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Enrichment of Functionally Distinct Mouse Hematopoietic Progenitor Cell Populations Using CD62L

Scott Cho* and Gerald J. Spangrude*†

The details of the bifurcation of the lymphoid and myeloid lineages following commitment by multipotent progenitor cells (MPP) remain a topic of controversy. We report that the surface glycoprotein CD62L can be characterized as a novel marker of this and other stages of early hematopoietic differentiation. Cell isolation and transplant studies demonstrated CD62Lneg/low long-term hematopoietic stem cells and CD62Lhigh MPP within the traditionally defined c-kitposLinneg/lowSca-1pos stem/progenitor cell population. Within the MPP population, previously defined as c-kitposLinneg/lowSca-1pos–Thy-1.1negFlt3pos, Sca-1 and CD62L resolved four populations and segregated Sca-1highCD62Lneg/low MPP from Sca-1highCD62Lhigh leukocyte-biased progenitors. Using a novel transplantation method that allows tracking of erythroid and platelet engraftment as an alternative to the classical method of in vitro colony formation, we characterized Sca-1highCD62Lneg/low cells as MPP, based on transient engraftment of these lineages. These data establish CD62L as a useful tool in the study of early hematopoiesis and emphasize the power of trilineage-engraftment studies in establishing the lineage potential of MPP subsets.

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In adult mammals, all blood cells originate from a pool of hematopoietic stem cells (HSC) residing in the bone marrow. These adult stem cells possess the prototypical stem cell characteristics: the ability to self-renew through mitosis and the capacity to generate cells of all hematopoietic lineages (1). As HSC mature and differentiate into progeny cells, their self-renewal ability becomes limited, and their multipotency is lost through lineage commitment. The early events of hematopoietic differentiation have been described to occur within a subset of immature cells in the bone marrow identified by a shared expression pattern of surface markers: coexpression of stem cell-associated markers c-kit and Sca-1 and no or only low-level expression of the mature cell markers collectively known as Lineage (Lin) (2, 3). This subset of hematopoietic stem and progenitor cells is routinely termed the KLS (c-kitposLinneg/lowSca-1pos) compartment.

Within the KLS compartment reside three distinct subpopulations that are considered to delineate early hematopoietic differentiation events. According to expression patterns of Flt3 and Thy1.1 surface markers, the three subpopulations are designated as Thy1.1posFlt3neg long-term HSC (LT-HSC), Thy1.1posFlt3pos short-term HSC (ST-HSC), and Thy1.1negFlt3pos multipotent progenitor (MPP) cells (4–6). Historically, Thy1.1 expression levels have been termed Thy1.1pos, Thy1.1neg, and Thy1.1high to distinguish stem/progenitor cells (Thy1.1pos and Thy1.1neg) from mature T cells (Thy1.1high). To avoid confusion caused by this nomenclature, in this study we refer only to Thy1.1pos and Thy1.1neg cells, because it is not necessary to distinguish Thy1.1pos stem/progenitor cells from Thy1.1high T cells. The LT-HSC subset includes the true HSC that initiates hematopoiesis. As LT-HSC differentiate, the Flt3 receptor is upregulated. Cells in the ST-HSC compartment are multipotent but possess a limited capacity for self-renewal, because transplantation studies showed the ST-HSC compartment to reconstitute the hematopoietic system of recipients only for ∼6–12 wk (5, 6). Finally, the last stage within the KLS compartment is the MPP stage that has lost self-renewal capability, accompanied by the loss of Thy1.1, but maintains multipotency.

The functional heterogeneity within the MPP compartment, as defined by Flt3-expressing KLS cells, has been the focus of recent discussions (7–11), mainly triggered by a study describing the existence of lymphoid-primed MPP (LMP) (7). The study identified LMP in the HSC compartment as the population of cells that expresses the highest level of Flt3, constituting a significant fraction of MPP (approximately the top 25% of KLS cells for Flt3 expression). Unlike MPP cells, which have significant output in all hematopoietic lineages, LMP cells generated insignificant numbers of platelets and RBCs, suggesting the loss of erythroid-megakaryocytic lineage (Meg/E) potential prior to cells exiting the HSC compartment and demonstrating the existence of oligopotent progenitors within the pool of true MPP. A subsequent study by another group showed that although LMP cells do have a detectable amount of Meg/E activity, it is significantly less than that of MPP, thereby contrasting the previous report’s claim of loss of Meg/E activity while confirming the existence of heterogeneity within the MPP population (9).

The MPP population has also been subfractionated using VCAM-1. In these studies, VCAM-1pos MPP generated cells of all lineages similar to traditional MPP cells, whereas VCAM-1neg MPP failed to generate Meg/E potentially as robustly as MPP cells.
or VCAM-1^pos MPP (10, 11). Consistent with the LMP study, the investigators observed that VCAM-1^high MPP cells expressed high levels of Flt3, whereas VCAM-1^pos MPP cells expressed both low and high levels of Flt3 (10). These observations suggested that Flt3 alone is insufficient to resolve committed subsets of MPP and that additional markers will be required to help identify functionally distinct subpopulations within MPP (8).

One issue with previous studies of the Meg/E potential of MPP is the prevalent use of the CD45 allelic system in transplant models since its introduction in 1988 (3). This model allows tracing of donor contributions to nucleated cell lineages by flow cytometry, a major advance over classical techniques that used electrophoresis to trace the origin of erythroid cells in transplant studies (12). More recent studies of Meg/E grafting have used surrogate markers or progenitor cell assays to infer platelet and erythrocyte engraftment because CD45 is not expressed by these lineages (13). The use of GFP-transgenic mice allows lineage tracing of platelets (9); however, application of the GFP-transgenic model to erythroid chimerism has been problematic because of the failure of most GFP-transgenic mouse strains to express the transgene in the erythroid lineage (14). As a result, the contributions by the MPP subsets to persistent erythroid engraftment in comparison with HSC in a transplant setting remain to be determined.

Our laboratory previously reported that the CD62L adhesion molecule can be used to fractionate the Thy.1.1^pos subset of KLS to identify a T cell-biased CD62L^high MPP and a CD62L^neg/low MPP with more generally distributed multipotency (15). These findings led us to hypothesize that CD62L is useful as an early marker of hematopoietic development. Our data demonstrated that, in a transplant setting, the CD62L^neg/low fraction of KLS contains highly enriched HSC, whereas the CD62L^high fraction contains MPP with a limited duration of output. We show that the CD62L^neg/low fraction contains HSC in both Sca-1^high and Sca-1^low subsets of KLS, with less HSC activity in the Sca-1^low subset, indicating a gradual population shift as Sca-1 is downregulated. We also present evidence that the primary source of HSC resides in the CD62L^neg/low fraction of the now widely accepted Flt3^posThy.1.1^pos KLS population. Furthermore, we present evidence that CD62L and Sca-1 can be used to isolate distinct subpopulations within the traditional MPP compartment, the Thy.1.1^posFlt3^pos KLS population. Within this MPP compartment, the CD62L^neg/lowSca-1^high KLS population contains the most primitive progenitor population, whereas the CD62L^highSca-1^low population contains the most mature progenitor population based on transplant studies that resolve trilineage engraftment. Fractionation of Meg/E potential from progenitors of nucleated lineages was achieved at the CD62L^highSca-1^high stage of development. These data indicated that CD62L is an effective marker for isolating functionally distinct MPP subpopulations, particularly in light of the restricted strain distribution of the Thy.1.1 allele (16) and the difficulty in confirming specificity of Flt3 staining because of the absence of sufficient numbers of Flt3^pos cells in normal mouse tissues.

Materials and Methods

Mice

Mice carrying homozygous Thy1^ and Ly5^ alleles on the C57BL background were generated and maintained in our animal facilities, as previously described (14). GFP-transgenic mice, generated by microinjection of C57BL/6 oocytes, were kindly provided by Dr. Masaru Okabe (Osaka University, Osaka, Japan) (17). These two strains were mated to generate C57BL mice with the GFP transgene on a Thy1a/bLy5a/b background, which served as transplant donors in all experiments, except as shown in Fig. 3, where the donor strain had homozygous Thy1^ and Ly5^ alleles on the C57BL background but lacked the GFP transgene. Mice congenic for the diffuse allele of the hemoglobin-δ (Hbb) chain on the B6 background (B6.Cg-Gpi1aHbb4 H1f1/Delh mice) (18) were kindly provided by Dr. David Harrison (Jackson Laboratory, Bar Harbor, ME) and were used as C57BL mice. All mice were kept in the animal facilities at the University of Utah under institutional animal care and use committee-approved protocols.

Abs

mAbs against CD2 (RM2.2), CD3 (KT3-1.1), CD5 (53-7.3), CD8 (53-6.7), CD11b (M1/70), Ly-6G (RB6-8C5), TER119, B220 (CD45R; RA3-6B2), and CD19 (1D3) were purified from the media of cultured hybridoma cell lines. PE-conjugated Sca-1 mAb was purchased from PharMingen (San Diego, CA). Conjugated (SC11) mAb was purified and conjugated to Alexa Fluor 647 in our laboratory. CD4 and CD8 mAbs were purified and conjugated to allopbyocyanin in our laboratory. Biotinylated Fli3, CD62L allophyocyanin-AF750, Thy1.1 PerCP-Cy5.5, Mac-1 PE, and Gr-1 PE Abs were purchased from eBioscience (San Diego, CA). Rat mAb 4A5, specific for mouse blood platelets (19), was kindly provided by Dr. S.A. Burstein (University of Oklahoma Health Sciences Center, Oklahoma City, OK) and was purified and conjugated to Alexa Fluor 647 in our laboratory.

Isolation of hematopoietic progenitor and stem cells

Bone marrow cells were harvested from young adult (6–12-wk-old) donor mice and incubated with a mixture of rat Abs to mature cell markers (CD2, CD3, CD5, CD8, CD11b, Ly-6G, TER119, B220, and CD19). Magnetic depletion of mature cells was performed by two successive incubations with magnetic bead-coupled anti-Thy-1.1 and anti-Ly5.1 Abs, as indicated in the legend, to electronically visualize and sort using a FACS Aria instrument (BD Immunocytometry Systems, San Jose, CA). A 0.3-μl solution of DAPI was used to discriminate dead cells from live cells.

Bone marrow transplantation

Recipient mice were lethally irradiated 1 d prior to transplant, using a [137Cs] source (Model Mark I-30; J.L. Shepherd & Associates, San Fernando, CA) to deliver 13 Gy, at a rate of 75 cGy/min, in two doses separated by 3 h. Isolated donor cells (genotype GFP^pos Thy1.1^ly5.1^Hbb^pos) were injected into recipients (genotype Thy1.1^Ly5.1^Hbb^pos) retro-orbitally. The recipient mice were anesthetized with isoflurane using the E-Z Anesthesia system (Euthanex, Palm Beach, FL) prior to injections.

Peripheral blood analysis

For posttransplant analysis, mice were anesthetized with isoflurane using the E-Z Anesthesia system (Euthanex, Palm Beach, FL) and peripheral blood samples were collected into acid citrate dextrose anticoagulant solution from the retro-orbitus sinus using heparinized capillary tubes. Immediately after the collection of blood samples, a volume of 10 μl blood/sample was added to diluted Alexa Fluor 647-conjugated 4A5 Ab, and an additional 10 μl sample was diluted in 10 μl DAPI stock solution (100 mg DAPI in 2.5 ml DMSO; it was stored at 4°C) and used as a control. BM samples were incubated with a [137Cs] source (Model Mark I-30; J.L. Shepherd & Associates, San Fernando, CA) to deliver 13 Gy, at a rate of 75 cGy/min, in two doses separated by 3 h. Isolated donor cells (genotype GFP^pos Thy1.1^Ly5.1^Hbb^pos) were injected into recipients (genotype Thy1.1^Ly5.1^Hbb^pos) retro-orbitally. The recipient mice were anesthetized with isoflurane using the E-Z Anesthesia system (Euthanex, Palm Beach, FL) prior to injections.

HPLC analysis of hemoglobin variants

An HPLC cation-exchange protocol was developed in our laboratory to discriminate and quantify Hbb^b and Hbb^c in the peripheral blood samples (14). A stock solution of 100 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving 100 mg DTNB in 2.5 ml DMSO; it was stored at −20°C. The BRC fraction was derivatized by adding 5 μl the BRC fraction into 250 μl 40 mM NaCl and 2 mM DTNB and incubating at room temperature for 30 min. Following centrifugation at 12,000 × g for 2 min, the supernatant was ana-
lyzed using a VARIANT hemoglobin testing system (Bio-Rad Laboratories, Hercules, CA) with an optimized β-thalassemia short program.

**Repopulating unit calculation**

The repopulating unit (RU) calculation is a commonly-used method to quantify the frequency of repopulating cells in comparison with a known quantity, often competitor cells of whole bone marrow (12, 20). The following formula was used for RU calculation: donor RU = % donor cells × C/(100 – % donor cells), where C is the number of competing RU (12). One competing RU is assumed to be equivalent to 10⁵ whole bone marrow cells (21, 22).

**Results**

**CD62L- and Flt3-expression patterns in the KLS pool are analogous**

To test the hypothesis that CD62L is a useful marker of the early stages of hematopoietic differentiation, the KLS pool of early hematopoietic progenitors was analyzed for CD62L expression (Fig. 1). The expression pattern (Fig. 1A) revealed a bimodal distribution of CD62L among KLS cells, with one subset expressing an intensity of CD62L expression equal to or exceeding that seen on spleen cells (CD62L⁹⁹ hereafter, representing 61 ± 2% of KLS cells) and a second subset ranging from negative to low (CD62Lneg/low hereafter, representing 39 ± 2% of KLS cells). Multiparameter flow cytometry comparing the distribution of CD62L with respect to Thy-1.1, which was shown to segregate Thy-1.1pos HSC from Thy-1.1neg progenitor cells (16, 23), validated the distinction between the CD62Lneg/low and CD62L⁹⁹ subsets (Fig. 1B). Visual examination of the data indicated a clear discrimination of four distinct populations, analogous to what is seen using Thy1.1 and Flt3 expression for the traditional definition of LT-HSC, ST-HSC, and MPP subsets (Fig. 1C). The expression pattern (Fig. 1). The fourth subset resolved by both marker sets as Thy-1.1neg Flt3neg accounted for ~15% of KLS cells and was not evaluated further in these studies; however, we previously characterized the Thy-1.1negCD62Lneg/low KLS subset as primarily a B lymphocyte progenitor population with some T lymphocyte and myeloid engraftment potential (15).

The CD62Lneg/low fraction of KLS contains the LT-HSC population

To investigate the presence of HSC in the CD62L⁹⁹ and CD62Lneg/low fractions in vivo, we performed a transplant experiment. GFP⁺/⁻ Hbb²/s mice were used as donors to allow for the tracking of platelets and WBC produced from the transplanted populations via flow cytometric analysis. RBC were tracked by the hemoglobin variant Hbb² using HPLC analysis. For each transplant recipient, 10⁵ donor KLS cells were sorted according to CD62L expression alone (Fig. 1A), without selection for Thy-1.1 or Flt3 expression. The donor cells were transplanted into lethally irradiated Hbb²/s recipient mice along with 10⁵ recipient bone marrow cells serving as competitors.

The transplant data showed that CD62Lneg/low cells reconstituted all three lineages of the hematopoietic system of the recipient mice strongly and persistently (Fig. 1C). In contrast, CD62L⁹⁹ cells failed to engraft persistently. Although cells of all three blood lineages were generated by CD62L⁹⁹ cells, the donor-derived cells diminished significantly during the weeks posttransplant. Donor-derived platelets were undetectable shortly after the transplant, whereas donor-derived RBC were not detected after week 9. WBC diminished to a very low level after initial engraftment but persisted throughout the entire observation period. The maximum number of RBC peaked prior to week 3 for CD62L⁹⁹ cells; however, CD62Lneg/low cells did not peak in RBC production until week 5 (Fig. 1C). Altogether, the data suggested that LT-HSC are restricted to the CD62Lneg/low population and are not present in the CD62L⁹⁹ population.

To confirm the usefulness of the CD62Lneg/low phenotype as a marker of LT-HSC, the bone marrow of the recipient mice was examined following the termination of the transplant experiment. Bone marrow samples harvested from recipients of CD62L⁹⁹ or CD62Lneg/low cells were Lin depleted and stained with c-kit and Sca-1 Abs to analyze the KLS compartment for GFP⁺ donor cells. Bone marrow cells of the CD62Lneg/low recipients showed a significant amount of GFP⁺ cells (54 ± 24% of KLS, range 35–89%), whereas in recipients of CD62L⁹⁹ cells only trace numbers of GFP⁺ cells were found (1.7 ± 0.6% of KLS; range, 1.1–2.4%; Fig. 2A). The GFP⁺ KLS cells were then isolated by FACS sorting and transplanted into another set of lethally irradiated hosts. Because...
CD62L<sup>high</sup> recipients generated only trace numbers of GFP<sup>+</sup> cells in their KLS fractions, all GFP<sup>+</sup> cells were pooled into one injection and given to one recipient. Five weeks later, peripheral blood samples were analyzed for donor-derived cells. Only mice receiving CD62L<sup>neg/low</sup> donor-derived GFP<sup>+</sup> KLS cells produced GFP<sup>+</sup> progenies in the secondary transplant recipients (Fig. 2B). This indicated that the CD62L<sup>high</sup> subset of KLS, which makes up <0.1% of the bone marrow, includes ~95% of the transient erythroid progenitor potential based on competition with 10<sup>5</sup> bone marrow cells. CD62L<sup>high</sup> progenitors also peaked in RBC and WBC production earlier than CD62L<sup>neg/low</sup> cells. WBC analysis showed results similar to the previous experiment, consistent with the interpretation that the CD62L<sup>neg/low</sup> and CD62L<sup>high</sup> populations contain LT-HSC and MPP, respectively (Fig. 3A).

To compare relative activity of the CD62L<sup>neg/low</sup> and CD62L<sup>high</sup> populations in the absence of normal marrow competitors and to investigate whether both populations of cells conferred radioprotection, 500 cells/CD62L fraction were transplanted. The results (Fig. 3B) showed that donor cells of both fractions successfully rescued all transplanted mice. However, persistent engraftment of donor-derived cells was observed only following transplantation of 10<sup>3</sup> cells for both CD62L<sup>neg/low</sup> and CD62L<sup>high</sup> fractions. To evaluate the presence of MPP, as defined by our ability to discriminate donor-derived cells in the peripheral blood of transplant recipients over time, we transplanted CD62L<sup>high</sup> cells at a dose 10-fold greater than in the previous experiment (10<sup>5</sup> cells/recipient). The one CD62L<sup>high</sup> transplant recipient did not produce a detectable number of GFP<sup>+</sup> cells in any lineage. These secondary transplantation results demonstrated that LT-HSC are restricted to the CD62L<sup>neg/low</sup> fraction and are not present in appreciable numbers within the CD62L<sup>high</sup> fraction.

**CD62L AS A MARKER OF HEMATOPOIETIC DIFFERENTIATION**

**FIGURE 2.** LT-HSC are confined to the CD62L<sup>neg/low</sup> fraction of the KLS compartment. The transplant recipients shown in Fig. 1C were sacrificed 13 wk posttransplant, and their bone marrow cells were analyzed for the presence of donor-derived cells within the KLS population. A. Lin-depleted bone marrow prepared from each of the five transplant recipients in each group was evaluated for GFP<sup>+</sup> cells within the KLS subset. One representative animal from each group is shown. Numbers represent mean ± SD values of each group. B. GFP<sup>+</sup> KLS cells from each of five CD62L<sup>neg/low</sup> transplant recipients were sorted and transplanted into one lethally irradiated secondary recipient. The average percentages of donor-derived cells are shown. UD, undetectable.

In the primary transplant of the experiment shown in Fig. 2, we transplanted 10<sup>5</sup> bone marrow cells along with an equal number of 10<sup>3</sup> cells for both CD62L<sup>neg/low</sup> and CD62L<sup>high</sup> fractions. To evaluate the presence of MPP, as defined by our ability to discriminate donor-derived cells in the peripheral blood of transplant recipients over time, we transplanted CD62L<sup>high</sup> cells at a dose 10-fold greater than in the previous experiment (10<sup>5</sup> cells/recipient). To evaluate the differential frequency of LT-HSC in the CD62L<sup>neg/low</sup> fraction, these cells were transplanted at a limiting dose of 10<sup>2</sup> cells. As before, both populations were transplanted competitively with 10<sup>5</sup> bone marrow cells. Analysis of RBC engraftment in this experiment confirmed and extended our previous findings. Transplantation of 10<sup>4</sup> CD62L<sup>high</sup> cells in competition with 10<sup>5</sup> bone marrow cells produced a wave of donor-derived RBC that diminished over time to a negligible percentage at week 16 (Fig. 3A).

**FIGURE 3.** LT-HSC are enriched in the CD62L<sup>neg/low</sup> fraction, and MPP are enriched in the CD62L<sup>high</sup> fraction of KLS. A. The CD62L<sup>neg/low</sup> fraction (10<sup>5</sup> cells) or the CD62L<sup>high</sup> fraction (10<sup>5</sup> cells) of the KLS compartment were each transplanted into groups of five lethally irradiated recipient mice along with 10<sup>5</sup> normal bone marrow cells. Donor animals in this experiment lacked the GFP transgene, therefore only erythrocytes (RBC) and leukocytes (WBC) were tracked over time by peripheral blood sampling based on analysis of Hbb and CD45 allelic markers. B. Each CD62L fraction of the KLS compartment was transplanted into four lethally irradiated mice, at a dose of 500 cells/mouse, without added competitive bone marrow cells. Donor cells lacked the GFP transgene. All transplant recipient mice survived; however, only recipients of CD62L<sup>neg/low</sup> cells showed persistent engraftment of both RBC and WBC lineages. Error bars indicate SEM.
of CD62L<sup>neg/low</sup> cells, whereas CD62L<sup>high</sup> cells provided only transient engraftment of erythroid and leukocyte lineages that was eclipsed by endogenous HSC activity by 16 wk posttransplant. Collectively, the data shown in Figs. 1–3 illustrate that CD62L expression levels can be a useful biomarker for separation of LT-HSC from MPP within the KLS bone marrow population.

The CD62L<sup>neg/low</sup> fraction of the KLS<sup>neg</sup> compartment includes most LT-HSC activity

Thy-1.1 and Flt3 have been characterized as markers for FACS sorting of LT-HSC. To evaluate the distribution of CD62L expression relative to LT-HSC potential among KLS cells in the context of Thy-1.1 and Flt3, we performed multiparameter flow cytometry to evaluate the Thy-1.1<sup>neg</sup>Flt3<sup>neg</sup> subset of KLS, previously shown to include LT-HSC, with respect to CD62L expression. Electronic gating on the Thy-1.1<sup>pos</sup>Flt3<sup>neg</sup> fraction of KLS (KLS<sup>neg</sup>) showed that the frequencies of the CD62L<sup>neg/low</sup> (78 ± 3%) and CD62L<sup>high</sup> (22 ± 3%) populations reversed with respect to that seen in the complete KLS population (Fig. 4A; compare with Fig. 1A). To evaluate LT-HSC activity, we transplanted 2 × 10<sup>5</sup> sorted cells (KLS<sup>neg</sup>CD62L<sup>neg/low</sup> and KLS<sup>neg</sup>CD62L<sup>high</sup>) into lethally irradiated recipients along with 10<sup>5</sup> normal bone marrow competitor cells.

Peripheral blood analysis for engraftment activity showed strong trilineage engraftment by the KLS<sup>neg</sup>CD62L<sup>neg/low</sup> fraction, reconstituting ~90% of platelets, erythrocytes, and leukocytes, despite competition from a 50-fold excess of unseparated bone marrow competitor cells.

Phenotypically distinct subpopulations suggest heterogeneity within the MPP population

To test the hypothesis that CD62L-based subfractionation can demonstrate heterogeneity within the MPP population, we analyzed the MPP subset of KLS using flow cytometry. The KLS cells were labeled with Flt3 and Thy1.1 Abs to display the traditional division of KLS into LT-HSC−, ST-HSC−, and MPP-enriched subsets (Figs. 1B, 5A). Combined labeling of Flt3 and CD62L revealed a high degree of coexpression, because 23 ± 1% of cells expressed neither marker, and 50 ± 2% of cells expressed both markers (Fig. 5A). This high degree of coexpression pattern is particularly interesting considering previous evidence demonstrating that variable expression levels of Flt3 correlated with variable capacity for multipotency among Flt3+ MPP (9, 10). Also, the presence of cells that are mutually exclusive for the expression of CD62L and Flt3 (27 ± 1% of KLS; Fig. 5A) demonstrated distinct populations that would not be identifiable without the use of CD62L as a marker.

To proceed with the isolation of subpopulations for functional analysis, the Thy-1.1<sup>neg</sup>Flt3<sup>pos</sup> MPP subset of KLS was used to subdivide the cells into four subsets based on Sca-1 and CD62L levels (Sca-1<sup>low</sup>/CD62L<sup>neg/low</sup>, CD62L<sup>neg/low</sup>/Sca-1<sup>high</sup>, Sca-1<sup>high</sup>/CD62L<sup>high</sup>, Fig. 5A). Based on previous studies, we would expect the least mature of these subsets to be Sca-1<sup>high</sup> and CD62L<sup>neg/low</sup> and the most mature subset to be Sca-1<sup>low</sup> and CD62L<sup>high</sup>. The four subsets were isolated from GFP<sup>tg</sup>/Hbb<sup>vd</sup> donor mice by cell sorting and reanalyzed to confirm purity, and 3 × 10<sup>6</sup> cells of each population were transplanted along with 2.5 × 10<sup>5</sup> Hbb<sup>dd</sup> whole bone marrow cells into lethally irradiated Hbb<sup>vd</sup> mice. Peripheral blood samples of the transplant recipients were periodically analyzed to identify and quantify progenies of specific lineages.

Over the course of 9 wk, the transplanted populations displayed varying amounts of Meg/E potential. CD62L<sup>neg/low</sup>Sca-1<sup>high</sup> cells, suspected to include the least mature progenitor population among the four, generated significantly more erythrocytes and platelets relative to the other three populations (Fig. 5B). As expected, all four progenitor populations generated platelets and erythrocytes only temporarily. The CD62L<sup>high</sup>Sca-1<sup>low</sup> population, suspected to include the most mature progenitors, generated the lowest number of erythrocytes and platelets among the four progenitor populations, and three of five recipients showed no platelet engraftment at any time after transplant (data not shown). CD62L<sup>neg/low</sup>Sca-1<sup>low</sup> and CD62L<sup>high</sup>Sca-1<sup>high</sup> cells generated intermediate num-

![FIGURE 4. The CD62L<sup>neg/low</sup> phenotype adds additional resolution to LT-HSC, as defined by Thy-1.1 and Flt3 expression. A, KLS cells were gated to select for the Flt3<sup>neg</sup>Thy-1.1<sup>neg</sup> LT-HSC subset. CD62L expression by the Thy-1.1<sup>neg</sup>Flt3<sup>neg</sup> fraction of KLS (KLS<sup>neg</sup>) is shown in the graph (right panel). The percentages indicate the frequency of the KLS<sup>neg</sup> subset of KLS and the frequencies of CD62L<sup>neg/low</sup> and CD62L<sup>high</sup> cells within the KLS<sup>neg</sup> compartment, determined as described in the legend for Fig. 1. B, The CD62L<sup>neg/low</sup> and CD62L<sup>high</sup> fractions of the KLS<sup>neg</sup> population were sorted, and 2 × 10<sup>5</sup> cells/fraction were transplanted into each of five lethally irradiated mice along with 10<sup>5</sup> competitor cells. Peripheral blood analysis was performed at the indicated times. Error bars indicate SEM.](http://www.jimmunol.org/issue_assets/M050852.jpg)
bers of platelets and erythrocytes compared with the other two populations, suggesting that the y represent transitional populations between the less mature CD62Lneg/lowSca-1high and the more mature CD62LhighSca-1low populations. The dramatic reduction of Meg/E potential observed as progenitors shifted from CD62Lneg/lowSca-1high to CD62LhighSca-1low is consistent with the idea that the CD62Lneg/lowSca-1high phenotype is uniquely associated with progenitor cells that retain Meg/E potential. This observation parallels other studies that also showed the reduction of Meg/E potential with the changing expression of other developmental Ags (Flt3 and VCAM-1) (7, 10).

The peripheral blood analysis of the transplant recipients also revealed differences in the numbers of WBC progenies generated from the subfractionated populations of the KLS MPP; however, these were only significant between subsets discriminated by Sca-1 expression levels and not by CD62L expression levels. Both Sca-1high populations generated robust numbers of myeloid and B lymphocyte cells 2 wk after transplantation, with B lymphocytes persisting to a greater extent compared with myeloid lineage cells (Fig. 5C). A similar pattern was observed for T cell development, although donor-derived T cell numbers were low and not significant until 9 wk posttransplantation (data not shown). At this time, the Sca-1high subset generated $0.33 \pm 0.09 \times 10^5$ T cells/µL of peripheral blood, whereas the Sca-1low subset generated $0.07 \pm 0.02 \times 10^5$ T cells/µL ($p = 0.007$). The observed difference in lineage potential seen in Fig. 5B and 5C suggests that the CD62Lneg/lowSca-1high subset includes true MPP but that upregulation of CD62L expression is accompanied by a decrease in Meg/E potential and a maintenance of WBC potential. Subsequently, a further loss of progenitor cell potential is identified by downregulation of Sca-1 expression.

**Discussion**

In this report, we characterized the usefulness of CD62L as a marker of hematopoietic differentiation. Although numerous markers for isolation of HSC subsets have been described, additional markers of differentiation add to our understanding of the complexity of the hematopoietic hierarchy. New advances in fluorescent probe and instrument technologies allow a deeper and more detailed view into the stages of development previously defined by a relatively small subset of surface Ags. The depth of our understanding of early hematopoietic development will depend on the availability and specificity of various markers. Additionally, the use of robust methods for distinction of transplanted donor cells from recipient cells is critical, because the inability to visualize engraftment in erythroid and platelet lineages using the CD45 allelic system has resulted in a general lack of good experimental evidence regarding the progenitor cells for these lineages. In vitro colony-forming assays have been used for this purpose, but these experiments are prone to artifacts and lack the in vivo relevance inherent in transplantation assays (24, 25).
The expression profile of Flt3 in conjunction with Thy1.1 has been useful in resolving the three early hematopoietic compartments (LT-HSC, ST-HSC, and MPP) that form the foundation of our current understanding of early hematopoietic events in adult mice (4–6). We have found that few commercially available anti-Flt3 reagents are suitable for high-quality resolution of LT-HSC from MPP, and the use of Flt3 is further complicated by the lack of suitable positive-staining control cell populations. As an additional marker, Thy1.1 is effective for the isolation of HSC within the KLS population but is limited to the few Thy1.1-expressing mouse strains because the more common Thy-1.2 allele is not expressed by most HSC (16). These issues emphasize the need for ongoing investigation of known markers, as well as exploration for new markers, to advance our ability to isolate HSC and to understand HSC biology. We presented in vivo evidence for CD62L as an attractive alternative, as well as a useful supplemental Ag, for use with Flt3 to improve isolation of HSC subsets. Abs against CD62L are available in a wide variety of conjugates, and expression of CD62L in normal mouse spleen is robust (Fig. 1A). Our transplant data demonstrated that CD62L fractionation of the KLS population yields a CD62L−neg population containing LT-HSC and a CD62Lhigh population devoid of LT-HSC and containing MPP. Further fractionation of MPP, as defined by the phenotype KLS-Thy-1.1neg Flt3pos, convincingly separates CD62L−neg/low MPP from more restricted progenitor subsets lacking Meg/E potential that are CD62Lhigh. Differential temporal kinetics and persistence of engraftment are also consistent with the usefulness of CD62L expression as a marker of LT-HSC versus MPP and later stages of progenitor cells.

In this report, we expanded upon previous findings describing CD62L as an effective marker for dividing the lymphoid progenitor subset (KLS-Thy-1.1neg) of bone marrow into a CD62Lhigh fraction, which resembled an early T-lineage progenitor, and a CD62L−neg/low fraction, which resembled the traditional MPP (15). Bone marrow transplant results from previous studies were restricted to the characterization of WBC engraftment only, because the approach used to differentiate the donor-derived hematopoietic cells from the cells of the recipient mice was allele-specific Ab labeling of CD45. As a result, previous studies could not discriminate Meg/E potential in the two fractions of MPP, as we showed in this article. The data presented in this study demonstrated subfractions of KLS MPP that are distinguishable by surface expression levels of CD62L and Sca-1 and are functionally distinct. Our in vivo transplant experiments demonstrated that the CD62Lneg/low Sca-1high KLS MPP population includes the most robust engraftment activity in all lineages, whereas the CD62Lhigh Sca-1low population exhibited significantly less production of all lineages. Interestingly, platelet and erythrocyte potential segregated from WBC potential at the CD62Lneg/low Sca-1high stage of development within the KLS MPP population. This finding is consistent with previous reports demonstrating that subpopulations within KLS MPP vary with respect to Meg/E activity.

Additional markers were reported to subfractionate the KLS compartment into distinct subpopulations. Several of the lineage markers that are typically used for depletion of mature hematopoietic cells have been applied in subfractionation strategies (26). Another set of markers, the Slam family proteins (CD150, CD244, and CD48), have also been reported to fractionate KLS into HSC and MPP (27, 28). A ligand for CD62L, the CD34 molecule, has also been used to isolate HSC and to fractionate KLS (29, 30). The coexpression patterns of these markers with CD62L is unknown. Future investigations into the relationship of CD62L expression with these other markers would be interesting and may yield new subpopulations that may represent acute transition stages, granting a better understanding of early hematopoietic events at higher resolution. Collectively, our data demonstrated that CD62L, despite having an expression pattern that is similar to that of Flt3, is able to identify functionally distinct subpopulations of MPP that would be impossible to resolve using Flt3 alone. Furthermore, the data supported the model of heterogeneous KLS MPP that sheds Meg/E potential, marked by the upregulation of CD62L, prior to the lymphoid and myeloid lineage separation through the formation of lymphoid- and myeloid-restricted progenitor cells.

Disclosures
The authors have no financial conflicts of interest.

References


