Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors

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The mechanism of lineage commitment from hematopoietic stem cells (HSCs) is not well understood. Although commitment to either the lymphoid or the myeloid lineage is popularly viewed as the first step of lineage restriction from HSCs, this model of hematopoietic differentiation has recently been challenged. The previous identification of multipotent progenitors (MPPs) that can produce lymphocytes and granulocyte/macrophages (GMs) but lacks erythroid differentiation ability suggests the existence of an alternative HSC differentiation program. Contribution to different hematopoietic lineages by these MPPs under physiological conditions, however, has not been carefully examined. In this study, we performed a refined characterization of MPPs by subfractionating three distinct subsets based on Flt3 and vascular cell adhesion molecule 1 expression. These MPP subsets differ in their ability to give rise to erythroid and GM lineage cells but are equally potent in lymphoid lineage differentiation in vivo. The developmental hierarchy of these MPP subsets demonstrates the sequential loss of erythroid and then GM differentiation potential during early hematopoiesis. Our results suggest that the first step of lineage commitment from HSCs is not simply a selection between the lymphoid and the myeloid lineage.

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Lineage commitment is a recurring theme throughout hematopoiesis, the process by which hematopoietic stem cells (HSCs) give rise to all classes of blood cells (1, 2). Multiple developmental intermediates downstream of HSCs have been prospectively isolated and characterized in the bone marrow, which enable us to gain insights into the sequential molecular events that occur during the process of maturation to different hematopoietic lineages (3, 4). Nonetheless, when and how the first step of lineage restriction occurs from HSCs remains obscure (5).

Early in their differentiation, long-term (LT)-HSCs lose their capacity for self-renewal, differentiating first into short-term (ST)-HSCs and subsequently to multipotent progenitors (MPPs). MPPs have very limited or no self-renewal activity but retain the potential for multilineage differentiation (6, 7). It was postulated that the first step of lineage restriction occurs at this developmental stage, where MPPs are specified to either the lymphoid or myeloid lineage, the two major branches of hematopoietic cells (8). Identification of common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs), which differentiate exclusively to specific cell lineages, supported this model of MPP differentiation (9, 10). Although lineage specification is widely thought to occur at the MPP stage (1), no clear evidence established the developmental hierarchy of MPPs and lineage-restricted progenitors. Recently, we demonstrated the ability of a subset of MPPs in giving rise to CMPs in vivo. In this previous study, we showed that the MPP population, defined as the $Flt3^{+}Thy\text{-}1.1^{-}$ cells in the $c\text{-}Kit^{hi}Lin^{-}Sca\text{-}1^{hi}$ (KLS) fraction of bone marrow, can be subdivided into two subpopulations based on vascular cell adhesion molecule 1 (VCAM-1) expression (11). Although $VCAM\text{-}1^{-}$ MPPs possess robust clonal granulocyte/macrophage (GM) and lymphoid differentiation potential in vitro, $VCAM\text{-}1^{-}$ MPPs preferentially become lymphocytes in vivo. $VCAM\text{-}1^{+}$ MPPs can give rise to CMPs and $VCAM\text{-}1^{-}$ MPPs, suggesting that $VCAM\text{-}1^{+}$ MPPs represent a branching point between the lymphoid and myeloid lineages (11). Interestingly, Adolfsson et al. (12) demonstrated that $Flt3^{+}KLS$ cells lack erythroid differentiation potential, but have potent lympho-myeloid

The online version of this article contains supplemental material.

differentiation potential in vitro. Their result challenged the hierarchy of MPPs and CMPs during differentiation and raised the possibility that the erythroid lineage diverges earlier than the GM lineage, or that there are multiple branching points for myeloid differentiation during hematopoiesis. However, the report by Adolfsson et al. (12) is inconsistent with our findings (11) and an earlier study by Christensen et al. (7), showing that Flt3$^+$KLS cells are either ST-HSCs or MPPs. These discrepancies prompted us to reevaluate the differentiation potential of VCAM-1$^+$ MPPs more carefully.

In this study, we found that all VCAM-1$^-$ MPPs homogeneously express high levels of Flt3, whereas VCAM-1$^+$ MPPs can be subdivided into Flt3^{lo} and Flt3^{hi} populations. Flt3^{lo}VCAM-1$^+$ MPPs have erythroid differentiation potential and can produce CMPs. In contrast, Flt3^{hi}VCAM-1$^+$ MPPs lack robust erythroid differentiation potential but retain a GM differentiation potential that is similar to Flt3^{lo}VCAM-1$^+$ MPPs in vivo. Flt3^{hi}VCAM-1$^-$ MPPs, conversely, give rise to erythroid and GM lineages at basal levels only and preferentially develop into lymphocytes in vivo. The inability of Flt3^{hi} VCAM-1$^+$ MPPs to give rise to CMPs implicates an alternative pathway for GM lineage differentiation independent of CMPs in vivo. Our results suggest that the divergence of the lymphoid and myeloid lineages during early hematopoiesis occurs asymmetrically at distinct progenitor stages.

RESULTS AND DISCUSSION

Purification of Flt3^{lo} and Flt3^{hi} MPPs

It has been shown that HSCs and MPPs can be separated in the KLS bone marrow fraction using the Thy-1.1 and Flt3 markers (7). LT-HSCs are highly enriched in the CD34$^{-/lo}$ fraction of the Thy-1.1^{lo}Flt3$^-$ KLS population (13, 14), whereas CD34$^+$ Thy-1.1^{lo}Flt3$^-$ KLS and Thy-1.1^{lo}Flt3$^+$ KLS cells represent ST-HSCs (7, 14). MPPs comprise the Thy-1.1$^-$Flt3$^+$ KLS cell population (7, 11). Although all HSCs are VCAM-1$^+$, the MPPs can be subdivided into three distinct populations based on Flt3 and VCAM-1 expression (Fig. 1 A). VCAM-1$^-$ MPPs uniformly express high levels of Flt3, whereas the VCAM-1$^+$ MPP population contains both Flt3^{lo} and Flt3^{hi} subsets. After two rounds of FACS sorting, we could isolate these MPP subsets to >95% purity (Fig. 1 B). c-Kit and Sca-1 expression levels were comparable among the three MPP subsets (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20060697/DC1). In addition, Flt3 expression by ST-HSC was similar to that of Flt3^{lo}-VCAM-1$^+$ MPPs, whereas LT-HSCs were Flt3$^-$ on FACS analyses (Fig. S1 B). We compared the differentiation potentials of these three purified subsets of MPPs to different hematopoietic lineages.

Erythroid differentiation potential is enriched in Flt3^{lo}VCAM-1$^+$ MPPs

We first evaluated the in vitro megakaryocyte/erythroid (MegE) differentiation potential of MPP subsets by comparing their colony-forming activities. MPPs were cultured in methylcellulose in the presence of stem cell factor, IL-3, IL-6, erythropoietin (Epo), and thrombopoietin. In these cultures, only Flt3^{lo}VCAM-1$^+$ MPPs produced obvious erythroid (CFU-E) or mixed (CFU-GEMM) colonies, at 4.3 and 8.7%, respectively (Fig. 2 A). Neither Flt3^{hi}VCAM-1$^+$ MPPs nor Flt3^{hi}VCAM-1$^-$ MPPs exhibited the capacity to generate erythroid or mixed colonies (<1%; Fig. 2 A). In addition, Flt3^{lo}VCAM-1$^+$ MPPs, but not Flt3^{hi}VCAM-1$^+$ MPPs or Flt3^{hi}VCAM-1$^-$ MPPs, expressed significant levels of GATA1 and EpoR (Fig. 2 B), suggesting that erythroid colony potential and expressions of erythroid lineage–affiliated genes are correlated.

To examine the MegE differentiation potential of MPP subsets in vivo, we purified each MPP type from enhanced GFP (EGFP) mice and injected it into lethally irradiated

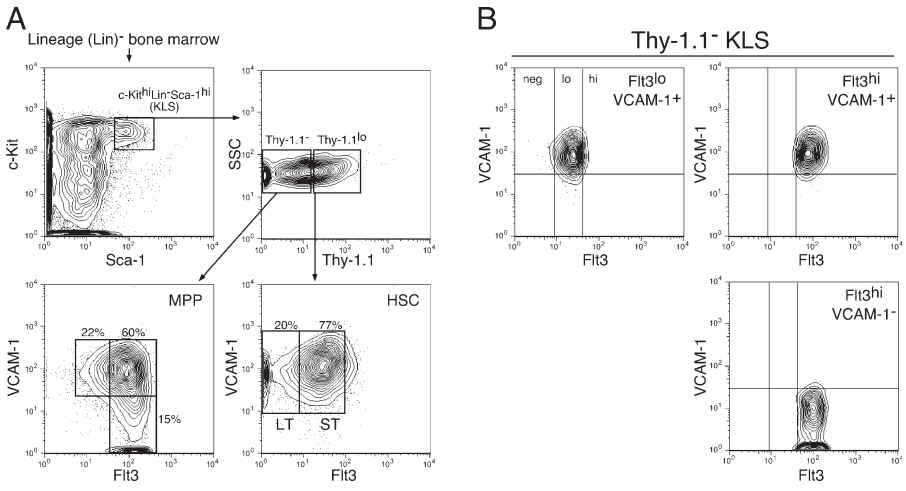

Figure 1. Subfractionation of MPPs by Flt3 and VCAM-1 expression. (A) Flt3 and VCAM-1 expression on HSCs and MPPs by FACS analysis. (B) FACS analysis of Flt3 and VCAM-1 expression levels on MPP subsets after two rounds of sorting. The purity of the sorted populations was >95%.

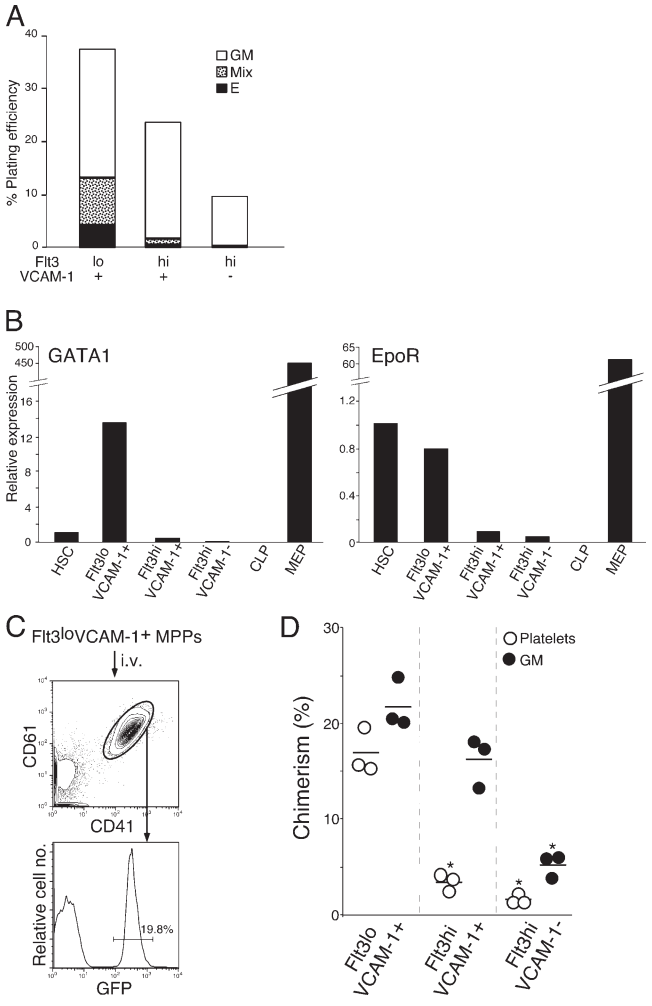

Figure 2. MegE differentiation potential is restricted to Flt3^{lo}-VCAM-1^{+} MPPs. (A) In vitro MegE and GM differentiation potential of MPP subsets assessed by colony-forming assay on methylcellulose. Data represent the average plating efficiency of triplicate methylcellulose cultures of each MPP population. (B) Comparison of MegE-affiliated GATA1 and EpoR expression levels in HSCs, MPP subsets, CLPs, and MEPs by quantitative PCR analysis. GATA1 and EpoR expression levels in HSCs were arbitrarily defined as unit 1. Data shown represent the mean value from triplicate reactions. (C) In vivo MegE differentiation by Flt3^{lo}VCAM-1^{+} MPPs. Peripheral blood from recipient mice injected with Flt3^{lo}VCAM-1^{+} MPPs was analyzed at 2 wk after injection to detect donor-derived (GFP^{+}) CD41^{+}CD61^{+} platelets. (D) Comparison of in vivo MegE and GM differentiation potential of MPP subsets. Peripheral blood from recipient mice injected with purified MPP subsets was analyzed at 2 wk after injection to evaluate the donor chimerism (GFP^{+}) of CD41^{+}CD61^{+} platelets (○) or Mac-1^{+} GM cells (●). Horizontal bars in the graph denote the average values from three mice analyzed in each group. *, $P < 0.05$, calculated by the Student's *t* test compared with percentage of chimerism of Flt3^{lo}VCAM-1^{+} MPPs.

hosts as described by NaNakorn et al. (15). In this assay system, MegE differentiation potential is evaluated based on the donor chimerism (GFP^{+}) of platelets in peripheral blood (Fig. 2 C). 2 wk after transfer, Flt3^{lo}VCAM-1^{+} MPPs contributed to 16–20% platelet chimerism. In contrast, transfer of Flt3^{hi}VCAM-1^{+} or Flt3^{hi}VCAM-1^{-} MPPs resulted in platelet chimerism of only 3.4 and 1.5%, respectively (Fig. 2 D). In the report by Adolfsson et al. (12), lymphoid-primed MPPs (LMPPs; Flt3^{+}KLS) lacking erythroid differentiation potential were characterized in the 25% highest Flt3-expressing cells of the KLS fraction. Our result is consistent with their findings that Flt3^{hi}KLS cells do not contribute significantly to erythroid differentiation. Nonetheless, we did observe low numbers of platelets derived from Flt3^{hi}VCAM-1^{+} and Flt3^{hi}VCAM-1^{-} MPPs (Fig. 2 D), perhaps due to the high sensitivity of our detection system or to the early determination of platelet chimerism. As MPPs have a limited self-renewal activity and can only reconstitute the hematopoietic system transiently (7), low frequencies of MegE lineage cells might not be present at later time points.

MPP subsets show different potentials for GM differentiation in vivo and define a developmental hierarchy

Although their potential for MegE differentiation was dramatically reduced, the GM potential of Flt3^{hi}VCAM-1^{+} MPPs was equivalent to Flt3^{lo}VCAM-1^{+} MPPs, as determined by chimerism at 2 wk after transfer (16 and 22%, respectively; Fig. 2 D). In contrast, Flt3^{hi}VCAM-1^{-} MPPs had little capacity for GM differentiation (4% chimerism; Fig. 2 D), as we demonstrated previously (11). All three MPP subsets could give rise to T and B cells in vivo, although the kinetics of lymphocyte production by Flt3^{lo}VCAM-1^{+} MPPs was delayed in comparison to Flt3^{hi}VCAM-1^{+} MPPs and Flt3^{hi}VCAM-1^{-} MPPs (Fig. 3 A). This delay suggests that Flt3^{lo}VCAM-1^{+} MPPs are less mature than either Flt3^{hi}VCAM-1^{+} or Flt3^{hi}VCAM-1^{-} MPPs. Given our previous demonstration that VCAM-1^{+} MPPs can give rise to VCAM-1^{-} MPPs (11), it is reasonable to conclude that Flt3^{hi}VCAM-1^{+} MPPs are more immature than Flt3^{hi}VCAM-1^{-} MPPs. We did not, however, observe any difference in B cell differentiation kinetics after reconstitution with Flt3^{hi}VCAM-1^{+} or Flt3^{hi}VCAM-1^{-} MPPs, perhaps because the transition between the two populations is rapid or because of the higher proliferative capacity of Flt3^{hi}VCAM-1^{+} MPPs. Collectively, our results suggest a developmental hierarchy among MPP subsets, with Flt3^{lo}VCAM-1^{+} MPPs being the most primitive with full multilineage differentiation potential. The more mature Flt3^{hi}VCAM-1^{+} MPPs lose the capacity for erythroid differentiation and in turn, differentiate into Flt3^{hi}VCAM-1^{-} MPPs that preferentially generate lymphocytes in vivo.

Lymphoid lineage priming in Flt3^{hi}VCAM-1^{-}, but not Flt3^{hi}VCAM-1^{+}, MPPs

Lineage priming, the promiscuous expression of lineage-affiliated genes, is thought to be important in the developmental plasticity of progenitor cells with the potential to produce multiple lineages (16–19). Yet despite their developmental potential for the lymphoid lineages, several

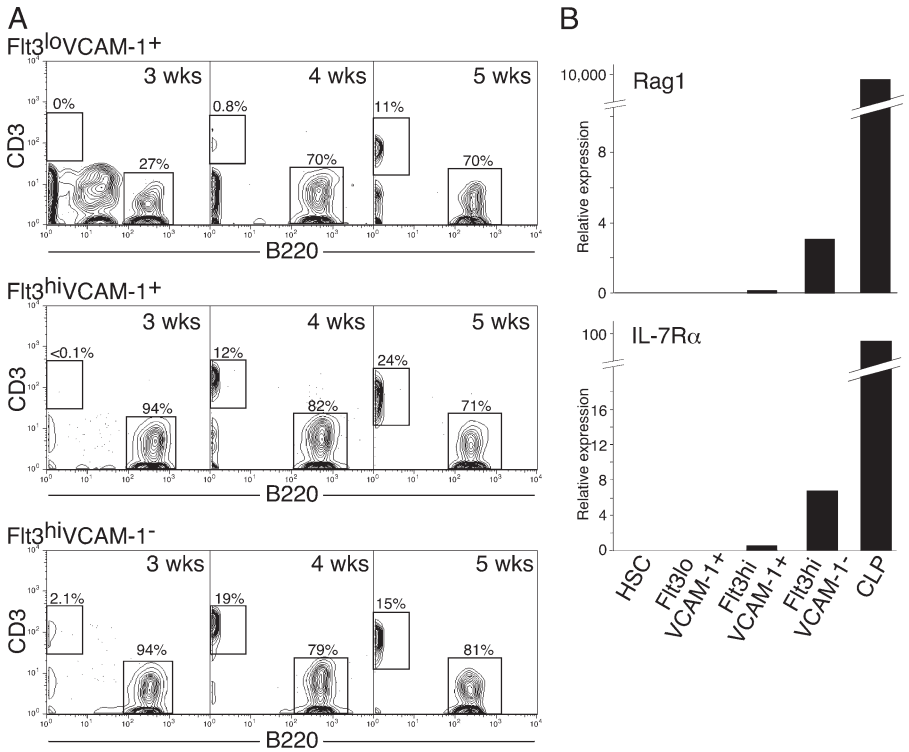

Figure 3. Lymphoid lineage differentiation potential and lymphoid lineage priming in MPP subsets. (A) All MPP subsets contributed to lymphoid lineage differentiation in vivo. Peripheral blood collected from reconstituted mice was analyzed for donor-derived $B220^+$ B cells and $CD3^+$ T cells at 3–5 wk after injection. FACS plot shown is pregated on $GFP^+CD45.1^-$ population. Almost all $B220^+$ cells are $CD19^+IgM^+$, and most $CD3^+$ cells are $TCR\beta^+$ in the peripheral blood (Fig. S2). It should be noted that due to the setting of the FACS machine, the $CD3^+$ population does not clearly appear in the plots. (B) Analysis of lymphoid-specific RAG1 and IL-7Rα expression levels in MPP subsets by quantitative PCR. Data shown represent the mean value from triplicate reactions. RAG1 and IL-7Rα expression levels in CLPs were arbitrarily set at 10,000 and 100, respectively.

studies show that myeloid, but not lymphoid, priming is observed in HSCs (20, 21). As some lymphoid-related genes are expressed in MPPs, the lymphoid lineage differentiation program might be activated in the MPP stage before commitment to the lymphoid lineages (18, 22). RAG1 and IL-7Rα are among the earliest lymphoid-related genes expressed in MPPs (11, 12, 22); therefore, we examined the RAG1 and IL-7Rα expression in HSCs and the MPP subsets. RAG1 and IL-7Rα transcripts were virtually absent in HSCs, $Flt3^{lo}VCAM\text{-}1^+$, and $Flt3^{hi}VCAM\text{-}1^+$ MPPs, but were up-regulated in $Flt3^{hi}VCAM\text{-}1^-$ MPPs (Fig. 3 B). We also found that a significant proportion (30–50%) of $VCAM\text{-}1^-$ MPPs expressed RAG1 by monitoring GFP expression in RAG1-GFP knock-in mice, whereas only a small fraction (<5%) of $VCAM\text{-}1^+$ MPPs are GFP^+ (unpublished data). These observations confirm our earlier finding that although some $VCAM\text{-}1^-$ MPPs are $IL\text{-}7R\alpha^+$, all $VCAM\text{-}1^+$ MPPs are $IL\text{-}7R\alpha^-$ (11). These data also suggest that initiation of lymphoid lineage priming and loss of erythroid differentiation potential do not occur simultaneously in MPPs. Upon loss of MegE differentiation ability, $Flt3^{hi}VCAM\text{-}1^+$ MPPs retain robust GM differentiation potential in vivo without lymphoid lineage priming. Subsequent activation of lymphoid-related gene expression in more mature $Flt3^{hi}VCAM\text{-}1^-$ MPPs potentiates their development into lymphocytes in vivo.

$Flt3^{lo}VCAM\text{-}1^+$ MPPs can give rise to CMPs

We previously demonstrated that $VCAM\text{-}1^+$ MPPs can give rise to CMPs (11). Consequently, to clarify their developmental relationship, we evaluated the ability of $Flt3^{lo}VCAM\text{-}1^+$ and $Flt3^{hi}VCAM\text{-}1^+$ MPPs to differentiate into CMPs. 6 d after the transfer of purified MPP populations, phenotypic CMPs were detected only in hosts given $Flt3^{lo}VCAM\text{-}1^+$ MPPs (Fig. 4 A). As we demonstrated previously, CMPs derived from $VCAM\text{-}1^+$ MPPs are functionally comparable to freshly isolated CMPs and have complete erythroid and GM differentiation potential (11). The high in vivo GM contribution but the lack of CMP (and GM progenitor [GMP]) differentiation from $Flt3^{hi}VCAM\text{-}1^+$ MPPs suggests that there may be an alternative GM differentiation pathway independent of CMPs or even GMPs. Because CMP could only be generated from $Flt3^{lo}VCAM\text{-}1^+$ MPPs, we redefine the MPP→CMP branching point to the $Flt3^{lo}VCAM\text{-}1^+$ MPP compartment. In addition, an alternative GM differentiation pathway branches from $Flt3^{hi}VCAM\text{-}1^+$ MPPs (Fig. 4 B).

Our detailed study of MPPs reveals that the initial step of lineage restriction during hematopoiesis is not simply lymphoid versus myeloid commitment. Instead, the first step in lineage restriction occurs in Flt3^{lo}VCAM-1^{+} MPPs, which are fully multipotent but possess limited or no self-renewal activity. We conclude that the earliest restriction in hematopoietic potential coincides with the loss of self-renewal ability in HSCs. Based on the results in our present and previous studies (11), we propose that there is a hierarchy in MegE, GM, and lymphoid differentiation during adult hematopoiesis (Fig. 4 B). In the course of early lymphoid differentiation, hematopoietic progenitors sequentially and gradually lose MegE and GM differentiation potential before lymphoid lineage commitment. The developmental hierarchy of the MPP subsets, based on their in vivo differentiation potential to erythroid, myeloid, and lymphoid lineages, supports the sequential differentiation model proposed by Brown et al. (23).

The LMPPs defined by Adolfsson et al. (12) express IL-7Rα and are most comparable to Flt3^{hi}VCAM-1^{-} MPPs characterized in our previous and current report (Fig. 3 B) (11). From the view of their physiological contribution in vivo, but not their differentiation potential in vitro, it may be most appropriate to consider Flt3^{hi}VCAM-1^{+} MPPs as bipotent progenitors for lymphoid and GM populations, even though lymphoid priming is not observed in these cells. Activation of lymphoid-related genes in the later LMPP or Flt3^{hi}VCAM-1^{-} MPPs directs them toward the lymphoid lineage, even though they can differentiate into GM cells in vitro with the appropriate cytokine signals (11). Because of limitation of the in vitro culture system, it is difficult to determine the proportion of clonal Flt3^{hi}VCAM-1^{-} MPPs with potentials for all three T, B, and GM lineages. It is possible that this MPP subset is heterogeneous, which may be composed of progenitors with various potentials, such as T/B/GM, T/B, T/GM, etc. Indeed, Flt3^{hi}VCAM-1^{-} MPPs are heterogeneous for gene expressions, such as IL-7Rα (11) and RAG1 (ELP-like cells in RAG1/GFP KI mice; unpublished data). Perhaps the promiscuous expression of both lymphoid and myeloid lineage genes in the LMPP or Flt3^{hi}VCAM-1^{-} MPPs, followed by the silencing of myeloid-affiliated genes, serves as a prerequisite to lymphoid lineage commitment (unpublished data). Analysis of MPP subsets at the molecular level should facilitate the investigation of how stochastic events at the molecular level and influence by extrinsic

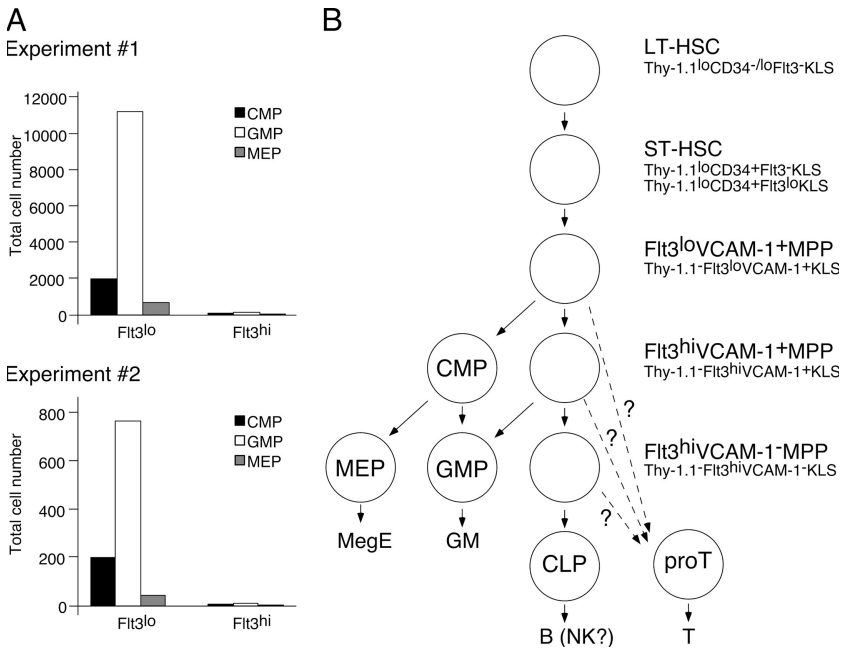

Figure 4. Flt3^{lo}VCAM-1^{+} MPPs, but not Flt3^{hi}VCAM-1^{+} MPPs, differentiate into CMPs in vivo. (A) Detection of CMPs, GMPs, and MEPs derived from Flt3^{lo}VCAM-1^{+} MPPs and Flt3^{hi}VCAM-1^{+} MPPs. Equal number of cells from the two MPP populations (CD45.2) were injected into CD45.1^{+} recipient mice. Bone marrow cells were harvested 6 d after injection for FACS analysis, and the total cell number of donor-derived CMPs, GMPs, and MEPs in recipient mice from two independent experiments is shown. FACS plots of these donor-derived bone marrow populations are shown in Fig. S3. Although the chimerism of donor-derived cells in the bone marrow varied between experiments, the difference in the contribution of CMPs, GMPs, and MEPs from Flt3^{lo}VCAM-1^{+} MPPs and Flt3^{hi}VCAM-1^{+} MPPs remained consistent. (B) Proposed model of hierarchy in MPP subsets and lymphoid- and myeloid lineage–restricted progenitors during hematopoiesis, based on in vivo differentiation potential of MPPs. Flt3^{lo}VCAM-1^{+} MPPs represent the first branching point during adult hematopoiesis, which diverge into CMPs and Flt3^{hi}VCAM-1^{+} MPPs. Flt3^{hi}VCAM-1^{+} MPPs represent the second branching point, diverging to lymphoid lineage and a GM differentiation pathway that is independent of CMPs. Because Flt3^{hi}VCAM-1^{-} MPPs do not contribute to GM lineages significantly in vivo, we consider these MPPs as lymphoid-specified progenitors before lymphoid lineage commitment in CLPs or pro–T cells in the thymus. Dotted lines represent potential migration of bone marrow progenitor candidates to the thymus for T cell development based on evidence by recent studies (reference 25).

factors are coordinated to determine lineage choice at these early branch points of hematopoiesis.

MATERIALS AND METHODS

Mice. Wild-type mice used in this study were C57BL/Ka-Thy-1.1 and C57BL/Ka-Thy-1.1-Ly5.2 (CD45.1). EGFP mice were generated as described previously (24) and backcrossed onto the C57BL/Ka-Thy-1.1 background for more than eight generations. For characterization and purification of bone marrow populations, 6–8-wk-old mice were used. Recipient mice used for reconstitution studies were 8–12 wk of age. All mice were maintained under specific pathogen-free conditions at the Duke University Animal Care Facility. All studies and procedures were approved by the Duke University Animal Care and Use Committee.

Flow cytometry. Antibodies used in this study are listed in supplemental Materials and methods, available at http://www.jem.org/cgi/content/full/jem.20060697/DC1. Preparation of single cell suspension, magnetic beads separation, and antibody staining of cells were performed as described previously (11). Cell sorting and cell surface phenotyping were performed on a FACSVantage SE with a DiVa option (488 nm argon, 599 nm dye, and 408 nm krypton lasers; BD Bioscience Flow Cytometry Systems), which is available in the FACS facility of Duke Comprehensive Cancer Center. To adjust the compensation of signals from different fluorescent channels, we performed single-color staining on splenocytes with anti-B220 antibodies conjugated to each fluorochrome. The stained splenocyte samples were analyzed on FACSVantage, and the DiVa software computationally determined compensation values. Data were analyzed with FlowJo software (Treestar). Dead cells were gated out from analyses and cell sortings as propidium iodide–positive cells.

Analysis of in vitro and in vivo lineage differentiation potentials. In vitro methylcellulose colony assays were performed as described previously (11). For analysis of in vivo differentiation potential to various hematopoietic lineages, 10^3 $Flt3^{lo}VCAM\text{-}1^+$, $Flt3^{hi}VCAM\text{-}1^+$, or $Flt3^{hi}VCAM\text{-}1^-$ MPPs ($EGFP^+CD45.2^+$) were injected into lethal dose (950 rad) –irradiated hosts (CD45.1) with 2×10^5 whole bone marrow cells (CD45.1) for radioprotection. Peripheral blood was obtained at various time points from reconstituted mice for FACS analysis as described above. For analysis of CMP, GMP, and megakaryocyte-erythroid progenitor (MEP) differentiation from MPP subsets, 2.5×10^4 $Flt3^{lo}VCAM\text{-}1^+$ or $Flt3^{hi}VCAM\text{-}1^+$ MPPs (CD45.2) were injected into lethally irradiated host (CD45.1). Bone marrow cells were harvested from femurs and tibiae 6 d after injection and subjected to FACS analysis as described above.

Quantitative PCR analysis. Cells were deposited directly into 1.5-ml microcentrifuge tubes with 1 ml TRIzol reagent (Invitrogen) during the second round of sorting. Total RNA was isolated according to the manufacturer's instructions. After treatment with DNase I (Invitrogen), first-strand cDNA synthesis was performed using Superscript III reverse transcriptase and oligo-dT primers (Invitrogen). Gene expression levels were quantified using the iCycler real-time PCR detection system (Bio-Rad Laboratories) and calculated and normalized to GAPDH expression level using the Relative Expression Software Tool provided by M. Pfaffl (Technical University of Munich, Munich, Germany). The PCR primers used are listed in supplemental Materials and methods.

Online supplemental material. Fig. S1 compares the surface expressions of c-Kit, Sca-1, and Flt3 on MPP subsets. Fig. S2 shows further characterization of $B220^+$ and $CD3^+$ cells in the peripheral blood, demonstrating that the majority of them are B and T cells, respectively. Fig. S3 shows the FACS plots of myeloid progenitors derived from $Flt3^{lo}VCAM\text{-}1^+$ and $Flt3^{hi}VCAM\text{-}1^+$ MPPs in vivo. Supplemental Materials and methods lists all the antibodies, definitions of bone marrow populations, and PCR primers used in this study. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20060697/DC1.

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