SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells

Resource

Mark J. Kiel,[1,3] Ömer H. Yilmaz,[1,3]
Toshihide Iwashita,[1,3] Osman H. Yilmaz,[1]
Cox Terhorst,[2] and Sean J. Morrison[1,*]
[1] Howard Hughes Medical Institute and
Departments of Internal Medicine and Cell
    and Developmental Biology
University of Michigan
Ann Arbor, Michigan, 48109
[2] Division of Immunology
Beth Israel Deaconess Medical Center
Harvard Medical School
Boston, Massachusetts 02215

Summary

To improve our ability to identify hematopoietic stem cells (HSCs) and their localization in vivo, we compared the gene expression profiles of highly purified HSCs and non-self-renewing multipotent hematopoietic progenitors (MPPs). Cell surface receptors of the SLAM family, including CD150, CD244, and CD48, were differentially expressed among functionally distinct progenitors. HSCs were highly purified as CD150+CD244−CD48− cells while MPPs were CD244+ CD150−CD48− and most restricted progenitors were CD48+CD244+CD150−. The primitiveness of hematopoietic progenitors could thus be predicted based on the combination of SLAM family members they expressed. This is the first family of receptors whose combinatorial expression precisely distinguishes stem and progenitor cells. The ability to purify HSCs based on a simple combination of SLAM receptors allowed us to identify HSCs in tissue sections. Many HSCs were associated with sinusoidal endothelium in spleen and bone marrow, though some HSCs were associated with endosteum. HSCs thus occupy multiple niches, including sinusoidal endothelium in diverse tissues.

Introduction

Two central and related questions in stem cell biology involve the identification of markers that distinguish stem cells from other progenitors and the identification of microenvironments ("niches") in which stem cells reside (Morrison et al., 1997a; Spradling et al., 2001). These issues have been studied extensively in the hematopoietic system (Lemischka, 1997; Phillips et al., 2000; Hackney et al., 2002; Ivanova et al., 2002; Ramalho-Santos et al., 2002), but our inability to rigorously identify HSCs using simple combinations of markers has compromised our ability to study HSC microenvironments in vivo.

The locations and identities of differentiated cells are often defined by the differential expression of individual

families of cell surface receptors. The locations and identities of olfactory sensory neurons and chemosensory neurons are distinguished by their differential expression of olfactory receptors (Buck and Axel, 1991) and Mrg family receptors (Dong et al., 2001), respectively. Although the gene expression profiles of multiple stem cell populations have been described (Ivanova et al., 2002; Ramalho-Santos et al., 2002; Easterday et al., 2003; Evsikov and Solter, 2003; Fortunel et al., 2003; Iwashita et al., 2003), no single family of cell surface receptors has yet been found in which members are differentially expressed in a way that correlates with primitiveness or developmental potential.

In the absence of simple combinations of markers that reliably purify HSCs, it has been necessary to use complex combinations of markers. HSCs have been highly enriched as Thy-1loSca-1+Lineage−c-kit+ cells or CD34−Sca-1+Lineage−c-kit+ cells using combinations of 10–12 surface markers (Spangrude et al., 1988; Morrison and Weissman, 1994; Osawa et al., 1996). But even using these complicated sets of markers, only 20% of intravenously injected cells gave long-term multilineage reconstitution in most studies (Morrison et al., 1995; Spangrude et al., 1995; Osawa et al., 1996; Wagers et al., 2002). By gating more restrictively on existing HSC markers, or by combining these with Hoechst exclusion, nearly homogeneous subsets of bone marrow HSCs have been isolated (Uchida et al., 2003; Matsuzaki et al., 2004; Takano et al., 2004), but these markers remain too complex for the identification of HSCs in tissue sections.

Studies of various transgenic mice have demonstrated the functional importance of osteoblasts in regulating bone marrow HSCs (Calvi et al., 2003; Zhang et al., 2003; Visnjic et al., 2004). Immunofluorescence studies with markers of primitive hematopoietic progenitors have suggested that HSCs interact with osteoblasts at the endosteum of bone marrow (Zhang et al., 2003; Arai et al., 2004; Wilson et al., 2004). However, not all HSCs can be associated with osteoblasts. The ability of cytokines to mobilize HSCs into circulation within minutes (Laterveer et al., 1995) has suggested that a subset of HSCs must be closely associated with blood vessels in the bone marrow (Heissig et al., 2002). Moreover, there are no osteoblasts in sites of extramedullary hematopoiesis, like the liver and spleen, where HSCs are maintained throughout adult life (Taniguchi et al., 1996). These observations indicate that the endosteum/osteoblast microenvironment is unlikely to be the sole supportive niche for HSCs.

Endothelial cells also regulate HSC function and could contribute to the creation of HSC niches. Endothelial cells are capable of maintaining HSCs in culture (Cardier and Barbera-Guillem, 1997; Ohneda et al., 1998; Li et al., 2004), and ablation of endothelial cells in vivo by administration of anti-VE-cadherin antibody leads to hematopoietic failure (Avecilla et al., 2004). Endothelial cells also create stem cell niches in other tissues such as the nervous system (Palmer et al., 2000; Capela and Temple, 2002; Louissaint et al., 2002; Shen

*Correspondence: seanjm@umich.edu
[3] These authors contributed equally to this work.

et al., 2004). Simple combinations of markers that identify HSCs with high reliability would make it possible to test whether HSCs interact with endothelial cells in vivo.

We have found that SLAM family receptors are differentially expressed among hematopoietic progenitors in a way that correlates with progenitor primitiveness. The SLAM family is a group of 10–11 cell surface receptors that are tandemly arrayed at a single locus on chromosome 1 (Engel et al., 2003; Sidorenko and Clark, 2003). SLAM family members regulate the proliferation and activation of lymphocytes (Howie et al., 2002; Wang et al., 2004). Our ability to purify HSCs using simple combinations of SLAM family members made it possible to image HSCs in tissue sections using markers that had been validated as yielding high HSC purity in functional assays. Many HSCs within the bone marrow and spleen were associated with sinusoidal endothelium. This reveals the importance of sinusoidal endothelial cells for the localization of HSCs throughout hematopoietic tissues.

Results

The Purity of Cells Used for Gene Expression Profiling

To identify genes that are tightly associated with HSC identity, we have compared the gene expression profiles of highly enriched populations of HSCs and transiently reconstituting multipotent progenitors (MPPs) (Morrison and Weissman, 1994; Morrison et al., 1997b). The quality of gene expression profiles depends upon the purity of cells used. HSCs were isolated as Thy-1^{lo}Sca-1$^+$Lineage$^-$c-kit$^+$ cells. One out of every 4.9 ± 2.5 (20%) intravenously injected Thy-1^{lo}Sca-1$^+$Lineage$^-$c-kit$^+$ cells long-term multilineage reconstituted irradiated mice in limit dilution competitive reconstitution assays (see Table S1 in the Supplemental Data available with this article online).

Thy-1^{lo}Sca-1$^+$Lineage$^-$c-kit$^+$ HSCs give rise to non-self-renewing Thy-1^{lo}Sca-1$^+$Mac-1^{lo}CD4^{lo} MPPs in vivo (Morrison et al., 1997b). We have enhanced the purity of this cell population by further selecting the B220$^-$ subset of Thy-1^{lo}Sca-1$^+$Mac-1^{lo}CD4^{lo} cells (Table S2). One out of every 4.0 intravenously injected Thy-1^{lo}Sca-1$^+$Mac-1^{lo}CD4^{lo}B220$^-$ cells (25%) reconstituted irradiated mice in competitive reconstitution assays, usually giving transient multilineage reconstitution (Table S2). Thy-1^{lo}Sca-1$^+$Mac-1^{lo}CD4^{lo}B220$^-$ cells are more highly enriched for MPPs than any previously characterized cell population.

Genes that Are Tightly Linked to HSC Identity

We isolated three independent 5000 cell aliquots of HSCs or MPPs or 8000 CD45$^+$ bone marrow cells (which include nearly all hematopoietic cells; more of these cells were used because they have a lower RNA content) and independently extracted and amplified RNA from each aliquot for gene expression profiling (Iwashita et al., 2003). The gene expression profiles were compared using Affymetrix oligonucleotide arrays (Tables S3 and S4). Variability was low among samples of the same type: Pearson correlation coefficient, $R^2 =$ 0.988 to 0.991 for untransformed data (Table S4). How-

ever, the variability between samples of different types (HSC versus MPP, $R^2 = 0.798 \pm 0.024$; HSC versus CD45$^+$, $R^2 = 0.558 \pm 0.009$) was significantly higher (p < 0.0005). Transcript expression was detected (present calls) at 46% of probe sets for HSCs, 46% of probe sets for MPPs, and 41% of probe sets for CD45$^+$ cells.

We identified genes for which signal intensities were at least 3-fold higher in HSCs, the difference was statistically significant (p < 0.05), and signals were significantly above background in at least one HSC sample (nonzero present call). We identified 1151 probe sets that satisfied these criteria for being upregulated in HSCs as compared to CD45$^+$ cells, and 46 probe sets in HSCs as compared to MPPs (out of 36,701 probe sets total). Twenty-seven of these 46 probe sets were expressed at higher levels in HSCs as compared to both MPPs and CD45$^+$ cells (Table 1). To further evaluate these genes, we compared their expression by quantitative (real-time) RT-PCR in at least two independent samples of HSCs, MPPs, and whole bone marrow cells. Of the 25 genes against which qPCR primers could be designed, all were confirmed as being expressed at >1.9-fold higher levels in HSCs as compared to MPPs and CD45+ cells (Table 1).

CD150 Is Expressed by HSCs but Not by MPPs

One of these genes encodes the homotypic cell surface receptor CD150 (SLAM), which was not previously identified as being expressed in stem cells or other hematopoietic progenitors. CD150 is the founding member of the SLAM family of cell surface receptors (Engel et al., 2003; Sidorenko and Clark, 2003). CD150 appeared to be 4- to 17-fold upregulated in HSCs as compared to MPPs and CD45$^+$ bone marrow cells by microarray analysis and qPCR (Table 1). Only 6.6% ± 1.7% of whole bone marrow cells were CD150$^+$ by flow cytometry (Figure 1A). Consistent with the trends observed at the RNA level (Table 1), CD150 was expressed by 46% ± 12% of cells within the Thy-1^{lo}Sca-1$^+$Lineage$^-$c-kit$^+$ HSC population but by only 0.9% ± 0.5% of cells in the Thy-1^{lo}Sca-1$^+$Mac-1^{lo}CD4^{lo}B220$^-$ MPP population (Figures 1B and 1C).

To test whether CD150$^+$ cells include HSCs, we performed competitive reconstitution assays in which CD150$^+$ or CD150$^-$ donor bone marrow cells were transplanted into lethally irradiated recipient mice along with a radioprotective dose of recipient-type whole bone marrow cells (Figure 1D). In each of two independent experiments, recipients of the CD150$^+$ cells were long-term multilineage reconstituted by donor cells (six of six mice), while recipients of CD150$^-$ cells almost always (eight of nine mice) exhibited transient multilineage reconstitution (one of nine mice was long-term multilineage reconstituted). These data indicate that HSCs are enriched in the CD150$^+$ fraction and depleted in the CD150$^-$ fraction of bone marrow cells. Recipients of CD150$^+$ bone marrow cells were always able to transfer long-term multilineage donor cell reconstitution to secondary recipients, while recipients of CD150$^-$ cells were not able to transfer donor cell reconstitution to secondary recipients (data not shown). These results identify CD150 as a new marker of HSCs.

Table 1. Genes that Were Expressed at Higher Levels in HSCs as Compared to MPPs and CD45+ Cells by Both Microarray Analysis and Quantitative PCR

Gene Symbol	Unigene Title	GenBank Accession	Microarray		qPCR	
			HSC/ MPP	HSC/CD45+	HSC/ MPP	HSC/WBM
Clca1	Chloride channel calcium activated 1	AF047838	8.3	9.1	32.8	3.3
Cpne8	Copine VIII	AV240111	7.0	11.0	3.8	29.9
Sdpr	Serum deprivation response	AI839175	7.0	7.0	116.7	40.3
Catnal1	Catenin alpha-like 1	AI152317	6.9	8.8	4.0	26.2
Prkcm	Protein kinase C mu	AV297026	6.4	7.0	ND	ND
Vwf	Von Willebrand factor homolog	AI843063	6.2	17.2	5.2	4.2
Est	RIKEN full-length library, clone:E330020H17	AI853427	5.7	5.7	4.9	2.8
Mjd	Machado-Joseph disease homolog	AV262417	5.2	5.9	1.9	2.3
Ly64	Lymphocyte antigen 64	AV204260	4.8	13.7	9.0	53.8
D10Ertd755e	DNA segment, Chr 10, ERATO Doi 755, expressed	AU019706	4.7	4.7	ND	ND
C530008M17Rik	RIKEN cDNA C530008M17 gene	AI851362	4.7	7.4	15.3	2.9
Est	Mus musculus transcribed sequences	AI666656	4.1	10.7	1.9	2.8
Slam	Signaling lymphocyte activation molecule	AI120008	4.0	4.0	5.8	17.0
Est	Mus musculus transcribed sequences	AV236645	3.6	9.7	5.6	7.0
Rnf125	Ring finger protein 125	AV361188	3.6	6.0	2.4	2.9
Peg12	Paternally expressed 12	AI413890	3.6	3.8	9.2	22.9
Bgn	Biglycan	AV016619	3.5	64.1	7.7	52.6
Exosc1	Exosome component 1	AI592141	3.4	7.8	2.5	2.6
Stub1	STIP1 homology and U-box containing protein 1	AW046544	3.4	5.0	2.5	2.2
Gemin4	Gem (nuclear organelle)-associated protein 4	AV341751	3.4	5.4	2.8	5.9
Tfpi	Tissue factor pathway inhibitor	AI480514	3.2	4.9	4.6	9.2
Est	Mus musculus transcribed sequences	AI482323	3.2	16.9	3.4	10.2
pbx1	Pre-B cell leukemia transcription factor 1	AI845678	3.1	4.3	3.5	15.6
Sdsl	Serine dehydratase-like	AI504310	3.1	14.7	7.0	6.1
Ppap2b	Phosphatidic acid phosphatase type 2B	AV346092	3.1	3.7	2.5	2.9
Armcx1	Armadillo repeat-containing, X-linked 1	AI846227	3.0	3.1	5.6	34.0
Phactr2	Phosphatase and actin regulator 2	AW123926	3.0	4.0	1.9	3.0

cRNA from Thy-1^{lo}Sca-1$^+$Lineage$^-$c-kit$^+$ HSCs, Thy-1^{lo}Sca-1$^+$Mac-1^{lo}CD4^{lo}B220$^-$ MPPs, or CD45+ bone marrow cells were hybridized to oligonucleotide arrays. The average untransformed probe intensities from three independent samples were used to calculate fold change (HSC/MPP; HSC/CD45$^+$). The table lists all of the genes that were expressed at significantly higher levels in HSCs as compared to MPPs and CD45+ cells by both microarray analysis (fold change >3.0) and quantitative (real-time) PCR (fold change >1.9).

CD244 Is Expressed by Transiently Reconstituting MPPs but Not by HSCs

To test whether other SLAM family members might be differentially expressed between hematopoietic progenitors, we examined the SLAM family member CD244, which was not detected in HSCs by microarray analysis (Table S1). At the protein level, only 8.9% ± 0.6% of bone marrow cells expressed CD244 by flow cytometry (Figure 2A). Little or no CD244 staining was detected among Thy-1^{lo}Sca-1$^+$Lineage$^-$c-kit$^+$ HSCs (Figure 2B), but 33% ± 10% of cells in the Thy-1^{lo}Sca-1$^+$Mac-1^{lo}CD4^{lo} MPP population were CD244$^+$ (Figure 2C). This suggested that at least some MPPs expressed CD244.

We performed two independent competitive reconstitution assays in which CD244$^+$ or CD244$^-$ donor bone marrow cells were transplanted into lethally irradiated recipient mice along with a radioprotective dose of recipient-type whole bone marrow cells (Figure 2D). Recipients of the CD244$^-$ cells were always long-term multilineage reconstituted by donor cells (eight of eight mice), while recipients of the CD244$^+$ cells were always transiently multilineage reconstituted (eight of eight mice). Consistent with this, recipients of CD244$^-$ cells were always able to transfer long-term multilineage reconstitution to secondary recipients, while recipients of CD244$^+$ cells were never able to transfer donor cell re-

constitution to secondary recipients (data not shown; transplants were performed 16 weeks after reconstitution of primary recipients). These data indicate that HSCs are contained within the CD244$^-$ fraction, while at least some transiently reconstituting MPPs are contained within the CD244$^+$ fraction of bone marrow cells.

CD48 Is Expressed by Restricted Progenitors but Not by HSCs or MPPs

By microarray analysis (Table S3), the SLAM family member CD48 was expressed at significantly higher levels on CD45$^+$ cells as compared to HSCs or MPPs (fold change >3). CD48 is a ligand for CD244 (Engel et al., 2003). To test whether CD48$^+$ cells were depleted of HSC activity, we performed competitive reconstitution assays in which CD48$^+$ or CD48$^-$ donor bone marrow cells were transplanted into lethally irradiated recipient mice along with a radioprotective dose of recipient-type whole bone marrow cells (Figure 3). Recipients of the CD48$^-$ cells were always long-term multilineage reconstituted by donor cells (five of five mice), while recipients of the CD48$^+$ cells were always reconstituted by B cells (six of six mice) but never by myeloid cells (zero of six) and rarely by T cells (one of six). These data indicate that CD48$^+$ cells include restricted B cell progenitors but that all HSCs and MPPs are CD48$^-$.

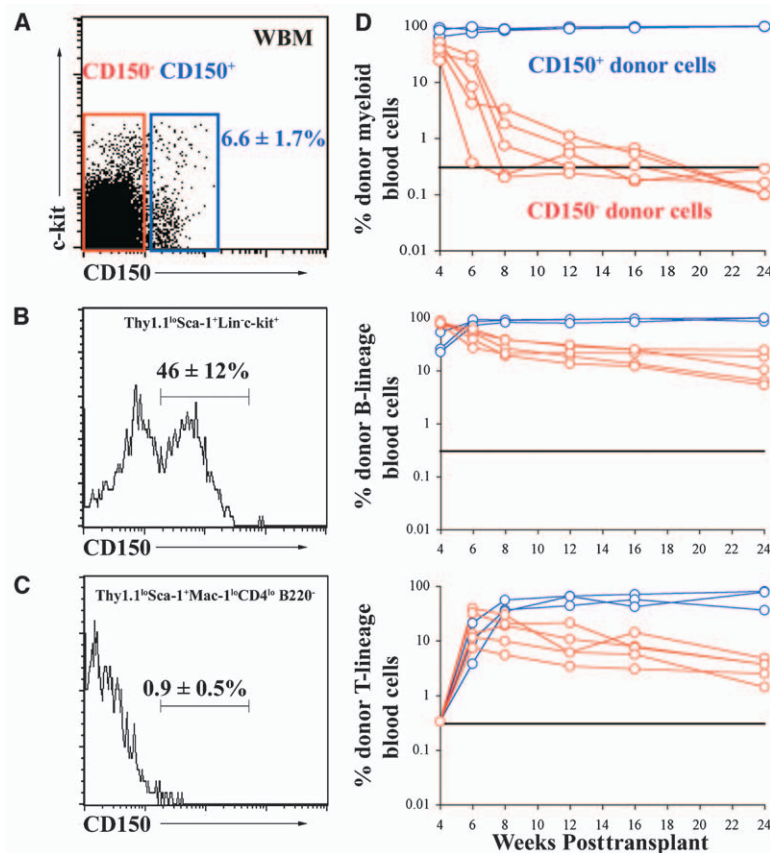Although CD48 was not expressed by multipotent

Figure 1. CD150+ Cells Are Enriched for HSCs while CD150− Cells Include Transiently Reconstituting MPPs

Only 6.6% of bone marrow cells express CD150 (A). CD150 expression was detected among Thy-1^lo^Sca-1+Lineage−c-kit+ HSCs (B) but not among Thy-1^lo^Sca-1+Mac-1^lo^CD4^lo^B220− MPPs (C). CD150+ bone marrow cells (20,000) gave rise to long-term multilineage reconstitution in all recipients (D, blue lines), while 180,000 CD150− bone marrow cells gave transient multilineage reconstitution (D, red lines). Cell doses were based on the fraction of 200,000 whole bone marrow cells that were CD150+ or CD150− as in prior HSC marker studies (Uchida and Weissman, 1992; Morrison et al., 1995). The black line at 0.3% represents the background threshold, meaning that reconstitution could not be detected in mice falling below this line. Data are from one of two independent experiments that gave similar results. Each line represents the frequency of donor-derived myeloid, B, or T cells in a single mouse.

progenitors, it was expressed by most progenitors that formed myeloerythroid colonies in culture in addition to B lineage progenitors (Figures 3D and 3E). Of all colony-forming progenitors from bone marrow, 83.4% ± 3.7% fell within the CD48+CD244+CD150− population, which accounts for only 5.1% ± 0.4% of bone marrow cells (Figure 3E). Some additional erythroid (BFU-E) and megakaryocyte (CFU-Meg) progenitors fell within the CD48+CD244−CD150− population (Figure 3E). While CD150 is expressed by HSCs and CD244 is expressed by transiently reconstituting MPPs, most colony-forming progenitors express CD48. Each of these markers is thus expressed at a different stage of the hematopoiesis hierarchy.

A SLAM Code for Hematopoietic Stem Cells

These results raised the possibility that HSCs and other progenitors could be isolated based on combinations of SLAM family members. HSC activity was contained in the CD150+ but rarely in the CD150− fraction (Figure 1D), the CD244− but not the CD244+ fraction (Figure 2D), and the CD48− but not the CD48+ fraction (Figure 3D). The CD150+CD48−CD244− fraction of bone marrow cells represented only 0.0084% ± 0.0028% of whole bone marrow cells. Since CD150+CD48− cells were uniformly CD244− (Figures 4A and 4B), we tested the reconstituting potential of CD150+CD48− cells. On average, one out of every 4.8 ± 2.7 (21%) injected cells engrafted and yielded long-term multilineage reconstitution (Figure 4C; Table S5). These results are similar to

those obtained with Thy-1^lo^Sca-1+Lineage−c-kit+ cells (one in 4.9 ± 2.5 engrafted and yielded long-term multilineage reconstitution; Table S1), indicating that the simple combination of CD150 and CD48 can highly enrich HSCs.

To test whether the combination of CD150 and CD48 with other markers might yield an even more highly enriched population of HSCs, we competitively reconstituted irradiated mice with single CD150+CD48−Sca-1+Lineage−c-kit+ cells in five independent experiments (Table 2). Only 0.0058% ± 0.0012% of bone marrow cells were CD150+CD48−Sca-1+Lineage−c-kit+. One CD150+CD48−Sca-1+Lineage−c-kit+ cell was sorted per well, and then the contents of each well were individually injected into the recipient mice. We visually confirmed the presence of a single cell per well prior to injection and functionally confirmed the presence of a single cell per well in control studies (Figure S1). One out of every 2.1 CD150+CD48−Sca-1+Lineage−c-kit+ cells (47%) engrafted and gave long-term multilineage reconstitution. In contrast, the CD150− subset of Thy-1^lo^Sca-1+Lineage−c-kit+ cells (Figure 1B) did not give long-term multilineage reconstitution (data not shown). The combination of CD150 and CD48 with previously identified HSC markers significantly increased HSC purity.

HSC SLAM Receptor Expression Is Conserved among Mouse Strains

One impediment in the identification of HSCs is that some of the best markers, including Thy-1 and Sca-1,
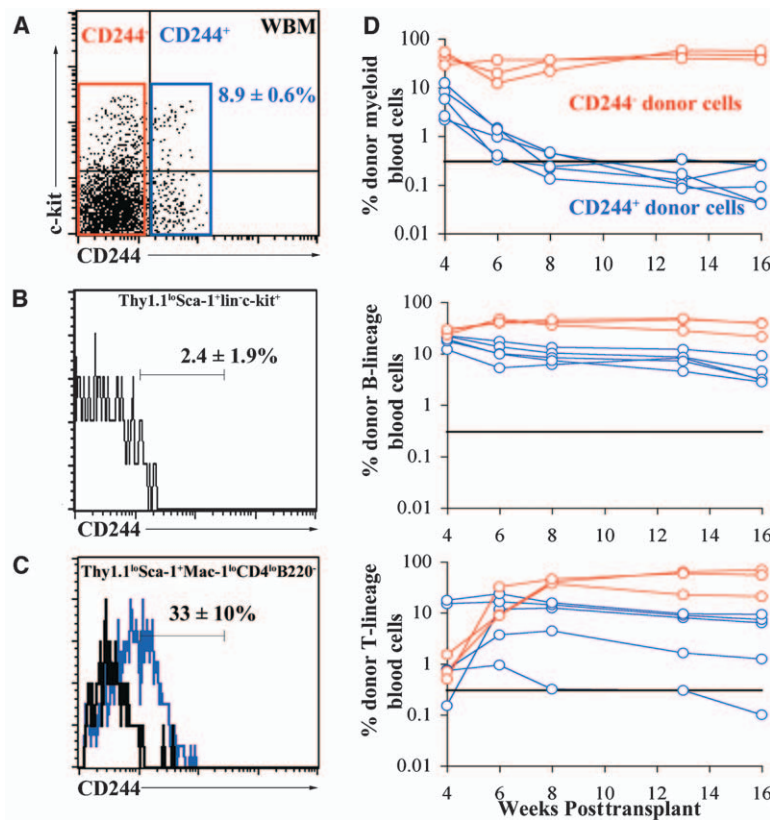
Figure 2. HSCs Are CD244$^-$ while Transiently Reconstituting Multipotent Progenitors Are CD244$^+$

Only 8.9% of bone marrow cells express CD244 (A). CD244 expression was not detected within the Thy-1^{lo}Sca-1$^+$Lineage$^-$c-kit$^+$ HSC population (B) but was detected on approximately 33% of Thy-1^{lo}Sca-1$^+$Mac-1^{lo}CD4^{lo}B220$^-$ MPPs (C). Note that the black histogram represents background fluorescence, while the blue histogram represents staining with the directly conjugated anti-CD244 antibody. CD244$^+$ bone marrow cells (20,000) gave transient multilineage reconstitution in all recipients (blue lines), while 180,000 CD244$^-$ bone marrow cells gave long-term multilineage reconstitution in all recipients (red lines). The data are from one of two independent experiments that gave similar results.

are not conserved among mouse strains (Spangrude and Brooks, 1992; Spangrude and Brooks, 1993). To test whether the HSC SLAM markers are conserved among mouse strains, we isolated CD150$^+$CD48$^-$ cells from the bone marrow of Balb/c and DBA/2 mice, which arise from distinct breeding lineages as compared to C57BL mice (Jackson Laboratory, 1997). CD150$^+$CD48$^-$ cells were rare in Balb/c and DBA/2 mice, representing 0.016% ± 0.002% or 0.028% ± 0.007% of bone marrow cells, respectively (Figures 5A and 5B). While Balb/c mice have a similar frequency of HSCs in their bone marrow as C57BL mice, DBA/2 mice have a 2- to 3-fold increase in HSC frequency (deHaan et al., 1997).

Ten male CD150$^+$CD48$^-$ cells from either strain were injected into lethally irradiated female recipients, along with a radioprotective dose of 200,000 female bone marrow cells. Blood chimerism was tested by quantitative (real-time) PCR using primers that amplify *SRY*, a Y chromosome (donor) marker. Sixteen weeks after transplantation, 11 out of 16 Balb/c recipients were reconstituted (>1.5%) by male cells, averaging 10.8% ± 14.3% of blood cells (Figure 5E). Seven out of 15 DBA/2 recipients were clearly reconstituted (>0.6%) by male cells, averaging 3.5% ± 3.8% of blood cells (Figure 5F). Lower levels of DBA/2 reconstitution were expected, given that DBA/2 mice have more competing HSCs in cotransplanted female bone marrow. Two additional DBA/2 mice exhibited very low levels of male cells (~0.2%) and were not considered HSC reconstituted.

To confirm that these mice exhibited multilineage reconstitution, three reconstituted mice and one unreconstituted mouse from each strain were sacrificed,

and myeloid (Mac-1$^+$B220$^-$CD3$^-$), B (B220$^+$CD3$^-$Mac-1$^-$), and T (CD3$^+$Mac-1$^-$B220$^-$) cells were isolated from their spleens. In each case, the reconstituted mice had male cells in all three lineages, while the unreconstituted mice did not (Figures 5E and 5F). These results correspond to 1 out of 9.1 Balb/c CD150$^+$CD48$^-$ cells (11%) and 1 out of 16.4 DBA CD150$^+$CD48$^-$ cells (6%) giving long-term multilineage reconstitution. HSCs from Balb/c and DBA/2 mice are also highly enriched within the CD150$^+$CD48$^-$ population.

Identifying HSCs using Simple Markers that Yield High Purity in Functional Assays

One out of 4.8 (21%) CD150$^+$CD48$^-$ cells from C57BL mice gave long-term multilineage reconstitution (Figure 4, Table S5). This raised the possibility of identifying HSCs in tissue sections using a simple two-color stain. Initial studies revealed that a subset of megakaryocytes also appeared CD150$^+$CD48$^-$ in sections (data not shown), partially explaining why not every CD150$^+$CD48$^-$ cell gave long-term multilineage reconstitution. To enhance our ability to reliably identify HSCs in tissue sections, we sought an additional marker that would distinguish HSCs from megakaryocytes. CD41 is a commonly used marker of megakaryocyte lineage cells (Phillips et al., 1988; Na Nakorn et al., 2003). Although CD41 is expressed by primitive HSCs, CD41 is downregulated by HSCs during the transition to definitive hematopoiesis, and most adult HSCs do not express CD41 (Ferkowicz et al., 2003; Mikkola et al., 2003). By flow cytometry, 37% ± 5% of CD150$^+$CD48$^-$ cells were CD41$^+$ (Figure S2A), and megakaryocytes (which can
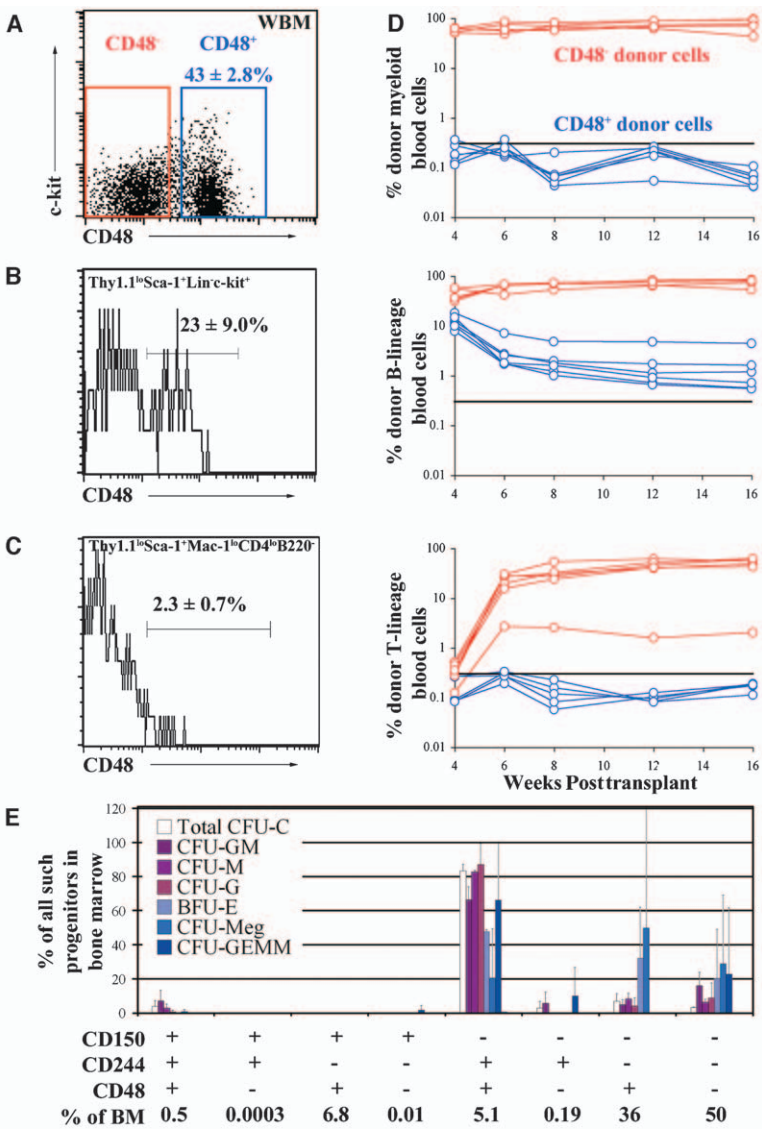
Figure 3. HSCs and MPPs Are Contained within the CD48− Fraction while Colony-Forming Progenitors Are Mainly in the CD48+ Fraction of Bone Marrow Cells

Forty-three percent of bone marrow cells express CD48 (A). CD48 expression was detected in the Thy-1^loSca-1^+Lineage^−c-kit^+ HSC population (B) but not in Thy-1^loSca-1^+Mac-1^loCD4^loB220^− MPPs (C). All multilineage reconstituting activity was contained within the CD48− cell fraction (D). CD48+ bone marrow cells (80,000) gave rise only to B cells in all recipients (blue lines), while 120,000 CD48− bone marrow cells gave long-term multilineage reconstitution in all recipients (red lines). CD48−c-kit^+ cells are highly enriched for HSCs but are not visible in (A) because they represent only 0.1% of bone marrow cells. The vast majority of bone marrow cells that formed myeloerythroid colonies in methylcellulose were CD48+CD244+CD150− (E). Each bar represents the percentage of total colony-forming progenitors from unfractionated bone marrow that are contained within the given cell population. For example, 83.4% of all colonies (CFU-C, white bar) formed by bone marrow cells originate from the CD48+CD244+CD150− population.

be recognized by their unique size and morphology) were excluded when sections were examined for CD150+CD48−CD41− cells (Figure 6C). The exclusion of CD41+ cells thus further enhances the purity of CD150+CD48− HSCs.

To confirm that adult HSCs were CD41−, even in extramedullary tissues, we sorted CD41+ and CD41− cells from the spleen after cyclophosphamide/G-CSF treatment (Figure S2B). Cyclophosphamide/G-CSF treatment leads to the mobilization of HSCs from the bone marrow, increasing the frequency of HSCs in the spleen (Morrison et al., 1997c). While CD41− cells always (five of five) gave long-term multilineage reconstitution in recipient mice, we never (zero of five) detected reconstitution from CD41+ cells (Figure S2C). Adult HSCs do not express detectable CD41 in mobilized spleen, consistent with previous studies of adult bone marrow.

To test the purity of CD150+CD48−CD41− cells, we isolated this population from normal bone marrow (where they represented 0.0065% ± 0.0009% of cells)

and mobilized spleen (where they represented 0.0063% ± 0.0005% of cells), and injected single cells into irradiated mice in competitive reconstitution assays. One out of every 2.2 bone marrow CD150+CD48−CD41− cells (45%) gave long-term multilineage reconstitution (Table 2). One out of every three CD150+CD48−CD41− cells from mobilized spleen (33%; four of 12 mice) gave long-term multilineage reconstitution (Table 2), a dramatic increase in purity relative to mobilized Thy-1^loSca-1^+Lineage^−c-kit^+ cells (Morrison et al., 1997c). This two-color stain thus simplifies and improves HSC isolation.

Extramedullary HSCs Associate with Sinusoidal Endothelium

Prior studies have imaged the interaction of primitive hematopoietic progenitors with osteoblasts in the endosteum of bone marrow (Zhang et al., 2003; Arai et al., 2004; Wilson et al., 2004) (see Figures S2D and S2E for schematic of bone marrow and spleen), but HSCs have not been imaged in sites of extramedullary hematopoi-
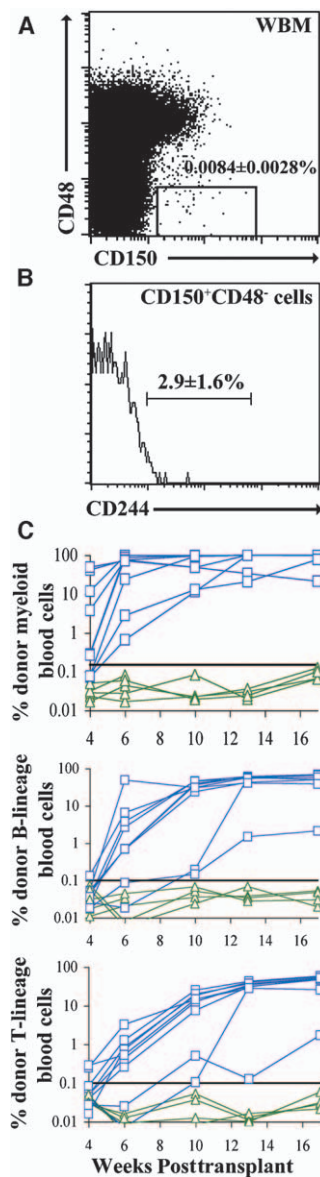
Figure 4. CD150$^+$CD48$^-$CD244$^-$ Cells Are Highly Enriched for Long-Term Reconstituting HSCs

CD150$^+$CD48$^-$ cells represented only 0.0084% of bone marrow cells (A), and these cells were uniformly negative for CD244 expression (B). Injection of three donor-type CD150$^+$CD48$^-$ cells into lethally irradiated recipient mice in a competitive reconstitution assay led to long-term multilineage reconstitution by donor cells in nine of 14 mice (blue lines) and no reconstitution in five of 14 mice (green lines).

esis. To address this, we have examined the spleens of cyclophosphamide/G-CSF-mobilized mice. CD150$^+$CD48$^-$CD41$^-$ cells were mainly associated with sinusoidal endothelium in parafollicular areas (red pulp) of the mobilized spleen. To be sure that rare differentiated cells could not appear to be CD150$^+$CD48$^-$CD41$^-$ by immunohistochemistry, we further stained these cells with lineage markers (Gr-1, Mac-1, B220, CD2, CD3, CD4, and CD8) to exclude myeloid, B, and T cells. Only

0.0059% ± 0.005% of cells in sections were CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ (identified by scanning 633,000 cells in sections from three independent spleens; a frequency comparable to that detected by flow cytometry), and all of these were found in the para-follicular red pulp. Moreover, these cells expressed Sca-1 and CD45, just like the CD150$^+$CD48$^-$CD41$^-$ cells identified by flow cytometry (Figure S3). Of the 37 CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells that were observed, 23 (62%) were in contact with sinusoidal endothelial cells (Figure 6A). Another 14 CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells (38%) were not visibly in contact with endothelium, though they were always near (<10 cell diameters) sinusoids in the red pulp (Figure 6B). Compared to other cells in red pulp, CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells were more than 3-fold more likely to be in contact with sinusoids, as 18.2% ± 2.2% of all nucleated cells in the red pulp were in contact with sinusoids. The proportion of cells associated with sinusoids in white pulp was much lower. These data suggest that most HSCs within the mobilized spleen are associated with sinusoidal endothelium.

Bone Marrow HSCs Associate with Sinusoidal Endothelium in Addition to Endosteum

We observed a total of 35 CD150$^+$CD48$^-$CD41$^-$ Lineage$^-$ cells in sections from three femurs. These cells represented 0.0067% ± 0.0016% of cells in the sections (identified by scanning 522,000 cells in sections from three independent bones; a frequency comparable to that detected by flow cytometry). As in the spleen, the CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells identified in sections expressed Sca-1 and CD45 (Figure S3). Most of these cells (20 of 35; 57%) were located in the trabecular zone, and the remaining cells were distributed throughout the diaphysis (shaft). Five of the cells (14%) were associated with endosteum, consistent with prior studies (Zhang et al., 2003; Arai et al., 2004; Wilson et al., 2004), and another nine cells were not associated with recognizable cell types. Most of the CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells that we observed (21 of 35; 60%) were in contact with sinusoidal endothelium (Figure 6C). Only 10.1% ± 1.4% of all nucleated bone marrow cells were in contact with sinusoids, indicating that CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells were 6-fold more likely to contact sinusoids. These data may underestimate the fraction of HSCs near endosteum because a minority of bone fragments peeled away from slides after sectioning, raising the possibility that some HSCs may have been lost. Nonetheless, many HSCs appear to localize to sinusoidal endothelium.

Discussion

By comparing the gene expression profiles of highly purified Thy-1^{lo}Sca-1$^+$Lineage$^-$c-kit$^+$ HSCs (Table S1), Thy-1^{lo}Sca-1$^+$Mac-1^{lo}CD4^{lo} MPPs (Table S2), and CD45$^+$ bone marrow cells, we found that SLAM family receptors were differentially expressed among hematopoietic stem and progenitor cells. CD150 was expressed by HSCs but not by MPPs or restricted hematopoietic progenitors (Figures 1 and 3). CD244 was expressed by MPPs and by some restricted pro-

Table 2. Competitive Reconstitution of Irradiated Mice with Single Cells from Various HSC Populations Reveals that Diverse HSC Populations Are Able to Engraft with High Efficiency in Lethally Irradiated Mice

HSC Population	Experiment	Cells that Engrafted	Engrafted Mice with Long-Term Multilineage Reconstitution	Cells that Long-Term Multilineage Reconstituted
BM CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$ c-kit$^+$	1	67% (ten of 15)	70% (seven of 10)	47% (seven of 15)
	2	67% (six of nine)	66% (four of six)	44% (four of nine)
	3	44% (eight of 18)	88% (seven of eight)	39% (seven of 18)
	4	53% (eight of 15)	88% (seven of eight)	47% (seven of 15)
	5	58% (seven of 12)	100% (seven of seven)	58% (seven of 12)
	Mean ±SD	58% ± 10%	82% ± 14%	47% ± 7% (one in 2.1)
BM CD150$^+$CD48$^-$CD41$^-$	1	30% (three of 10)	100% (three of three)	30% (three of ten)
	2	100% (five of five)	60% (three of five)	60% (three of five)
	Mean	65%	80%	45% (one in 2.2)
Mobilized spleen CD150$^+$CD48$^-$CD41$^-$	1	42% (five of 12)	80% (four of five)	33% (one in 3.0)

Single cells were sorted into different wells of 96 well plates, and the wells were visually inspected to confirm that only a single cell was sorted (see Figure S1). Then the contents of each well were individually injected along with 300,000 recipient CD150$^-$ bone marrow cells.

genitors but not by HSCs (Figure 2). CD48 was expressed by restricted B lineage and myeloerythroid lineage progenitors but not by multipotent progenitors (Figure 3). This demonstrates that SLAM family members are differentially expressed among hematopoietic progenitors in a way that correlates with primitiveness. This is the first example of a single family of cell surface receptors that is precisely differentially expressed among stem and progenitor cells in a way that predicts developmental potential.

Improving HSC Purification
SLAM family members are so precisely differentially expressed that HSCs are very highly enriched within the CD150$^+$CD48$^-$ cell population, which represents only 0.0084% ± 0.0028% of C57BL bone marrow cells (Figure 4). Twenty-one percent of CD150$^+$CD48$^-$ cells (one in 4.8) that were intravenously injected into irradiated mice gave long-term multilineage reconstitution in limit dilution reconstitution assays (Table S5). This demonstrates that two SLAM family markers yield HSC enrichments that are comparable to much more complex combinations of markers (Morrison et al., 1995; Spangrude et al., 1995; Osawa et al., 1996; Wagers et al., 2002; Benveniste et al., 2003; Chen et al., 2003). Moreover, 47% of single CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$c-kit$^+$cells (one in 2.1) and 45% of single CD150$^+$CD48$^-$CD41$^-$ cells (one in 2.2) yield long-term multilineage reconstitution in irradiated mice (Table 2). SLAM family markers simplify and enhance HSC purification and are conserved among mouse strains (Figure 5).

Most of the best protocols for HSC isolation yield populations from which 20% of intravenously injected cells engraft and give long-term multilineage reconstitution (Morrison et al., 1995; Spangrude et al., 1995; Osawa et al., 1996; Wagers et al., 2002; Benveniste et al., 2003; Chen et al., 2003). This raised the question of whether the maximum efficiency with which HSCs are able to engraft after intravenous transplantation is only around 20% (one in five) or whether available markers only yield populations of HSCs that are 20% pure (Benveniste et al., 2003). Recently, HSC purities of 40%–96% were achieved by gating more restrictively on pre-

viously identified markers (Matsuzaki et al., 2004; Takano et al., 2004). However, many HSCs are excluded from these very restrictive gates, raising the possibility that highly efficient engraftment is a property of only a subset of HSCs (Christopherson et al., 2004). The fact that we have achieved 47% functional HSC purity using new markers that include most or all bone marrow HSCs suggests that highly efficient engraftment is a property of nearly all HSCs in young adult bone marrow.

The ability of SLAM family members to improve HSC purity is even more dramatic in the mobilized spleen. Only 1% of Thy-1^{lo}Sca-1$^+$Lin$^-$c-kit$^+$ cells (one in 100) from cyclophosphamide/G-CSF-mobilized spleen give long-term multilineage reconstitution, raising the possibility that mobilized HSCs might engraft less efficiently than HSCs from normal bone marrow (Morrison et al., 1997c). The fact that 33% of CD150$^+$CD48$^-$CD41$^-$ cells (one in 3.0) from the mobilized spleen were able to give long-term multilineage reconstitution indicates that even mobilized HSCs are able to reconstitute with high efficiency.

We have not yet detected an HSC defect in *CD150*-deficient mice (Wang et al., 2004). Hematopoiesis appeared normal in *CD150$^{-/-}$* mice, as the cellularity and composition of the bone marrow, blood, spleen, and thymus were grossly normal (Figure S4A). We did not observe any differences between *CD150$^{-/-}$* mice and littermate controls in terms of complete blood cell counts (data not shown) or the frequency of colony-forming progenitors in the bone marrow (Figure S4B). We also did not detect any difference in HSC frequency or reconstituting potential upon transplantation into irradiated mice (Figures S4C and S4D). Thus CD150 is not required for HSC maintenance or function in young adult mice but could regulate more subtle aspects of HSC function.

The mechanism by which the tandemly arrayed genes at the SLAM locus are differentially expressed among primitive hematopoietic progenitors is uncertain. Loss of *CD150* did not detectably affect the expression pattern of CD48 or CD244 on HSCs or on bone marrow as a whole (data not shown). Thus CD150 did not appear to repress CD48 or CD244. Understanding the mechanisms responsible for the differential expres-
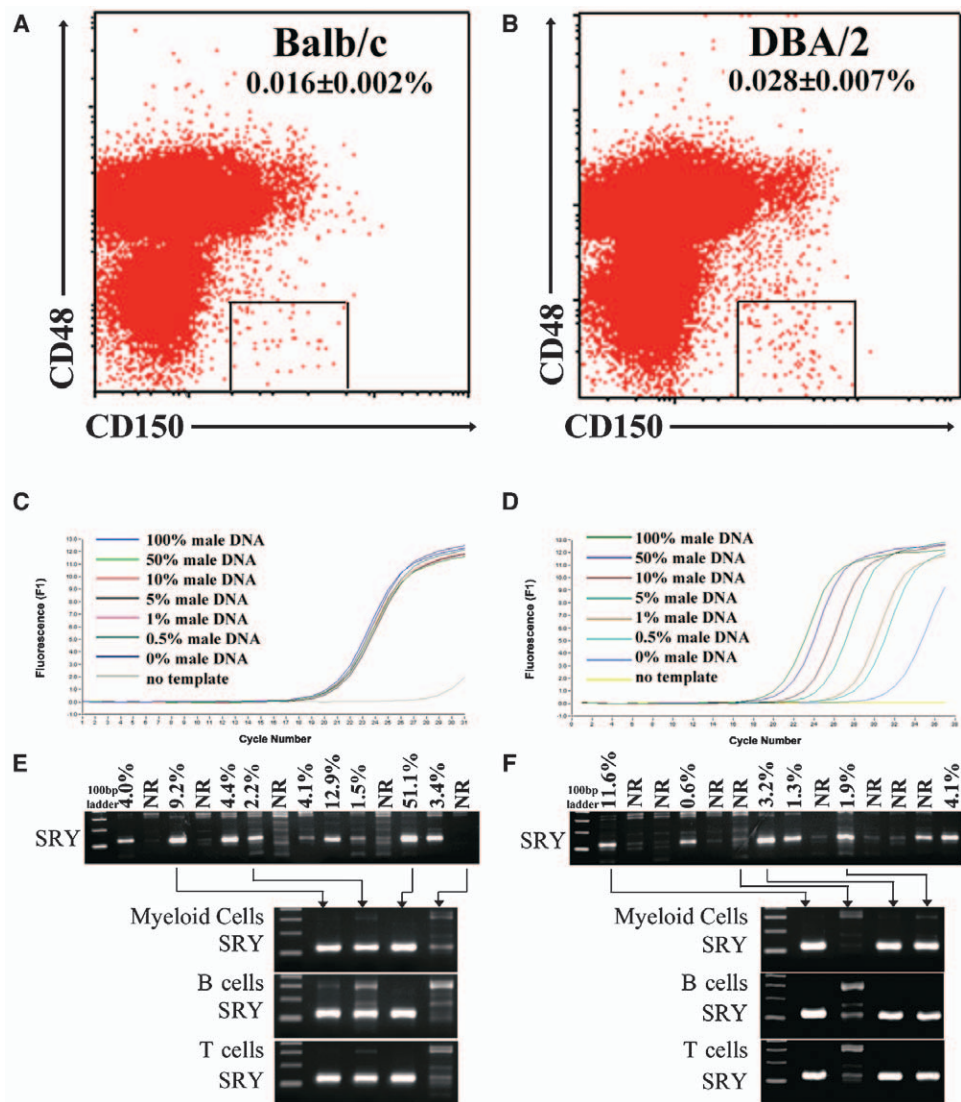
Figure 5. Balb/c and DBA/2 HSCs Are Enriched in the CD150⁺CD48⁻ Population

CD150⁺CD48⁻ cells are rare in Balb/c (A) and DBA/2 (B) bone marrow. Ten CD150⁺CD48⁻ cells from male donors were injected into lethally irradiated female recipients in competitive reconstitution assays. At week 16, DNA from peripheral leukocytes was extracted and subjected to qPCR using primers specific for genomic SRY to determine the relative contribution of male cells to the peripheral blood of female recipients. Control DNA from untreated male mice was diluted into control DNA from untreated female mice as indicated (C and D) to construct a standard curve. DNA content was normalized based on genomic β-actin amplification (C), and the level of male DNA (SRY) in each sample was determined by qPCR (D). Eleven out of 16 Balb/c recipients were clearly reconstituted by male cells ([E]; 14 mice are shown). Seven out of 15 DBA/2 recipients were clearly reconstituted by male cells ([F]; 14 mice are shown). Splenic myeloid (Mac-1⁺B220⁻CD3⁻), B (B220⁺CD3⁻Mac-1⁻), and T (CD3⁺Mac-1⁻B220⁻) cells were isolated and examined for donor cell chimerism in three reconstituted mice and one unreconstituted mouse from each strain. In each case, the reconstituted mice showed multilineage reconstitution.

sion of SLAM genes might provide important insights into the determination of stem cell identity, given that SLAM receptors are differentially expressed in a way that correlates with primitiveness.

Identifying HSC Niches in Tissue Sections
The identification of a simple combination of markers that includes most or all HSCs made it possible for the first time to examine HSC localization in bone marrow and in extramedullary tissues using markers that were validated in functional assays to yield high HSC purity.

Many HSCs appeared to be in contact with sinusoidal endothelium in bone marrow, while other HSCs appeared to be associated with endosteum (Figure 6C). The precise proportion of bone marrow HSCs in each location is uncertain, given that half of CD150⁺CD48⁻CD41⁻ cells failed to give long-term multilineage reconstitution in irradiated mice. HSCs that localized to endosteum were presumably associated with osteoblasts, consistent with prior studies (Calvi et al., 2003; Zhang et al., 2003; Arai et al., 2004; Visnjic et al., 2004). Since bone marrow cells enter circulation through sinusoids,
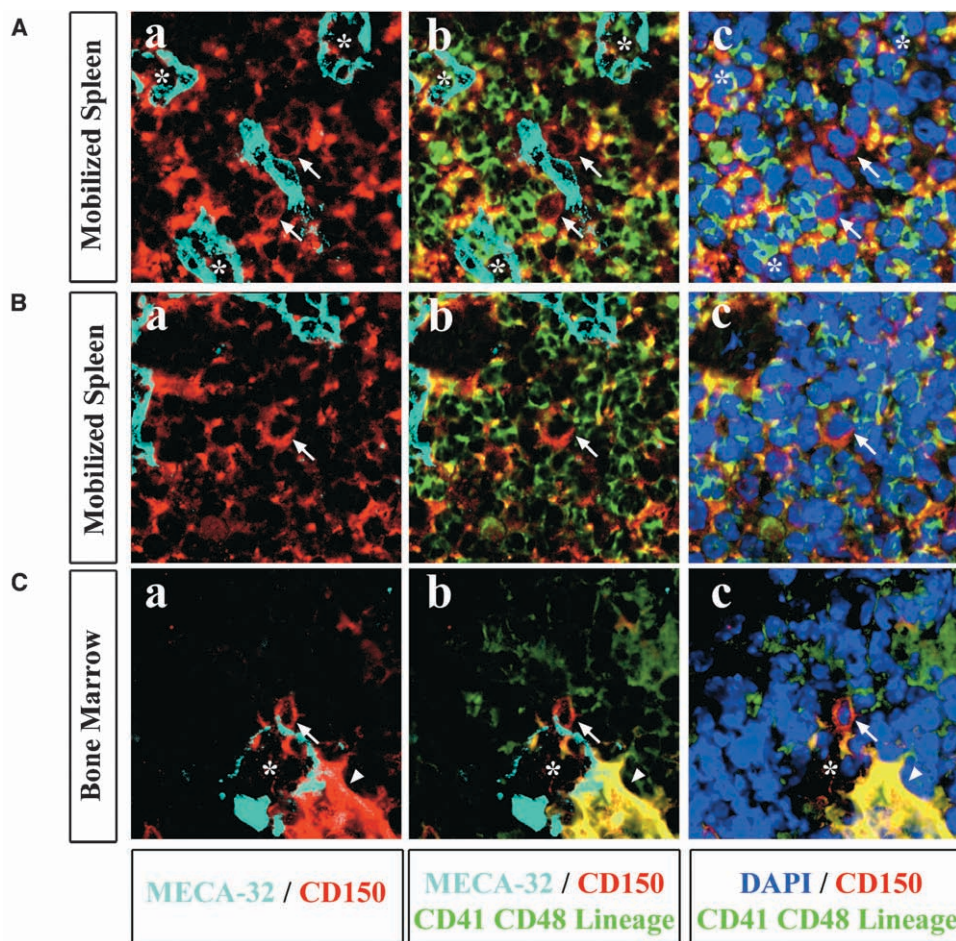
Figure 6. HSCs Are Associated with Sinusoidal Endothelial Cells in the Spleen and Bone Marrow

In the cyclophosphamide/G-CSF mobilized spleen, CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells represented 0.0059% ± 0.005% of cells in sections. Twenty-three of 37 (62%) of these cells were in contact with sinusoidal endothelial cells ([A], arrows; * indicates the sinusoid lumen). Another 38% (14 of 37) of CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells were located in parafollicular regions that were often near sinusoids but not visibly in contact ([B], arrow). It is unclear whether these cells are migrating to/from sinusoids or whether there are multiple niches within the spleen. In normal bone marrow, CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells represented 0.0067% ± 0.0016% of cells in sections. Some of these cells were closely associated with endosteum (data not shown). However, most of these cells contacted sinusoidal endothelium ([C], arrow). Note the large megakaryocyte that was also associated with the sinusoid ([C], arrowhead). These images each represent a single optical section, but a series of images through each cell is shown in Figure S6. Platelets are evident as red (CD150$^+$) and green (CD41$^+$) specks throughout the images from spleen.

the association of HSCs with sinusoidal endothelium explains how HSCs could be mobilized into circulation within minutes of treatment with certain cytokines (Laterveer et al., 1995). Bone marrow HSCs appear to localize to at least two distinct niches, defined by the association of HSCs with sinusoidal endothelial cells and endosteum in different locations within the bone marrow.

We found no heterogeneity within the CD150$^+$ CD48$^-$CD41$^-$ HSC population that correlated with the difference in localization. For example, only 3.8% of CD150$^+$CD48$^-$CD41$^-$ cells in normal bone marrow were in S/G2/M phases of the cell cycle (Figure S5), consistent with previous studies reporting that HSCs are mainly quiescent (Morrison and Weissman, 1994; Cheshier et al., 1999). This means that the vast majority of CD150$^+$CD48$^-$CD41$^-$ cells associated with both sinus-

oids and endosteum must be in the G0/G1 phase of the cell cycle.

HSCs in the spleens of mice treated with cyclophosphamide/G-CSF appeared to usually associate with sinusoidal endothelium as well (Figure 6A). However, since two-thirds of single CD150$^+$CD48$^-$CD41$^-$ cells failed to give long-term multilineage reconstitution in irradiated mice, it is not possible to infer the precise proportion of HSCs associated with sinusoids as compared to other sites. These results suggest that sinusoidal endothelial cells create a niche that sustains HSCs in extramedullary tissues. These HSCs are unlikely to be migrating into circulation because the number of HSCs in the spleen continues to increase for several days after the onset of mobilization (Morrison et al., 1997c). Moreover, most of the CD150$^+$CD48$^-$CD41$^-$ Lineage$^-$ cells we observed in normal adult bone mar-

row were also in contact with endothelial cells. The association of many HSCs with sinusoidal endothelium during steady-state hematopoiesis suggests that this is not a transient interaction.

The observation that HSCs interact with sinusoidal endothelial cells in bone marrow and extramedullary tissues is consistent with observations that endothelial cells express factors that regulate HSC maintenance and function. Definitive HSCs first arise during embryonic development among endothelial cells in the dorsal aorta and have a very close developmental relationship with the endothelial lineage (Kennedy et al., 1997; North et al., 2002; Oberlin et al., 2002; Kubo and Alitalo, 2003). Coculture of HSCs with vascular endothelial cells from hematopoietic and nonhematopoietic tissues maintains the repopulating capacity of HSCs under conditions in which HSCs would otherwise differentiate or die (Cardier and Barbera-Guillem, 1997; Ohneda et al., 1998; Li et al., 2004). This indicates that endothelial cells express factors that promote the maintenance of HSCs. Endothelial cells appear to regulate the function of primitive hematopoietic progenitors via multiple mechanisms in vivo (Heissig et al., 2002; Avecilla et al., 2004). These observations suggest that endothelial cells create a niche in hematopoietic tissues that sustains a substantial fraction of the HSC pool.

Neural stem cells are also thought to localize to vascular niches (Palmer et al., 2000; Capela and Temple, 2002; Louissaint et al., 2002), and endothelial cells can support the self-renewal of neural stem cells in culture (Shen et al., 2004). This raises the possibility that endothelial cells are generally important in the construction of mammalian stem cell niches and that sinusoidal endothelium represents a specialization adapted for the maintenance of HSCs.

SLAM family markers represent an important new resource for studying HSC biology. The use of SLAM markers in future studies should refine our understanding of stem cell identity and the role of the environment in regulating HSC function in vivo.

Experimental Procedures

All mice used in this study were housed in the Unit for Laboratory Animal Medicine at the University of Michigan. Donor hematopoietic cells were obtained from adult (6- to 8-week-old) C57BL/Ka-CD45.2:Thy-1.1 mice. Recipient mice in reconstitution assays were adult C57BL/Ka-CD45.1:Thy-1.2 mice.

Flow-Cytometric Isolation of Stem and Progenitor Cells
Bone marrow cells were flushed from the long bones with Hank's buffered salt solution without calcium or magnesium, supplemented with 2% heat-inactivated calf serum (GIBCO, Grand Island, New York; HBSS[+]). Cells were triturated and filtered through nylon screen (45 μm, Sefar America, Kansas City, Missouri) to obtain a single cell suspension.

Thy-1^{lo}Sca-1^{+}Lineage^{−}c-kit^{+} HSC and Thy-1^{lo}Sca-1^{+}Mac-1^{lo}CD4^{lo}B220^{−} MPPs were isolated as previously described (Morrison and Weissman, 1994; Morrison et al., 1997b). For isolation of Thy-1^{lo}Sca-1^{+}Lineage[−]c-kit^{+} cells, whole bone marrow cells were incubated with unconjugated monoclonal antibodies to lineage markers including B220 (6B2), CD3 (KT31.1), CD5 (53-7.3), CD8 (53-6.7), Gr-1 (8C5), and Ter119. Following dilution, pelleted cells were resuspended in anti-rat IgG-specific F(ab)$_2$ conjugated to phycoerythrin (PE; Jackson ImmunoResearch, West Grove, Pennsylvania). Cells were then stained with directly conjugated antibodies to Sca-1 (Ly6A/E-APC), c-kit (2B8-biotin), Thy-1.1 (19XE5-FITC), Mac-1

(M1/70-PE), and CD4 (GK1.5-PE). Progenitors were often enriched by preselecting for Sca-1^{+} or c-kit^{+} cells using paramagnetic microbeads (Miltenyi Biotec, Auburn, California) and autoMACS. For isolation of Thy-1^{lo}Sca-1^{+}Mac-1^{lo}CD4^{lo}B220^{−} MPPs, the directly conjugated antibodies described above were combined with anti-B220-Tricolor (6B2, Caltag, Burlingame, California).

Cells sorted based on CD150 expression were incubated with unconjugated antibody to CD150 (26D12; DNAX, Palo Alto, California) and subsequently stained with goat anti-rat IgG F(ab)$_2$ fragment conjugated to FITC, PE, or APC (Jackson ImmunoResearch). When CD150 was combined with lineage markers, directly conjugated antibodies were used to stain lineage markers. Cells sorted according to CD41, CD48, or CD244 expression were stained with directly conjugated anti-CD41 (MWReg30-FITC) or anti-CD48 (HM48-1-FITC or PE) or with directly conjugated CD244.2 (2B4-FITC). Cells were resuspended in 2 μg/ml 7-AAD (Molecular Probes) or DAPI to discriminate live from dead cells. All flow cytometry was performed on a FACS Vantage dual laser flow cytometer (Becton-Dickinson, San Jose, California).

Long-Term Competitive Reconstitution Assays
Adult recipient mice were irradiated with an Orthovoltage X-ray source delivering approximately 300 rad/min. C57BL and DBA/2 recipient mice received two doses of 550–570 rad, delivered at least 2 hr apart. Balb/c recipients received two doses of 495rad. When HSCs/MPPs were tested for reconstituting potential, the donor (CD45.2[+]) population was sorted, and the number of cells to be injected per mouse was resorted into individual wells of a 96-well plate containing 200,000 CD45.1[+] whole bone marrow cells in HBSS[+]. The contents of individual wells were injected into the retroorbital venous sinus of individual lethally irradiated CD45.1[+] recipients. For at least 16 weeks after transplantation, blood was obtained from the tail veins of recipient mice, subjected to ammonium-chloride potassium red cell lysis (Morrison and Weissman, 1994), and stained with directly conjugated antibodies to CD45.2 (104, FITC), B220 (6B2), Mac-1 (M1/70), CD3 (KT31.1), and Gr-1 (8C5) to monitor engraftment.

Immunofluorescence Analysis of Hematopoietic Tissue Sections
Femurs from 6- to 12-week-old wild-type mice were embedded in 8% gelatin (Sigma) in phosphate buffer and snap frozen in –80°C N-methylbutane chilled in a slurry of ethanol and dry ice. Sections (7 μm) were generated using the CryoJane system (Instrumedics, Hackensack, New Jersey) with coated slides and a tungsten carbide blade. Details of the staining procedure can be found in Supplemental Experimental Procedures. To obtain spleens from cyclophosphamide/G-CSF-mobilized mice, mice were injected intraperitoneally with 4 mg of cyclophosphamide ($\sim$200 mg/kg; Bristol-Myers Squibb) and then on successive days with 5 μg of human G-CSF by subcutaneous injection ($\sim$250 μg/kg per day; Amgen Biologicals). Mice were sacrificed after 4 days of G-CSF treatment, and their spleens were dissected, sectioned, and fixed.

RNA Amplification for Microarray Analysis
RNA extraction, amplification, and microarray analysis were performed as described (Iwashita et al., 2003). See Supplemental Experimental Procedures for details.

Supplemental Data
Supplemental data include six figures, five tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/121/7/1109/DC1/.

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Supplemental Data

SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells

Mark J. Kiel, Ömer H. Yilmaz, Toshihide Iwashita, Osman H. Yilmaz, Cox Terhorst, and Sean J. Morrison

Supplemental Experimental Procedures

Methylcellulose Culture

Bone marrow cells, or single resorted hematopoietic progenitors were plated in wells of 96-well plates (Corning, Corning NY) containing 100μl 1.0% methylcellulose (Stem Cell Technologies, Vancouver BC) as previously described (Morrison et al., 1996). The methylcellulose was supplemented with 20% charcoal absorbed fetal bovine serum (Cocalico, Reamstown PA), 1% BSA (Sigma), 1% penicillin/streptomycin (Gibco) 50ng/ml stem cell factor (SCF), 10ng/ml interleukin-3 (IL-3), 10ng/ml interleukin-6 (IL-6), 3U/ml erythropoietin (Epo), 10ng/ml Flt-3 and 10ng/ml thrombopoietin (Tpo). All cytokines were obtained from R&D Systems (Minneapolis MN). Colonies were maintained at 37°C in humidified chambers containing 6% CO_2. Colony formation was scored after 10-14 days of culture.

RNA Amplification for Microarray Analysis

Methods for RNA extraction, amplification, and microarray analysis were as described (Iwashita et al., 2003). Briefly, total RNA was extracted from 3 independent, freshly isolated aliquots of 5,000 Thy-1^{lo}Sca-1$^+$Lineage$^-$c-kit$^+$ cells, 5,000 Thy-1^{lo}Sca-1$^+$Mac-1^{lo}CD4^{lo}B220$^-$ cells, or 8,000 CD45+ cells using Trizol with 250μg/ml glycogen (Roche Diagnostic Corporation, Indianapolis IN). The extracted RNA (30μl volume) was treated for 20min at 37°C with 2μl of RNase-free DNaseI (2U/μl; Ambion, Austin TX) in the presence of 2μl of RNase inhibitor (10U/μl) (Invitrogen). The RNA was then purified with RNeasy Mini Kit (Qiagen, Valencia CA) according to the manufacturer's instructions and washed 3 times with 500μl of RNase-free water in a Microcon YM-100 (Millipore, Bedford MA). After adding 0.025μg T7-d(T)$_{24}$ primer (containing a T7 RNA polymerase binding sequence; 5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG(T)24,; Proligo, Boulder CO), the RNA was dried down to 2.5μl. RNA was amplified through two consecutive rounds of amplification using a modified version of the method of Baugh et al. (Baugh et al., 2001). To make cDNA, first strand was synthesized using T7-d(T)$_{24}$ primer. After second strand synthesis, complementary RNA (cRNA) was generated by T7 RNA polymerase (Promega, Madison WI). For the second round of amplification, first strand cDNA was synthesized using random hexamers and second strand was synthesized using the T7-d(T)$_{24}$ primer. The double stranded cDNA was resuspended with 22μl RNase-free water and transcribed to cRNA with the biotin labeling kit (BioArray Highyield RNA transcript labeling kit (T7), Enzo Diagnostics, Farmingdale NY) for 12 hr. cRNA was purified using the RNeasy Mini Kit. 60μg of biotinylated cRNA were obtained from two rounds of RNA amplification from 5,000 cells.

Hybridization and Data Analysis

After fragmentation, 15μg of HSC cRNA were hybridized per chip to Mouse Genome U74 Arrays (version 2 Chips A, B and C; Affymetrix). The chips were hybridized and scanned according to the manufacturer's instructions. Signal intensities were analyzed as described previously (Iwashita et al., 2003). To measure fold changes, all values less than 100 were set to 100. The significance of differences in signal intensity for each probe set was evaluated by T-test using the log$_{10}$ transformed values from 3 independent replicates per cell type.

Quantitative Real Time-PCR (qPCR)

2,000 to 10,000 cells were directly sorted into 400ul Trizol (Ambion, Austin TX) containing 250ug/ml glycogen (Roche, Indianapolis IN). RNA was extracted according to manufacturer's instructions. The extracted RNA (30ul volume) was treated for 20 min at 37°C with 2ul RNase-free DNase-1 (2U/ul; Ambion) in the presence of 2ul RNase inhibitor (10U/ul; Invitrogen). The RNA was then purified using an RNeasy Mini Kit (Qiagen, Valencia CA) according to manufacturer's instructions and washed three times with 500ul RNase-free water. The RNA was used for making cDNA by reverse transcription with 1ug random hexamer. The cDNA was extracted with phenol-

chloroform and precipitated with 20ug glycogen. After dissolving the cDNA with RNase-free water, cDNA equivalent to 200 cells was used for each PCR reaction. qPCR was performed using at least two independent cell samples. Primers were designed to have a Tm of ~59°C and to generate short amplicons (100-150bp). The PCR reactions were performed using a LightCycler (Roche Diagnostic Corporation) according to the manufacturer's instructions. The RNA content of samples compared by qPCR was normalized based on the amplification of hypoxanthine phosphoribosyl transferase (HPRT). In addition to confirming the specificity of the qPCR reactions by examining the melting curves of the products, qPCR products were separated in 2% agarose gels to confirm the presence of a single band of the expected size. To estimate the difference in the expression levels of individual RNAs between samples, we assumed that one cycle difference in the timing of amplification by qPCR was equivalent to a 1.9-fold difference in expression level (90% amplification efficiency), a typical value (Fink et al., 1998).

Experiments to examine the engraftment of female Balb/c and DBA/2 mice by male HSCs involved quantitative PCR of extracted genomic DNA rather than cDNA but otherwise similar methods as described above were employed. Genomic DNA was extracted from blood cells or splenocytes and resuspended at 10-50ng/ul. The level of male cell chimerism was tested by qPCR using primers that amplified genomic *SRY* and *β-actin* (to normalize DNA content). Known dilutions of male DNA into female DNA were used to establish standards. Individual samples were normalized to the 100% male reference standard according to the following formula: $1.9 \exp (C_T SRY^{100\%} - C_T SRY)/1.9 \exp(C_T actin^{100\%} - C_T actin)$, where $C_T SRY^{100\%}$ represents the *SRY* crossing point for the reference sample and $C_T actin^{100\%}$ the *β-actin* crossing point for the reference sample. Level of chimerism in experimental animals was estimated by linear regression using all control samples.

CD150-Deficient Mice
CD150-deficient mice (Wang et al., 2004) were backcrossed for more than ten generations onto the C57BL/6 background. Genotyping was performed using the primers: *CD150$^{+/+}$*-F (5′CAC CCC AGG CAC TTC ACC AAG TCC CAG AGC) and *CD150$^{+/+}$*-R (5′GCT GGC TGT GAA CTC CCA TCC CAT CCT TG); *CD150$^{-/-}$*-F(5′GAA AAT TGG GTC AGG AAG TAA ACG CAG) and *CD150$^{-/-}$*-R(5′GGG CCA GCT CAT TCC TCC CAC).

Immunofluorescence Analysis of HSC Localization in Tissue Sections
Freshly dissected undecalcified femurs from 6-12 week old wild-type C57BL mice or spleens from 6-12 week old mice treated with CY/G-CSF were embedded in 8% gelatin (Sigma) in 0.1M phosphate buffer pH7.4 and snap-frozen in N-methylbutane chilled in a slurry of ethanol and dry ice. Sections were generated using the CryoJane tape transfer system (Instrumedics, Hackensack NJ) with methacrylate coated slides, a tungsten carbide blade (Diamond Knives, Wilmington DE) and Bright Cryostat at -24°C. The 7um sections were air-dried overnight at room temperature and subsequently fixed in -20°C acetone for 15 min. Slides were then blocked with 20% goat serum in 0.1M phosphate buffer (pH 7.4) for 40 min prior to antibody staining. Slides were first incubated in 0.03ug/ul 26D12 anti-CD150 antibody (DNAX, Palo Alto CA) for 1 hr and rinsed. Goat anti-rat IgG conjugated to Alexa555 (Molecular Probes) was added at 1/200 dilution and rinsed. Slides were then incubated in 0.1ug/ul rat IgG (Sigma-Aldrich) for 10min and rinsed. To avoid the problem of other cells in the tissue sections appearing to be CD150$^+$CD48$^-$CD41$^-$ in a way that was not detected by flow-cytometry we included the additional panel of lineage markers in the tissue immunofluorescence stains. By themselves these lineage markers should exclude differentiated cells, providing redundancy within the panel of markers for the exclusion of non-HSCs. Unconjugated anti-CD48 and FITC-conjugated anti-CD41 antibodies (each 1/100 dilution) as well as FITC-conjugated lineage markers including anti-B220 (stains B cells), CD2 (T cells), CD4 (T cells), CD8a (T cells), Gr-1 (myeloid cells) and CD11b (myeloid cells) antibodies (each 1/200 dilution), and biotinylated pan-endothelial cell antigen (MECA-32) at a 1/25 dilution were then incubated for 1hr in 0.1ug/ul rat IgG and rinsed. CD48 was visualized by incubation with FITC-conjugated goat anti-Armenian hamster (Jackson ImmunoResearch) at a 1/200 dilution for 30min. Pan-endothelial cell antigen was visualized with Alexa647-conjugated streptavidin (Molecular Probes) at a 1/200 dilution. Finally, slides were rinsed twice for 5 min each and mounted without drying using Prolong Antifade (Molecular Probes). To ensure the accuracy of antibody labeling of HSCs, control slides were separately stained as above without addition of CD150 primary antibody. All antibody incubations took place in blocking buffer. All rinse steps were 2 times for 3 min each with blocking buffer unless otherwise indicated. All antibodies were purchased from Becton Dickinson unless otherwise noted. The nuclear dye DAPI was included in all stains to evaluate nuclear morphology and to exclude debris (lacking a nucleus), or dying cells (with fragmented nuclei). Microscopy was performed using an Olympus BX51 florescence microscope or an Olympus FV-500 confocal microscope.

Controls to Ensure that the CD150⁺CD48⁻CD41⁻Lineage⁻ Cells Identified in Tissue Sections Were the Same as the Functionally Characterized CD150⁺CD48⁻CD41⁻ Cells Isolated by Flow Cytometry

We performed a number of controls to confirm that the CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells identified by immunofluorescence in tissue sections were the same as the CD150$^+$CD48$^-$CD41$^-$ HSCs isolated by flow-cytometry. If non-stem cells were included in the CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ population identified in sections, then the frequency of these cells in sections should be higher than the frequency of CD150$^+$CD48$^-$CD41$^-$ cells identified by flow-cytometry. The CD150$^+$CD48$^-$CD41$^-$ population in mobilized spleen by flow-cytometry was $0.0063 \pm 0.0005\%$ whereas the frequency of CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells in spleen sections was 0.0059%. The frequency of CD150$^+$CD48$^-$CD41$^-$ cells in bone marrow by flow-cytometry ($0.0065 \pm 0.0009\%$) also did not differ from the frequency of CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells identified within bone marrow sections ($0.0067 \pm 0.0016\%$). These results demonstrate that the frequency of CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells in sections was not higher than the frequency of CD150$^+$CD48$^-$CD41$^-$ cells identified by flow-cytometry.

To test whether the CD150$^+$CD48$^-$CD41$^-$ cells identified by flow-cytometry represent the same population identified in tissue sections as CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells we examined two independent markers of HSC, Sca-1 and CD45. The HSC activity within the CD150$^+$CD48$^-$ population was also Sca-1$^+$Lineage$^-$c-kit$^+$ (Table 2), consistent with prior studies of HSC marker expression. By flow-cytometry, we found that $88 \pm 3\%$ of CD150$^+$CD48$^-$CD41$^-$ cells were Sca-1$^+$ and in tissue sections we found that 71% of CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells were detectably Sca-1$^+$ (Suppl. Figure 3). This may underestimate the true frequency of Sca-1$^+$ cells in tissue sections since the flow-cytometer is more sensitive than the eye to lower levels of staining, and the Sca-1 stain that we were forced to use in this multi-color analysis was dim. Furthermore, essentially all of the CD150$^+$CD48$^-$CD41$^-$ cells identified by flow-cytometry as well as the CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells identified in tissue sections were CD45$^+$ (Suppl. Figure 3). This demonstrates that most of the cells identified in tissue sections expressed two independent HSC markers also seen on the CD150$^+$CD48$^-$CD41$^-$ population by flow-cytometry.

Supplemental Figure S1.
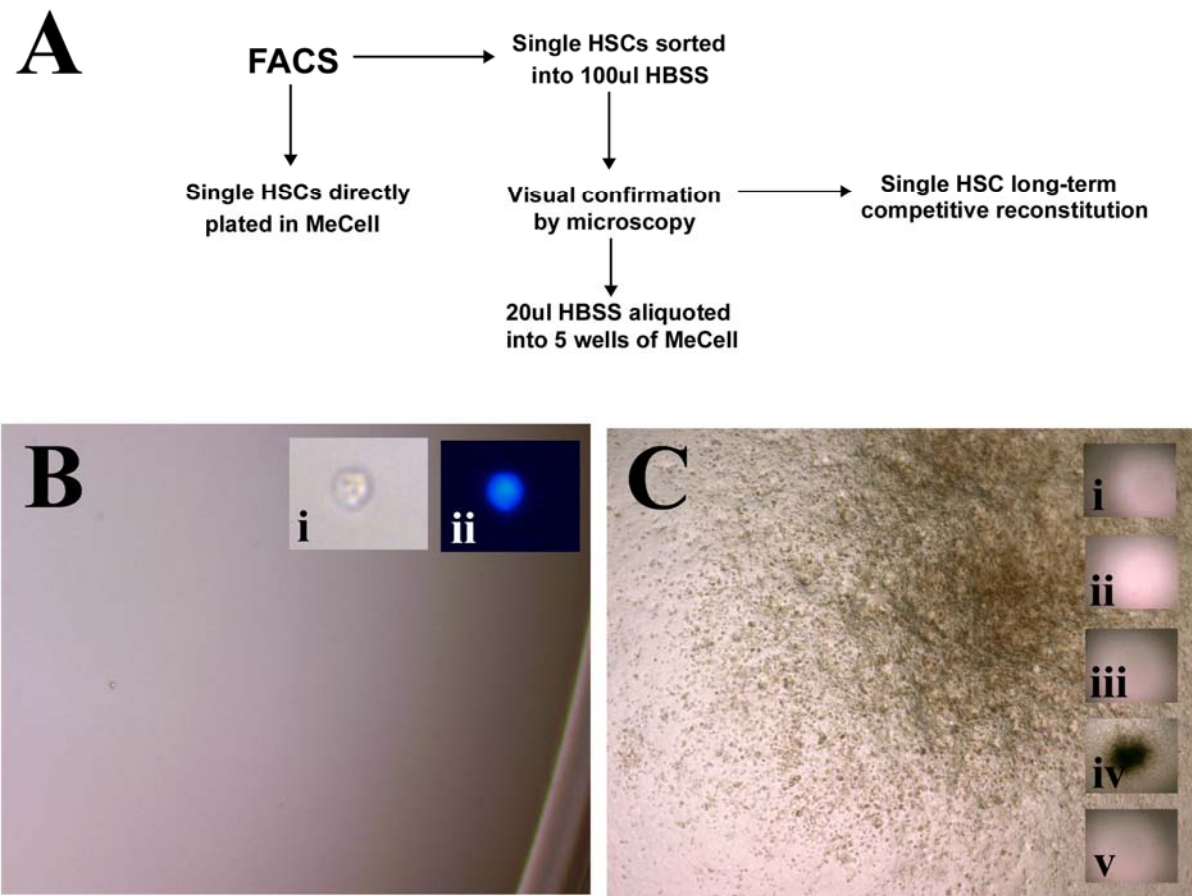
Single $CD150^+CD48^-Sca-1^+Lin^-c-kit^+$ HSCs were sorted and then resorted by flow-cytometry (FACSVantage SE) on counter mode using doublet discrimination (A). Single cells were deposited into 100ul of sterile HBSS buffer containing 2.5ug/ml Hoechst 33342 (Sigma) and 5.0ug/ml verapamil (Sigma) in individual wells of a 96-well plate (B). After the presence of a single cell was assessed using light microscopy (i) and confirmed using UV florescence microscopy (ii), the contents of each well were injected into lethally irradiated recipients along with a radioprotective dose of 300,000 recipient-type $CD150^-$ bone marrow cells. In none of more than 150 wells inspected was more than one cell seen. In control studies to functionally test whether there was only a single cell per well, the contents of each well were divided into five equal volumes and plated into separate wells of methylcellulose and allowed to form colonies (C). In three separate experiments, $90.0 \pm 10.0\%$ of the sets of five methylcellulose wells contained a single hematopoietic colony and in no circumstance (0/48) was more than one colony observed. When single cells were directly plated into methylcellulose, $93.3 \pm 7.6\%$ of single cells formed hematopoietic colonies. There was thus no difference between the clonogenicity of directly plated HSCs or of diluted HSCs (p=0.67). The contents of a representative set of five methylcellulose cultures is shown in the inset (i-v). These results confirm that only a single cell was sorted per well in these experiments.

Supplemental Figure S2. Excluding CD41$^+$ Cells Increases the Purity of HSCs in the CD150$^+$CD48$^-$ Fraction, Allowing Localization of HSCs within the Bone Marrow and Spleen
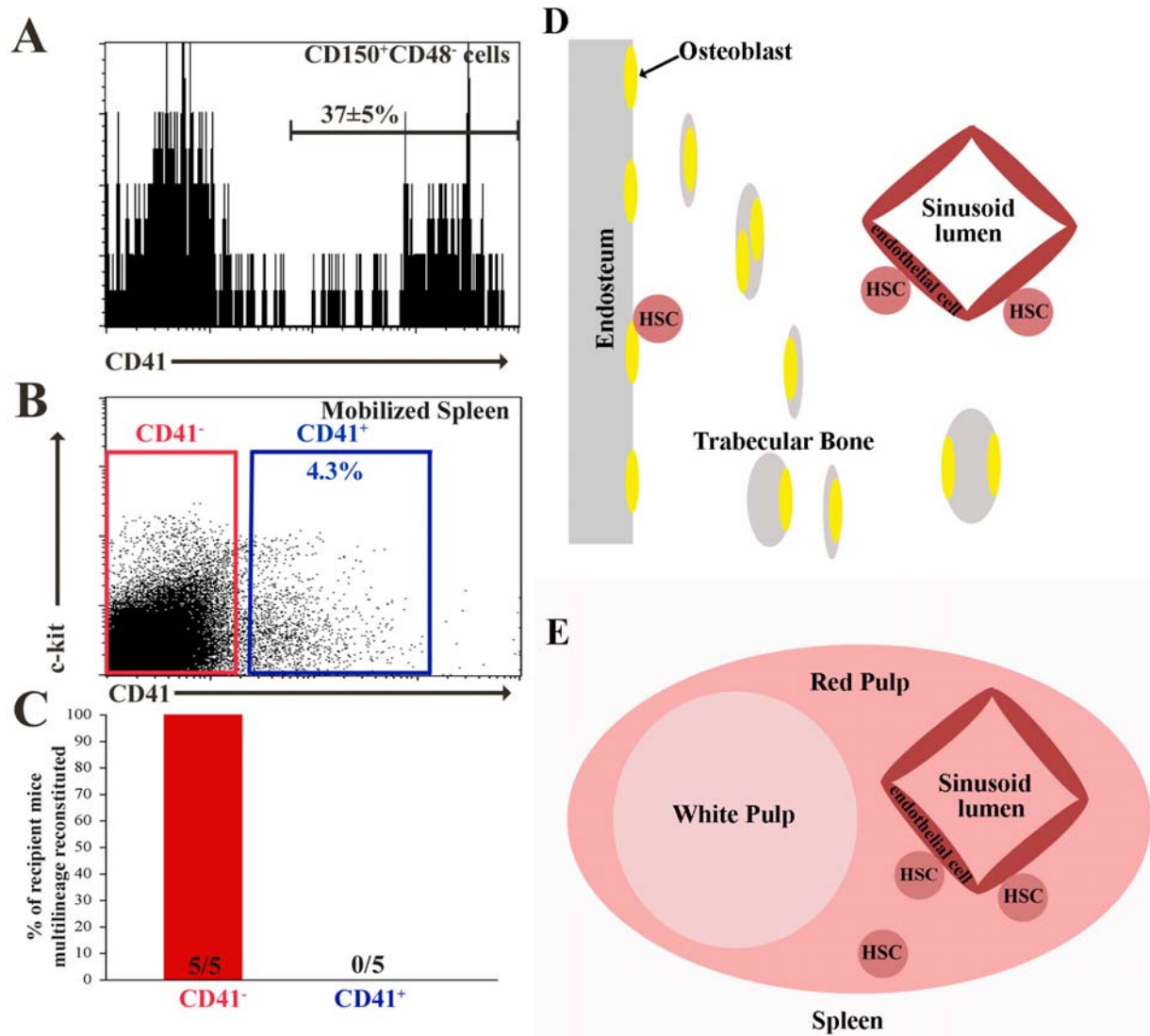
A) $37 \pm 5\%$ of CD150$^+$CD48$^-$ bone marrow cells express CD41. (B) Only 4.3% of splenocytes in cyclophosphamide/G-CSF mobilized mice express CD41. (C) 10,000 CD41$^+$ mobilized splenocytes gave no detectable reconstitution in any recipients (0 of 5 mice, blue), while 200,000 CD41$^-$ mobilized splenocytes gave long-term multilineage reconstitution in all recipients (5 of 5 mice, red). Gross anatomy of bone marrow and spleen are schematized in panels D and E to summarize the interpretation of data presented in Figure 6. HSCs were associated with sinusoidal endothelial cells in the bone marrow (D) and in the parafollicular red pulp of the spleen (E). A subset of HSCs in the bone marrow also localized to the interface of bone and bone marrow (A, the endosteum) where osteoblasts also localize (yellow).

Supplemental Figure S3. CD150$^+$CD48$^-$CD41$^-$ Cells Express CD45 and Sca-1 in Tissue Sections and By Flow Cytometry
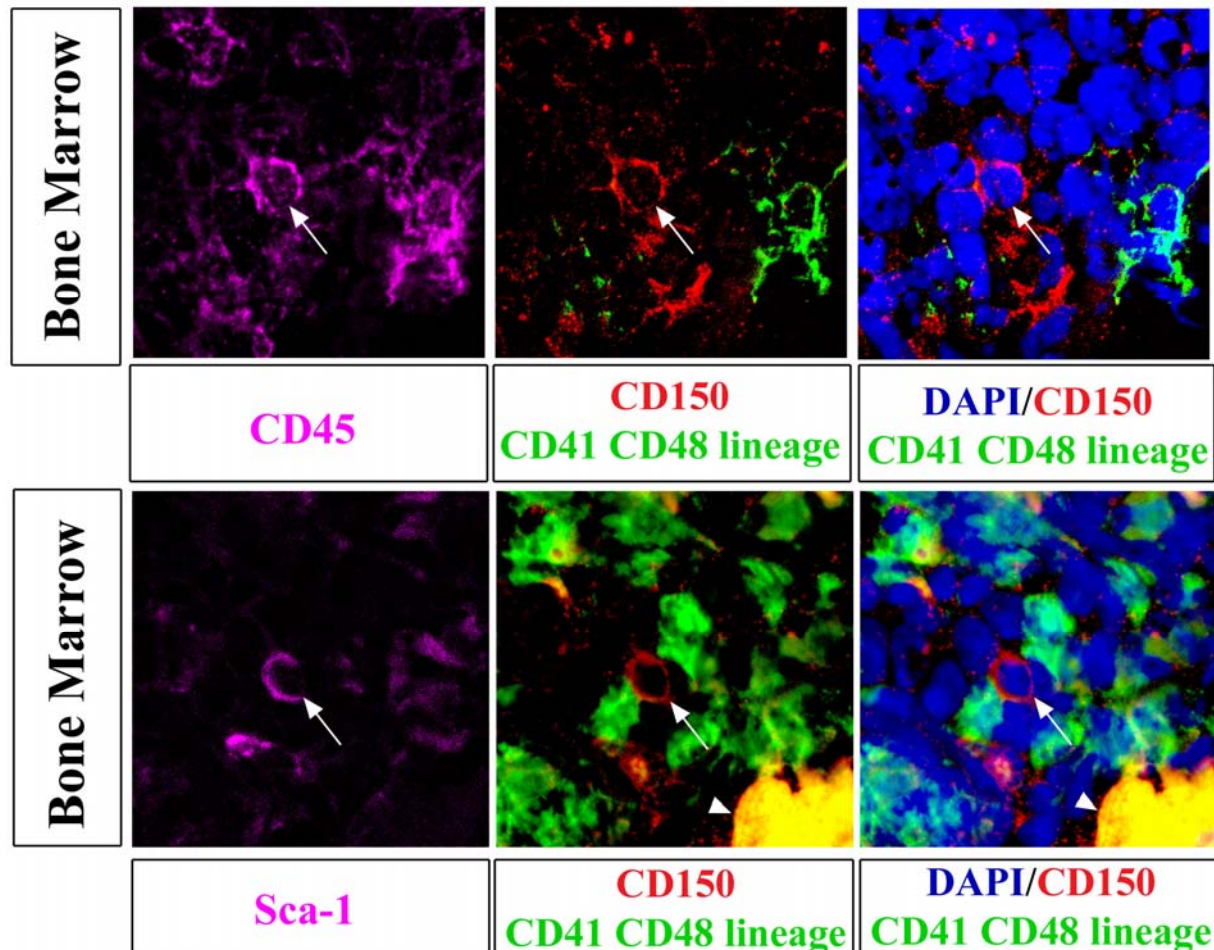

CD150$^+$CD48$^-$CD41$^-$ cells isolated by flow-cytometry were highly enriched for long-term multilineage reconstituting HSCs, and were present at very similar frequencies as CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells identified in tissue sections. Bone marrow CD150$^+$CD48$^-$CD41$^-$ cells isolated by flow-cytometry expressed both CD45 (98% CD45$^+$) and Sca-1 (89 ± 3.5% Sca-1$^+$). CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells identified in bone marrow sections also expressed CD45 (100% CD45$^+$; A) and Sca-1 (71% Sca-1$^+$; B). CD150$^+$CD48$^-$CD41$^-$Lineage$^-$ cells identified in spleen sections also expressed CD45 (100% CD45$^+$) and Sca-1 (80% Sca-1$^+$) (data not shown). The co-expression of these additional HSC markers on both the flow-cytometrically isolated cells and on the cells identified in tissue sections strongly supports the conclusion that this is the same cell population identified by both methods.

Supplemental Figure S4. *CD150*-Deficient Mice Exhibit Normal Hematopoiesis, Colony-Forming (CFU-C) Progenitor Activity, and HSC Frequency and Function
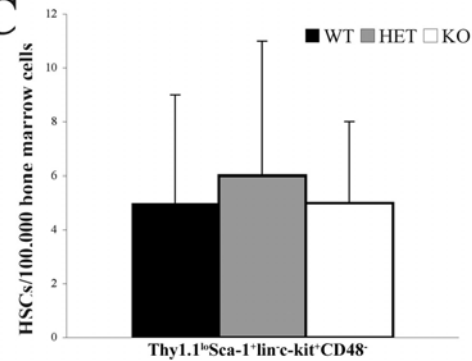
A

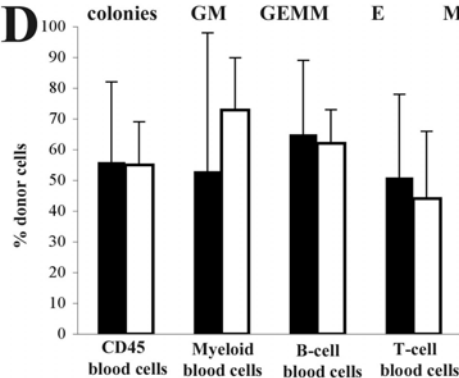|		Bone Marrow		Spleen	
		$CD150^{+/+}$	$CD150^{-/-}$	$CD150^{+/+}$	$CD150^{-/-}$
Cellularity	[$\times 10^7$ cells]	3.2±0.9	3.0±0.4	12±7	11±1
B cells	B220+sIgM+ (%)	9.8±2.3	10.3±2.3	30.0±1.4	30.8±2.2
	B220+sIgM- (%)	13.6±4.1	14.6±3.5	2.2±0.5	2±0.5
pro-B cells	B220+sIgM-CD43lo (%)	5.3±2.2	5.8±1.4		
pre-B cells	B220+sIgM-CD43- (%)	3.1±1.3	3.4±1.2		
T cells	CD3+ (%)	1.7±0.4	1.6±0.3	6.4±0.7	7.3±0.6
Erythroid Progenitors	Ter119+ (%)	22.2±4.9	18.1±2.7	4.4±4.6	2.7±1.5
Myeloid Progenitors	Mac-1+Gr-1+ (%)	28.2±7.1	29.8±4.6	0.7±0.2	0.7±0.2
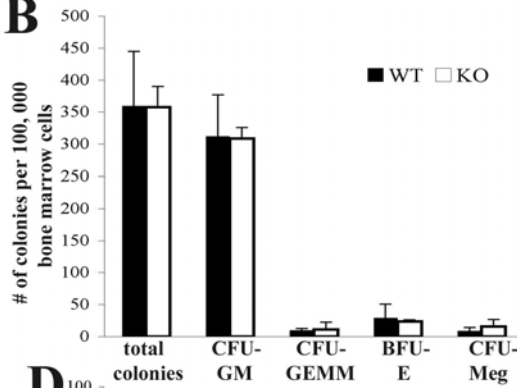
(A) No statistically significant differences were observed in overall cellularity or in the frequencies of myeloid, B, T, or erythroid populations in the bone marrow or spleens of adult *CD150*-deficient mice. There was also no difference in the overall cellularity or frequency of progenitor populations within the thymus (data not shown). Each statistic represents mean ± standard deviation for five mice per genotype. (B) Colony forming assays reveal no differences between $CD150^{+/+}$ and $CD150^{-/-}$ littermates in the frequency of restricted hematopoietic progenitors in the bone marrow (4 independent experiments). (C) No statistically significant difference in the frequency of Thy-1^{lo}Sca-1$^+$lin$^-$c-kit$^+$CD48$^-$ HSCs in $CD150^{+/+}$ (black bars), CD150$^{+/-}$ (gray bars), and $CD150^{-/-}$ (white bars) littermates (n=5 to 7 for each genotype). (D) 200,000 $CD150^{+/+}$ or $CD150^{-/-}$ donor type bone marrow cells were transplanted into irradiated recipient mice along with 200,000 recipient-type bone marrow cells. No statistically significant differences were observed at 16 weeks post-transplantation in the level of donor-type CD45, myeloid, B, or T cells in recipient mice that received $CD150^{+/+}$ or $CD150^{-/-}$ bone marrow cells.

Supplemental Figure S5. Few CD150$^+$CD48$^-$CD41$^-$ Cells Are in Cycle
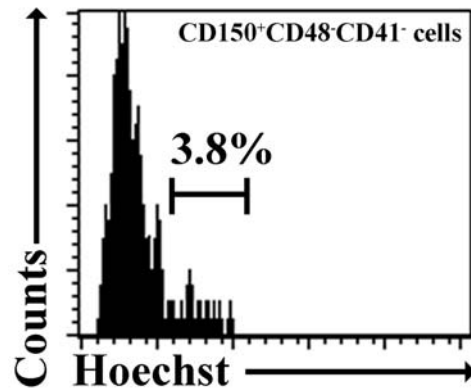
Only 3.8% of bone marrow CD150$^+$CD48$^-$CD41$^-$ cells were in S/G2/M phases of the cell cycle based on Hoechst 33342 staining of DNA content. This is consistent with prior studies that found most adult bone marrow HSCs are quiescent (Morrison and Weissman, 1994; Cheshier et al., 1999).

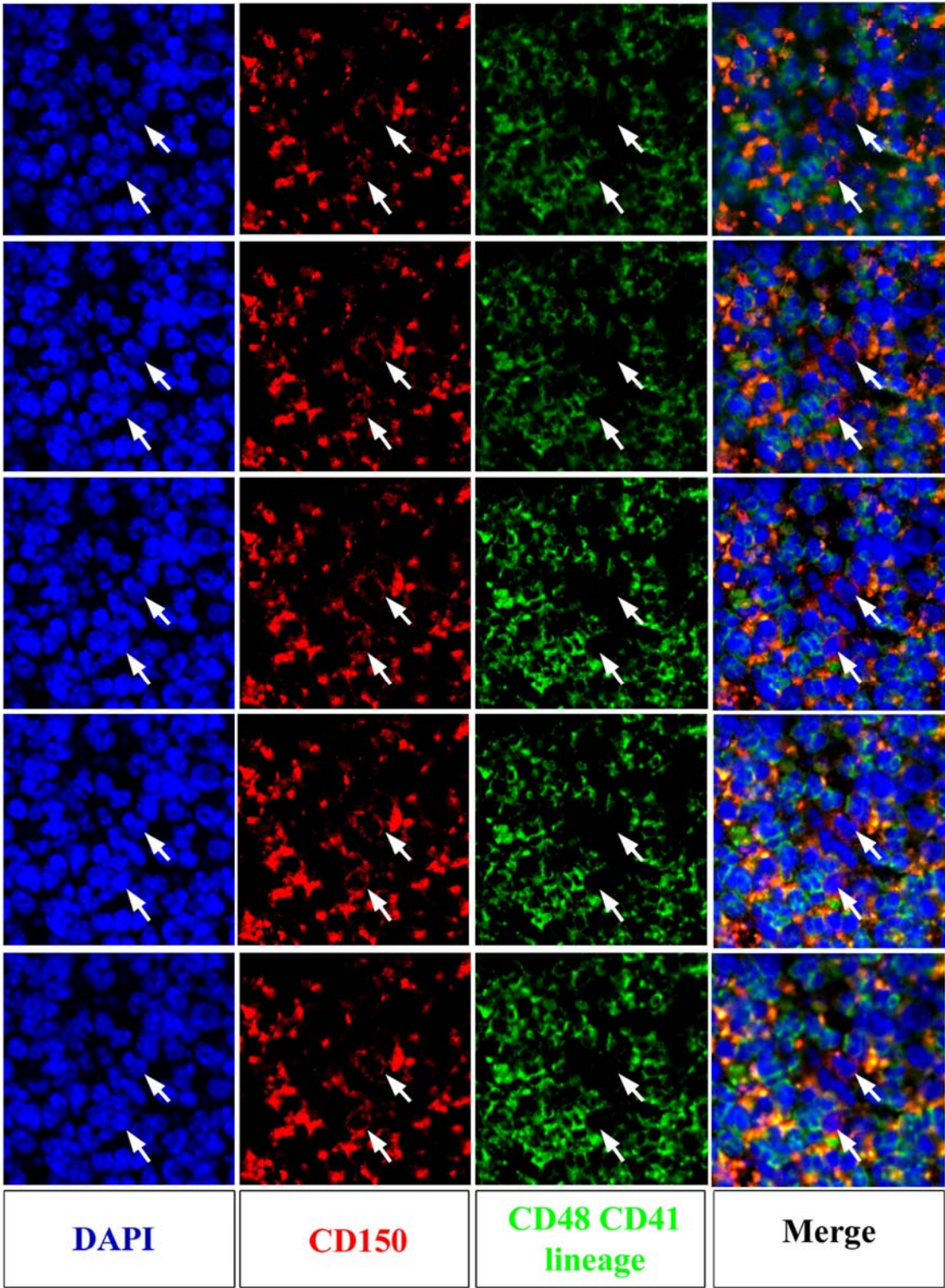Supplemental Figure S6. Serial Optical Sections through the Cells Shown in Figure 6

| DAPI | CD150 | CD48 CD41 lineage | Merge |

Figure 6A

| DAPI | CD150 | CD48 CD41 lineage | Merge |

Figure 6B
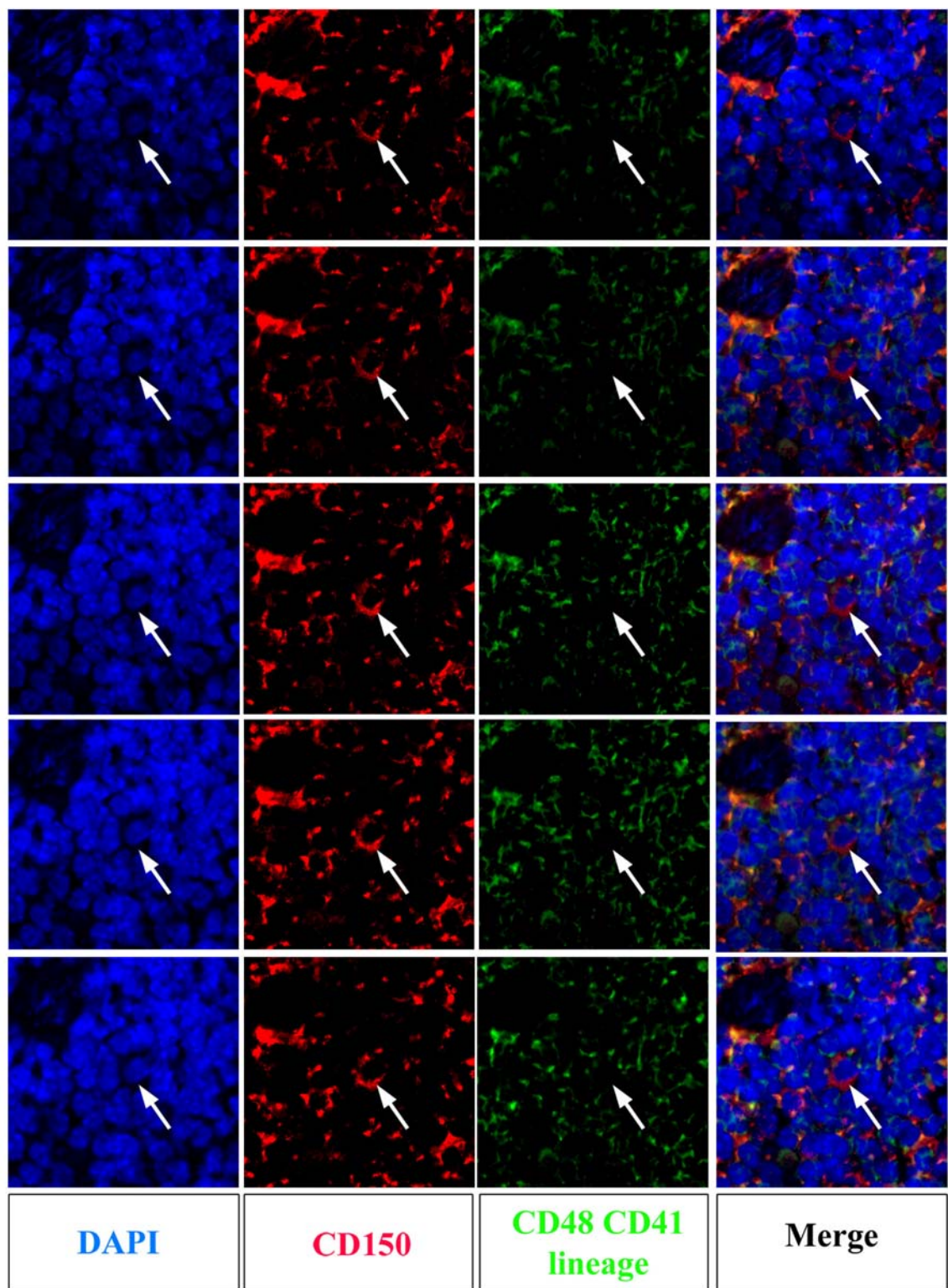
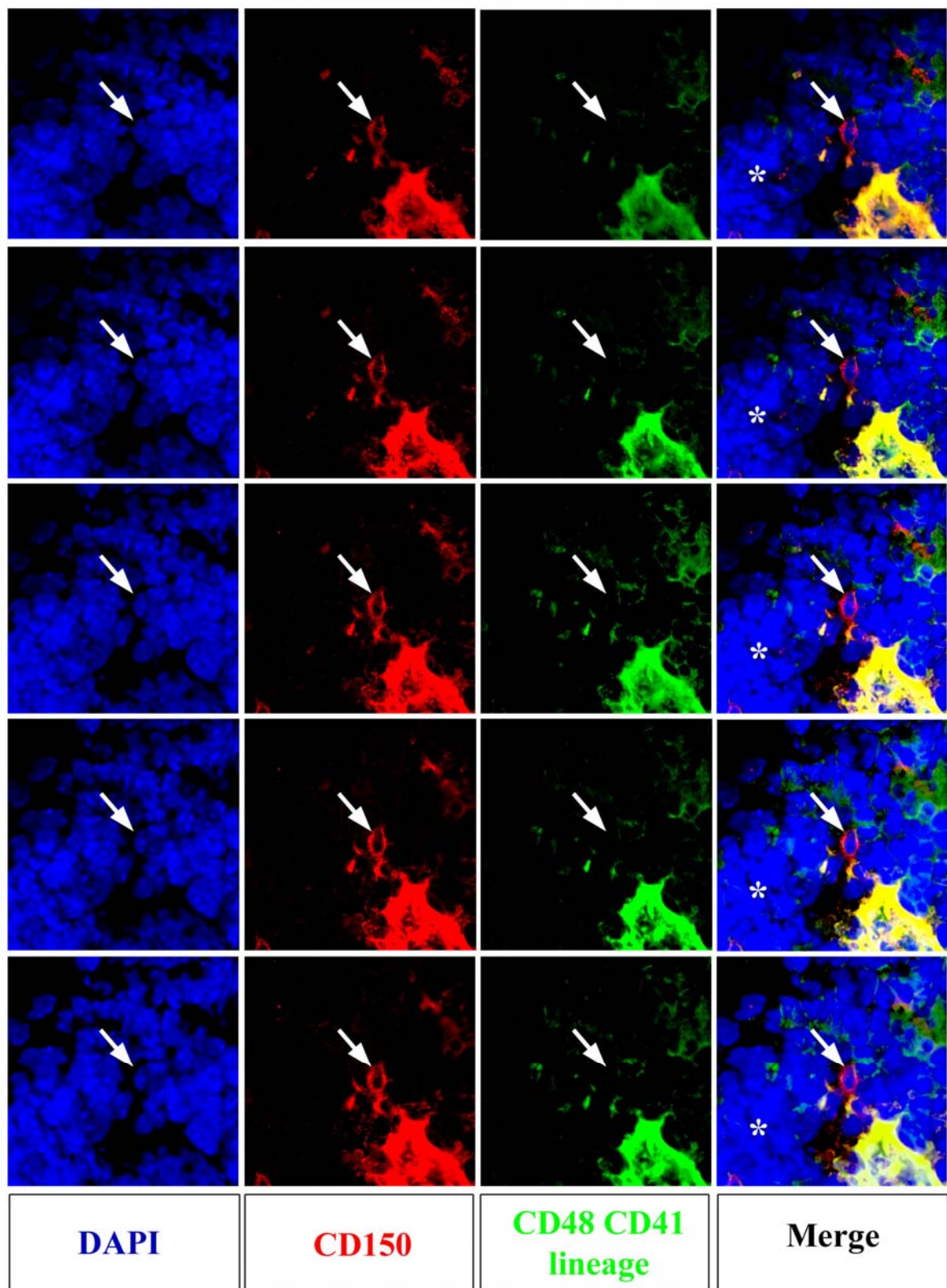| **DAPI** | **CD150** | **CD48 CD41 lineage** | **Merge** |

Figure 6C

A series of optical sections through the images from Figures 6A (A), 6B (B), and 6C (C) are shown such that each row represents a single optical section. White arrows point to the $CD150^+CD48^-CD41^-Lineage^-$ cells in each image. The spleen contains a higher frequency of $CD150^+$ cells than the bone marrow as some lymphocytes, in addition to HSCs, express CD150. Each image, particularly from the spleen, contained 2 to 4 µm particles lacking nuclei that stained for both CD150 (red) and CD41 (green). These appear to be platelets, which are $CD150^+CD41^+$ and are present throughout hematopoietic tissues, particularly in the spleen. Note that HSCs in the bone marrow were sometimes adjacent to clusters of cells that failed to stain with lineage markers (* in C), presumably reflecting clusters of other early hematopoietic progenitors near HSC niches.

Supplemental Table S1. Thy-1^{lo}Sca-1$^+$Lineage$^-$c-kit$^+$ Cells Are Highly Enriched for Long-Term Reconstituting, Multipotent HSCs

Cells Injected	Mice that Engrafted	Frequency of Cells that Engrafted	Engrafted Mice with Long-Term Multilineage Reconstitution	Frequency of Cells that Long-Term Multilineage Reconstituted (HSCs)
10	24/28	1 in 5.7	88% (21/24)	1 in 7.7 (21/28)
5	13/15	1 in 3.0	100% (13/13)	1 in 3.0 (13/15)
4	25/34	1 in 3.5	92% (23/25)	1 in 4.1 (23/34)
Mean±SD	82 ± 7%	1 in 4.1 ± 1.4	93 ± 6%	1 in 4.9 ± 2.5 (20%)

The indicated number of donor-type (CD45.2$^+$) Thy-1^{lo}Sca-1$^+$Lineage$^-$c-kit$^+$ cells was transplanted intravenously into lethally irradiated recipients (CD45.1$^+$) along with 200,000 recipient-type (CD45.1$^+$) whole bone marrow cells for radioprotection. Recipients were considered engrafted by donor cells if any CD45.2$^+$ cells were detected in their peripheral blood (above background: >0.1-0.3% of myeloid cells or >0.1-0.15% of lymphoid cells). The frequency of cells that engrafted was calculated based on limit-dilution (Poisson) statistics (Smith et al., 1991). Mice were considered long-term multilineage reconstituted if donor-type myeloid, B, and T cells were present for more than 16 weeks after reconstitution.

Supplemental Table S2. Thy-1^{lo}Sca-1^{+} Mac-1^{lo}CD4^{lo}B220^{-} Cells Are Highly Enriched for Transiently Reconstituting, Multipotent Progenitors

Cells injected	Mice that engrafted	Frequency of cells that engrafted	Engrafted mice with short-term multilineage reconstitution	Engrafted mice with only B and T lineage reconstitution	Engrafted mice with B-only lineage reconstitution
4	69% (11/16)	1 in 4.0	64% (7/11)	27% (3/11)	9% (1/11)

In previous studies, only 26% of Thy-1^{lo}Sca-1^{+}Mac-1^{lo}CD4^{lo} cells formed myeloerythroid colonies in methylcellulose cultures, and many clones gave rise to only B lineage reconstitution in vivo (Morrison and Weissman, 1994; Morrison et al., 1997b). This raised the question of whether this population contained multipotent progenitors, or a mixture of multipotent and lymphoid committed progenitors. To resolve this question we searched for sources of heterogeneity within the Thy-1^{lo}Sca-1^{+}Mac-1^{lo}CD4^{lo} population, and found that 55±14% of these cells expressed the B cell marker B220. The B220^{+} subset of Thy-1^{lo}Sca-1^{+}Mac-1^{lo}CD4^{lo} cells lacked the ability to form colonies in methylcellulose or to give multilineage reconstitution in vivo (data not shown), while the B220^{-} subset was more highly enriched for transiently reconstituting multipotent progenitors.

Supplemental Table S4. Summary of the Microarray Analyses of Thy-1^{lo}Sca-1^{+}Lineage^{-}c-kit^{+} HSCs, Thy-1^{lo}Sca-1^{+}Mac-1^{lo}CD4^{lo} MPPs, and CD45^{+} Bone Marrow Cells

A. Genes that were differentially expressed among HSCs, MPPs, and CD45+ cells

based on an analysis of oligonucleotide arrays containing 36,701 probe sets

Probe sets with fold change>3, P<0.05, non zero present call

HSC>MPP	47 probe sets
HSC<MPP	236 probe sets
HSC>CD45+	1151 probe sets
HSC<CD45+	835 probe sets

B. Squared Pearson correlation coefficients (R^2 values) between similar or different samples

		Mean±SD
Among replicate HSC samples, N=3	log transformed	0.894 ± 0.012
	non-log transformed	0.991 ± 0.002
Among replicate MPP samples, N=3	log transformed	0.894 ± 0.003
	non-log transformed	0.988 ± 0.003
Among replicate CD45+ samples, N=3	log transformed	0.859 ± 0.012
	non-log transformed	0.991 ± 0.0004
Between HSCs and MPPs, N=9	log transformed	0.851 ± 0.011
	non-log transformed	0.798 ± 0.024
Between HSCs and CD45+ samples, N=9	log transformed	0.686 ± 0.013
	non-log transformed	0.558 ± 0.009

To calculate the squared Pearson's correlation coefficient between two groups, we transformed each value to the base 10 logarithm ($\log_{10}$). $\log_{10}$ transformation is statistically preferred because the Pearson's correlation coefficient should be calculated based on normally distributed data, and the untransformed data are not normally distributed.

Supplemental Table S5. CD150$^+$CD48$^-$ Bone Marrow Cells Are Highly Enriched for Long-Term Self-Renewing, Multipotent HSCs Based on the Ability of Three or Five CD150$^+$CD48$^-$ Cells to Competitively Reconstitute Lethally Irradiated Mice

Cell dose	Mice that engrafted	Frequency of cells that engrafted	Engrafted mice with long-term multilineage reconstitution	Frequency of cells that long-term multilineage reconstituted (HSCs)
5	14/15	1 in 2.4	93% (13/14)	1 in 3.1 (13/15)
3	4/6	1 in 3.2	50% (2/4)	1 in 7.9 (2/6)
3	9/14	1 in 3.4	100% (9/9)	1 in 3.4 (9/14)
Mean±SD		1 in 3.0 ± 0.5	81 ± 27%	1 in 4.8 ± 2.7 (21%)

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SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity

Ömer H. Yilmaz, Mark J. Kiel, and Sean J. Morrison

Recent advances have increased the purity of hematopoietic stem cells (HSCs) isolated from young mouse bone marrow. However, little attention has been paid to the purity of HSCs from other contexts. Although Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from young bone marrow are highly enriched for HSCs (1 in 5 cells gives long-term multilineage reconstitution after transplantation into irradiated mice), the same population from old, reconstituted, or cytokine-mobilized mice engrafts much less efficiently (1 in 78 to 1 in 185 cells gives long-term multilineage reconstitution). To test whether we could increase the purity of HSCs isolated from these contexts, we examined the SLAM family markers CD150 and CD48. All detectable HSCs from old, reconstituted, and cyclophosphamide/G-CSF–mobilized mice were CD150$^+$CD48$^-$, just as in normal young bone marrow. Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old, reconstituted, or mobilized mice included mainly CD48$^+$ and/or CD150$^-$ cells that lacked reconstituting ability. CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old, reconstituted, or mobilized mice were much more highly enriched for HSCs, with 1 in 3 to 1 in 7 cells giving long-term multilineage reconstitution. SLAM family receptor expression is conserved among HSCs from diverse contexts, and HSCs from old, reconstituted, and mobilized mice engraft relatively efficiently after transplantation when contaminating cells are eliminated. (Blood. 2006;107:924-930)

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Introduction

Hematopoietic stem cells (HSCs) are self-renewing, multipotent progenitors that give rise to all types of blood and immune system cells.[1,2] Recent studies have improved the purity with which HSCs can be isolated from young adult mouse bone marrow, to the point where 40% to 96% of single cells from various populations can give long-term multilineage reconstitution in irradiated mice.[3-5] This proves that HSCs from normal young bone marrow can engraft highly efficiently after intravenous transplantation into irradiated mice.[6]

Comparatively little attention has been paid to the purity of HSCs isolated from other contexts and it remains to be determined whether HSCs from other contexts can also engraft efficiently after transplantation. One (20%) of every 5 Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells isolated from young adult mouse bone marrow gives long-term multilineage reconstitution upon transplantation into irradiated mice.[7-10] However, HSCs isolated from other contexts have exhibited much poorer reconstituting efficiencies. One (1.3%) in 78 Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells isolated from old mouse bone marrow gave long-term multilineage reconstitution in irradiated mice.[11] One (0.7%) in 150 to 1 (0.5%) in 185 Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells isolated from reconstituted or cyclophosphamide/G-CSF–mobilized mice gave long-term multilineage reconstitution in irradiated mice.[12,13] Others have also reported the reduced engraftment efficiency of HSCs isolated from old or cytokine-mobilized mice.[14-16] These observations raise the question of whether HSCs from old, reconstituted, and mobilized mice exhibit large defects in their ability to home/engraft after transplantation, or whether Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from these contexts contain more contaminating non-HSCs. If HSCs from old, reconstituted, and mobilized donors are intrinsically less able to engraft after transplantation, it could have important implications for clinical transplants in these settings.

We recently discovered that HSCs from young adult mice and cytokine-mobilized mice can be more highly purified with the use of SLAM family markers, either by themselves or in combination with other HSC markers.[3] In particular, the SLAM family receptors CD150 and CD48 enhance HSC purity, as 47% of single CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ bone marrow cells (1 in 2.1) give long-term multilineage reconstitution. The fact that SLAM family members are differentially expressed among young adult bone marrow hematopoietic progenitors in a way that correlates with primitiveness, and that HSCs can be highly enriched using only CD150 and CD48, suggested that HSCs can be identified based on a SLAM code (CD150$^+$CD48$^-$).[3]

This raises the question of whether these markers also enhance the purity of HSCs in other contexts. Cyclophosphamide/G-CSF–mobilized HSCs are also highly enriched within the CD150$^+$CD48$^-$ population,[3] though we did not test whether all mobilized HSCs fell

within this population and therefore could not assess the overall engraftment efficiency of mobilized HSCs. It has not been tested whether HSCs from old or reconstituted mice are CD150$^+$ or CD48$^-$. The conservation of these markers between HSCs in different mouse strains suggests they might be robust stem cell markers.[3] However, gene expression profiling showed that CD48 appeared to be up-regulated in an enriched HSC population after 5-fluorouracil treatment.[17] Although this study did not functionally test whether the CD48$^+$ cells were HSCs or contaminating cells, these results raised the possibility that CD48 may change its expression on HSCs under certain circumstances.

To test whether the CD150$^+$CD48$^-$ markers that identify young adult bone marrow HSCs[3] are conserved among HSCs in other contexts, we examined HSCs from old, mobilized, and reconstituted mice. In each case, all detectable HSCs were CD150$^+$ and CD48$^-$, emphasizing the robustness of these markers. The use of these new markers substantially increased the purity of HSCs from old, mobilized, and reconstituted mice, such that relatively efficient engraftment was observed from CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$ c-kit$^+$ cells in each case (on average 1 in 3.6 to 1 in 6.9 cells engrafted and gave long-term multilineage reconstitution in each case). These data are consistent with our previous analysis of old HSCs[11] and with the recent functional analysis of old HSC engraftment by Liang et al,[14] in suggesting that old HSCs may exhibit a 3-fold engraftment defect relative to young adult HSCs. However, the data do not support the possibility of more profound engraftment defects. By substantially increasing the purity with which HSCs can be isolated from old, cytokine-mobilized, and reconstituted mice, SLAM family markers enhance the precision with which HSCs can be studied.

Materials and methods

All mice used in this study were housed in the Unit for Laboratory Animal Medicine at the University of Michigan. Donor hematopoietic progenitors were obtained from adult C57BL/Ka-CD45.2:Thy-1.1 mice or C57BL/Ka-CD45.1:Thy-1.2 mice as specified. Old donor mice were at least 22 months of age and had normal spleen size. Young adult mice were 6 to 8 weeks of age. Prior to being used in these experiments, reconstituted mice were long-term multilineage reconstituted for at least 20 weeks after receiving transplants with either donor whole bone marrow cells or enriched donor HSCs. When donor cells were retransplanted from these mice into secondary recipients, CD45.1 (clone A20.1) or CD45.2 (clone 104) was included in the lineage cocktail to exclude recipient-type cells. As described previously,[13] mobilized mice were injected with cyclophosphamide (200 mg/kg; Bristol-Myers Squibb, New York, NY) and then on each of 7 subsequent days with 5 μg human G-CSF (250 μg/kg per day; Amgen, Thousand Oaks, CA) before being killed to obtain splenocytes for analysis. Throughout the paper, these "mobilized" cells are referred to as day-7 cyclophosphamide/G-CSF–mobilized splenocytes. Recipient mice in reconstitution assays were adult C57BL/Ka-CD45.2:Thy-1.1 mice or C57BL/Ka-CD45.1:Thy-1.2 mice as specified.

Flow-cytometric isolation of stem and progenitor cells

Bone marrow cells were flushed from femurs and tibias with Hanks buffered salt solution without calcium or magnesium, supplemented with 2% heat-inactivated calf serum (HBSS$^+$; Gibco, Grand Island, NY). Cells were triturated and filtered through nylon screen (45 μm; Sefar America, Kansas City, MO) to obtain single-cell suspensions.

Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ HSCs were isolated as previously described.[12,18] Briefly, whole bone marrow cells were incubated with unconjugated monoclonal antibodies to lineage markers including B220 (6B2), CD3 (KT31.1), CD5 (53-7.3), CD8 (53-6.7), Gr-1 (8C5), and Ter119.

Following dilution, pelleted cells were resuspended in anti–rat IgG specific F(ab)$_2$ fragment conjugated to phycoerythrin (PE; Jackson ImmunoResearch, West Grove, PA). Cells were subsequently stained with directly conjugated antibodies to Sca-1 (Ly6A/E-APC), c-kit (2B8-biotin), Thy-1.1 (19XE5-FITC), Mac-1 (M1/70-PE), CD4 (GK1.5-PE), and, when specified, CD48 (HM48-1-PE; Pharmingen, San Diego, CA). Progenitors were often enriched by preselecting for Sca-1$^+$ or c-kit$^+$ cells using paramagnetic microbeads (Miltenyi Biotec, Auburn, CA) and selecting on a Miltenyi autoMACS prior to flow cytometry.

Cells sorted based on CD150 expression were incubated with unconjugated antibody to CD150 (26D12; a gift of DNAX, Palo Alto, CA), and subsequently stained with goat anti–rat IgG F(ab)$_2$ fragment conjugated to FITC (Jackson ImmunoResearch). Cells sorted according to CD48 were stained with directly conjugated anti-CD48 (FITC or PE). Cells were resuspended in 2 μg/mL 7-AAD (Molecular Probes, Eugene, OR) to discriminate live from dead cells. Only live (7-AAD$^-$) cells were included in analyses and sorts. All flow cytometry was performed on a FACS Vantage dual laser flow-cytometer (Becton Dickinson, San Jose, CA).

Methylcellulose culture

Methylcellulose cultures were performed as previously described.[11] Briefly, unfractionated bone marrow cells, or single resorted hematopoietic progenitors were plated in wells of 96-well plates (Corning, Corning, NY) containing 100 μL 1.0% methylcellulose (Stem Cell Technologies, Vancouver, BC, Canada). The methylcellulose was supplemented with 20% charcoal-absorbed fetal bovine serum (Cocalico, Reamstown, PA), 1% BSA (Sigma), 1% penicillin/streptomycin (Gibco), 50 ng/mL stem cell factor (SCF), 10 ng/mL interleukin-3 (IL-3), 10 ng/mL interleukin-6 (IL-6), 3 U/mL erythropoietin (Epo), 10 ng/mL Flt-3, and 10 ng/mL thrombopoietin (Tpo). All cytokines were obtained from R&D Systems (Minneapolis MN). Colonies were maintained at 37°C in fully humidified chambers containing 6% CO$_2$. Colony formation was scored after 10 to 14 days of culture.

Long-term competitive reconstitution assays

Adult recipient mice were lethally irradiated with an Orthovoltage x-ray source delivering approximately 3 Gy/min. The mice received 2 doses of 5.5 to 5.7 Gy, delivered at least 2 hours apart. For transplantation of sorted cell populations, CD45.2$^+$ stem or progenitor cells were sorted and then resorted into individual wells of a 96-well plate containing 200 000 CD45.1$^+$ whole bone marrow cells in 100 μL HBSS$^+$. In some experiments, 300 000 recipient-type CD150$^-$ bone marrow cells were used for radioprotection. The contents of individual wells were drawn into a 500-μL insulin syringe (Becton Dickinson) and injected into the retro-orbital venous sinus of lethally irradiated, anesthetized CD45.1 recipients. Mice were maintained on antibiotic water (1.1 g neomycin sulfate and 10^6 U/L polymixin B sulfate; Sigma) ad libitum. Starting at 3.5 weeks after transplantation and continuing for at least 16 weeks after transplantation, peripheral blood was obtained from the tail veins of individual recipient mice, subjected to ammonium-chloride potassium red cell lysis,[18] and stained with antibodies to CD45.2 (clone 104) or CD45.1 (clone A20.1), and B220 (6B2), Mac-1 (M1/70), CD3 (KT31.1), and Gr-1 (8C5).

Results

Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old, reconstituted, and mobilized mice contain CD48$^+$ cells with no multilineage-reconstituting activity

To determine whether CD48 might enhance the purity of HSCs from old, reconstituted, or mobilized mice, we examined CD48 expression on Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from each of these contexts. While only 22 ± 3% of young adult Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ bone marrow cells expressed CD48, 61 ± 30% of Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ bone marrow cells from 26- to

28-month-old mice, $70 \pm 2\%$ of Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ splenocytes from day-7 cyclophosphamide/G-CSF–treated mice, and $86 \pm 4\%$ of Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ bone marrow cells from reconstituted mice expressed CD48 (Figure 1). The observation that most Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old, reconstituted, or mobilized mice expressed CD48 was reminiscent of the increase in CD48 expression by an enriched HSC population after 5-FU treatment.[17] This raised the question of whether HSCs gain CD48 expression in old, reconstituted, and mobilized mice, or whether the CD48$^+$ cells are contaminating non-HSCs, just as they were previously observed to lack HSC activity in young adult bone marrow.[3]

To test this, we performed competitive reconstitution assays in which CD48$^+$ or CD48$^-$ cells from old, reconstituted, or mobilized mice were transplanted into lethally irradiated recipients along with a radioprotective dose of 200 000 recipient-type whole bone marrow cells (Table 1). In each context, recipients that received CD48$^-$ cells were usually long-term multilineage reconstituted by donor cells (11/11 from old mice, 4/7 from reconstituted mice, and 5/5 from mobilized mice), but recipients that received CD48$^+$ cells were rarely or never long-term multilineage reconstituted by donor cells (1/11 from old mice, 0/5 from reconstituted mice, and 0/5 from day-7 Cy/G-CSF–treated mice). These data indicate that most or all of the detectable HSC activity is in the CD48$^-$ fractions of old bone marrow cells, reconstituted bone marrow cells, and mobilized splenocytes.

This suggested that it might be possible to significantly improve HSC enrichment in each of these contexts by excluding the CD48$^+$ cells from the Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ population. To further test this possibility using highly enriched HSCs, we isolated the CD48$^+$ and CD48$^-$ subsets of Flk2$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ cells. Flk2 was used in place of Thy1.1 because Flk2$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ cells are highly enriched for HSC activity,[20] and Flk2 could be added to the lineage panel, freeing up a channel to sort based on CD48. We transplanted 15 Flk2$^-$Sca-1$^+$Lineage$^-$c-kit$^+$CD48$^-$ cells or 15 Flk2$^-$Sca-1$^+$Lineage$^-$c-kit$^+$CD48$^+$ cells from old bone marrow, mobilized spleen, or reconstituted bone marrow into lethally irradiated recipient mice along with 200 000 recipient-type whole bone marrow cells (Table 2). While virtually all recipients of Flk2$^-$Sca-1$^+$Lineage$^-$c-kit$^+$CD48$^-$ cells became reconstituted and

Table 1. HSCs are enriched within the CD48$^-$ fraction but not the CD48$^+$ fraction of old bone marrow, reconstituted bone marrow, and cyclophosphamide/G-CSF–mobilized spleen

Source of cells	No. donor-type cells transplanted	No. mice with long-term multilineage engraftment/no. mice total
Old bone marrow		
CD48$^+$	60 000	1/11
CD48$^-$	140 000	11/11
Mobilized splenocytes		
CD48$^+$	30 000	0/5
CD48$^-$	170 000	5/5
Reconstituted bone marrow		
CD48$^+$	35 000	0/5
CD48$^-$	165 000	4/7

Old bone marrow cells were obtained from 26- to 28-month-old C57BL mice. Mobilized splenocytes were obtained from mice that had been treated with cyclophosphamide followed by 7 daily injections of G-CSF.[13] Reconstituted bone marrow cells were obtained from mice that had been long-term multilineage reconstituted for 20 to 24 weeks by highly enriched HSCs. Donor cells from reconstituted mice were selected for donor cell origin (CD45.1$^+$) in addition to CD48. The indicated number of donor-type (CD45.1$^+$) cells was transplanted intravenously into lethally irradiated recipients (CD45.2$^+$) along with 200 000 recipient-type (CD45.2$^+$) whole bone marrow cells. The dose of CD48$^+$ or CD48$^-$ donor cells was based on the number of cells from each population contained in 200 000 old bone marrow, mobilized spleen, or reconstituted bone marrow cells as done in previous studies of marker expression on HSCs.[3,10,13,19] Mice were considered long-term multilineage reconstituted if donor-type myeloid, B, and T cells were present for at least 16 weeks after transplantation.

most of these mice exhibited long-term multilineage reconstitution, none of the recipients of Flk2$^-$Sca-1$^+$Lineage$^-$c-kit$^+$CD48$^+$ cells showed any engraftment by donor-type cells. These data further support the conclusion that HSCs from old, mobilized, and reconstituted mice are CD48$^-$ and that HSCs are further enriched by excluding the contaminating CD48$^+$ cells.

Table 2. All HSC activity from the Flk2$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ population is contained within the CD48$^-$ subset of cells

Donor	Cell dose	No. mice that engrafted/no. mice total	No. mice with long-term multilineage reconstitution/no. mice total	Frequency of cells that long-term multilineage reconstituted
Young				
FSLK48$^-$	10	5/5	5/5	CNBC
FSLK48$^+$	10	0/6	0/6	NA
Old				
FSLK48$^-$	15	8/8	6/8	1 in 11.0
FSLK48$^+$	15	0/8	0/8	NA
Reconstituted				
FSLK48$^-$	15	5/5	2/5	1 in 29.9
FSLK48$^+$	15	0/7	0/7	NA
Mobilized				
FSLK48$^-$	15	3/4	2/4	1 in 22.1
FSLK48$^+$	15	0/8	0/8	NA

The indicated number of donor-type (CD45.2$^+$) FLK2$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ cells from young adult bone marrow, old adult bone marrow, reconstituted bone marrow, and day-7 cyclophosphanide/G-CSF–mobilized splenocytes were transplanted intravenously into lethally irradiated recipients (CD45.1$^+$) along with recipient-type 200 000 whole bone marrow cells. Recipients were considered engrafted by donor cells if any CD45.2$^+$ cells were detected in their peripheral blood (above background: > 0.3 of myeloid cells or > 0.1-0.15 of lymphoid cells). Mice were considered long-term multilineage reconstituted if donor-type myeloid, B, and T cells were present for at least 16 weeks after reconstitution. The frequency of cells that gave long-term multilineage reconstitution (HSCs) was calculated based on limit-dilution Poisson statistics.[21] NA indicates not applicable because no HSC activity was detected; CNBC, could not be calculated because all mice were LTMR.
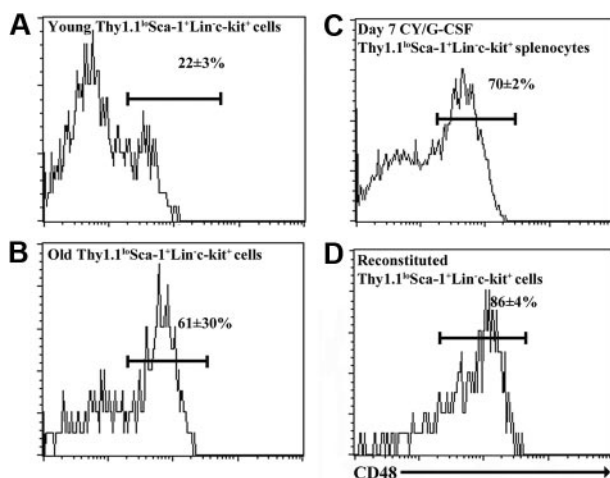
Figure 1. CD48 is heterogeneously expressed by Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from young, old, cyclophosphamide/G-CSF–mobilized, and reconstituted mice. Cells were derived from bone marrow in each case except mobilized mice in which they were obtained from the spleen.

Selecting CD48⁻ cells improves HSC purity from old, mobilized, and reconstituted mice

To test the extent to which HSCs were enriched by excluding CD48$^+$ cells, we transplanted varying doses of either Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells or Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$CD48$^-$ cells from old, reconstituted, and mobilized mice into lethally irradiated recipients along with a radioprotective dose of 200 000 recipient-type whole bone marrow cells. In each case, the exclusion of CD48$^+$ cells by adding CD48 to the lineage panel led to an increase in the frequency of HSCs based on limit-dilution analysis.[21] The frequency of long-term multilineage-reconstituting HSCs from old donors appeared to increase about 2-fold by excluding CD48$^+$ cells, while the frequency of long-term multilineage-reconstituting HSCs from reconstituted or mobilized donors appeared to increase 3- to 8-fold (Table 3). The precise magnitude of the increases in HSC purity cannot be calculated solely from the experiments in Table 3 because the confidence intervals around the calculated HSC frequencies remain large. Nonetheless, when combined with the observation that most Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells in old, mobilized, and reconstituted mice are CD48$^+$ (Figure 1), and that these CD48$^+$ cells lack reconstituting potential (Tables 1-2), the data in Table 3 indicate that HSC purity can be significantly increased in each of these contexts by excluding CD48$^+$ cells.

HSCs from old, reconstituted, and mobilized mice are CD150$^+$ and not CD150$^-$

The other SLAM family member that facilitated the purification of young adult HSCs and that was expressed by at least some cytokine-mobilized HSCs was CD150.[3] Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old bone marrow, reconstituted bone marrow, and mobilized spleen were heterogeneous for CD150 expression. While 30% ± 14% of young adult Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ bone marrow cells expressed CD150 in these experiments, 33% ± 13% of Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ bone marrow cells from 26- to 28-month-old mice, 44% ± 2% of Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ splenocytes from day-7 cyclophosphamide/G-
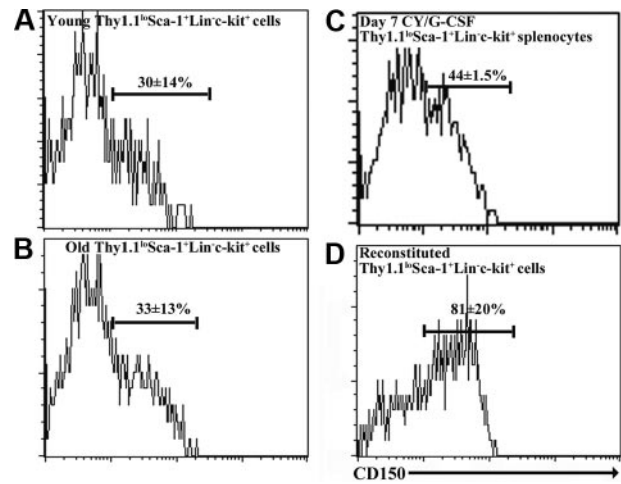

Figure 2. CD150 is heterogeneously expressed by Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from young, old, cyclophosphamide/G-CSF–mobilized, and reconstituted mice. Cells were derived from bone marrow in each case, except mobilized mice in which they were obtained from the spleen.

CSF–treated mice, and 81% ± 20% of Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ bone marrow cells from reconstituted mice expressed CD150 (Figure 2). If HSCs are CD150$^+$ and not CD150$^-$ in each of these contexts, as was previously observed in young adult bone marrow,[3] then CD150 might further enhance the purification of these HSCs.

To test whether HSCs from old and reconstituted mice were also CD150$^+$, and whether all mobilized HSCs were CD150$^+$, we performed competitive reconstitution assays in which CD150$^+$ or CD150$^-$ donor cells from each of these contexts were transplanted into lethally irradiated mice along with 200 000 recipient-type whole bone marrow cells. CD150$^+$ cells accounted for about 10% of cells in the bone marrow of old and reconstituted mice, as well as around 10% of splenocytes in cytokine-mobilized mice (data not shown). These frequencies of CD150$^+$ cells were similar to that observed in the bone marrow of young adult mice (7%).[3] Irrespective of the source of cells, recipients of CD150$^+$ cells were almost always long-term multilineage reconstituted (7/8 for old donors, 3/4 for reconstituted donors, and 8/9 for mobilized donors), while recipients of CD150$^-$ cells were never long-term multilineage reconstituted (0/9 for old donors, 0/5 for reconstituted donors, 0/10 for mobilized donors) (Table 4). Especially in view of the fact that CD150$^-$ cells represent around 90% of all cells in old bone marrow, reconstituted bone marrow, and cytokine-mobilized spleen, these data indicate that HSCs are enriched in the CD150$^+$ fraction and depleted in the CD150$^-$ fraction in each of these contexts.

SLAM family members improve the purity of HSCs from different contexts

These results suggest that by further selecting the CD150$^+$CD48$^-$ subset of Sca-1$^+$Lineage$^-$c-kit$^+$ cells that it might be possible to significantly increase the purity of HSCs isolated from old, reconstituted, and mobilized mice. To test this possibility, we transplanted 3 CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old bone marrow, reconstituted bone marrow, or day-7 cyclophosphamide/G-CSF–mobilized spleen into lethally irradiated mice along with a radioprotective dose of recipient-type bone marrow cells. This donor population engrafted and gave long-term multilineage reconstitution much more efficiently than observed from

Table 3. The CD48⁻ subset of Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ (TSLK) cells is further enriched for long-term multilineage-reconstituting HSCs

Donor	Cell dose	No. mice that engrafted/no. mice total	No. mice with long-term multilineage reconstitution/no. mice total	Frequency of cells that long-term multilineage reconstituted
Old				
TSLK	5	6/9	4/9	1 in 9.0
TSLK	5	3/7	2/7	1 in 15.4
TSLK48⁻	5	8/9	7/9	1 in 3.8
Reconstituted				
TSLK	10	9/11	1/11	1 in 105
TSLK	20	2/4	1/4	1 in 70.0
TSLK48⁻	5	6/9	2/9	1 in 20.4
Mobilized				
TSLK	30	8/9	3/9	1 in 74.5
TSLK48⁻	5	14/18	8/18	1 in 9.0

The indicated number of donor-type (CD45.2$^+$) Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old bone marrow, reconstituted bone marrow, or day-7 cyclophosphanide/G-CSF–mobilized splenocytes were transplanted intravenously into lethally irradiated recipients (CD45.1$^+$) along with 200 000 recipient-type whole bone marrow cells. The frequency of cells that gave long-term multilineage reconstitution (HSCs) was calculated based on limit-dilution (Poisson) statistics.[21] Mice were considered long-term multilineage reconstituted if donor-type myeloid, B, and T cells were present for at least 16 weeks after reconstitution.

Table 4. All HSC activity is in the CD150$^+$ fraction of old bone marrow, reconstituted bone marrow, and day-7 cyclophosphamide/G–CSF-treated splenocytes

Source of cells	No. donor-type cells transplanted	No. mice with long-term multilineage engraftment/no. mice total
Old bone marrow		
CD150$^+$	20 000	7/8
CD150$^-$	180 000	0/9
Mobilized splenocytes		
CD150$^+$	20 000	8/9
CD150$^-$	180 000	0/10
Reconstituted bone marrow		
CD150$^+$	20 000	3/4
CD150$^-$	180 000	0/5

The indicated number of donor-type (CD45.1$^+$) cells was transplanted intravenously into lethally irradiated recipients (CD45.2$^+$) along with 200 000 recipient-type (CD45.2$^+$) whole bone marrow cells. The dose of CD150$^+$ or CD150$^-$ donor cells was based on the number of cells from each population contained in 200 000 old bone marrow, mobilized spleen, or reconstituted bone marrow cells as done in previous studies of marker expression on HSCs.[3,10,13,19] Mice were considered long-term multilineage reconstituted if donor-type myeloid, B, and T cells were present for at least 16 weeks after transplantation.

Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells (Table 3). Previously, we reported that only around 1 in every 150 Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from day-7 cyclophosphamide/G-CSF–mobilized spleen engrafted and gave long-term multilineage reconstitution after intravenous transplantation into irradiated mice.[13] In this study, we found that 1 in 74.5 Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from mobilized spleen engrafted and gave long-term multilineage reconstitution (Table 3). In contrast, 1 in 9.0 CD48$^-$Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells (Table 3) or 1 in 3.6 CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ cells from mobilized spleen engrafted and gave long-term multilineage reconstitution (Table 5), suggesting a dramatic increase in HSC purity.

Previously, we reported that only 1 of every 185 Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from reconstituted mice gave long-term multilineage reconstitution after intravenous transplantation into irradiated mice.[12] In this study, we found that 1 in 70.0 to 1 in 105 Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from reconstituted bone marrow engrafted and gave long-term multilineage reconstitution (Table 3). In contrast, 1 in 20.4 CD48$^-$Thy-1^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells (Table 3) or an average of 1 in 5.1 CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ cells from reconstituted bone marrow engrafted and gave long-term multilineage reconstitution in several independent experiments (Table 5). This again suggests a dramatic increase in HSC purity in this population from reconstituted bone marrow.

Previously, we found that only 1 of every 78 intravenously injected Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old mice was able to engraft and give long-term multilineage reconstitution.[11] By gating more stringently on Lineage markers in this experiment, we found that an average of 1 in 12.2 Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old mice engrafted and gave long-term multilineage reconstitution (Table 3). On average, 1 in 6.9 ± 1.1 CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old bone marrow engrafted and gave long-term multilineage reconstitution (Table 5). While the reconstitution assays using old Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells were not repeated enough to determine whether this difference is statistically significant, the data demonstrating the presence of contaminating CD48$^+$ and CD150$^-$ cells within the old Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ population suggest that the selection of CD150$^+$CD48$^-$ cells does contribute to increased purity in this population.

Discussion

Although a number of recent studies have identified new markers or strategies for improving HSC purification from young adult bone marrow,[4,5,19,22-24] little attention has been paid to other contexts in which HSCs must be studied. This is despite the fact that standard HSC markers, such as Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$, do a much poorer job of enriching long-term multilineage-reconstituting cells from old mice, reconstituted mice, or cytokine-mobilized mice.[11-16] It is possible that newly identified HSC markers might enhance the enrichment of old, reconstituted, or mobilized HSCs. However, the fact that HSC markers change their expression under a variety of circumstances, including after 5-fluorouracil treatment,[25] at different stages of development,[10,26,27] and after cytokine mobilization,[16] makes it necessary to confirm that HSC markers identified in one circumstance are also expressed under other circumstances.

In this study, we have demonstrated that SLAM family receptors exhibit similar expression patterns on HSCs isolated from old, reconstituted, and mobilized mice compared with their expression on HSCs from young adult bone marrow. We recently showed that SLAM family receptors including CD150, CD48, and CD244 are differentially expressed among stem and progenitor cells at different stages of the hematopoiesis hierarchy in a way that correlates with primitiveness.[3] The reconstituting potential of primitive hematopoietic progenitors can be predicted based upon the combination of SLAM family members they express (SLAM codes). CD150 is expressed by HSCs in young adult bone marrow, while CD244 is expressed by non–self-renewing multipotent progenitors, and CD48 is expressed by restricted progenitors. These surface receptors are so precisely differentially expressed that HSCs can be isolated from young adult bone marrow as CD150$^+$CD48$^-$ cells, with a similar degree of purity

Table 5. CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old, reconstituted, and cyclophosphamide/G-CSF-mobilized mice are highly enriched for long-term self-renewing, multipotent HSCs

Donor/experiment	No. mice that engrafted/no. mice total	No. mice with long-term multilineage reconstitution/no. mice total	Frequency of cells that long-term multilineage reconstituted
Young			
1	8/8	7/8	1 in 2.0
Old			
1	4/7	3/7	1 in 5.9
2	6/12	5/12	1 in 6.1
3	5/9	3/9	1 in 7.9
4	4/9	3/9	1 in 7.9
Reconstituted			
1	3/6	3/6	1 in 4.8
2	12/13	10/13	1 in 2.6
3	7/9	3/9	1 in 7.9
Mobilized			
1	5/8	5/8	1 in 3.6

Three (CD45.1$^+$) CD50$^+$CD48$^-$Sca-1$^+$lin$^-$c-kit$^+$ cells from old, reconstituted, or day-7 cyclophosphamide/G-CSF–mobilized splenocytes were transplanted intravenously into lethally irradiated recipients (CD45.2$^+$) along with 300 000 CD150$^-$ recipient-type cells or 200 000 whole bone marrow cells for radioprotection. There was no apparent difference in the frequency of donor cells that long-term multilineage reconstituted based on the nature of cells used for radioprotection. Recipients were considered long-term multilineage reconstituted if donor-type myeloid, B, and T cells were present for at least 16 weeks after transplantation.

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BLOOD, 1 FEBRUARY 2006 • VOLUME 107, NUMBER 3   SLAM FAMILY MARKERS IMPROVE HSC PURITY   929

as in the Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ population. Our observation that HSCs from old, reconstituted, and mobilized mice are CD150$^+$CD48$^-$, just like HSCs from young adult bone marrow, further emphasizes the robustness of these HSC markers.

A recent study reported that CD48 was up-regulated on enriched HSCs isolated from 5-fluorouracil–treated bone marrow cells[17] and proposed that CD48 is part of the molecular signature of activated HSCs. However, the reconstituting potential of CD48$^+$ cells was not tested in this study, and the functional purity of the cell populations used for gene expression profiling was not indicated. We observed that long-term multilineage-reconstituting cells from young adult bone marrow, old adult bone marrow, reconstituted bone marrow, and mobilized spleen (a context in which HSCs are activated[13]) are always CD48$^-$ despite the consistent presence of contaminating CD48$^+$ cells that lack multilineage-reconstituting activity. Indeed, we previously found that CD48 was preferentially expressed by restricted colony-forming progenitors but not HSCs or transiently reconstituting multipotent progenitors in young adult bone marrow.[3] This suggests that the CD48$^+$ cells from 5-fluorouracil–treated bone marrow may also be non–HSC-contaminating cells, though the reconstituting potential of these cells must be tested. These observations emphasize the importance of functionally confirming markers identified by gene expression profiling to ensure that they do not reflect changes in contaminating cells.

The use of SLAM family markers has improved the purity with which HSCs can be isolated from old, reconstituted, or mobilized mice. Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells from each of these contexts were heterogeneous for their expression of CD48 (Figure 1) and CD150 (Figure 2), with most cells in these populations being CD48$^+$ and/or CD150$^-$. Yet, these CD48$^+$ cells or CD150$^-$ cells had no long-term multilineage-reconstituting activity upon transplantation into irradiated mice (Tables 1-4). Indeed, the CD48$^+$ cells from within the Flk2$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ population had no detectable reconstituting activity of any type (Table 2). This demonstrates that the Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ population from old, reconstituted, and cytokine-mobilized mice contains mainly contaminating cells that lack HSC activity. When these cells were eliminated by selecting the CD150$^+$CD48$^-$ subset of Sca-1$^+$Lineage$^-$c-kit$^+$ cells, the frequency of cells that gave long-term multilineage reconstitution increased, sometimes dramatically.

The dramatic increase in purity afforded by the SLAM family markers should greatly increase the precision with which HSCs can be studied in old, reconstituted, and mobilized mice. The low rate of reconstitution from Thy^{low}Sca-1$^+$Lineage$^-$c-kit$^+$ cells isolated from these contexts had previously raised the possibility that these cells were grossly defective in their ability to home to bone marrow and engraft following intravenous transplantation into irradiated mice (4- to 20-fold less than young adult cells).[11-16] The increased reconstituting efficiency that was obtained with the addition of SLAM family markers rules out the possibility of dramatic (> 4-fold) homing defects in these cells.

Nonetheless, the data in this paper remain consistent with the possibility of smaller engraftment defects. CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ cells from young mice gave long-term multilineage reconstitution approximately 3-fold more efficiently than CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old or reconstituted mice (Table 5). This is consistent with the engraftment defect that was previously inferred based on reconstitution experiments with enriched HSCs from old mice.[11] These data are also consistent with the observed 3-fold reduction in the engraftment efficiency of old compared with young competitive repopulating units.[14] While our data leave open the possibility that CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ cells from old, reconstituted, and mobilized mice may still contain more contaminating non-HSCs than the same population from young mice, the functional analysis of engraftment efficiency performed by Liang et al[14] strongly suggests that purified HSCs from old mice exhibit a somewhat attenuated ability to engraft after transplantation. If so, the CD150$^+$CD48$^-$Sca-1$^+$Lineage$^-$c-kit$^+$ population from old mice may contain a similar degree of HSC purity as observed in the same population from normal young adult mice.[3]

We and others had reported previously that HSCs from old mice and cyclophosphamide/G-CSF–mobilized mice are increased in frequency and mitotic activity relative to young adult bone marrow HSCs.[11,13,28,29] Examination of the more highly purified HSC populations using SLAM family markers continues to support these conclusions. For example, we find that even using the more highly purified HSCs described in this paper, we continue to observe a 3-fold increase in HSC frequency and a 3-fold increase in the percentage of HSCs in S/G$_2$/M phases of the cell cycle in C57BL mice older than 22 months of age (data not shown).

The use of SLAM family markers to enhance the purification of HSCs from diverse contexts should continue to refine our understanding of the biology of these cells by increasing the purity with which the HSCs can be isolated.

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mortality if unchecked. Survival in the rodent SMA model may occur because the spleen in rodents is a major erythropoietic organ, whereas in primates it is not. Alternatively, it may be that rodent SMA models lack certain features of SMA that occur in *P falciparum* infections in humans and its nonhuman primate models. Clearly, continued investigations into SMA mechanisms in both the rodent models and the nonhuman primate models are important to gain fresh insight leading to new therapeutic interventions for SMA. ■
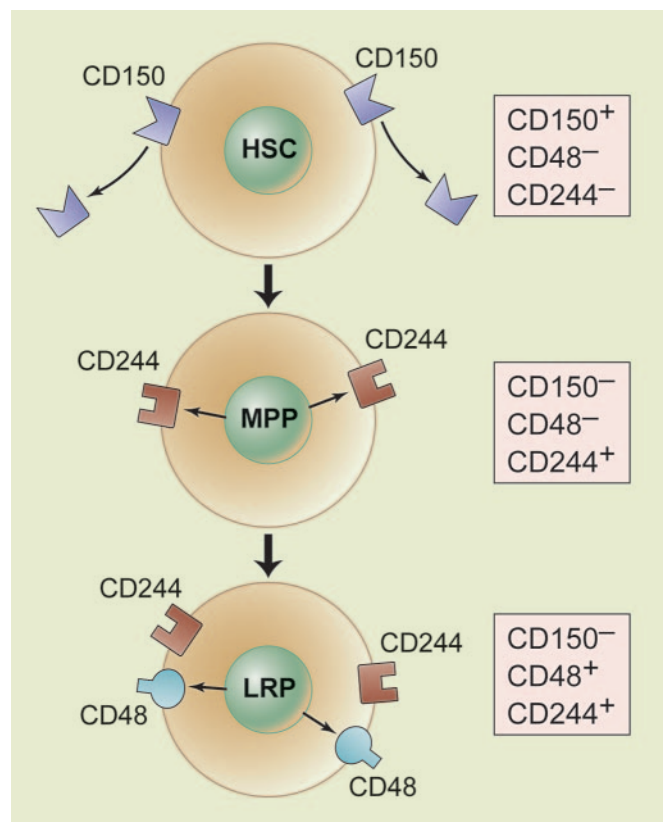
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be attributable to several possibilities. One is that expression of the traditional panel of markers is altered on cells either during aging, mobilization, or prior transplantation such that the staining profile is no longer inclusive of stem cells and/or is no longer exclusive of contaminating cells. A second possibility is that the requisite steps leading to long-term engraftment—that is, homing of stem cells to the marrow and the maintenance of long-term hematopoiesis—are less efficient in stem cells derived from contexts other than young bone marrow. Compelling data in the present paper demonstrate that the bulk of the disparity is accounted for by the presence of cells in the population identified as stem cells (Lin$^-$, Sca-1$^+$, c-Kit$^+$, Thy1^{lo}) that demonstrably lack stem cell function. In contrast, the population of CD150$^+$, CD48$^-$ cells from all hematopoietic sources tested showed dramatically improved stem-cell purities, although not quite to the

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● ● ● HEMATOPOIESIS

Comment on Yilmaz et al, page 924

Stem cell markers: less is more!

Gary Van Zant UNIVERSITY OF KENTUCKY SCHOOL OF MEDICINE

Yilmaz and colleagues present a simplified technique for the prospective identification and purification of hematopoietic stem cells; unlike previous methods, their method is effective in a variety of contexts, including old marrow, mobilized peripheral blood, and recipients of long-term engrafted transplants.

In this issue of *Blood*, Yilmaz and colleagues address a sine qua non of stem cell biology—the prospective identification and purification of viable hematopoietic stem cells. The Morrison lab determined that a simple combination of 2 cell-surface markers, CD150 and CD48, is sufficient to identify all of the long-term repopulating activity from not only young mouse bone marrow, but also, importantly, from bone marrow of old mice, from long-term engrafted radiation chimeras, and from mobilized stem cells found in the spleen. This feat has not been possible with other stem cell purification techniques. In a paper in *Cell* earlier this year,[1] this group detailed the use of members of this SLAM family of cell receptors to characterize specific developmental steps in the stem and progenitor cell hierarchy in bone marrow of young mice and arrived at the simple SLAM "code" for stem cells as CD150$^+$ and CD48$^-$/CD244$^-$ (see figure).

Heretofore, typical methods have relied on a combination of up to 10 cell-surface markers to characterize hematopoietic stem cells. Despite its difficulty and nuances, this technique has been in successful practice, and has advanced our understanding of stem cell biology, since its original publication in 1988.[2] As interest in hematopoietic stem cells from different aged animals and from different hematopoietic sources has grown, limitations have become apparent, the most significant of which has been the issue of whether the stem cell markers that were used maintained their stem-cell fidelity. In earlier studies, Morrison et al showed that the same technique that yielded a population in which 1 in every 5 cells purified from young bone marrow had long-term engrafting potential yielded dramatically lower purities of *functional stem cells* from other sources.[3,4]

The disparity in apparent purities could

SLAM cell-surface markers delineate differentiation steps in early hematopoiesis. Originating with pluripotent hematopoietic stem cells (HSCs), differentiation steps include multipotent progenitor cells (MPPs) and lineage-restricted progenitor cells (LRPs). Each is characterized by a different complement of SLAM markers: HSCs are CD150$^+$CD48$^-$CD244$^-$; MPPs are CD150$^-$CD48$^-$CD244$^+$; LRPs are CD150$^-$CD48$^+$CD244$^+$. It should be noted that CD48 is a ligand for CD244, thus CD150$^+$CD48$^-$ is sufficient to distinguish HSCs from MPPs and LRPs. Illustration by Kenneth Probst.

highest level of enrichment obtained in populations purified from young bone marrow. With respect to aged stem cells, the difference between apparent young and old purities is probably accounted for by an approximately 3-fold less efficient homing of old stem cells to the marrow microenvironment.[3,5]

The findings of Yilmaz et al should significantly simplify stem cell purification, while at the same time improving purity. The results also raise important considerations for studies in which stem cells purified from different contexts are contrasted. For example, in comparing gene or protein expression profiles between stem cells from mice of different ages, it is crucial that the populations compared contain equivalent enrichments for cells with stem cell *function*. Purifications using the proper SLAM code should ensure that apples are compared to apples. ■

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● ● ● ● **HEMOSTASIS** ▰▰▰▰

Comment on Huang et al, page 980

Portable tissue factor to the rescue

Gary E. Gilbert VA BOSTON HEALTHCARE SYSTEM

When the knife slips, sometimes the bleeding does not stop. A novel type of tissue factor has been engineered to home to the injury and stop the bleeding.

Some patients have life-threatening bleeding caused by blood coagulation abnormalities that cannot be treated by replacement of a missing protein. Examples include hemophilia patients who have developed antibodies to factor VIII, patients with massive hemorrhage, and patients in whom anticoagulants complicate hemostasis. At this time, recombinant factor VIIa is the most efficacious therapy available. However, it carries a significant risk of thrombosis, particularly stroke.

Tissue factor is the primary physiologic instigator of normal blood coagulation. Tissue factor forms a complex with activated factor VII (factor VIIa). This complex (see figure) activates factor X, and also factor IX, driving the common pathway of blood coagulation. In theory, delivery of active tissue factor to the site of bleeding could be a potent and specific mechanism for staunching hemorrhagic blood loss.

Tissue factor is a transmembrane protein that is primarily expressed on extravascular cells, exposed to liquid blood only after it flows from a ruptured blood vessel. However, a small amount of tissue factor circulates in blood,[1] probably located primarily on microvesicles derived from the plasma membranes of activated monocytes.[2] This circulating tissue factor is apparently a driving force in thrombus formation within the lumen of minimally injured blood vessels.[3]

The activity of tissue factor that is exposed to blood is tightly controlled both because its activity is encrypted on a quiescent cell and also because the active form is quickly inhibited. The de-encryption mechanism includes exposure of plasma membrane phosphatidylserine that enhances binding of factor VIIa to tissue factor and activity of the assembled factor VIIa–tissue factor complex toward factor X.[4,5] Thus, phosphatidylserine exposure regulates tissue factor activity, and data suggest that it is exposed at sites of vascular injury.

In this issue, Huang and colleagues report that they have joined the procoagulant extramembrane fragment of tissue factor to the phosphatidylserine-binding protein, annexin V. The strategy is based upon the assumptions that cells at the site of vascular injury will expose a phosphatidylserine target for annexin V and that the novel, membrane-bound protein will have clot-instigating potency similar to native, transmembrane tissue factor.

The results in their report must have gratified Huang et al, indicating that the chimeric molecule retains the phosphatidylserine-binding properties of annexin V and the procoagulant properties of the extramembrane fragment of tissue factor. While in solution, the tissue factor–annexin V exhibits very little procoagulant activity. However, upon binding to a membrane with exposed phosphatidylserine, the chimeric protein has procoagulant activity equivalent to intact tissue factor (see figure). In vivo experiments indicate that the tissue factor–annexin V chimera shortens the bleeding time following tail transection in mice. Following heparin treatment, administration of the chimeric protein shortens the prolonged bleeding time toward the normal range. Thus, it appears that this novel therapeutic agent is able to target procoagulant activity to the site of vascular injury.

As always, important questions remain. How safe is the tissue factor–annexin V chimera with regard to thrombosis, particularly
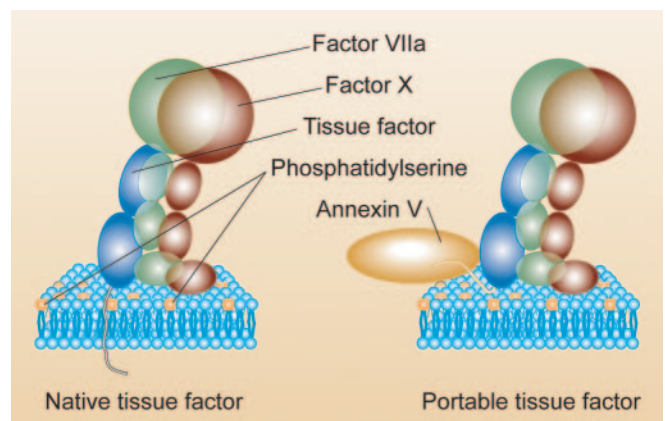
Enzyme complexes containing native tissue factor versus portable tissue factor. Native tissue factor contains a transmembrane tail that anchors it to the membrane. Portable tissue factor is a soluble, engineered protein that uses annexin V to come to a phosphatidylserine-containing membrane. Exposed phosphatidylserine enhances binding of factor VIIa to tissue factor and of factor X to the complex of factor VIIa with tissue factor. Illustration by Paulette Dennis.