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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-1^{lo}Sca-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). However, the variability between samples of different types (HSC versus MPP, R^2 = 0.798 ± 0.024; HSC versus CD45^{+}, R^2 = 0.558 ± 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

			Microarray		qPCR	
Gene Symbol	Unigene Title	GenBank Accession	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 reconstitution 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

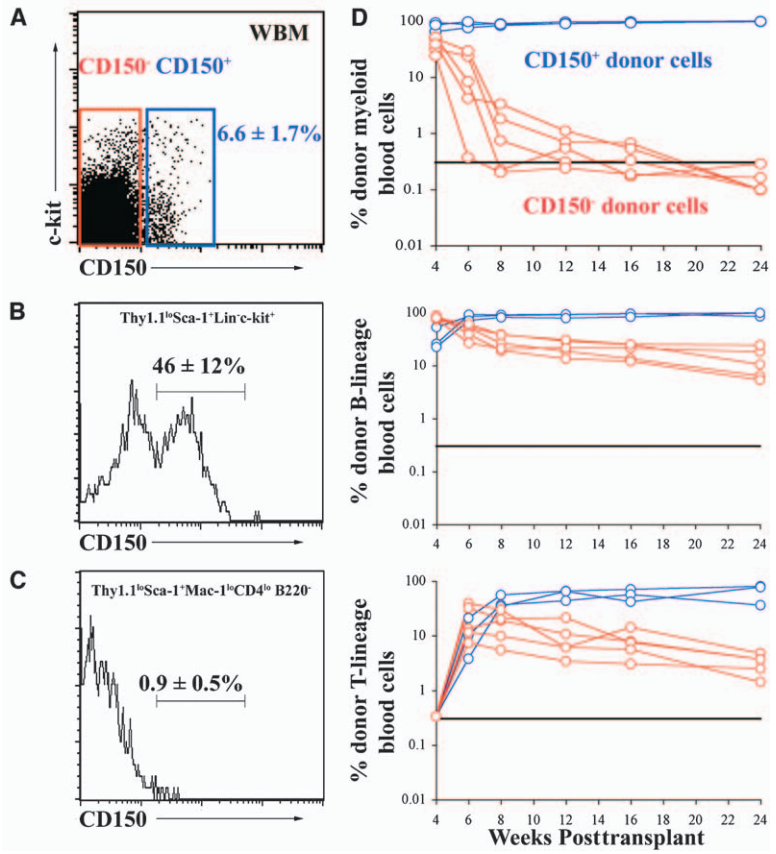

Figure 1. CD150+ Cells Are Enriched for HSCs while CD150− Cells Include Transiently Reconstituting MPPs

Only 6.6% of bone marrow cells express CD150 (A). CD150 expression was detected among Thy-1loSca-1+Lineage−c-kit+ HSCs (B) but not among Thy-1loSca-1+Mac-1loCD4loB220− 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-1loSca-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-1loSca-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,

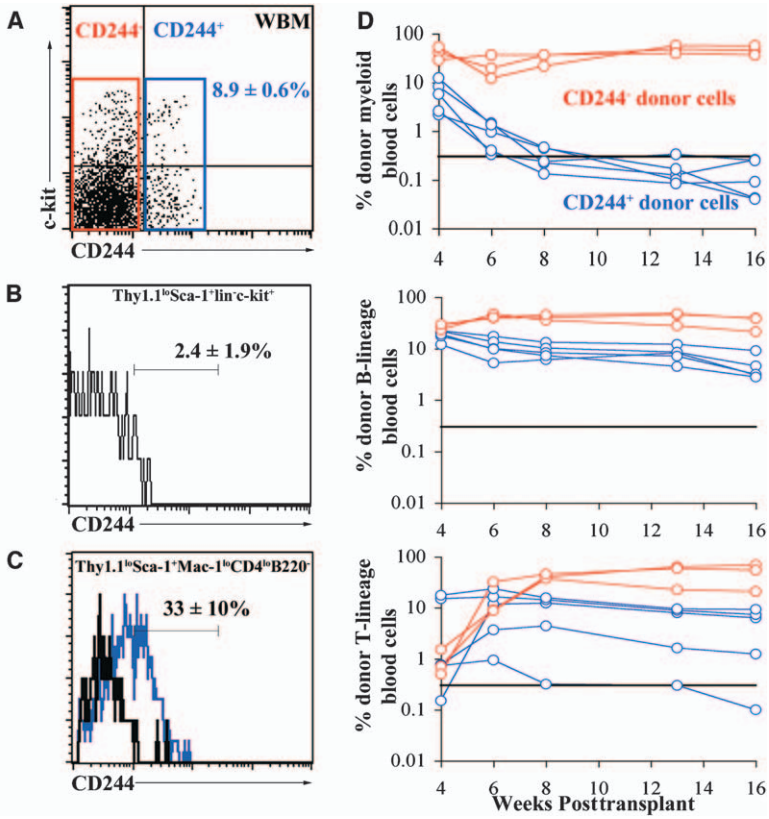

Figure 2. HSCs Are CD244⁻ while Transiently Reconstituting Multipotent Progenitors Are CD244⁺

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

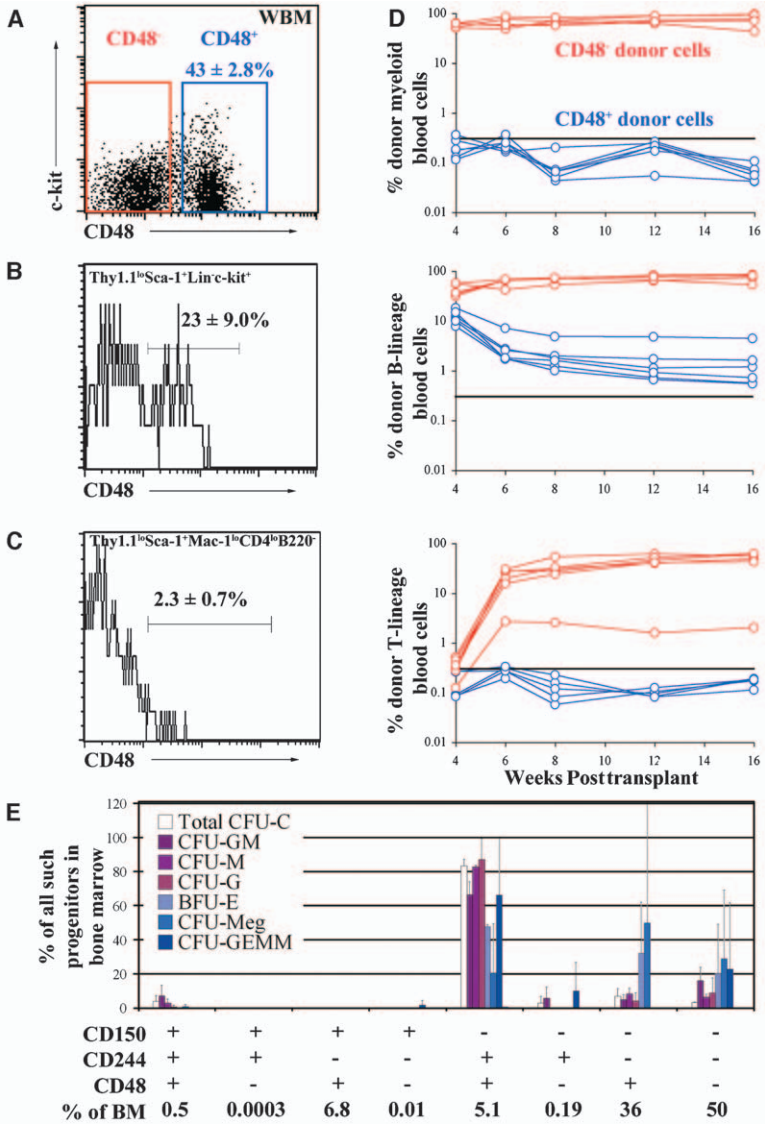

Figure 3. HSCs and MPPs Are Contained within the CD48⁻ Fraction while Colony-Forming Progenitors Are Mainly in the CD48⁺ Fraction of Bone Marrow Cells

Forty-three percent of bone marrow cells express CD48 (A). CD48 expression was detected in the Thy-1^{lo}Sca-1^{+}Lineage^{-}c-kit^{+} HSC population (B) but not in Thy-1^{lo}Sca-1^{+}Mac-1^{lo}CD4^{lo}B220^{-} 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^{lo}Sca-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-

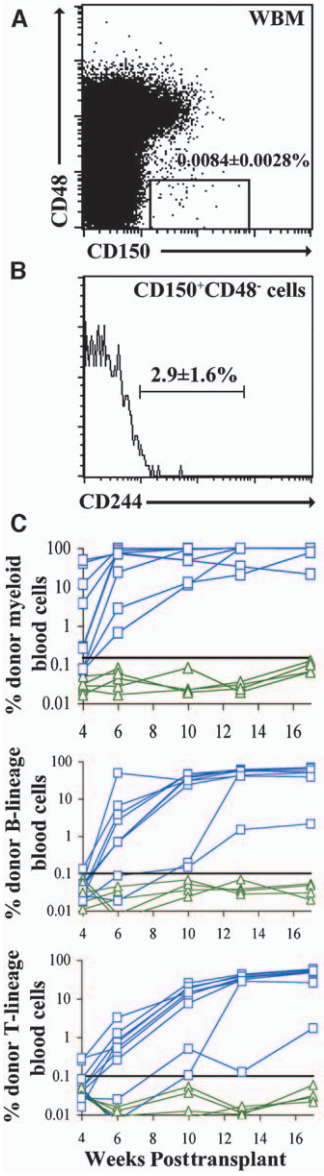

Figure 4. CD150$^+$CD48$^-$CD244$^-$ Cells Are Highly Enriched for Long-Term Reconstituting HSCs

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 parafollicular 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 $CD150^+CD48^-Sca\text{-}1^+Lineage^-$ 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 $CD150^+CD48^-Sca\text{-}1^+Lineage^-$ 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 previously 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-$1^{lo}Sca\text{-}1^+Lin^-$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-

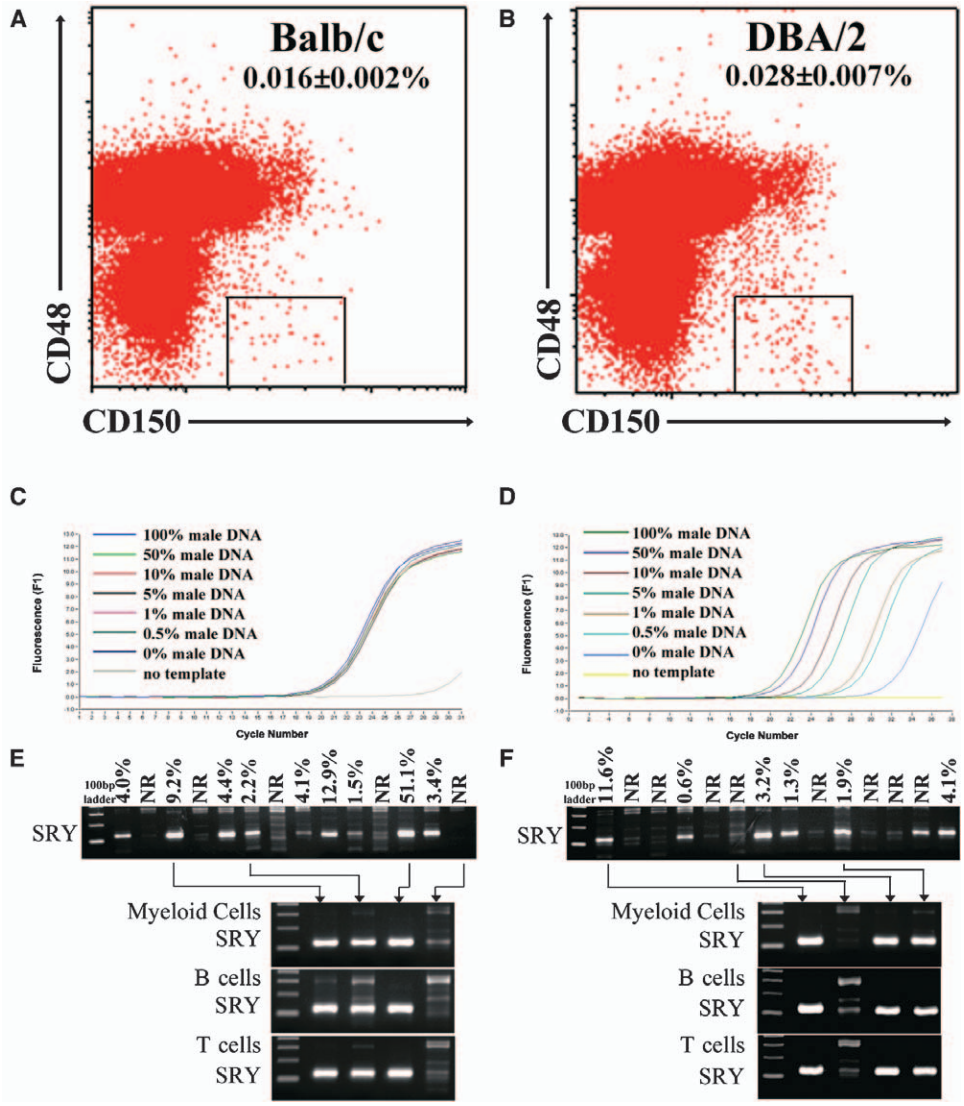

Figure 5. Balb/c and DBA/2 HSCs Are Enriched in the CD150$^+$CD48$^-$ Population

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,

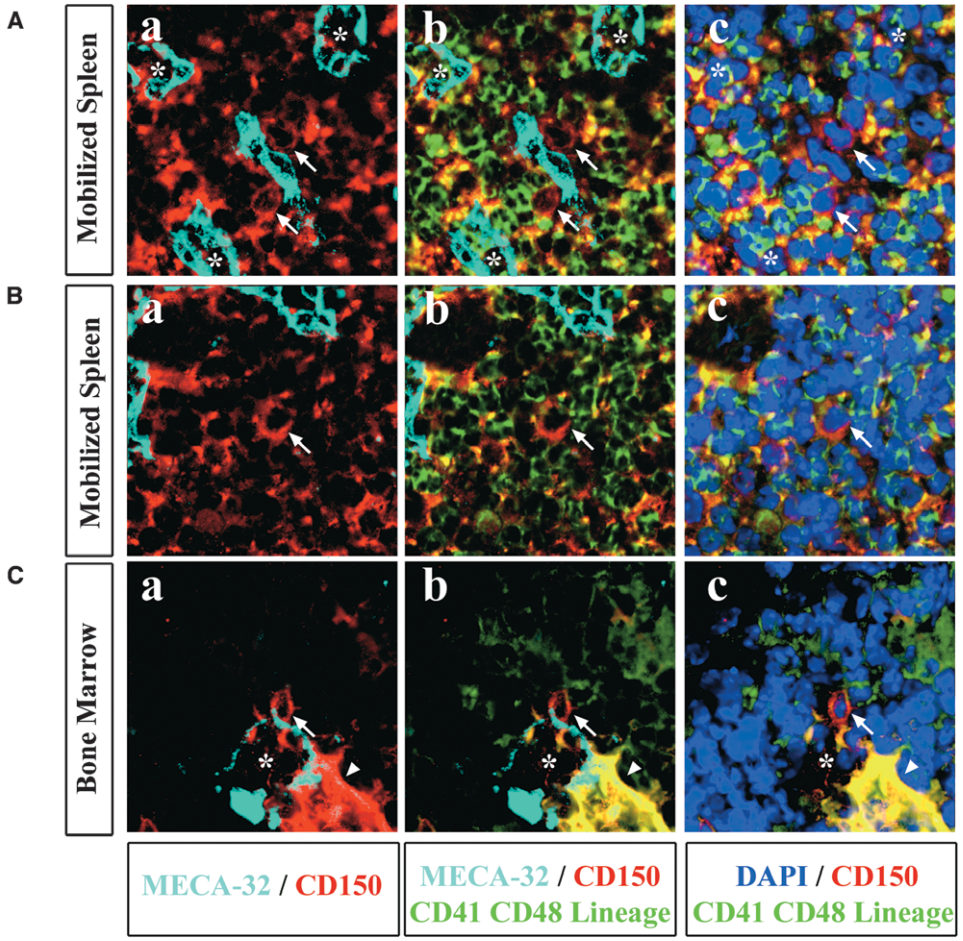

Figure 6. HSCs Are Associated with Sinusoidal Endothelial Cells in the Spleen and Bone Marrow

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 sinusoids 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 (~200 mg/kg; Bristol-Myers Squibb) and then on successive days with 5 μg of human G-CSF by subcutaneous injection (~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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