IMPACT OF ALCAM (CD166) ON HOMING OF HEMATOPOIETIC STEM AND PROGENITOR CELLS

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ABSTRACT

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IMPACT OF ALCAM (CD166) ON HOMING OF HEMATOPOIETIC STEM AND PROGENITOR CELLS

The potential of hematopoietic stem cells (HSC) to home and to anchor within the bone marrow (BM) microenvironment controls the ability of transplanted HSCs to establish normal hematopoiesis. Activated Leukocyte Cell Adhesion Molecule (ALCAM; also identified as CD166), which participates in homophilic interactions, is expressed on a group of osteoblasts in the hematopoietic niche capable of sustaining functional HSC *in vitro*. Since we could also detect ALCAM expression on HSC, we suspect that ALCAM may play a role in anchoring primitive hematopoietic cells to ALCAM expressing components of the hematopoietic niche via dimerization. We investigated the role of ALCAM on the homing abilities of hematopoietic stem and progenitor cells (HSPC) by calculating recovery frequency of Sca-1+ALCAM+ cells in an *in vivo* murine bone marrow transplantation model. Our data supports the notion that ALCAM promotes improved homing potential of hematopoietic Sca-1+ cells. Recovery of BM-homed Sca-1+ cells from the endosteal region was 1.8-fold higher than that of total donor cells. However, a 3.0-fold higher number of Sca-1+ALCAM+ cells homed to the endosteal region compared to total donor cells.

Similarly, homed Sca-1+ALCAM+ cells were recovered from the vascular region at 2.1-fold greater frequency than total homed donor cells from that region, compared to only a 1.3-fold increase in the recovery frequency of Sca-1+ cells. In vitro quantitation of clonogenic BM-homed hematopoietic progenitors corroborate the results from the homing assay. The frequency of in vitro clonogenic progenitors was significantly higher among endosteal-homed Sca-1+ALCAM+ cells compared to other fractions of donor cells. Collectively, these data demonstrate that engrafting HSC expressing ALCAM home more efficiently to the BM and within the BM microenvironment, these cells preferentially seed the endosteal niche.

Mark G. Goebl, Ph.D., Chair

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ALCAM	Activated Leukocyte Cell Adhesion Molecule
BM	Bone Marrow
BMMC	Bone Marrow Mononuclear Cells
CFU	Colony Forming Units
CTV	Cell Trace Violet
FITC	Fluorescein Isothiocyanate
HN	Hematopoietic Niche
HSC	Hematopoietic Stem Cells
HSPC	Hematopoietic Stem and Progenitor Cells
Marrow ECM	Marrow Extracellular Matrix
MSC	Mesenchymal Stem Cells
OB	Osteoblasts
PE	Phycoerythrin
PT	Post transplantation
RBC	Red Blood Cells
Sca-1	Stem Cell Antigen 1

INTRODUCTION

Hematopoietic stem cells are multipotent progenitor cells that reside within a unique environment[1] in the bone marrow, namely the hematopoietic niche (HN). Multiple components of the HN contribute to the regulation[14] of HSC function, including self-renewal, homing, trafficking, proliferation and differentiation. While murine HSC have been well defined[44, 46, 52], the complexity of their niche is still for the most part not fully understood[50]. Attempts to define this compartment anatomically have been widely debated during recent years[14].

The endosteal surface and its elements, credited by many as the hematopoietic niche[2, 4, 12, 30], consists of osteolineage cells, vascular endothelium, bone marrow "stromal" cells (fibroblasts, macrophages, adipocytes), CXCL12-expressing reticular cells and extracellular matrix proteins[2]. Others have provided genetic models[47], whose design has been critisized[45], but nevertheless, implicating perivascular and endothelial cells as the components of a "vascular" niche, responsible for regulating HSC behavior and function[2, 47]. Yet, no significant difference between the regulatory functions of these two bone marrow niches has been fully depicted[23].

It is reasonable to suspect that the wide spectrum of hematopoietic activities characterizing HSC may require different microenvironments[19], which coexist in a functionally dynamic and physically interconnected setting[14, 23, 32, 42]. Therefore, anatomical segregation of sinusoids and endosteal surfaces is

rendered somewhat invalid when considering the proximity of these two "functional units"[2, 14].

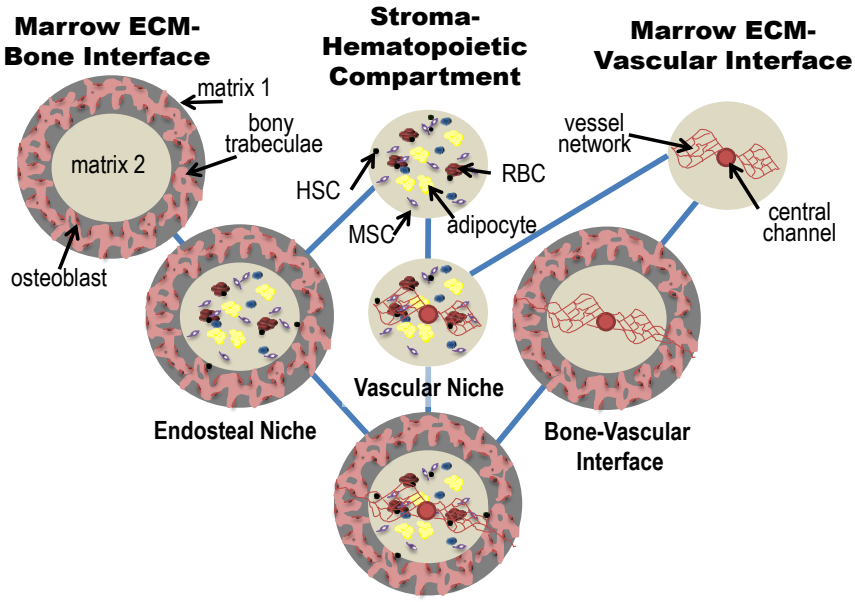

Figure 1. Integrated Bone Marrow Microenvironment Model. Adopted from Chitteti et al., 2010.

When infused in a conditioned recipient, hematopoietic stem and progenitor cells (HSPC) are quickly cleared from the peripheral circulation and migrate rapidly towards the BM vasculature[11]. Before anchoring to specialized niches of the BM microenvironment, HSC must go through adhesion to vascular endothelium, trans-endothelial migration, trans-marrow migration, and finally, lodgement in the HN[33]. These steps describe the process of homing. Despite accumulation of experimental murine and human xenotransplantation studies, events surrounding homing, migration, and trafficking of HSC remain ambiguous [4, 15, 59]. Various data on the subject have emerged. Some studies argue that

HSC trafficking is random and homing is not specific[4]. However, multiple adhesion molecules expressed on primitive hematopoietic progenitors and their cognant receptors present on BM cellular components have been recognized to have a role in homing and engraftment of HSC[5, 15, 35, 44].

Taichman and Emerson first suggested that osteoblasts (OB) may play an important role in the regulation of human hematopoietic progenitors [25], which was later proven by Calvi et al.[7] Accumulating evidence[12, 19, 26] has been supporting direct association of these cells with enhanced hematopoietic function. Others doubt the involvement of OB as a critical component of the HN, where vascular and perivascular cells[41], as well as mesenchymal stem cells[20] have been recognized in maintaining function of HSC.

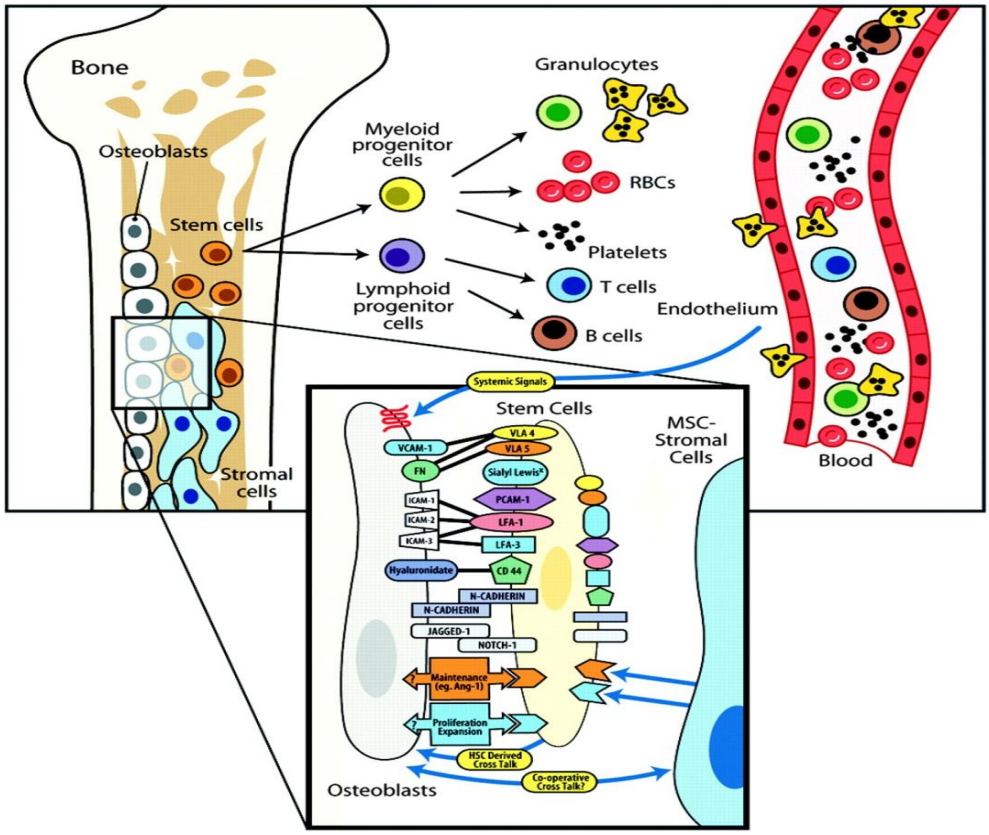

Figure 2. Model of support for hematopoietic stem cells by osteoblasts.[19]

Despite the continued expansion of the list describing cellular components of the HN[21], OB play an important role in homing. Osteopontin secreted by these cells has a regulatory effect on the trans-marrow migration and lodgement of HSC[18]. In addition, recent findings suggest that a particular subset of OB, expressing a novel surface molecule - ALCAM, upregulates homing-related genes in HSPC[22] and is strongly associated with enhanced hematopoietic activity[12, 26].

Activated leukocyte cell adhesion molecule (ALCAM, or CD166), also known as hematopoietic cell antigen (HCA) is a member of the cell surface immunoglobulin superfamily and is involved in homophilic (ALCAM-ALCAM) as well as heterophilic (ALCAM-CD6) binding[6]. ALCAM expression was first discovered on thymic epithelial cells[35], and activated leukocytes[6]. It is also expressed in most primitive $CD34^+$ hematopoietic cells (rhodamine 123^{lo}, Thy-1^+, $CD38^{-/lo}$) and myeloid progenitors[37], perichondrium (mesenchymal stem cells) [30], endothelial cells[29], OB[38], stromal cells[8], and melanoma cells[60]. Recent evidence had recognized the role of this molecule in the homeostatic control of growth saturation[3] and vascular invasion[30], and therefore, placed significance on ALCAM when studying metastasis and tumor progression[27, 31]. In addition, on account of its homophilic adhesion function, tightly regulated through the actin cytoskeleton[36], ALCAM has been suspected to serve as a key adhesion molecule between primitive $CD34^+$ hematopoietic cells and ALCAM expressing cells within the HN.

Thus, it is possible that OB may play a more direct role in anchoring and expanding HSC within the endosteal region of the BM via homophilic binding, since they also express ALCAM[12, 19, 22]. Considering the evidence of enhanced hematopoietic function mediated by ALCAM+ OB[12, 22], we examined whether ALCAM plays a role in homing of hematopoietic stem and progenitor cells and if it does, is there a preferential lodgement in the endosteal niche via homophilic ALCAM interactions with ALCAM-expressing OB. We investigated the role of ALCAM on the homing abilities of HSC by calculating recovery frequency of Sca-1+ALCAM+ cells in an *in vivo* murine BM transplantation model. To minimize added complexity from proliferation[10] and to assess homing capabilities more accurately, we analyzed only short-term recovery (16 hours after injection). In order to examine whether cells isolated from the endosteal region of recipients had increased percentage of functional donor progenitors and to investigate association with ALCAM, we conducted an *in vitro* clonogenic assay for the quantitation of colony forming units in parallel to a homing assay for the phenotypic identification of the ALCAM+Sca1+ population.

We hypothesized that ALCAM expression enhances homing of Sca1+ HSPC through anchoring of these hematopoietic cells to ALCAM expressing cells of the HN via dimerization. Furthermore, we also hypothesized that HSC lodgement within the endosteal region may be more enhanced due to homophilic adhesion between ALCAM+ hematopoietic cells and ALCAM expressing OB. Our analysis permits observations of early HSPC homing characteristics in

regards to the impact of ALCAM expression on their homing and engraftment capabilities.

METHODS

Mice

Adult (6- to 8-week-old) male B6.SJL-PtrcaPep3b/BoyJ (BoyJ) mice were used in all experiments. In one experiment, as denoted in Figure 2, an F1 mouse (C57BL/6 X BoyJ) was used for the procurement of donor cells. Animals were housed in the animal facility at Indiana University. All studies were reviewed and approved by the Laboratory Animal Research Center of the Indiana University School of Medicine.

Irradiation

Recipient mice received a lethal dose of ionizing irradiation (950 cGy) from a cesium source in one fraction immediately prior to transplantation. In some experiments as noted in Figure 1, some mice were not irradiated prior to transplantation.

Isolation of Hematopoietic Cells from Different Marrow Regions

Mice were killed by carbon dioxide inhalation followed by cervical dislocation. Limbs and pelvises were cleaned from muscle and connective tissues and femurs, tibias, humeri and iliac crests were collected for extraction of BM cells. A ratio representing these bones as a percent of total bone marrow mass per mouse[39] was used when calculating the absolute number of total BM cells present within a single mouse.

Vascular Marrow Isolation. For the isolation of cells from vascular marrow, bones were flushed using 27-gauge needle and 13-30 ml of Iscove

modified Dulbecco medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (1000U/ml of penicillin, 1000 ug/ml of streptomycin), and 1% L-glutamine (GlutaMAX I, 200mM, Invitrogen). Cells were washed by centrifuging at 2000 rpm for 10 minutes at 5°C. Low-density cells were collected by Ficoll centrifugation (GE Healthcare). Throughout the text, these cells would be referred to as vascular niche cells.

Endosteal Marrow Isolation. After flushing, bones were cut into less than 1-mm segments and digested by 200 U/ml of collagenase (Worthington Biochemical Corp., Lakewood, NJ) for 30 minutes in 37°C water bath. Cells were then filtered using 30 um filter (Miltenyi Biotec, Inc., Auburn, CA) and 2-3 ml of PBS (Sigma-Aldrich Corp., St. Louis, MO), and washed in 20 ml of Hank's Balanced Salt Solution (Invitrogen) supplemented with 10% bovine calf serum, 1% penicillin/streptomycin (1000U/ml of penicillin, 1000 ug/ml of streptomycin). Low-density cells were collected by Ficoll centrifugation (GE Healthcare). Throughout the text, these cells would be referred to as endosteal niche cells.

Labeling of Donor Cells with Cell Trace Violet

Low-density bone marrow cells were labeled with CellTrace™ Violet Cell Proliferation Kit in order to detect donor cells in the recipients' cell populations (Invitrogen, http://probes.invitrogen.com/media/pis/mp34557.pdf). Cells were labeled *in vitro* according to manufacturer's specifications.

Stem Cell Antigen (Sca-1) and ALCAM Labeling

Cells were washed with stain wash (PBS, 1% bovine calf serum, and 1% penicillin-streptomycin) and stained with phycoerythrin (PE)-conjugated ALCAM

(eBioscience, San Diego, CA) and fluorescein isothiocyanate (FITC)-conjugated Sca-1 (BD Pharmingen, San Diego, CA) for 15 minutes on ice in the dark, and then washed again with cold stain wash.

Sca-1 is a well-established marker for the enrichment and characterization of many classes of progenitor cells including fetal and adult mouse HSC[48, 51, 53]. This is why it is the basis of many models of phenotypic characterization of murine HSC[46]. In our studies, use of additional markers to identify subsets of Sca1+ cells may have been problematic due to the very low[16] yield of recovered donor BM-homed cells. Thus, the utility of a single marker selecting for both hematopoietic stem and progenitor cells, such as Sca-1, allowed for a valid evaluation of homing potential of HSPC, represented by Sca-1+ALCAM+ cells.

Flow Cytometric Analysis and Cell Sorting

CTV-labeled donor cells were sorted on BD FACSAria (BD Biosciences). Low-density total bone marrow cells from donors and both fractions of the recipients' marrow (from vascular and endosteal niche), were gated and analyzed for the presence of Sca-1+ALCAM+ cells on a BD LSRIII (BD Biosciences). Recovery of Sca-1+ALCAM+ cells was calculated using the following equation:

$$\text{Recovery} = \frac{\text{Absolute number of recovered donor cells with indicated phenotype}}{\text{Absolute number of injected donor cells with indicated phenotype}} \times 100$$

Since marrow cells contained in bones analyzed from each mouse represent 38% of total body marrow cellularity[39], total BM-homed cells were estimated to be the number of CTV+ cells recovered from the bones analyzed multiplied by 2.63 (100/38). $ALCAM^-$ cells have been documented to gain expression of the surface molecule *in vitro*[30]. However, the possibility of this phenomenon to interfere with our results was excluded by our methods, since we did not culture prior to flow cytometric analysis.

Progenitor Cell Assay

Total bone marrow cells flushed from the donor's vascular marrow were plated in duplicate in 3-cm Petri dishes (BD Discovery Labware, Franklin Lakes, NJ) containing 1 mL of methylcellulose with cytokines (MethoCult GF M3434; StemCell Technologies). This was done before and after CTV staining pre-transplantation using 100,000 cells/plate in order to control for the effect of CTV staining on the viability of cells. Plates were incubated at 37°C in a humidified incubator at 5% carbon dioxide. Total donor BM cells recovered from both the vascular and the endosteal regions of recipient mice 16 hours post transplantation were sorted for CTV^+ cells, and cells from both fractions were plated at 10,000 cells/plate as described above. Colonies were counted on an inverted microscope 6-7 days after plating.

Homing Assay

CTV^+ low-density bone marrow mononuclear cells ($< 1.084 \pm 0.001$ g/ml) were transplanted in 0.2 ml of Iscove modified Dulbecco medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine by

injection into the lateral tail vein of lethally irradiated or non-irradiated male BoyJ recipients as detailed in Results. Between 1.7 x 10^7 and 3.5 x 10^7 cells were transplanted into each recipient immediately after irradiation. Percentages of donor cells recovered in marrow less than 20 hours after injection are a more accurate reflection of the homing potential of transplanted cells rather than their descendants[10]. Thus, we designed the homing assay for analysis between 16 and 20 hours post-transplantation. We will therefore refer throughout the dissertation to the time at which homing was assessed in transplanted mice as 16 hours post-transplantation (16h PT). At 16h PT, mice were killed and bone marrow cells flushed and collected for flow cytometric cell sorting of CTV^+ cells and flow cytometric analysis of Sca-1+ and ALCAM+ populations. Cells recovered from flushed bones represented cells that homed to the vascular niche of the BM. Flushed bones were digested with collagenase as described above to release HCS that were anchored to elements of the endosteal region. Cells recovered after collagenase digestion represented cells that homed to the endosteal niche of the BM. Cells released from the digested bones were also sorted and analyzed.

Statistical Analysis

Differences between groups were analyzed using an unpaired Student's *t*-Test, where a probability value of less than 0.05 was considered statistically significant. Data are expressed as the mean plus or minus SD. Excel 2003 program (Microsoft) was used for all statistical evaluations. A minimum of 3 experiments were performed for each set of results.

RESULTS

Sca-1+ALCAM + cells display an enhanced homing potential

Homing of HSC to the BM is a very rapid process that can be captured within the first 20 hours after injection of donor cells. Transplanted stem and progenitor cells migrate to the BM to anchor within specialized niches and begin dividing, thus, repopulating the hematopoietic system. For the short-term analysis of homing events, we transplanted CTV-labeled total donor cells to determine their absolute and relative retention within the host's bone marrow. Since our hypothesis states that ALCAM promotes anchoring of HSC to elements of the hematopoietic niche via homophilic cell-to-cell adhesion, we expected to observe preferential recovery of BM-homed Sca-1+ALCAM+ cells relative to other cells contained in the graft.

In order to determine the frequency of Sca-1+ALCAM+ donor population that homed successfully and whether spatial preference of homing does exist within the marrow of irradiated and nonirradiated recipient 16h PT, we analyzed the cells collected from the endosteal and from the vascular marrow separately. We examined the absolute and percent recovery of Sca-1+ALCAM+ donor cells and compared those to the recovery of three other phenotypes: total donor bone marrow mononuclear cells, total ALCAM+ cells, and total Sca-1+ cells. As illustrated in Figure 1A, Sca-1+ALCAM+ cells in the vascular region of the BM showed increased recovery (8.35% ± 3.70) compared to total donor cells (4.0% ± 2.0 , $p < 0.05$), total ALCAM+ cells (4.2% ± 1.8, $p < 0.05$), and total Sca-1+ cells

(5.2% ± 2.4, $p < 0.05$). The same pattern of enhanced recovery of the Sca-1+ALCAM + phenotype was observed within the endosteal region, where recovery of double positive cells was 2.2% ± 1.2. This was significantly higher than the corresponding recovery of total cells (0.7% ± 0.3, $p < 0.05$), total ALCAM+ cells (1.0% ± 0.6, $p < 0.05$), and total Sca-1+ cells (1.3% ± 0.5, $p < 0.05$). When recoveries from both the vascular and endosteal regions of the marrow were combined, we observed the same general outcome. Recovery of Sca-1+ALCAM+ cells was significantly higher than that of all four phenotypes: total donor cells ($p < 0.01$), total ALCAM+ ($p < 0.01$), Sca-1+ALCAM + ($p <0.01$), and total Sca-1+ ($p < 0.01$). These data support the notion that ALCAM expression on Sca-1+ cells enhances homing to and retention of these hematopoietic cells in the BM.

Figure 1A.

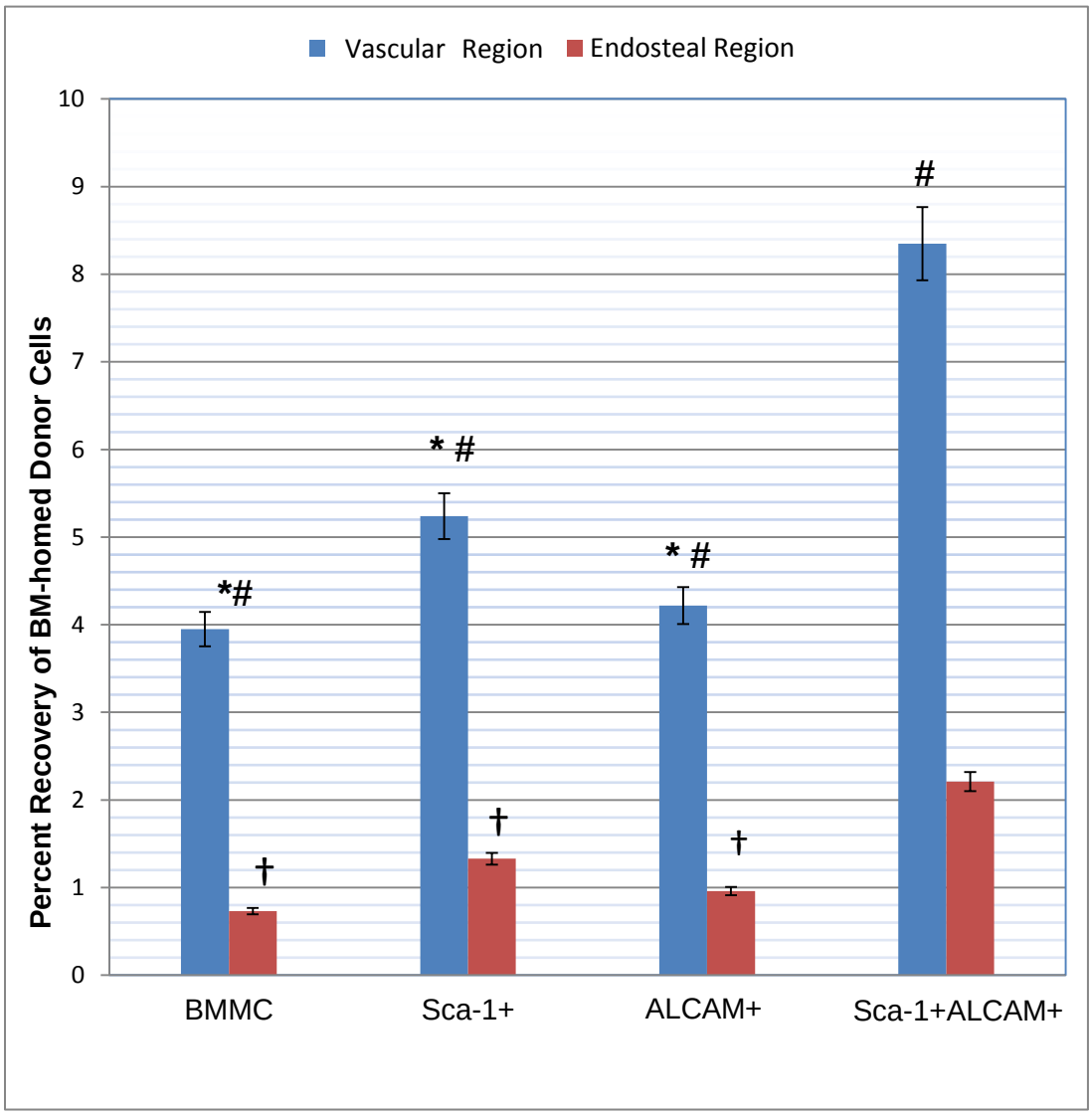
Vascular Region
Endosteal Region
Percent Recovery of BM-homed Donor Cells
10
9
8
7
6
5
4
3
2
1
0
*#
†
* #
†
* #
†
#
BMMC
Sca-1+
ALCAM+
Sca-1+ALCAM+

Figure 1A. Enhanced homing of Sca-1+ALCAM+ cells.

Frequency of recovered phenotypes in the vascular (blue) and endosteal (red) region of the bone marrow 16h PT of total BM cells into lethally irradiated recipients. Sca-1+ALCAM+ cells within both regions of the marrow showed prominently enhanced recovery. Recovery frequencies are calculated relative to the corresponding phenotype contained in the graft prior to transplantation to reveal the homing capability of individual phenotypically defined subsets of cells in the original graft. Thus, the percentage of recovered total donor cells appears artificially lower than the percentage of the three phenotypes under consideration. In one experiment, an F1 mouse (C57BL/6 X BoyJ) was used for the procurement of donor cells. In two experiments some mice were not irradiated prior to transplantation. Data are presented as mean ± SD (* $p < 0.05$, relative to Sca-1+ALCAM+ cells from the Vascular region; † $p < 0.05$ relative to Sca-1+ALCAM+ cells from the Endosteal region; # $p < 0.01$ relative to Endosteal counterpart). n = 8, BMMC = bone marrow mononuclear cells.

The different approaches utilized in harvesting BM-homed cells from both regions introduced a substantial difference in yield of HSPC. Thus, we represented data from Figure 1A as a fold increase in recovery of specific phenotypes compared to total donor cells within each BM region (Figure 1B). Fold increase in recovery was calculated as such:

$$\textbf{Fold increase in recovery} = \frac{\textbf{Recovery frequency of indicated phenotype within a BM region}}{\textbf{Recovery frequency of total donor cells within a BM region}}$$

Dividing results from all phenotypes by the same denominator, as opposed to the input frequencies of different phenotypes in Figure 1A, gave a clearer visualization of phenotype-related spatial preference exhibited by hematopoietic progenitor cells in homing. The frequencies of BM-homed donor-derived ALCAM+Sca-1+, total ALCAM+, and total Sca-1+ cell populations obtained from the endosteum were 3.0,1.3, and 1.8-fold respectively higher than the recovery frequency of total donor cells within that BM region. In contrast, the same phenotypes of donor cell populations obtained from flushing the vascular marrow, were 2.1,1.1, and 1.3-fold higher than the frequency of total donor cells recovered in that region. These findings support speculations of many previous studies[10, 28, 33] by indicating that HSC in particular and primitive hematopoietic progenitor cells in general home and dock within the endosteal region more efficiently than within the vascular marrow. Furthermore, our results illustrate that this pattern of spatial localization in homing may be contingent on ALCAM, since Sca-1+ALCAM + cell populations derived from the endosteal region of the

recipient animal displayed a prominent enrichment (3.0-fold) of progenitors amongst all other populations.

Figure 1B.

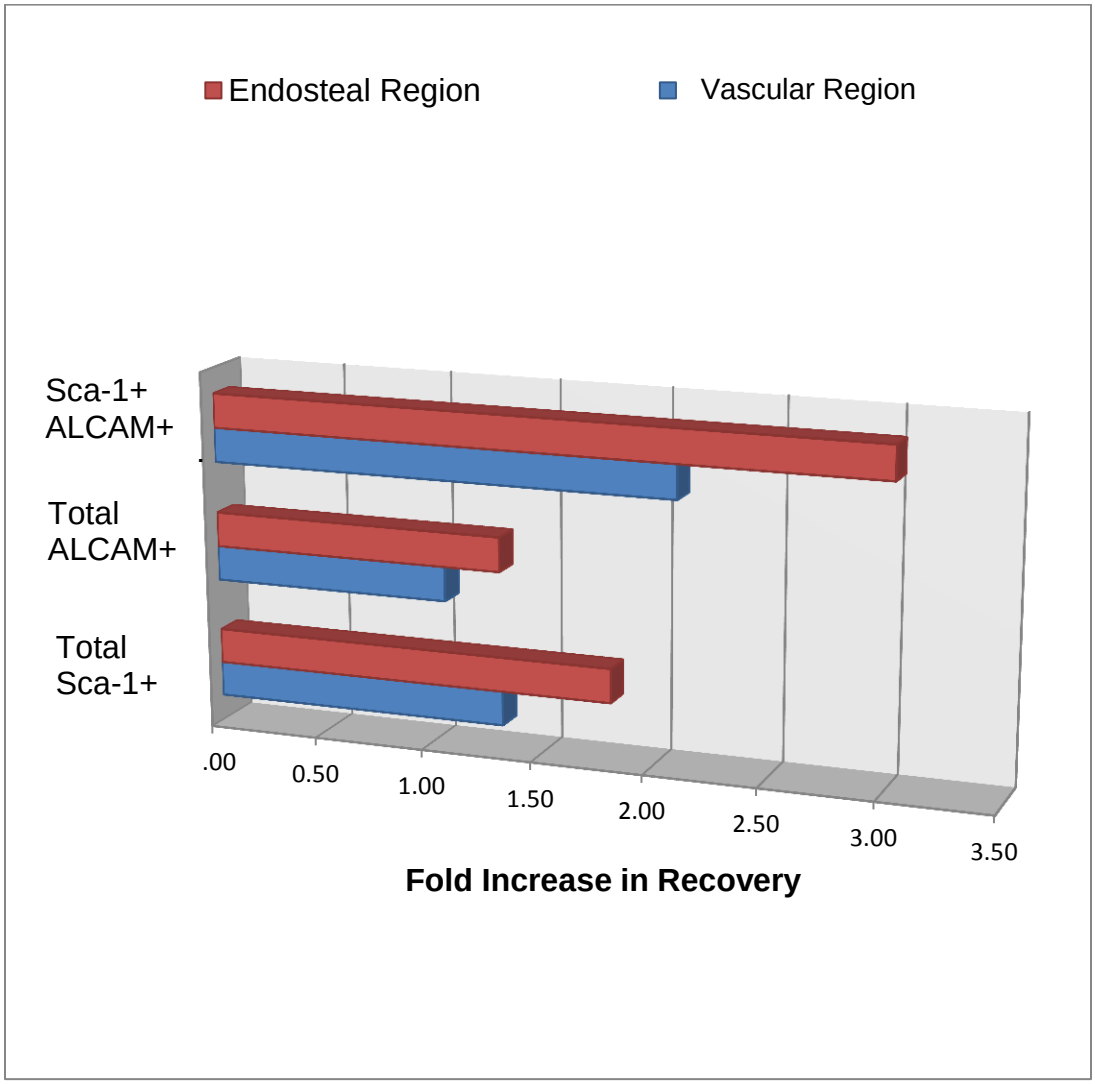
Endosteal Region
Vascular Region
Sca-1+ ALCAM+
Total ALCAM+
Total Sca-1+
.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
Fold Increase in Recovery

Figure 1B. Donor derived hematopoietic progenitors preferentially home to the endosteal region of the hematopoietic niche.

Recovery of all three indicated phenotypes; Sca-1+ALCAM+, total ALCAM+, and total Sca-1+ cells, calculated against total recovered donor cells in each BM fraction. The equation used to calculate fold increase in recovery is described on page 17. Endosteal region (red); vascular region (blue).

To better quantify successfully retained cells in the BM of recipient mice 16h PT in relationship to percentages present in the graft, we evaluated relative homing efficiency of each phenotype using the following equation:

$$\textbf{Relative homing efficiency} = \frac{\textbf{Mean percent recovery of indicated phenotype}}{\textbf{Absolute percentage of recovered phenotype in BM-homed cells}}$$

This representation of our data (Figure 1C) illustrated preferential homing relative to the graft itself within each region and further supported our hypothesis by demonstrating the prominently increased efficiency with which the BM successfully and specifically retains ALCAM expressing Sca-1+ hematopoietic precursors at 16h PT. In this calculation, the homing efficiency for total BM mononuclear cells and total ALCAM+ cells is low due to the high denominator value for these groups of cells because of the wide representation of these cells in the graft. When interpreted in the context of homing efficiency, these data indicate that the large number of BMMC and all ALCAM-expressing cells are much slower to reach the HN and home there. Whereas, the less frequent Sca-1+ALCAM+ group of cells which is also enriched for functional progenitors contributes to an enhanced relative homing efficiency.

Figure 1C.

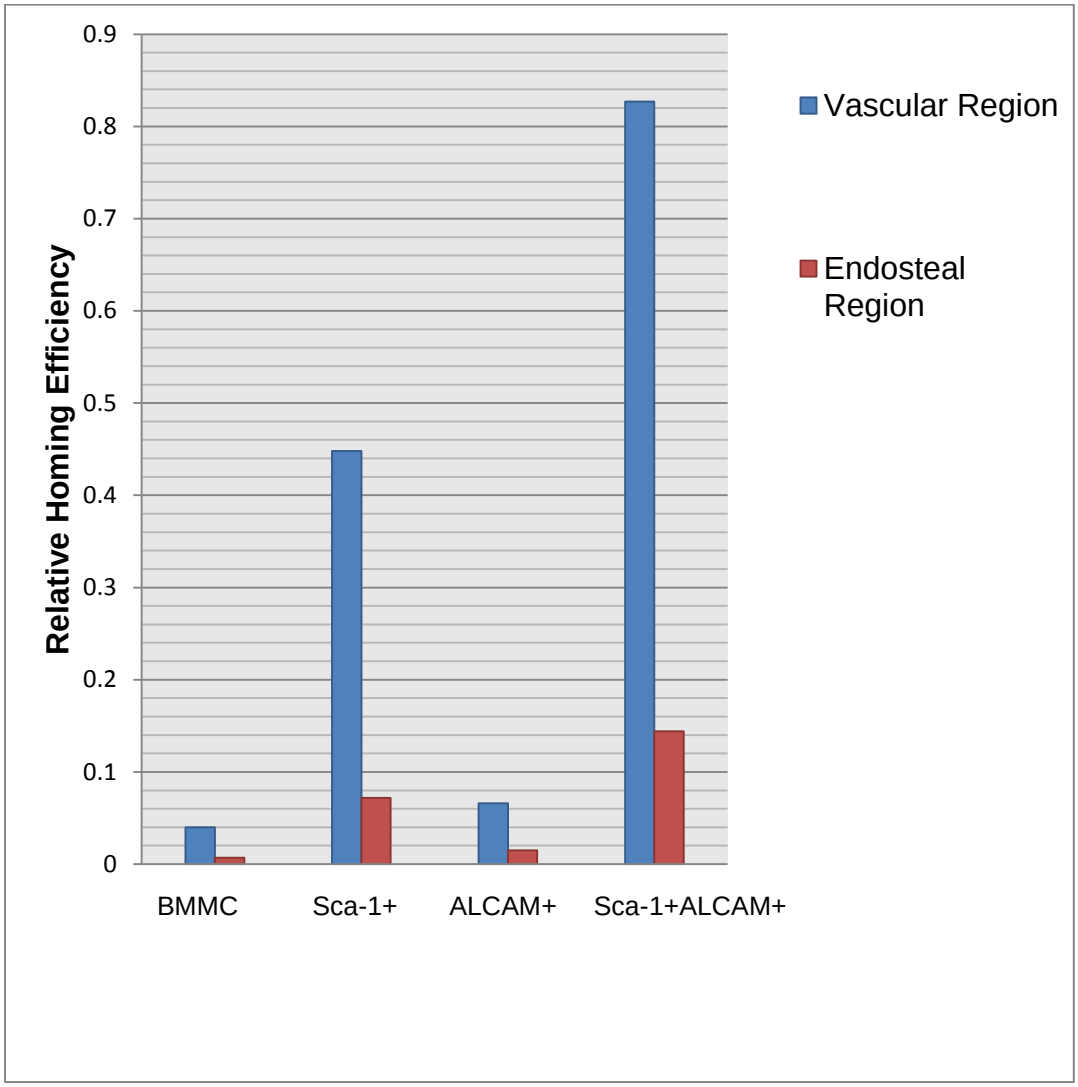
Relative Homing Efficiency
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
BMMC
Sca-1+
ALCAM+
Sca-1+ALCAM+
Vascular Region
Endosteal Region

Figure 1C. Evaluation of phenotype specific homing abilities.

Relative homing efficiency of indicated phenotypes in the vascular (blue) and endosteal (red) region of the bone marrow. Sca-1+ALCAM+ cells within both regions of the marrow showed prominently enhanced homing abilities. n = 8, BMMC = bone marrow mononuclear cells.

In vitro quantitation of colony forming units (CFU) contained in phenotypically defined groups of BM-homed cells

Speculations about cells from the osteoblastic lineage playing a significant role in the regulation of hematopoiesis arose long time ago[25]. Involvement of OBs in supporting HSC function was later proven in genetic models demonstrating that OBs are in fact active participants of the HN *in vivo*[7]. Hematopoietic precursors are thought to localize in close proximity to the endosteal surfaces[9] possibly due to the presence of immature OB possessing strong hematopoiesis enhancement activity[26] and as a result of cell-cell adhesive interactions[19] between HSC and these immature OB. To investigate whether transplanted cells isolated from the endosteal region of recipients had increased percentage of homed ALCAM+ progenitors, we conducted an *in vitro* clonogenic assay. Total donor cells were plated in Methylcellulose before transplantation (Day 0) at a density of 10^5 cells per dish. To determine the number and frequency of CFU in the graft, total donor cells from vascular marrow and from endosteum were recovered 16h PT (Day 1) through sorting of CTV^+ cells and plated at 10^4 cells per dish. Colonies were counted after 6-7 days and the number of colonies obtained at that time was used to express plating efficiency for the post-transplantation fractions (Day 1). Pre-transplantation colonies were normalized to the plating cell density of the two post-transplantation fractions for comparison.

A significant increase in CFU content (Figure 2) was observed in the fraction isolated from the recipient's vascular marrow (10 ± 1 CFU/10^4 plated

cells) when compared to the pre-transplantation donor cells (7 ± 0 CFU/10^4 plated cells , $p < 0.05$). However, the greatest significant increase in CFU content was displayed by the fraction isolated from the host's endosteal BM (15 ± 1 CFU/10^4 plated cells) in comparison to both pre-injection ($p < 0.05$) and post-injection vascular marrow ($p < 0.05$) donor cell fractions.

In parallel to these experiments we conducted a homing assay to corroborate the results obtained *in vitro* and better examine homing abilities of hematopoietic progenitor cells in relation to ALCAM expression. The same populations were analyzed by flow cytometry to determine the absolute frequency of Sca-1+ALCAM+ cells. Consistent with the results obtained *in vitro*, the endosteal fraction of donor cells in the recipient showed the highest percentage (4.9% ± 0.6) of double positive cells when compared to recovered donor cells from the vascular region (3.5% ± 0.8, $p < 0.05$), and to cells before transplantation (2.1% ± 0.8, $p < 0.05$). The comparison between Sca-1+ALCAM+ frequencies in the Day 0 and the vascular Day 1 cell fractions was also significant, $p < 0.05$.

These results illustrate a strong positive relationship between number of homed progenitors and frequency of Sca-1+ALCAM+ cells, which suggests that hematopoietic precursors were retained better within the host BM microenvironment when associated with ALCAM expression. Moreover, these data demonstrated a selective preference for homing to the endosteum, which resulted in the highest enrichment of functional donor-derived progenitors in this niche.

It’s important to mention that while some investigators[24] argued that irradiation of hosts affects distribution patterns of transplanted cells, we and others used nonirradiated[9, 10] and irradiated recipients and still observed a consistent preferential distribution of hematopoietic progenitor cells within the endosteum (data not shown).

Figure 2.

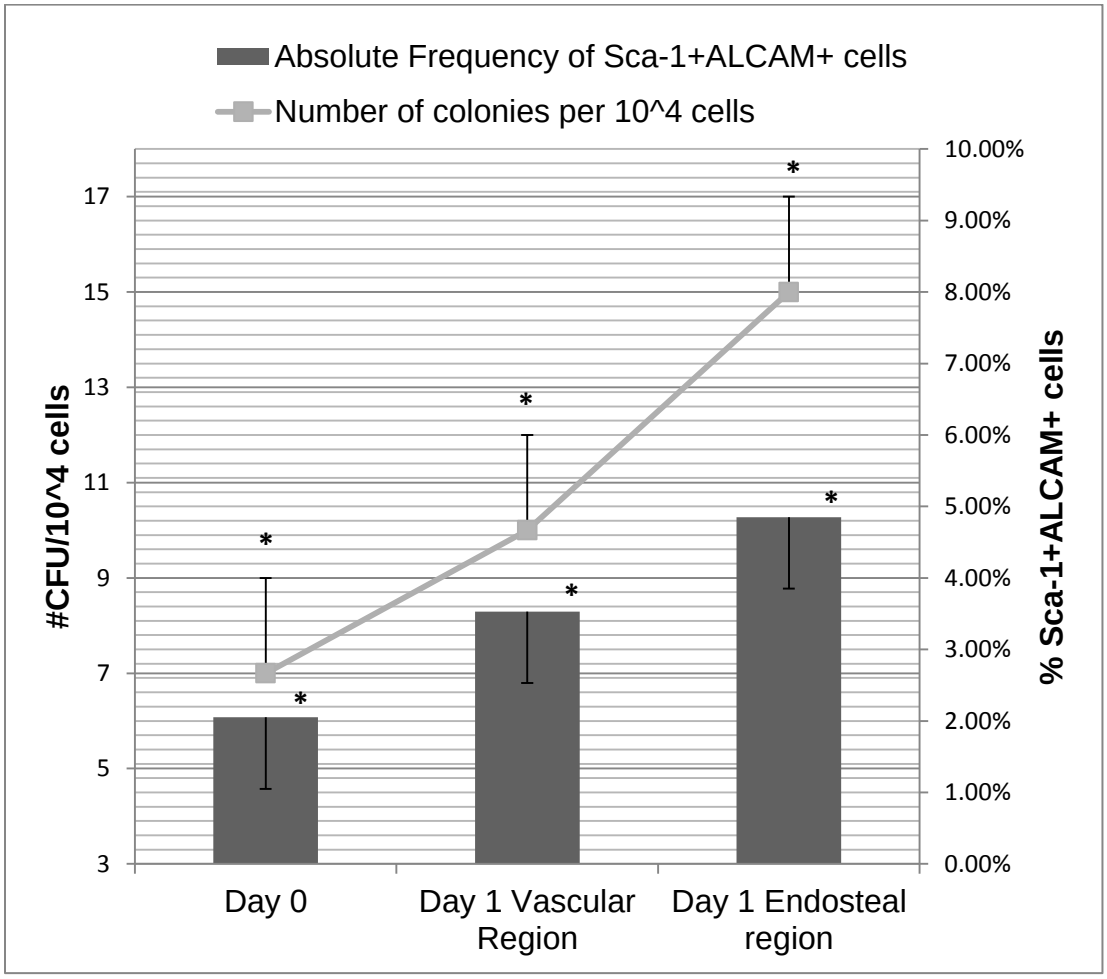
Absolute Frequency of Sca-1+ALCAM+ cells
Number of colonies per 10^4 cells
#CFU/10^4 cells
% Sca-1+ALCAM+ cells
17
15
13
11
9
7
5
3
10.00%
9.00%
8.00%
7.00%
6.00%
5.00%
4.00%
3.00%
2.00%
1.00%
0.00%
Day 0
Day 1 Vascular Region
Day 1 Endosteal region

Figure 2. Increase in homed progenitors associated with increase in Sca-1+ALCAM+ frequency with preference for the endosteal region of the BM. Absolute percentage (bars) of donor Sca-1+ALCAM+ cells pre-transplantation (Day 0), and 16 hours post-transplantation (Day 1) isolated from vascular and endosteal marrow were determined by FACS analysis. Plating efficiency (line graph) of the same cell populations was expressed as number of colonies observed on day 7 of culture in Methylcellulose at 10,000 cells per dish (the actual plating density for the post-transplantation fractions; the pre-transplantation cells were normalized to that plating density). In one experiment an F1 mouse (C57BL/6 X BoyJ) was used for the procurement of donor cells. Data are presented as mean ± SD ($p < 0.05$, $n = 3$).

DISCUSSION

The BM microenvironment provides hematopoietic precursors with the factors necessary for their regulation and balance between cycling and quiescence to maintain a steady state of HSC self-renewal and differentiation of cells into all blood lineages. The complexity of the relationship between HSC and their niche prompted many studies searching for major factors contributing to the competence of the HN. However, complete understanding of the dynamics of HSC-HN interactions and how the HN participates in the regulation of HSC function remains illusive. Investigations on the subject over the years has presented both polarizing and unifying evidence of the coexistence of two HSC niches – an endosteal niche comprised of functionally heterogeneous bone-lining cells[41] and a perivascular niche consisting of the marrow vascular endothelium[61]. Initiation of hematopoiesis in the transplantation setting by infused donor cells relies upon their successful engraftment within the recipient's marrow, which in turn depends on the homing of these donor HSC to the BM and their lodgement within appropriate niches[4]. Currently, most of the steps and factors involving these processes are not fully understood and delineated.

Work on several adhesion molecules expressed on HSC has implicated their involvement in homing to the BM[5]. However, limited investigation on the novel surface molecule ALCAM has been performed and its full impact on homing via homophilic interactions of ALCAM expressed on HSC and cellular elements of their supporting environment has not yet been defined. We therefore

attempted to elucidate the role of ALCAM in homing of HSC and their retention in the vascular and endosteal regions of the BM microenvironment.

Supporting evidence of adhesive interactions between primitive human hematopoietic precursors, namely $CD38^{-/lo}$, $CD34^{+}$, $Thy1^{+}$, $rho123^{lo}$, and stromal cells in adult BM mediated by their mutual expression of ALCAM has been previously reported[8]. Attempts at studying homing, and consequently the role of ALCAM in homing, however have been hampered by the inability to precisely determine when homing should be assessed after transplantation or which exact regions of the BM should be investigated for the presence of homed hematopoietic cells. Even if these issues were resolved, an important parameter to examine in any homing study is the nature and functional capacity of BM-homed hematopoietic cells.

While pursuing definitive evidence, the scientific community presently has accepted a hypothesis that associates the primitive long-term repopulating quiescent HSC with cells of the endosteum, and the more mature short-term repopulating HSPC with the sinusoidal endothelium[7, 9, 10, 12, 17, 19, 22, 28]. This notion of spatial localization of HSC within the mouse endosteum was proposed by early studies on clonogenic progenitors and spleen colony-forming units (CFU-S)[54]. Despite the fact that these cells are nowadays considered more differentiated than HSC, their increased enrichment in close proximity to the inner surface of the femur, paralleled by a low frequency of committed granulocytic progenitors[54], underscored the relationship between location and developmental stage of hematopoietic cells. Nilsson et al. extended that argument by

demonstrating that highly purified, primitive, rhodamine 123/Hoechst 33342 dull (Rh/Hoe^{dull}) stem cells transplanted in nonablated syngeneic recipients displayed strong spatial association for the endosteal BM region[9]. Even though this study further supported an intimate association of hematopoietic early precursors with bone's inner surface, it did not offer insight of the early post-transplantation events since it only described long-term engraftment ranging from six weeks to six months after injection. In order to exclude the possibility that donor cells initially homed to a different location and distributed to a new area post-proliferation, an early post-infusion analysis was required. In addition, cell cycle analysis has shown that the first 40 hours post transplantation depict only events relating to homing alone[4], since hematopoietic progenitor cells remain quiescent within that time window. Thus, any observations collected within the first 20 hours after transplanting donor cells would eliminate the complexity of proliferation. For that reason we designed all of our experiments so that analysis was performed 16 hours post transplantation, thus ensuring we are evaluating only homing ability alone in association with ALCAM.

Nilsson et al. later confirmed that even at 15 hours after injection, transplanted marrow hematopoietic progenitors selectively distributed within the endosteal region, whereas more mature and lineage-committed cells were mainly found in the vascular region of the recipient's marrow[10]. Even though our analysis did not incorporate detailed phenotypic differentiation status, but only relied on identification of early hematopoietic precursors by Sca-1 expression, our data are consistent with these previous studies by demonstrating *in vitro* that

early post-transplantation donor progenitors distributed with greatest frequency within the endosteum. Results from our clonogenic assay illustrated significant enrichment of donor-derived progenitors obtained from the recipient's endosteal region 16h PT (Figure 2). These cells contained a 1.5-fold higher number of clonogenic progenitors than their counterparts from the vascular region of the host's BM. Our data also suggest that spatial distribution of transplanted marrow cells is not a random phenomenon, but is rather dependent on phenotype. The marked enrichment of ALCAM+ progenitors we observed *in vitro* in cells derived from the endosteum indicate that this region might facilitate an improved engraftment and retention of immature hematopoietic precursors.

While the components of the BM microenvironment contributing to the process of homing are still being investigated, several studies have actually suggested that the origin of HSC plays a role in their homing efficiency[28, 33]. Lineage- Sca-1+ c-Kit+ (LSK) HSC isolated from the endosteal region displayed superior homing efficiency when compared with their counterparts from the vascular BM[28, 33]. Our protocol differed from the one utilized in the latter studies in the cells used for transplantation – we only injected cells derived from flushing the vascular marrow of the donor. Nevertheless, we still observed superior retention of progenitors within the endosteum. Our results suggest that a fraction of vascular marrow cells retain the ability to efficiently home to the endosteal niche. Whether these cells possess an enhanced marrow repopulating potential relative to cells that home to the vascular niche, remains to be determined. It is also reasonable to suspect that both the endosteal microenvironment and HSC

share a biological property that leads to a higher homing potential within that region.

In particular, adhesion molecules on cells of the osteoblastic lineage were suspected by Taichman et al. to be instrumental in the homing and tethering of HSPC to the endosteum[19]. The importance of OB in regulating HSC function and numbers[27, 55, 57, 58] through the expression of multiple factors[18, 25] has already been established. Evidence of the required intimate cell-to-cell contact for the survival of HSC in coculture with OB has been presented[56] and the mechanisms of the homotypic ALCAM-ALCAM interactions[36] have been proposed. It is therefore logical to speculate that a surface marker, such as ALCAM, expressed on both cell types – OB and hematopoietic progenitors, acts as a homophilic adhesive molecule and is mediating a mechanism for high homing efficiency.

Indeed, two reports have illustrated elegantly the significance of ALCAM expressing OB in the competence of the HN[12, 22]. Chitteti et al. demonstrated that OB exercise positive regulatory effect on LSK HSC function[12]. Nonhematopoietic Sca-1-ALCAM+ cell populations were established as the immature phenotype within the osteoblastic lineage[12], which was associated with greater support of hematopoietic function[26] in comparison to the more mature ALCAM- OB. In the analysis of nonhematopoietic, nonendothelial BM endosteal cell populations, Nakamura et al. confirmed that Sca-1-ALCAM+ cells have the most robust enhancing activity for HSC, when compared to Sca-1-ALCAM- subset of OB[22]. The latter report went even further into demonstrating higher

levels of expression of cell adhesion- and homing-related genes in hematopoietic precursors promoted by ALCAM+ OB.

Our data support the findings of both published reports[12, 22] by establishing a clear relationship between enhanced homing of hematopoietic primitive cells and their expression of ALCAM. We illustrate that recovery frequencies of Sca-1+ hematopoietic cells from both regions of the BM are prominently increased in association with ALCAM expression (Figure 1A, B, and C). These observations were significantly different from recovery frequencies of total ALCAM+ and total Sca-1+ cell populations, either of which when evaluated separately contributed slightly to an enhanced homing ability.

FUTURE DIRECTIONS

While providing valuable insights on the impact of ALCAM on the distribution and homing of hematopoietic precursors within appropriate niches of the BM early post transplantation, our study did not examine long-term repopulating activity. Hence, further analysis must follow in that direction to fully reveal the mechanism of HSC' dependence on ALCAM. Current research is being conducted (B.R.C., E.F.S.) to demarcate engraftment capability of HSC based on ALCAM expression by incorporating *in vivo* transplantation models with ALCAM knockout recipients and/or donors. Early results confirm ALCAM+ HSC as superior in their engraftment potential compared to ALCAM- HSC (data not published). In addition, the lab has demonstrated (unpublished) that LSK cells from ALCAM knockout mice do not engraft efficiently in wild type recipients.

Many more questions concerning ALCAM's mechanism of action remain to be answered in the future. Further investigation through *in vivo* imaging and gene silencing studies could reveal the pathways and major factors involved in regulation of ALCAM expression, which is a promising approach to manipulation of the HN in the direction of improved long term hematopoietic reconstitution in the context of BM transplantation. Establishing the exact steps in the process of homing – transmigration through the BM endothelium, trans-marrow migration or anchoring and retention in the HN, that are indeed directly affected by ALCAM's action, is also an intriguing aspect of this research. Such discovery could not only improve clinical outcomes in BM transplantation, but may provide a target

for suppression of interactions between leukemic stem cells and their supporting environment.

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