 SIGNALING IN LYMPHOPOIESIS


