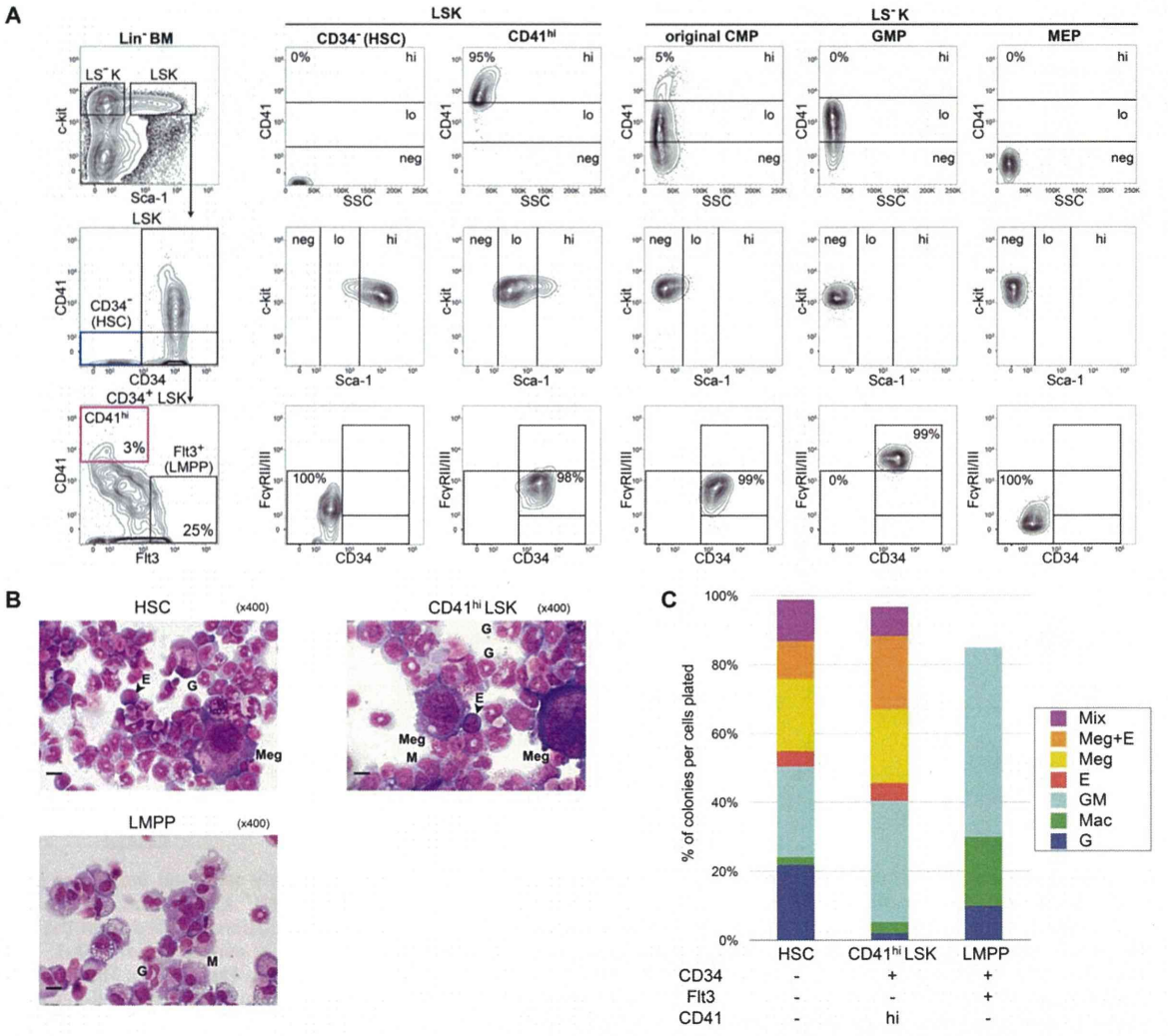

Figure 2. The phenotype of CD41^{hi} LSKs, and their myelo-erythroid differentiation potential in vitro. **(A):** Expression of CD41 and other surface markers in HSC and CD41^{hi} population in the LSK fraction, and CMP, GMP, and MEP populations in the Lin$^-$Sca-1$^-$c-Kit$^+$ (LS$^-$K) fraction. CD41^{hi} cells are almost exclusively found within the LSK fraction. The CD41^{hi} LSKs expressed FcγRII/III^{lo} and CD34$^+$ whose phenotype is similar to that of the original CMP, but expressed Sca-1 at a low level. **(B):** Morphology of cells derived from HSCs, CD41^{hi} LSKs, and LMPPs 12 days after the initiation of culture (May-Giemsa staining, ×400). HSCs and CD41^{hi} LSKs gave rise to both GM- and MegE-lineage cells, including granulocytes (G), monocytes (M), erythroblasts (E), and megakaryocytes (Meg). Bars = 10 μm. **(C):** Results of clonogenic myelo-erythroid colony assays of purified stem/progenitor cells. CD41^{hi} LSKs showed differentiation potential into GM- and MegE-lineage with high plating efficiency. One hundred and twenty cells per population were analyzed in each experiment. Data shown are representative of three independent experiments. Abbreviations: BM, bone marrow; CMP, common myeloid progenitor; HSC, hematopoietic stem cell; LMPP, lymphoid-primed multipotent progenitor; LSK, Lineage$^-$Sca-1$^+$c-Kit$^+$.

Results

Identification of Cell Surface Markers Specifically Expressed on GATA-1$^+$ LSKs

We have found that in mice harboring transgenic GFP driven by a GATA-1 promoter, a fraction of LSKs expressing GFP has potent CMP activity [10]. In order to prospectively purify the GATA-1-GFP$^+$ LSK fraction (Fig. 1A) in wild-type mice, we tried to identify a surface marker specific for this population. GATA-1-GFP$^+$ LSKs were contained within the CD34$^+$Flt3$^-$ LSK fraction (Fig. 1A). In addition to GATA-1-GFP$^+$ and GATA-1-GFP$^-$ populations within the CD34$^+$Flt3$^-$ LSK fraction, CD34$^-$Flt3$^-$ LSKs (HSCs) [4], CD34$^+$Flt3$^+$ LSKs (LMPPs) [8], and the original CMPs [5] were purified. These populations were subjected to a cDNA microarray analysis to find a GATA-1-GFP$^+$ LSKs-specific surface molecule (six replicates for HSC and four replicates for all other populations) (Fig. 1B).

In the hierarchical clustering analysis (Supporting Information Fig. S1), GATA-1-GFP$^+$ LSKs displayed a gene expression pattern close to that of the original CMPs, while showing the most distinctive difference from that of LMPPs. We sought for genes highly expressed in GATA-1-GFP$^+$ LSKs but not in GATA-1-GFP$^-$ LSKs, LMPPs, or HSCs. Figure 1B shows representative genes with such expression patterns. Then, the expression of these candidate cell surface proteins was analyzed by a flow cytometer to test whether their expression levels correlated with GFP levels in the LSK fraction. Among the 18 proteins tested, only CD41 (*Itga2b*) was exclusively expressed in GATA-1$^+$ LSKs at a high level (Fig. 1C) with exceeding sensitivity, specificity, and positive predictive value (Supporting Information Table S1).

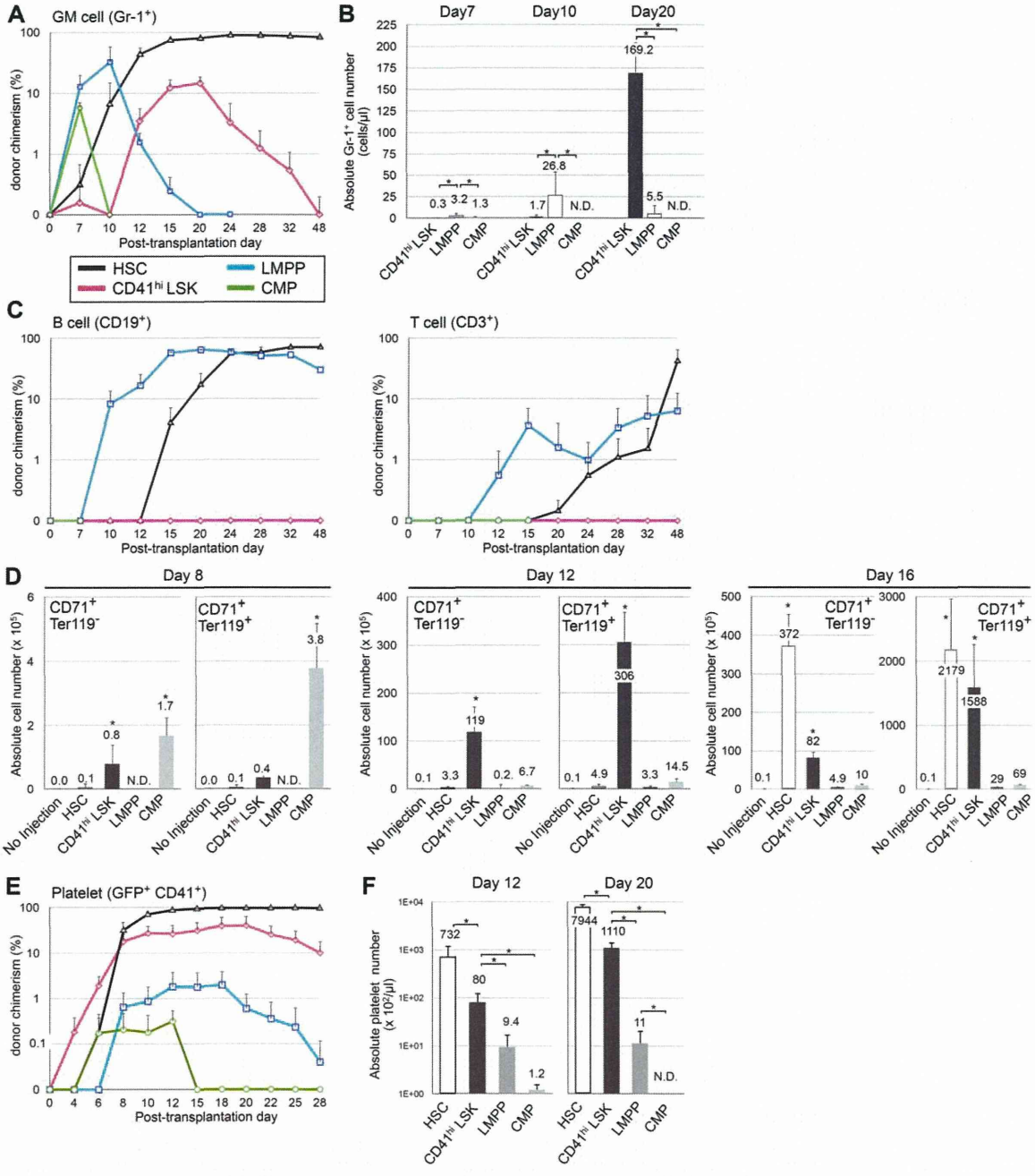

Figure 3. Lineage potential of CD41^{hi} LSKs in competitive reconstitution assays. **(A):** Chronological analysis of GM progeny after transplantation of purified stem/progenitor populations. Five hundreds cells of each population (Ly5.1) were intrafemorally transplanted with 250 Ly5.2 LSKs into lethally irradiated congenic Ly5.2 mice. The peak of GM progeny was delayed in CD41^{hi} LSKs (red, on day 20) as compared to those in CMPs (green, on day 7) or LMPPs (blue, on day 10). **(B):** The absolute numbers of GM progeny on days 7, 10, and 20 in the blood. CD41^{hi} LSKs displayed the strongest myeloid potential on day 20. **(C):** Lymphoid potential of stem/progenitor cell populations. CD41^{hi} LSKs did not differentiate into B or T cells. **(D):** The absolute numbers of proerythroblasts (CD71^{+}/Ter119^{-}) and erythroblasts (CD71^{+}/Ter119^{+}) in the spleen on days 8, 12, and 16 after transplantation. **(E, F):** Platelet production from stem/progenitor populations. Each population was purified from CAG-EGFP mice, and donor-derived GFP^{+} platelets were counted on days 12 and 20. Bars indicate mean ± SD. All experiments shown are representative of two to three independent experiments ($n = 3$ in each population). *, $p < .05$ (Turkey HSD). N.D.: not detectable. Abbreviations: CMP, common myeloid progenitor; GFP, green fluorescent protein; GM, granulocyte/monocyte; HSC, hematopoietic stem cell; LMPP, lymphoid-primed multipotent progenitor; LSK, Lineage^{-}Sca-1^{+}c-Kit^{+}.

Figure 2A shows the distribution of CD41 in early hematopoiesis. CD41 was expressed in CD34^{+} LSKs, and CMPs and GMPs, but not MEPs or CLPs. However, a high level expression of CD41 was found only in 3%–5% of CD34^{+} LSKs and the original CMPs. The CD34^{+} CD41^{hi} LSK constitutes ~0.01% of nucleated cells in the BM, whose frequency is higher than that of CD34^{-} long-term HSCs (~0.005%). The detailed surface phenotype of purified CD41^{hi} LSKs is shown in Figure 2A. Similar to the original CMPs [5], CD41^{hi} LSKs are FcγRII/III^{lo}CD34^{+}. Interestingly, the expression level of Sca-1 in CD41^{hi} LSKs is low, as compared to the high level of Sca-1 in HSCs. Thus, CD41 is upregulated at the transition between

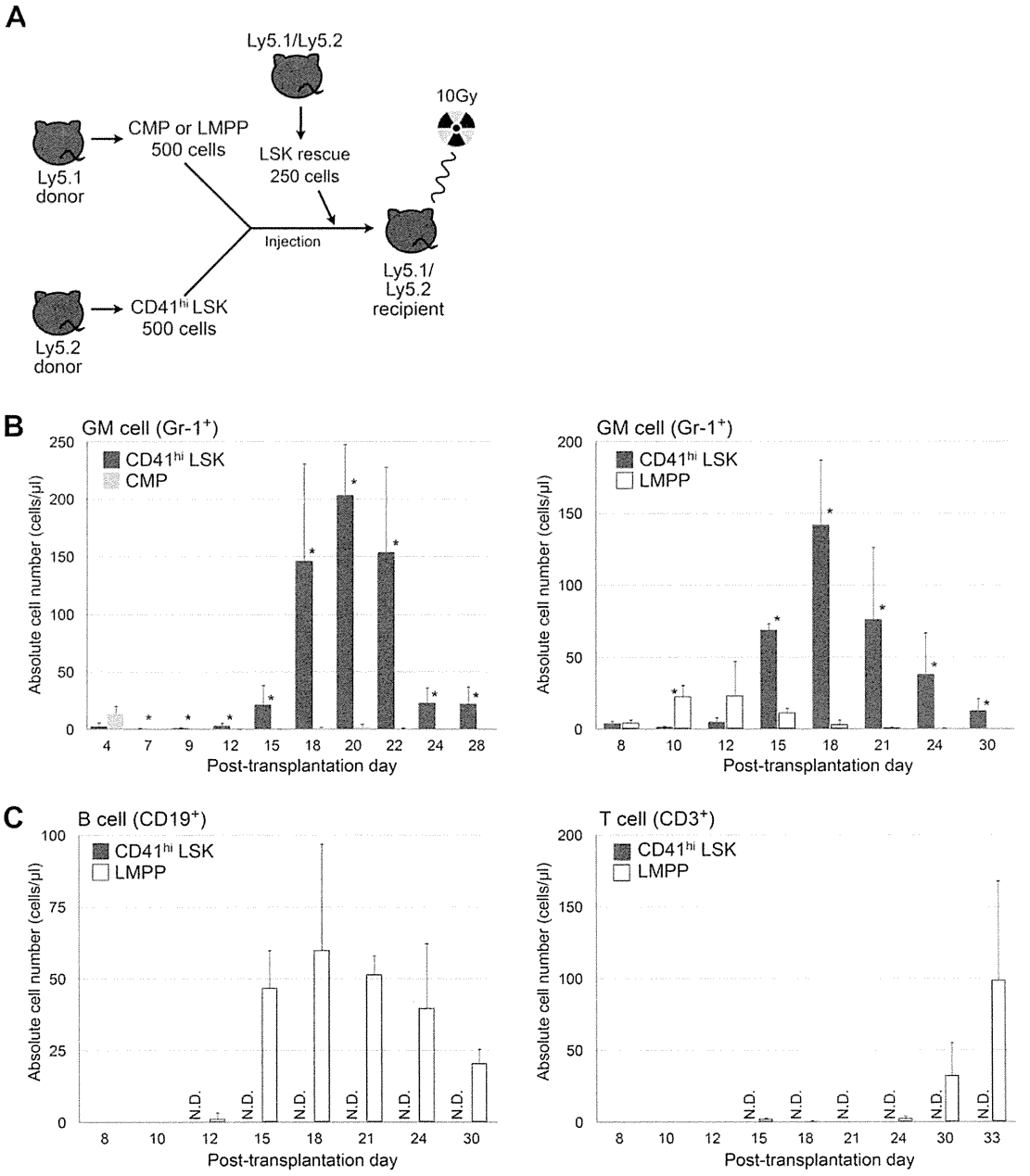

Figure 4. Direct comparison between progeny numbers from CD41^{hi} LSKs and those from either CMPs or LMPPs. **(A):** The scheme of this experiment. Five hundreds of CD41^{hi} LSKs (Ly5.2) and the same number of either CMPs or LMPPs (Ly5.1) are coinjected into lethally irradiated Ly5.1/Ly5.2 recipient mice. **(B):** Sequential analysis of blood GM progeny from CD41^{hi} LSK cell versus the original CMP, or CD41^{hi} LSK cell versus LMPP experiments. CD41^{hi} LSKs showed more potent and prolonged production of GM progeny than the original CMP or LMPP did. **(C):** Sequential analysis of blood CD19^{+} B cell and CD3^{+} T cell progeny in the same experiment. Note that CD41^{hi} LSKs were incapable of production of lymphocytes. Bars indicate mean ± SD of two to three independent experiments ($n = 3$ in each population) *, $p < .05$ (Student's t test). Abbreviations: CMP, common myeloid progenitor; GM, granulocyte/monocyte; LMPP, lymphoid-primed multipotent progenitor; LSK, Lineage^{-}Sca-1^{+}c-Kit^{+}.

the LSK HSC and the Lin^{-}Sca-1^{-}c-Kit^{+} (LS^{-}K) original CMP stages, in accordance with the initial upregulation of GATA-1 in early hematopoiesis. According to previous reports regarding subpopulations of LSK classified by SLAM family receptor expression patterns [21, 22], the CD41^{hi} LSK population constitutes a fraction of the CD150^{+} CD48^{dim-+} hematopoietic progenitor-2 (HPC-2) population. Although the majority (~80%) of HPC-2 is CD244^{+}, the CD41^{hi} LSK fraction corresponds to ~20% of HPC-2 that do not express CD244 (Supporting Information Fig. S1B).

CD41^{hi} LSKs Can Differentiate into GM and MegE Lineages but Not into Lymphoid Cells In Vitro

We evaluated in vitro differentiation capability of CD41^{hi} LSKs (Fig. 2B, 2C). CD41^{hi} LSKs and HSCs gave rise to both GM and MegE cells, whereas LMPPs differentiated only into GM cells as reported (Fig. 2B). Single-cell myelo-erythroid colony assay also showed that CD41^{hi} LSKs generated colonies of MegE or GM lineages, as well as colonies containing both MegE and GM cells (CFU-Mix), whose frequencies were almost equal to

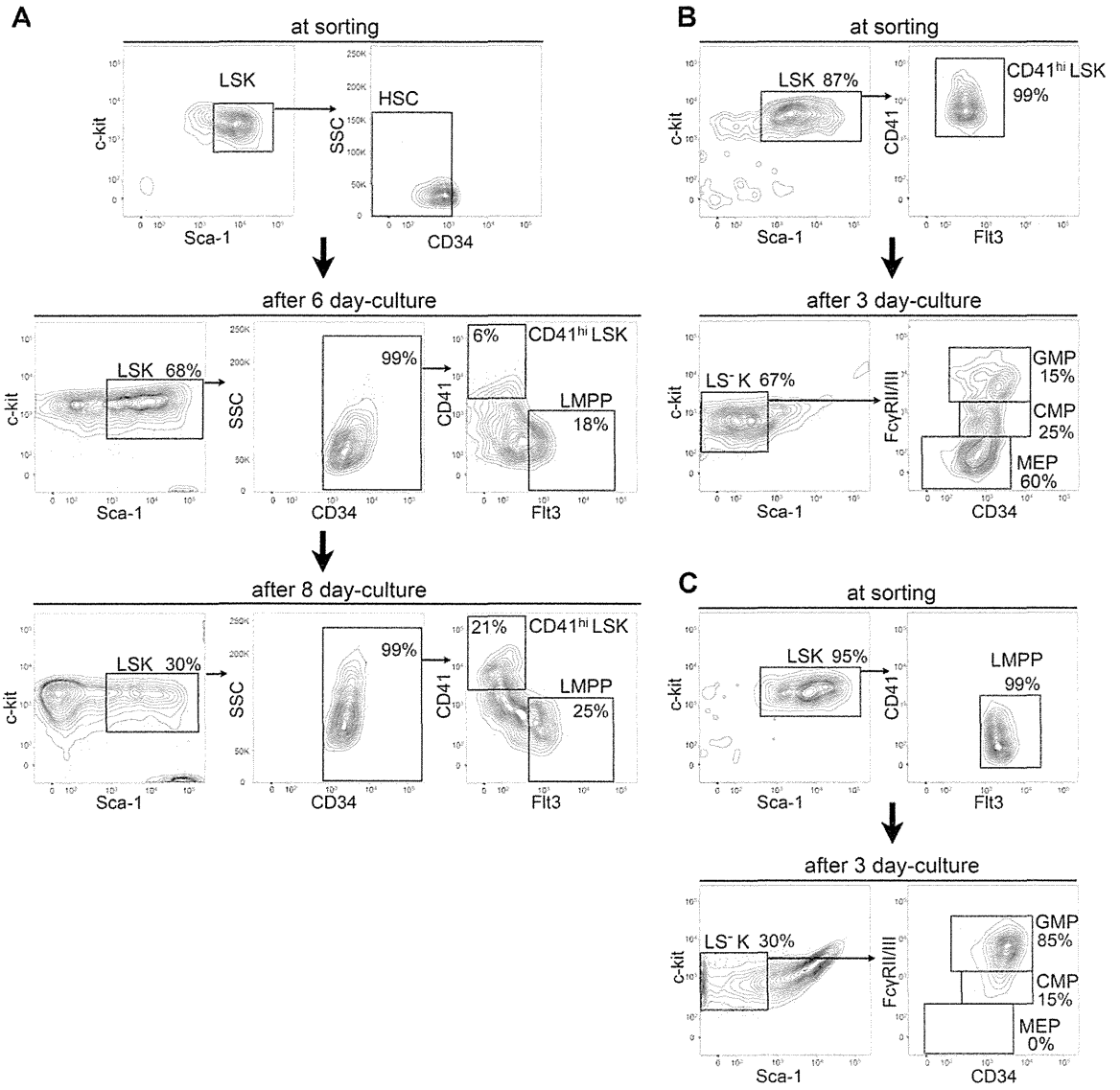

Figure 5. Lineal relationships among HSC, CD41^{hi} LSK, LMPP, the original CMP, GMP, and MEP. **(A):** Short-term culture of CD34^{-} LSK HSCs. HSCs gave rise to both CD41^{hi} LSKs and LMPPs. **(B):** Short-term culture of CD41^{hi} LSKs. CD41^{hi} LSKs gave rise to cells of CMP, GMP, and MEP phenotype. **(C):** Short-term culture of LMPPs. In contrast to CD41^{hi} LSKs, LMPPs gave rise mainly to GMPs. Representative fluorescence-activated cell sorting dot plots of three independent experiments are shown. Abbreviations: CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; LMPP, lymphoid-primed multipotent progenitor; LSK, Lineage^{-}Sca-1^{+}c-Kit^{+}; MEP, megakaryocyte-erythrocyte progenitor.

those of HSCs (Fig. 2C). In contrast, LMPPs always gave rise only to GM-related colonies.

To test the T and B lymphoid potential, 1,000 cells of purified each population were cultured on OP9-DL1 or OP9 stromal cell monolayer, respectively, for 21 days (Supporting Information Fig. S2). LMPPs and CD41$^{-/lo}$ LSKs showed robust T and B lymphoid potential, but CD41^{hi} LSKs did not. These in vitro experiments strongly suggest that CD41^{hi} LSKs have been committed to the myelo-erythroid lineage, and have lost lymphoid potential.

CD41^{hi} LSKs Display Potent and Prolonged GM and MegE Reconstitution as Compared to the Original CMPs or LMPPs

We then evaluated in vivo differentiation potential of CD41^{hi} LSKs. Five hundred cells of each population were purified from donor (Ly5.1) mice, and were transplanted into lethally irradiated recipient (Ly5.2) mice with 250 Ly5.2 LSKs for rescue. After transplantation, percentages of donor-derived Ly5.1^{+} cells in the blood were serially evaluated. As shown in Figure 3A, CD41^{hi} LSKs displayed prolonged GM (Gr-1^{+}) cell reconstitution with its peak at days 20–24. In contrast, GM progeny from the original CMPs reached its peak on day 7, and disappeared by day 10. The peak of GM progeny from LMPPs was day 10, but they rapidly disappeared by day 20. We next evaluated the absolute number of GM progeny (Fig. 3B), since the chimerism could be affected by the number of Ly5.2^{+} cells, mainly derived from the rescue cells. The peaks of GM progeny number from the original CMPs, LMPPs and CD41^{hi} LSKs were 1 cells per microliter (day 7), 27 cells per microliter (day 10), and 169 cells per microliter (day 20), respectively, indicating that CD41^{hi} LSKs possess the most potent and prolonged myeloid

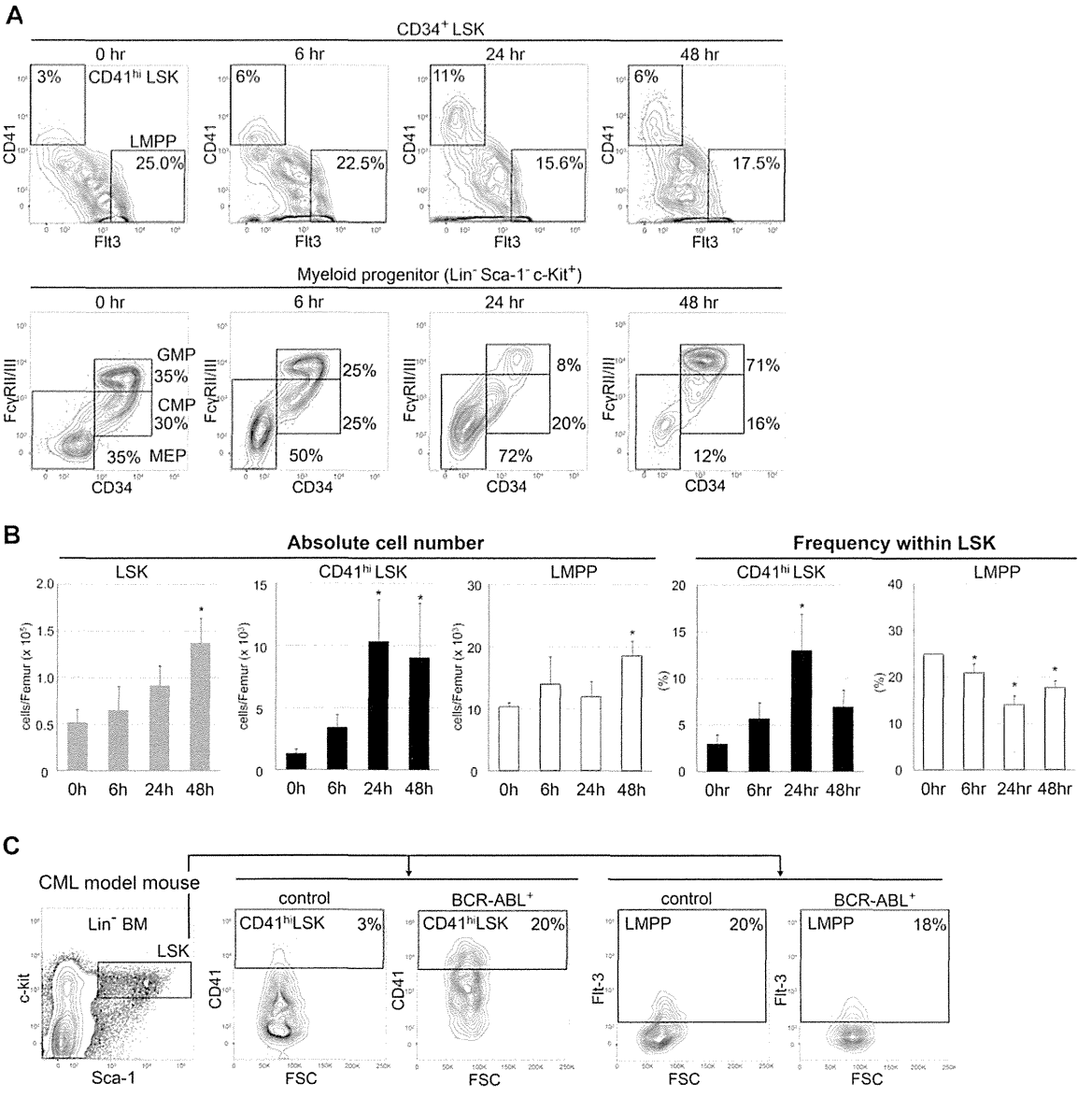

Figure 6. The CD41^{hi} LSK stage is critical in emergent and malignant granulopoiesis. **(A, B):** The changes in the frequencies and absolute numbers of CD41^{hi} LSKs and LMPPs in the setting of bacterial infection are shown. After the CeLP procedure, the CD41^{hi} LSK population expanded quickly and dramatically. **(C):** Changes in CD41^{hi} LSK and LMPP populations in CML model mice generated by retroviral transduction of BCR-ABL oncogene are shown. The CD41^{hi} LSK population of BCR-ABL^{+} cells increased dramatically, but there was no change in the LMPP population. Representative fluorescence-activated cell sorting analysis (A, C) and summarized data of three to four independent experiments (B) are shown. Bars indicate mean ± SD. *, $p < .05$ (Dunnett's test; 0 hour value was set as a control). Abbreviations: CML, chronic myelogenous leukemia; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; LMPP, lymphoid-primed multipotent progenitor; LSK, Lineage^{-}Sca-1^{+}c-Kit^{+}; MEP, megakaryocyte-erythrocyte progenitor.

potential among these progenitors. CD41^{hi} LSKs, however, were not able to produce B or T cells after transplantation, whereas LMPPs rapidly differentiated into B and T cells (Fig. 3C).

Figure 3D shows the absolute number of erythroid progeny in the spleen. It has been shown that CD71^{+}/Ter119^{-} and CD71^{+}/Ter119^{+} cells are proerythroblasts and more mature erythroblasts, respectively [23–25]. On day 8, the original CMPs showed the strongest erythroid potential, and CD41^{hi} LSKs produced only ~2- and ~10-fold less numbers of proerythroblasts and late erythroblasts than those from the original CMPs, respectively. On day 12, however, CD41^{hi} LSKs produced >20-fold numbers of these erythroid progeny as compared to the original CMPs. Mice injected with HSCs exhibited the strongest erythroid potential among LSK subpopulations on day 16. In contrast, LMPPs produced only a small number of erythroid cells on day 12 and day 16.

To evaluate platelet progeny, we used CAG-EGFP mice as a donor whose platelets are visible by GFP expression. As shown in Figure 3E, CD41^{hi} LSKs displayed strong and prolonged platelet production peaked at day 20, whereas the peak of platelet progeny was on day 12 in the original CMPs. CD41^{hi} LSKs produced >50- and >1,000-fold higher numbers of platelets on day 12 and day 20, respectively, as compared to the original CMPs (Fig. 3F). Interestingly, LMPPs possessed considerable platelet potential (Fig. 3E, 3F) [26].

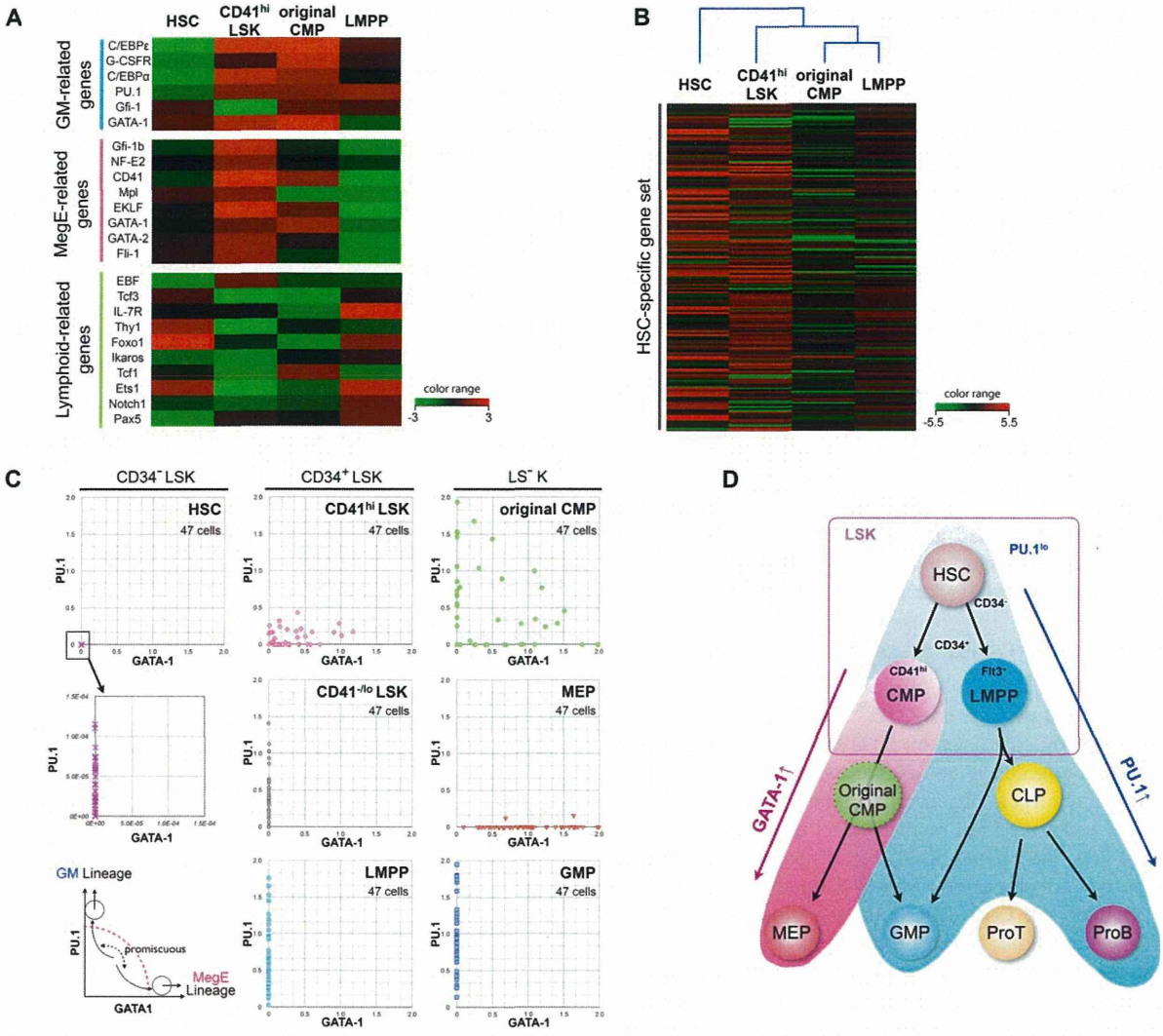

Figure 7. Molecular signatures at the commitment step of the myelo-erythroid versus the myelo-lymphoid pathway. **(A):** The expression of lineage-related genes in stem/progenitor populations. CD41^{hi} LSKs as well as the original CMPs coexpress both GM- and MegE-related genes (blue and red), but not lymphoid-related genes (green), presumably reflecting their priming state to differentiate into these lineages. **(B):** The hierarchical clustering analysis of 159 genes predominantly expressed in HSCs. The expression patterns in CD41^{hi} LSKs were close to those in HSCs, whereas the original CMPs have lost the expression of these HSC-specific genes (Supporting Information Table S2). **(C):** Quantification of PU.1 and GATA-1 mRNA at the single-cell level in stem/progenitor populations. Each cell is plotted on scatter diagrams as a dot with its mRNA level. The promiscuous expression of PU.1 and GATA-1 initiates at CD41^{hi} LSKs but not CD41$^{-/lo}$ LSKs, and the original CMP further upregulates both PU.1 and GATA-1 (Supporting Information Table S3). **(D):** The revised model of the hematopoietic development with initial bifurcation into CD41^{hi} myelo-erythroid CMP and myelo-lymphoid LMPP. Abbreviations: CMP, common myeloid progenitor; GM, granulocyte/monocyte; GMP, granulocyte-macrophage progenitor; HSC, hematopoietic stem cell; LMPP, lymphoid-primed multipotent progenitor; LSK, Lineage^{-}Sca-1^{+}c-Kit^{+}; MEP, megakaryocyte-erythrocyte progenitor.

Thus, CD41^{hi} LSKs possess stronger and more prolonged myelo-erythroid differentiation activity than the original CMPs, but lack lymphoid differentiation, suggesting that they are the early CMPs residing upstream of the original CMPs [5].

Head-to-Head Comparison of Reconstitution Capability Shows an Exceedingly Strong Myeloid Potential of CD41^{hi} LSKs

To test differences in reconstitution potential of these progenitor populations more rigorously, we transplanted 500 Ly5.2 CD41^{hi} LSKs and the same number of either Ly5.1 CMPs or LMPPs into Ly5.1/5.2 hosts, with 250 Ly5.1/5.2 LSKs for rescue (Fig. 4A). This system enabled us to compare the reconstitution potential of two types of donor cells under the same condition, minimizing possible bias that could be involved in independent experiments.

As shown in Figure 4B, the number of GM progeny from CD41^{hi} LSKs again peaked at days 18–20, whereas those of original CMPs and LMPPs peaked at days 4 and day 10, respectively. Strikingly, the absolute numbers of GM progeny of CD41^{hi} LSKs were about 15- and 6-fold higher than those of CMPs and LMPPs at their own peaks, respectively. In contrast, T- and B-cell progeny were only produced from LMPPs, but not from CD41^{hi} LSKs (Fig. 4C). Thus, this head-to-head comparison experiment shows that the CD41^{hi} LSK population possesses much more potent and sustained GM producing activity than the original CMP or LMPP does. These data suggest that the CD41^{hi} LSK stage constitutes the major pathway for GM cell development.

CD41^{hi} LSK Is the Precursor for the Original CMP

To test the lineal relationship between HSC, CD41^{hi} LSK, LMPP, and other myelo-erythroid progenitors, we cultured these stem/progenitor populations for a short-term and tracked their differentiation in vitro (Fig. 5). As shown in Figure 5A, during 6–8 days after initiation of culture, CD34^{-} LSK HSCs differentiated into CD34^{+} LSKs and progressively gave rise to both CD41^{hi} LSK and LMPP populations. Interestingly, the expression of CD41 and Flt3 in the CD34^{+} LSK progeny was mutually exclusive as observed in fresh BM. When we cultured purified CD41^{hi} LSKs, they shut down Sca-1 expression, and differentiated into cells of the original CMP, GMP, and MEP phenotypes in 3 days (Fig. 5B). In contrast, purified LMPPs gave rise mainly to cells of the GMP phenotype (Fig. 5C). The purified secondary myeloid progenitors derived from CD41^{hi} LSKs had in vitro GM, MegE, and/or mixed colony-forming activity in accordance with the original progenitor definitions (Supporting Information Fig. S3). These data strongly suggest that HSC gives rise to both CD41^{hi} LSK and LMPP in a mutually exclusive manner, and that the CD41^{hi} LSK population is the precursor for the original CMP [5].

CD41^{hi} LSK Is the Critical Stage for Pathological Myelopoiesis

We wished to test whether the CD41^{hi} LSK stage is physiologically and pathologically critical for myelo-erythroid cell production in vivo. Bacterial infection is one of the most effective stimuli for granulocyte production. The cecal ligation and puncture (CeLP) model is a well-established murine model of sepsis by purulent peritonitis [15]. The kinetics of CD41^{hi} LSKs and myeloid progenitors after the CeLP procedure is shown in Figure 6A, 6B. Strikingly, 24 hours after the CeLP procedure, the absolute number of CD41^{hi} LSK dramatically expanded by eightfold in response to a demand for myeloid cells. GMP frequency increased after 48 hours, preceded by temporal decrease presumably reflecting its consumption. However, the absolute number of LMPP did not significantly increased during severe infection. These differences could be observed as the shift in the balance of CD41^{hi} LSK and LMPP frequencies within the LSK population. These observations suggested that the CD41^{hi} LSK population should contribute mainly to emergent granulocyte production.

We also tested whether the CD41^{hi} LSK stage is involved in expansion of myelo-erythroid cells in myeloproliferative neoplasm. To establish a murine CML model, we transduced a retrovirus carrying BCR-ABL chimeric gene and a GFP reporter [27] into purified LSKs, and transplanted these LSKs into lethally irradiated congenic mice as we reported previously [28]. As shown in Figure 6C, in mice which displayed malignant myeloproliferation, donor cells expressing GFP showed significant expansion of CD41^{hi} LSK but not LMPP population, as compared to these populations of control cells. These data strongly suggest that the CD41^{hi} LSK population is a critical stage for malignant myeloproliferation.

The Priming of Early Myelo-Erythroid Versus Myelo-Lymphoid Gene Expression Is Evident in CD41^{hi} LSKs and LMPPs

We performed the cDNA microarray analysis of 5,000 cells each of HSC, CD41^{hi} LSK, the original CMP, and LMPP in wild-type mice. Figure 7A shows the expression of representative transcription factors related to GM, MegE, or lymphoid lineages [9, 16]. Similar to the original CMPs, CD41^{hi} LSKs expressed both the GM- and MegE-related, but not lymphoid-related transcription factors. In contrast, LMPPs coexpressed GM- and lymphoid-related transcription factors, but not MegE-related genes. Coexpression of the GM and MegE lineage-related transcription factors in CD41^{hi} LSK and original CMP population has been interpreted to represent their "lineage priming" to maintain bipotency to these lineages [29–31].

We then extracted 159 genes predominantly expressed in HSCs (Supporting Information Table S2) using a web-based open platform, Gene Expression Commons [17], and analyzed the expression patterns of these HSC-affiliated genes in each population. The hierarchical clustering analysis revealed that the expression pattern in CD41^{hi} LSKs and then in LMPPs were close to the pattern in HSCs, whereas the original CMPs have mostly downregulated the expression of these HSC-specific genes (Fig. 7B). Thus, the molecular signature of CD41^{hi} LSKs might be consistent with their myelo-erythroid activity (Fig. 7A), and with their more immature biological characteristics than those in the original CMP (Fig. 7B).

CD41^{hi} LSK Population Is the Earliest GATA-1/PU.1 Priming Stage to Achieve CMP Activity

To understand molecular event at the LMPP versus the CD41^{hi} LSK branchpoint in terms of lineage-instructive competition between PU.1 and GATA-1, we quantified mRNA levels of PU.1 and GATA-1 in stem/progenitor populations at the single-cell level (Fig. 7C). The expression levels of PU.1 and GATA-1 in each cell were designated as a relative value to the median of PU.1 and GATA-1 mRNA amount in single GMPs and MEPs, respectively (Supporting Information Table S3). The conceptual scheme of lineage priming is shown at left bottom of Figure 7C [7, 9]. The most primitive HSCs expressed very low levels of PU.1 (mean $\pm$ SD: $2.6 \times 10^{-5} \pm 3.2 \times 10^{-5}$) but not detectable GATA-1. Within the CD34^{+} LSK progenitor fraction, CD41^{hi} LSKs expressed a low level of GATA-1 (mean $\pm$ SD: 0.29 ± 0.29) but the remaining CD41$^{-/lo}$ LSK and LMPP fraction did not, indicating that GATA-1 transcription initiates at the CD41^{hi} LSK stage. PU.1 was upregulated in CD41^{hi} LSKs (mean $\pm$ SD: 0.08 ± 0.11) as compared to HSCs, and ~50% of CD41^{hi} LSKs expressed both PU.1 and GATA-1, resulting in promiscuous expression of PU.1 and GATA-1 at this stage. In contrast, CD41^{-} Flt3^{+} LMPPs expressed ~10-fold higher levels of PU.1 (mean $\pm$ SD: 0.90 ± 0.85) than those in CD41^{hi} LSKs, but did not express detectable GATA-1. Thus, the upregulation of PU.1 in LSK populations might lead to the acquisition of LMPP activity, whereas GATA-1 upregulation might be critical for multipotent cells to acquire the CMP activity. The original CMPs further upregulated GATA-1 (mean $\pm$ SD: 0.50 ± 0.59) and PU.1 (mean $\pm$ SD: 0.62 ± 0.78) whose levels were approximately two- and sevenfold higher than those in CD41^{hi} LSKs, and most of these cells expressed both GATA-1 and PU.1, supporting the fact that the original CMPs are descendant of CD41^{hi} LSKs toward further MegE or GM lineage commitment. After commitment into either GM or MegE lineage, GMPs expressed only PU.1 (mean $\pm$ SD: 1.21 ± 0.74), whereas MEPs reciprocally expressed only GATA-1 (mean $\pm$ SD: 1.07 ± 0.51), whose levels were further upregulated up to approximately twofold higher than original CMPs. Collectively, these results show that the levels and patterns of GATA-1 and PU.1 expression faithfully reflect the

lineage potential of hematopoietic stem/progenitor cells, and from this view, the CD41^{hi} LSK population should be the earliest CMP that initiates priming of both GATA-1 and PU.1 transcription factors in early hematopoiesis.

Discussion

In this study, within the primitive CD34^{+} LSK progenitor population, we identified the CD41^{hi} LSK population as the earliest CMP that is prospectively isolatable with cell surface markers. This population initially upregulates GATA-1 transcription factor in early hematopoiesis. CD41^{hi} LSK does not produce lymphoid cells, but has robust and prolonged GM and MegE lineage-restricted reconstitution potential, and the number of their progeny enormously exceeds that of the original CMP that resides outside of the LSK fraction. CD41^{hi} LSKs can generate the original, functional CMPs in vitro, downregulating both Sca-1 and CD41. These data strongly suggest that CD41^{hi} LSK is the precursor for the original CMP, and therefore should represent the earliest, true CMP. Thus, together with LMPP, CD41^{hi} LSK population constitutes a critical branchpoint for the myelo-erythroid versus the myelo-lymphoid lineage commitment in early hematopoiesis. Accordingly, the definition of CMP in our original report [5] should be revised. The proposed new scheme of hematopoietic development is shown in Figure 7D. Hereafter, we call the CD41^{hi} LSK population as the CD41^{hi} CMP.

The competitive reconstitution analysis showed that myelo-erythroid potential was profoundly concentrated in CD41^{hi} CMPs (Fig. 3). The critical question is which LSK subpopulation contributes mainly to production of GM cells in vivo, the myelo-erythroid CD41^{hi} CMP or the myelo-lymphoid LMPP population. In our hands, CD41^{hi} CMPs produced sixfold higher numbers of GM cells as compared to LMPPs in head-to-head competitive reconstitution assays (Fig. 4B). Furthermore, only CD41^{hi} CMPs expanded after the CeLP procedure or after the occurrence of malignant myeloproliferation (Fig. 6). Collectively, these results strongly suggest that CD41^{hi} CMPs are the major source for myelo-erythroid cells in both physiological and pathological myelopoiesis.

CD41 (glycoprotein IIb or integrin αIIb) is a member of integrin family protein, which forms a heterodimer with integrin β3, CD61. It has been shown that CD41 is expressed during early embryonic definitive hematopoiesis [32]. We found that in mice within 16 weeks old, CD41 expression is upregulated in CD34^{+} LSK progenitor fraction, and reaches its highest level at the CD41^{hi} CMP stage (Fig. 2A), synchronizing with the GATA-1-GFP reporter activation (Fig. 1C). The expression of CD41 could be controlled at least in part by GATA-1, since several consensus sequences for GATA-1 binding exist in the promoter region of mouse and human CD41 genome, and disruption of these binding sites reduced the transcription of CD41 [33, 34]. A recent study has shown that in aged mice, CD41 is upregulated in a fraction of the CD34^{-} LSK HSC population, and this CD34^{-}CD41^{+} population possessed myeloid-biased differentiation [35]. Our data also showed that aged mice >20-week-old have a fraction of CD34^{-} LSKs expressing CD41 (Supporting Information Fig. S4). They also demonstrated that the frequency of CD41-positive cells in HSCs increased with age and that CD41^{+} HSCs upregulated master transcription factors for myelo-erythroid lineage such as Gata1, Zfpm1 (FOG-1), Gfi1b, Klf1 as a reflection of their myeloid-biased output [35]. The regulation of CD41 expression is apparently affected by aging, and therefore, the CD41^{hi} CMP should be used as a functionally homogeneous population probably only in young adult mice.

Single-cell quantitative PCR analysis showed that the expression of PU.1 starts at the most primitive HSCs, and all the LSK populations express PU.1. However, only CD41^{hi} CMPs, but not CD41$^{-/lo}$ LSKs, upregulate GATA-1 within LSKs (Fig. 7C). Thus, we directly showed that the CD41^{hi} CMP is the first population that upregulates GATA-1 to promiscuously express GATA-1 and PU.1. The original CMPs also coexpressed GATA-1 and PU.1, but their expression levels were approximately two- to sevenfold higher than those in the CD41^{hi} CMPs (Fig. 7C and Supporting Information Table S3). In contrast, LMPPs, myelo-lymphoid progenitors, expressed PU.1 at a level similar to that in original CMPs, but did not express detectable GATA-1. Collectively, CD41^{hi} CMP and LMPP constitute the earliest myelo-lymphoid versus myelo-erythroid lineage branchpoint [10], and the expression patterns of GATA-1 and PU.1 in these populations faithfully reflect their lineage potential.

However, LMPP is not absolutely restricted to myelo-lymphoid lineages, because this population produced a considerable amount of erythroid cells and platelets in vivo (Fig. 3D–3F) as previously reported [26]. In PU.1-GFP knockin reporter mouse [11], a population expressing a high level of GFP was found in 20% of LMPP, and this population was more strictly restricted to GM and lymphoid lineages (granulocyte/monocyte/lymphoid-restricted progenitor: GMLP) [10]. It should be critical to identify another surface marker to isolate the GMLP in wild-type mice, as a paired population of the CD41^{hi} CMP, in future studies.

A recent study showed that CD34^{-} LSK fraction contains cells with long-term, but myeloid and/or erythroid lineage-restricted reconstitution potential, and their restricted lineage potential is preserved even after secondary transplantation [36]. It is possible that these putative self-renewing myeloid-restricted cells as well as multipotent HSCs could produce CD41^{hi} CMPs. However, since this population is unable to be prospectively purified, it is difficult to argue their lineal relationship at this moment. This finding suggests that in long-term HSCs, lineage restriction can occur without losing self-renewal potential. Since our analysis shows that CD34^{-} LSKs never expressed GATA-1, but expressed only a very low level of PU.1 at the single-cell level (Fig. 7C), this early lineage-restriction might not depend upon the interplay of these transcription factors, but presumably by cell-intrinsic epigenetic mechanism.

Conclusions

We have successfully identified the earliest, isolatable CMP population within the LSK population, and proposed to revise the developmental map in early hematopoiesis (Fig. 7D). Together with the LMPP, the CD41^{hi} CMP constitutes the myelo-erythroid and myelo-lymphoid branchpoint in early hematopoiesis. This population appears to be critical for physiological and pathological myelopoiesis. The redefined, prospectively isolatable CMP population in normal mouse BM should be useful for the analysis to understand the developmental mechanisms of normal and malignant hematopoiesis.

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Author Contributions

K.M.: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; Y.A. and H.I.: data analysis and interpretation and manuscript writing; K.K.: provision of study material or patients and data analysis and interpretation; H.T.: collection and/or assembly of data; T.I. and K.T.: provision of study material or patients; T.S. and Y.K.: data analysis and interpretation; T.M.: administrative support and data analysis and interpretation; K.A.: conception and design, financial support, manuscript writing, and final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Dectin-2 Is a Direct Receptor for Mannose-Capped Lipoarabinomannan of Mycobacteria

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SUMMARY

Mycobacteria possess various immunomodulatory molecules on the cell wall. Mannose-capped lipoarabinomannan (Man-LAM), a major lipoglycan of *Mycobacterium tuberculosis*, has long been known to have both inhibitory and stimulatory effects on host immunity. However, the direct Man-LAM receptor that explains its pleiotropic activities has not been clearly identified. Here, we report that a C-type lectin receptor Dectin-2 (gene symbol *Clec4n*) is a direct receptor for Man-LAM. Man-LAM activated bone-marrow-derived dendritic cells (BMDCs) to produce pro- and anti-inflammatory cytokines, whereas it was completely abrogated in *Clec4n*$^{-/-}$ BMDCs. Man-LAM promoted antigen-specific T cell responses through Dectin-2 on DCs. Furthermore, Man-LAM induced experimental autoimmune encephalitis (EAE) as an adjuvant in mice, whereas *Clec4n*$^{-/-}$ mice were resistant. Upon mycobacterial infection, *Clec4n*$^{-/-}$ mice showed augmented lung pathology. These results demonstrate that Dectin-2 contributes to host immunity against mycobacterial infection through the recognition of Man-LAM.

INTRODUCTION

Mycobacteria possess various cell wall components that influence host immune responses, such as trehalose-6,6′-dimycolate (TDM), mycolate, phosphatidyl-*myo*-inositol mannosides (PIMs), lipomannnan (LM), and lipoarabinomannan (LAM). LAM is a major lipoglycan and important virulence factor of mycobacteria (Mishra et al., 2011), enabling mycobacteria to infect host organisms and survive within host cells. Ethambutol, an inhibitor of LAM synthesis, is widely used as an antimycobacterial drug (Belanger et al., 1996). LAM consists of four components: a mannosyl-phosphatidyl-*myo*-inositol (MPI) anchor, a mannose backbone, an arabinan domain, and capping moieties. The capping moieties located at the terminal extremity of the arabinan domain differ among mycobacterial species, such as mannose-capped LAM (Man-LAM), phosphoinositol-capped LAM (PI-LAM), and noncapped LAM (Ara-LAM). Among them, Man-LAM has been intensively studied because it exerts pleiotropic effects on host immunity (Mishra et al., 2011).

Pathogenic species, including *Mycobacterium tuberculosis*, possess Man-LAM, which has been shown to suppress host immune system (Briken et al., 2004) and phagosome-lysosome fusion (Fratti et al., 2003). Although various inhibitory mechanisms have been proposed thus far, one of the key events is the production of immunosuppressive cytokine interleukin-10 (IL-10). On the other hand, Man-LAM also potentiates immunostimulatory responses, such as nitric oxide release and secretion of proinflammatory cytokines (Chan et al., 2001; Gringhuis et al., 2009; Mazurek et al., 2012).

C-type lectin receptors (CLRs) have been recently identified as pattern recognition receptors (PRRs) for a wide variety of pathogens. A member of CLRs, dendritic-cell (DC)-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN, also called CD209) and its putative murine homologs SIGN-related 1 (SIGNR1, also called CD209b) and SIGNR3 (CD209d) are reported to recognize Man-LAM and mediate its immunosuppressive activities (Geijtenbeek et al., 2003; Schlesinger et al., 1994; Tailleux et al., 2003). Macrophage mannose receptor (MMR, also called CD206) is also a candidate for inhibitory receptor for LAM, because it delivers a negative signal to attenuate DC activation (Nigou et al., 2001). In addition to these reports regarding inhibitory functions, engagement of SIGNR3 by Man-LAM also induces the secretion of IL-6 and tumor necrosis factor (TNF) in macrophages transfected with SIGNR3 (Tanne et al., 2009). Moreover, the scavenger receptor CD36 enhances the stimulatory activity of Man-LAM leading to TNF release in lipopolysaccharide (LPS)-stimulated macrophage cell line (Józefowski et al., 2011). Although many proteins have been proposed as a receptor for Man-LAM, none of these receptors fully explain its divergent functions, both stimulatory and inhibitory effects,