Introduction

The ability of hematopoietic stem cells (HSCs) to exercise their function is vested in their interactive relationships with their neighbor cells and matrix in the place of their residence. This molecularly interactive unit is referred to as “stem cell niche” and its influence on stem cell fate decisions has enjoyed an upsurge of attention lately. Although HSCs on their developmental journey recruit new anatomic sites for their development and are thereby interacting with different “niches,” it is only the bone marrow (BM) niche in adults that has received the most research attention. Genetic mouse models have been instrumental in uncovering critical cellular components of the niche or critical molecular pathways important in supporting niche function. However, the precise anatomic localization or the precise cellular elements of the niche in BM have sparked a continuous debate. Newer data aimed to address or redress the challenges have not led to resolution of divergent views. For example, whether quiescent stem cells reside on the endosteum or near sinusoids and mostly in central areas is not clear. Likewise, whether the coalescence or constellation of few critical cellular components versus 1 or 2 is required to constitute the niche is currently debated. Thus, endosteum cells or sinusoidal cells, or CXCR4-abundant reticular cells, or osteoprogenitor or sinusoidal progenitor cells all have been considered as pivotal cellular contributors to niche formation. Whether these coalesce in specific domains of BM or whether cellular contact between HSCs and these cells is necessary has also been not resolved. Stem cells could respond to gradients of factors generated by the niche or only through their cellular contact with niche cells, but some of the molecular pathways described as responsible for contact (Tie2/Ang-1, N-cadherin) have been convincingly challenged recently.

Major technical difficulties in these studies stem from the very small numeric recoveries after transplantation of few highly purified HSCs used as donor cells. Because of the aforementioned limitations (few total sections, few total cells evaluated), we made an effort to provide rigorous quantitative data with normal cells. In addition, we are presenting novel data with α4-deficient cells in many comparative experiments. We believe that our data in aggregate complement and extend current knowledge in the field.

Methods

Animals

B6x129 or C57Bl/6 wild-type mice were used as donors or recipients for most experiments. In addition, mice lacking membrane-bound kit-ligand (SISIP, Jackson Laboratories) served as recipients for some experiments. Tie2+cre4loxP mice (deleted for kit ligand) and Tie2+cre4loxP/4f/f littermate controls were used as donors for some experiments. Animals were housed at the University of Washington Comparative Medicine Specific Pathogen-Free vivarium, with autoclaved chow and water ad libitum. All procedures were done in agreement with protocols approved by the University of Washington Institutional Animal Care and Use Committee (IACUC).

Transplantation

Recipient mice were conditioned by lethal irradiation with a single dose of 1150 cGy or not conditioned, as indicated. Donor cells (7-30 × 10^6) were transplanted by injection into the lateral tail vein within 4 hours of irradiation. Mice were painlessly killed 20 hours after transplantation, and
Table 1. Quantitative microanatomic distribution analysis of wild-type or α4 integrin-deficient Lin− c-kit+ donor cells in nonirradiated or irradiated compact or trabecular bone

<table>
<thead>
<tr>
<th>Compact bone (femur diaphyses)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of experiments</td>
<td>No. of sections</td>
<td>No. of cells</td>
<td>Zone 1, % cells</td>
<td>Zone 2, % cells</td>
<td>Zone 3, % cells</td>
</tr>
<tr>
<td>α4+/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nonirradiated</td>
<td>3</td>
<td>261</td>
<td>12 379</td>
<td>10.19 ± 0.96</td>
<td>35.60 ± 1.51</td>
</tr>
<tr>
<td>irradiated</td>
<td>6</td>
<td>595</td>
<td>7825</td>
<td>20.58 ± 2.71</td>
<td>38.37 ± 1.88</td>
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<td>α4−/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nonirradiated</td>
<td>4</td>
<td>489</td>
<td>10 042</td>
<td>8.69 ± 1.04</td>
<td>27.62 ± 1.81</td>
</tr>
<tr>
<td>irradiated</td>
<td>5</td>
<td>465</td>
<td>4305</td>
<td>8.98 ± 1.44</td>
<td>36.44 ± 2.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trabecular bone (epiphyses, sternum)</th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of experiments</td>
<td>No. of sections</td>
<td>No. of cells</td>
<td>Zone 1, % cells</td>
<td>Zone 2, % cells</td>
<td>Zone 3, % cells</td>
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<td>α4+/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nonirradiated</td>
<td>3</td>
<td>312</td>
<td>61 630</td>
<td>17.97 ± 1.85</td>
<td>82.03 ± 1.85</td>
</tr>
<tr>
<td>irradiated</td>
<td>7</td>
<td>638</td>
<td>45 869</td>
<td>18.54 ± 1.18</td>
<td>81.46 ± 1.18</td>
</tr>
<tr>
<td>α4−/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nonirradiated</td>
<td>3</td>
<td>325</td>
<td>11 141</td>
<td>9.44% ± 1.85</td>
<td>90.56 ± 1.87</td>
</tr>
<tr>
<td>irradiated</td>
<td>4</td>
<td>348</td>
<td>8049</td>
<td>10.66 ± 2.09</td>
<td>89.34 ± 2.09</td>
</tr>
</tbody>
</table>

Summary of all data from nonirradiated or irradiated recipients of transplanted normal (α4+/−) or α4 integrin-deficient HSC populations: between 60 and 150 sections were analyzed in each experiment. Donor cells given were from 7 to 30 × 10^6 per recipient. Indicated are, for each of the modalities, the number of experiments, the cumulative number of sections and total cells analyzed, and the relative distribution of homed cells to distinct zones within BM (percentage, mean ± SEM). Statistically significant differences between nonirradiation versus irradiation conditioning in compact bone and between α4+/− and α4−/− in trabecular bone are indicated by boldface. A relative preference for endosteal seeding in irradiated marrow in compact bone was observed for wild-type HSCs, whereas α4 integrin-deficient cells distributed randomly, as in nonirradiated hosts. Microscopic images illustrating the different microanatomic distribution of transplanted wild-type cells in hosts with/without irradiation are shown in Figure 2G and H.

*P < .005; †P = .001; ‡P = .02; §P = .03; ††P = .006.

Cells

To generate donor cell populations, cohorts of isogenic donor mice were painlessly killed using approved protocols, and BM cells were harvested from long bones. Cells were pooled and then either enriched for c-kit-expressing cells or depleted for lineage marker-expressing cells using immunomagnetic isolation with commercially available reagents (biotinylated anti–c-kit antibody [2B8], biotinylated lineage-depletion antibody cocktails, anti–biotin-immunomagnetic beads [Miltenyi Biotec]) following the manufacturer’s protocols. Prior to transplantation, purity of the resulting cell populations was routinely assessed; c-kit enrichment regularly resulted from long bones. Cells were pooled and then either enriched for c-kit–expressing cells or depleted for lineage marker–expressing cells using cell-permeable dyes carboxyfluorescein succinimidyl ester (CFSE) (green; Molecular Probes) or red (SNARF) labeled cells. A labeling efficiency of >95% was regularly achieved. Label crossovers were confirmed using equal homing efficiency of green (carboxyfluorescein succinimidyl ester [CFSE]) or red (SNARF, red; Molecular Probes). A labeling efficiency of >95% was achieved. Label crossovers were confirmed using equal homing efficiency of green (carboxyfluorescein succinimidyl ester [CFSE]) or red (SNARF, red) labeled cells.

Homing experiments

For homing studies the proportions of colony-forming unit cell (CFU-C) populations recovered from femurs, peripheral blood, and spleen of irradiated recipients given α4+/− and α4−/− cells were assessed 24 hours after transplantation relative to the inoculum (CFU-C) transplanted. In addition to cells recovered from flushing femurs in these experiments, cells recovered from fragmented femur segments following collagenase I treatment (3 mg/mL collagenase type I [Sigma-Aldrich]) were also evaluated for CFU-C recovery at 24 hours (27 × 10^6 BM donor cells) and 8 days after transplantation (3 × 10^6 BM donor cells).

Migration assay

Transwell migration of Kit+/α4+/− or Kit+/α4−/− BM cells was performed as previously described. Briefly, to engage the α4 integrin, immunomagnetically purified kit+ cells were first incubated in medium containing 10% fetal calf serum, 10% Wehi-conditioned medium from Walter and Eliza Hall Institute–3 cells, and stem cell factor (100 ng/mL) for 2 hours, then transferred to RetroNectin-coated Transwells in the presence or absence of stromal-derived factor 1α (SDF-1α; 100 ng/mL; Peprotech) in the bottom chamber. Spontaneous or SDF-1α–directed migration was assessed after 4 hours by quantitating the number of migrated cells recovered from the lower chamber as a proportion of the input cells.

Preparation of bone sections, image acquisition, and analysis

Recipient mice were prefixed in 4% paraformaldehyde. Bone sections representing compact bone (entire femur shafts) or trabecular bone (proximal and distal ends of 2 femurs [epiphyses] or sternum) were cut into 3 to 5 segments, embedded without decalcification in OCT (Tissue-Tek; Sakura Finetechinal Co), and frozen in acetone/dry ice. Embedded bony fragments were cut into 4- to 5-μm sections (transverse for compact bone, longitudinal for trabecular bone) using a cryostat microtome (CM1850; Leica) equipped with a tungsten carbide knife (FM16 cm/d; Leica) and a CryoThin tape-transfer system (Instruments, Inc). Sections were mounted with DAPI (4,6 diamidino-2-phenylindole; blue nuclear stain) mounting medium (Vectashield; Vectorlabs). Images were acquired at room temperature using a Leica DMLB fluorescence microscope (with appropriate filters) outfitted with a Leica 10×0.30 PH1 HC-PL FLUOTAR objective and a Spot RT Riliader camera using SPOT Advanced software (Version 4.6; Diagnostic Instruments Inc). For cell counting an unbiased observer counted green (or red) cells relative to their spatial distribution within coded sections using a Nikon Eclipse E800 fluorescence microscope. Three zones were defined within the BM cavity for compact bone: endosteal (≤3 cell diameters from endosteal surface: zone 1), subendosteal (4–14 cell diameters from endosteal surface: zone 2), or central (≥14 cell diameters from endosteal surface: zone 3, and 2 zones were defined for trabecular bone: endosteal (≤3 cell diameters from endosteal surface) or nonendosteal (≥3 cell diameters from endosteal surface). Sixty to 150 sections were analyzed from either compact or trabecular bone (femur shafts or epiphyses/sternum). Results are expressed as either relative frequency of homed cells in each of the zones among cells homed in all zones (microanatomic
Figure 1. Definition of endosteal, subendosteal, and central zones in BM and demonstration of cell imaging. (Ai-i) Definition of endosteal, subendosteal, and central zones in BM sections of femur diaphysis representing compact bone. Regions in each transverse bone section were defined as endosteal (≤ 3 cell diameters from endosteal surface; zone 1, depicted in red), subendosteal (4-14 cell diameters from endosteal surface; zone 2, depicted in yellow), or central (≥ 14 cell diameters from endosteal surface; zone 3). The average relative surface area of each of the 3 regions was calculated, using image analysis software, as 8% ± 0.63%, 31% ± 1.31%, and 61% ± 1.85%, for zones 1, 2, and 3, respectively. (Bi-ii) For trabecular bone sections, 2 zones were evaluated: endosteal in red and the rest. (C) Depiction of homed donor Lin−kit+ cells to irradiated BM. Transplanted cells were labeled with CFSE (green); red-stained cells (anti–CD31-PE) are vascular cells and megakaryocytes (white arrow). (D) Competitive homing of α4-deficient (CFSE, green) and α4 integrin–deficient (CFSE, green) labeled hematopoietic Lin−kit+ cells in irradiated BM. The 2 populations were given at a ratio 0.7 (red):1 (green) (see Table 2 for details). The white interrupted line in panels C and D represents the endosteal border.

homing distribution), or as absolute numbers of cells from all sections counted (Table 1), or of cells recovered per section, normalized to the number of transplanted cells (Figure 3 and Table 3).

Immunohistochemical staining

For analysis of CD31 expression, frozen sections were blocked with 10% goat serum followed by subsequent staining with anti-CD31 (MEC13.3; BD Biosciences) or isotype control and Alexa Fluor 594 goat anti–rat immunoglobulin G (Molecular Probes). Slides were mounted and images acquired as described in “Preparation of bone sections, image acquisition, and analysis.”

Statistical analysis

Homing efficiency and relative distribution to the different zones for wild-type versus α4 integrin-deficient cells, or nonirradiated versus irradiated hosts, were compared using Student t test. Mean plus or minus standard error of the mean (SEM) was calculated using Student t tests in Excel Software (Microsoft). A P value less than .05 was considered statistically significant.

Results

Cell recovery in trabecular versus compact bone before and after irradiation

As indicated in “Preparation of bone sections, image acquisition, and analysis,” for compact bone (femoral diaphysis) we chose to use transverse sections of the entire femur shaft because the distances from the center of the bone to the endosteu are maintained through a large number of such consecutive sections. To assure the presence of an adequate number of donor cells in each section, we infused several millions (7 to 30) of CFSE-labeled Lin−Kit+ cells and made our evaluation in an unbiased fashion using a rigid set of criteria (see Figure 1Ai-ii for anatomic zones) chosen to allow comparisons with previously published data. Furthermore, using the same pool of donor cells, observations were made in both irradiated and nonirradiated recipients. To represent trabecular and nontrabecular bones we also evaluated sections from femur epiphyses and sternal bones, adapting a modified set of criteria (2 zones instead of 3, Figure 1Bi-ii). Several experiments were performed and 60 to 150 sections were evaluated from 2 femurs or from proximal and distal epiphyses from each recipient (Table 1). Thus, the entire femur shafts (representing compact bone) or entire epiphyses or sternal bones (representing trabecular bone) were sectioned. Cellular images of nonirradiated and irradiated bone sections are shown in Figure 2A through F. Donor cells seeded in irradiated and nonirradiated tibiae are shown in Figure 2G through H. Total cells recovered per section (from either compact or trabecular bone) were enumerated and normalized to the number of CFSE-labeled donor cells transplanted (Figure 3).

Two striking differences are immediately apparent: the recovery of intravenously transplanted cells was significantly higher in trabecular bone areas (femoral epiphyses, sterna) than in femoral shafts. Nearly 5-fold differences were seen between the former versus the latter bones in nonirradiated recipients and about 4-fold in irradiated recipients. Overall these data suggest that trabecular bone is more conducive to the entrapment of intravenously infused cells.

Equally important is the conclusion that the total recovery of cells (per section/1 × 10^6 cells) is much higher in nonirradiated
than in irradiated recipients. This difference was more pronounced in compact bone than in trabecular bone. Although the reasons for these quantitative differences before and after irradiation are not immediately apparent, the anatomic changes in cellular density after irradiation may be of relevance. After irradiation there are prominent differences in cellular density in both compact and trabecular bone. In addition to the general decrease in cellular density regularly seen after irradiation (Figure 2), small acellular areas, especially near the center, are not infrequent after irradiation. Large acellular areas were separately evaluated through imaging analysis and found to account for 9% plus or minus 1% of the total space in each section (n/H11005/20). It is thus possible that cellular density, modified by irradiation, influences at least partially and indirectly cellular retention after transplantation. Furthermore, irradiation-induced changes, that is, increase in SDF-1 elaboration, in extramedullary organs are also possible, as these could additionally siphon transplanted cells off into nonhematopoietic tissues thus reducing the number of cells available for BM uptake.

Microanatomic distribution of transplanted normal cells in nonirradiated versus irradiated trabecular or compact bone

In addition to cellular recoveries, in each section we evaluated in detail the microanatomic partitioning of CFSE-labeled Lin^−kit^+ cells into the 3 designated zones in compact bone (Figure 1Aii) or the 2 zones (endosteal and rest) in trabecular bone (Figure 1Bii). Femur shafts were divided into 3 to 5 segments and from each,
24 to 32 serial sections were prepared and evaluated. Given the thickness of these sections a few cells may have been counted in more than one section, so the error would affect data equally before and after irradiation. Furthermore, in our counting we avoided the recording of small fluorescent events without a nucleus (DAPI stained). Qualitative distribution of donor cells is seen in Figures 1 and 2C. A solitary rather than a clustered pattern is observed and cumulative data from all experiments are presented in Table 1 and Figure 4. In nonirradiated recipients, a consistent proportion of 10.19% plus or minus 0.96% in irradiated bone marrow (3 cells from endosteum), of 35.6% plus or minus 1.51% in subendosteal bone, and of 54.21% plus or minus 0.55% in compact bone is seen. To test whether these values deviated from expected random distribution according to the anatomic area represented by each zone, we evaluated in many sections (by image analysis) the relative anatomic surface area occupied by each zone. The values from each zone (zone 1: 8.0% ± 0.6%; zone 2: 31.0% ± 1.3%; zone 3: 61.0% ± 2.0%) are not considerably different from the proportional cell distribution mentioned 7 to 9 lines above and evaluated independently in each section. This would suggest that, in nonirradiated recipients, the distribution of incoming cells (Lin–kit+) is close to random. In contrast to the near-random distribution seen in nonirradiated hosts, transplanted cells in irradiated hosts showed a preferential endosteal lodgment (Table 1, Figure 2, and supplemental Figure 3, available on the Blood website; see the Supplemental Materials link at the top of the online article). At the same time there was a significant reduction of cells recovered from the central area. Thus it would appear that a shift in population distribution occurring after irradiation (increase near endosteum vs decrease in central zone) should yield a relative change in endosteum (zone 1) or subendosteum (zone 2) or both. To what extent preferential seeding to endosteal zones was due to other than the relative changes mentioned, that is, initial random distribution with subsequent migration (chemotactic?) to endosteal zones and/or enhanced retention in this region because of better preservation of endothelial cells after irradiation,15 was not clear from the data with normal cells and was subsequently addressed using donor cells with deficient adhesion and/or migration properties. In trabecular bone, no significant distribution differences were recorded after irradiation even though the overall cell recovery was also reduced after irradiation as in compact bone (Figures 2-3). However, there are some inherent limitations in interpreting the data with 2-dimensional sections of trabecular bone. Depending on cell size, a few cells scored in zone 2 in one section may be scored to zone 1 in a consecutive section. Although this may alter the frequency of cells with “endosteal” placement, our conclusions with trabecular bone data, that is, differences before and after irradiation or total cell recovery compared with compact bone, would not be altered.

Recovery and microanatomic localization of α4 integrin-deficient cells in trabecular versus compact bone before and after irradiation

Having established a template of microanatomic distribution and recovery with normal cells, we then explored the behavior of α4-deficient cells in all parameters studied with normal cells. Our previous studies, confirmed by a number of investigators, documented reduced homing using α4-deficient donor cells, but the ability of these cells to partition to different anatomic zones within BM was not previously tested. Beyond the comparison of α4+/cre+ cells (α40/cre−) with α4−/− cells we also conducted successful experiments in which both types of donor cells, labeled with different fluorochromes, were given to the same recipients, that is, a “competitive” biodistribution assay (Figure 1D). Quantitative data from these experiments are shown in Table 2 and the variability in cell recovery per section for each zone is shown in Table 3.

As depicted in Figure 3 showing cell recoveries for all experiments with α4−/− cells, a significant decrease in cell recovery with α4−/− cells was found compared with normal cells, both in compact and trabecular bone, either before or after irradiation. This difference is consistent with all previous data on homing with these deficient cells using standard homing assays (Scott et al13 and references therein) and reassuring that our current evaluation reflects these changes faithfully. Furthermore, insightful data were obtained in terms of microanatomic distribution of α4−/− cells (Table 1 and Figure 5). In nonirradiated recipients the distribution of α4−/− cells in the 3 anatomic zones was essentially the same as that of α4+/+ cells, and both types of cells had a distribution not significantly deviating from random, estimated by image analysis (P = .63 for zone 1, P = .13 for zone 2, and P = .42 for zone 3 for α4−/− cells). These data would suggest that α4-deficient cells are capable of a random interstitial transmigration within BM. However, in irradiated hosts, a number of differences are seen with these cells: (1) The preferential distribution to the endosteal region, which was observed with α4+/+ donor cells, was not seen when α4−/− cells were transplanted. Thus, values of 8.69% plus or minus 1.04% before irradiation and 8.98% plus or minus 1.44% after irradiation were observed in endosteal zone 1, and both of these values were very similar to the surface...
area estimated, suggesting a random distribution. (2) In zone 2, more cells were recovered after irradiation (27.82% ± 1.82% vs 36.44% ± 2.02%), whereas and similarly to normal cells, significantly fewer cells were seen in zone 3 (central zone) after irradiation (63.49% ± 2.4% vs 54.58% ± 2.35%). These changes are likely consequent to alterations in cell density seen after irradiation, although one could interpret the increase in zone 2 as a sluggish response of α4−/- cells to stimuli from endosteum. The microanatomic distribution with α4−/- cells gives added credence to our chosen narrow definition of endosteal placement, compared with a more broad one (ie, 12-cell distance from endosteum) used previously.10 In trabecular bone, as in normal cells, no changes were recorded after irradiation.

To test whether the findings with α4-deficient cells can be reproduced in an independent manner, we also did the following: In standard homing assays using α4+/+ and α4−/- cells we recovered not only cells flushed from femur shafts 24 hours later, but also cells from cleaned and fragmented bones treated with collagenase to collect cells from endosteal areas. We cultured each sample and compared the proportional recovery of injected CFU-Cs from flushed femurs and from cells recovered from endosteal areas. The same studies were also repeated 8 days after transplantation. The data (supplemental Figure 1) show again impaired total recovery of α4-deficient cells compared with controls, not only from flushed femurs as expected, but also from endosteal areas, both at 24 hours and 8 days after transplantation. These data reaffirm the compromised retention of α4-deficient cells in endosteal areas.

### Microanatomic localization of pertussis toxin–treated donor cells or of normal cells in Sl/SF recipients before and after irradiation

As described in the previous section, α4-deficient cells do not preferentially accumulate at the endosteal surfaces (zone 1) after irradiation. One of the possibilities responsible for this outcome is their failure to migrate to a putative SDF-1 gradient existing near endosteum.17 A prerequisite for this hypothesis is that donor cells treated with pertussis toxin (PTX) ex vivo would behave similarly. Gi protein signaling inhibition by pertussis toxin induces mobilization similar to that seen in α4-deficient cells18 and when used in combination (ie, pertussis toxin treatment of α4−/- cells) the homing is synergistically impaired.19 When PTX-treated donor cells (Lin−kit+) were given to irradiated recipients, we found that in femur diaphysis sections representing compact bone (no. of sections = 120), 9.5% plus or minus 2.3% of cells were in the endosteal area (zone 1), 38.5% plus or minus 6.3% were in subendosteum (zone 2); and 48.04% plus or minus 7.3% were in the central area (zone 3; Figure 4). Thus the endosteal distribution of PTX-treated donor cells in irradiated recipients was significantly different from the concurrently studied non–PTX-treated control cells given to another recipient (supplemental Table 1A), and was similar to α4-deficient cells, unlike all other normal cells (Figure 4).

In addition to the cross-talk between α4 integrins and SDF-1 signaling, cooperative signaling was previously demonstrated between α4 integrin and kit/kit ligand (KL).20-22 To study the behavior of normal cells into transplanted hosts lacking membrane bound KL, that is, Sl/SF− mice, we assessed their microanatomic

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### Table 2. Competitive biodistribution of α4+/+ and α4−/- cells

<table>
<thead>
<tr>
<th>Cells counted</th>
<th>Zone 1, %</th>
<th>Zone 2, %</th>
<th>Zone 3, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irradiated recipients</td>
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<td></td>
</tr>
<tr>
<td>11.5 × 10^6 green α4+/+ cells + 11.8 × 10^6 red α4−/- cells</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CB</td>
<td>100</td>
<td>906 green α4+/+ cells</td>
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<tr>
<td></td>
<td></td>
<td>277 red α4−/- cells</td>
<td>10.12 ± 1.12</td>
</tr>
<tr>
<td>CB</td>
<td>118</td>
<td>4509 green α4+/+ cells</td>
<td>21.29 ± 3.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>910 red α4−/- cells</td>
<td>12.53 ± 2.2</td>
</tr>
<tr>
<td>Nonirradiated recipients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.25 × 10^6 green α4+/+ cells + 8.5 × 10^6 red α4−/- cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB</td>
<td>140</td>
<td>26 green α4+/+ cells</td>
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<tr>
<td></td>
<td></td>
<td>1212 red α4−/- cells</td>
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<td>1942 green α4+/+ cells</td>
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Cumulative data from irradiated and nonirradiated recipients of both α4+/+ and α4−/- cells labeled with different fluorochromes. Note that the data from these competitive distribution experiments are in line with data shown in Table 1 regarding differences between α4+/+ and α4−/- cells in both cell recoveries and microanatomic distribution after irradiation in compact bone. (The numbers for α4−/- cells in “Nonirradiated recipients” are not included in Table 1.)

CB indicates compact bone; Tr B, trabecular bone.

Boldface indicates significant differences between α4+/+ and α4−/- cells.
distribution in nonirradiated or irradiated Sl/Sld recipients. We found that in nonirradiated recipients (90 sections, compact bone) the distribution across the 3 zones was 8.01% plus or minus 1.3% (zone 1), 34.29% plus or minus 2.9% (zone 2), and 57.71% plus or minus 4.2% (zone 3), whereas in irradiated recipients (106 sections, compact bone) it was 14.2% plus or minus 2.5% (zone 1), 51.2% plus or minus 2.4% (zone 2), and 34.55% plus or minus 4.76% (zone 3) (Figure 4 and supplemental Table 1B). A modest increase was seen in zone 1 in irradiated Sl/Sld hosts compared with cells given to normal irradiated recipients but this was at the lowest levels seen in normal recipients (Figure 4). Thus, these data taken together suggest that the α4 integrin, the G protein–dependent, and to a lesser degree the kit/KL-dependent signaling pathways play a role in microanatomic partitioning of cells near the endosteum.

Discussion

Hematopoietic stem cell localization at or near the endosteum was presented more than 30 years ago by Lord et al23 but refuted by Maloney et al.24 A number of recent studies have resurrected the endosteal issue of the niche16 and many subsequent enthusiastic supporters provided static or in vivo color images for its support.6,8,9,25-27 However, immunofluorescence images were not usually supplemented with adequate quantitative data and neither the criteria of distance from endosteum (from 2 cells25 to 12 cells6,26) nor the donor cells used were the same (Lin−, LSK CD150+/48−, Flt3+, etc). Nevertheless, despite these inconsistencies, reported studies indicate that there are modest differences, if any, in anatomic sublocalization between populations highly enriched in repopulating stem cells versus progenitor cells. Both the latter16,25 and the highly enriched cells26 were found to be preferentially localized near endosteum, albeit with different criteria defining distance from endosteum.

To provide large-scale quantitative data we decided to use populations of cells and evaluation criteria that allowed comparisons with previously presented data. The differences we observed with Lin−/kit+ cells in irradiated versus nonirradiated recipients are qualitatively similar to those of more purified cells recently reported by Li et al3 and Lo Celso et al,26 although anatomic differences encountered in irradiated BM and potentially quantitatively influencing the results were not considered in these studies. Furthermore, the random distribution (Figure 5) documented in nonirradiated recipients is reminiscent to the one found with highly enriched stem cells (CD150+/48−) described by Kiel et al17 in steady-state hematopoiesis. Such a distribution is also consistent with the random spread across BM of CXCL12-abundant reticular cells supporting stem cells.10 A preferential endosteal localization of highly purified HSCs compared with progenitor enriched cells was reported by Lo Celso et al26 in irradiated recipients, although the differences were modest and, on the average, less than 1 cell diameter. Similar population distinctions were not made, however, in nonirradiated recipients in which the data presented (8 cells from 3 recipients) show no endosteal placement for highly purified LSK CD48−Flk2− cells. (They were all 30 to 50 μm away from the endosteum.) In contrast, in another recent study, 11% (5 cells) of transplanted Lin−/Sca+“kit” cells were in direct contact with endosteum (and 95% were within 20 μm from endosteum), whereas only 4% (3.9 cells) of Lin−/Sca+“kit” cells did contact endosteum.28 These data led the authors to suggest “distinct” niches for Lin−/Sca+“kit” versus Lin−/Sca+“kit” cells, although endosteal placement for differentiated cells (macrophages and dendritic

Figure 5. Microanatomic distribution of transplanted α4+ and α4− cells. (A) Flow cytometric analysis of donor cell populations. Lineage-depleted (using antibodies, magnetic beads, and column) cells were labeled with anti–c-kit (top 2 panels) and with anti-α4 Ab (bottom panels). Kit positivity was 82.8% in α4+ donor cells and 77.3% in α4− donor cells. Shaded areas represent isotype control. α4 integrin was expressed on >90% of normal donor cells and virtually absent on α4-ablative donor cells. (B) Relative distribution of transplanted cells from normal or α4 integrin-deficient donors to nonirradiated or irradiated normal hosts to endosteal regions of trabecular bone. The relative seeding to endosteal regions of trabecular bone was not affected by irradiation, and did not differ significantly between normal and α4 integrin-deficient donor cells. (□), normal donor cells; (■), α4− donor cells; mean ± standard error of the mean; see Table 1 for values.) (C) Relative microanatomic distribution of transplanted cells from normal or α4 integrin–deficient donors to nonirradiated or irradiated normal hosts to endosteal, subendosteal, and central regions of compact bone. In each graph represent the relative surface area estimated by image analysis of each of the 3 zones (Figure 1A). Relative distribution frequencies equivalent to these values would thus indicate random distribution of donor cells throughout the marrow mass. Such random distribution was observed in nonirradiated hosts (□), irrespective of donor type, and in irradiated recipients (■) given α4− cells. In contrast, normal cells transplanted into irradiated hosts distributed preferentially to the endosteal region (top panel). Asterisks indicate statistically significant differences between normal and α4− cells and between no-irradiation (□) and irradiation (■) conditioning.
cells) was also documented. Thus, a diversity in the microdistribution of HSCs versus hematopoietic progenitor cells has been presented depending on the approach used. Furthermore, it is worth noting that the previously reported homing data (assessed as cells recovered from flushed bones in recipients up to 24 hours later) did not differ between highly purified cells\(^4\) (Hoechst\(^{lo}\) and Rhodamine\(^{ho}\)) and progenitor-enriched cells.\(^{30}\)

Thus the weight of the evidence would point to at least no sharp differences in anatomic partitioning between transplanted stem cell–enriched versus progenitor-enriched donor cells, although their responses to niche stimuli may be quite different because of their intrinsic repertoire of factors.

Using the same protocols, contrasting data were obtained with \(\alpha 4\)-deficient cells. A significant overall decrease in \(\alpha 4^{lo}\) cell recoveries was observed (Figure 3, Table 2) consistent with previous data on reduced homing (Scott et al\(^{13}\) and references therein). However, unlike normal cells, preferential endosteal seeding following irradiation was not observed. The significance of these data is 2-fold. First, it was unclear with normal cells to what extent the increased endosteal frequency in irradiated hosts was the result of a preferential migratory shift of transplanted cells toward the endosteal region, or whether it was indirect and consequent to cellular shifts in other areas after irradiation. Clearly the failure of \(\alpha 4^{lo}\) cells to preferentially locate to the endosteal region, even in the presence of changes in other areas, favors the former. Second, the inability of \(\alpha 4^{lo}\) cells to preferentially seed to the endosteal region may be due to their impaired responsiveness to putative chemotactants emanated by the radiation-damaged endosteal regions, or to the absence of \(\alpha 4\)-dependent retention signals in endosteal sinusoids, as previously shown for calvarial sinusoids,\(^{31}\) or in BM sinusoids for immature B cells.\(^{32}\)

An impaired ability of \(\alpha 4^{lo}\) lymphocytes\(^{34}\) to migrate in vitro toward an SDF-1–dependent chemotactic gradient was previously documented and impairment was also seen with \(\text{kit}^{+}\alpha 4^{lo}\) cells (supplemental Figure 2). Although these in vitro data are consistent with our in vivo findings, it is acknowledged that these in vitro static data cannot be extrapolated to the in vivo functional behavior of cells, especially when integrins can alter their function quickly, depending on their environmental context. Because there is a cross-talk between \(\alpha 4\) and G-protein–coupled receptor–dependent signaling, it is theoretically expected that other cells with impaired responses to an SDF-1 gradient, that is, PTX-treated cells, may behave similarly to \(\alpha 4^{lo}\) cells in vivo. Following this reasoning we used PTX-treated cells in our studies and found that these cells behave similarly to \(\alpha 4^{lo}\) cells after irradiation, that is, their endosteal placement was at levels similar to their random preirradiation distribution. Although these data provide support for the presence of an active SDF-1 gradient near endosteum after irradiation as previously suggested,\(^{33}\) they do not necessarily explain the \(\alpha 4\) data, nor do they exclude scattered increases in SDF-1 throughout bone marrow. Furthermore, our data strictly concern \(\alpha 4^{lo}\) progenitor enriched cells, and whether highly purified stem cell populations would display different functional behavior remains to be seen. In this context, the recent findings of Köhler et al\(^{38}\) suggesting an inverse correlation between expression of CD49d and distance from endosteum in Lin ‘Sca’‘kit’ cells versus Lin ‘Sca’‘kit’ cells. However, a positive correlation was found for young versus aged Lin ‘Sca’‘kit’. These determinations were recorded in cells ex vivo and may not account for functional differences or for ligand-induced modulation of functional integrin when the cells encounter the in vivo environment.

![Figure 6. Microanatomic distribution of normal donor cells in irradiated Sli/Sli recipients. Transplanted HSCs display a qualitatively similar preferential lodging to endosteal regions in irradiated Sli/Sli recipients (blue: DAPI, green: CFSE-labeled wild-type HSCs, red: anti-CD31, top panel: tibia, bottom panel: femur), as normal cells do.](image)

What are the implications of these findings in terms of short- and/or long-term hematopoietic reconstitution? It was previously argued that an impaired endosteal portioning of incoming cells within nonirradiated BM could account for a decrease in transplantation outcomes observed in irradiated recipients, especially when traditional homing assays did not show any significant deficit.\(^{16,25,28,34}\) It is difficult to reconcile these data with our data, as well as those of Kiel et al,\(^{7}\) Sugiyama et al,\(^{10}\) and Xie et al,\(^{27}\) all of which suggest random distribution of either highly enriched or progenitor-enriched cells in a nonirradiated environment. Moreover, our data further suggest that the capacity for endosteal placement after irradiation, together with reduced homing, as occurs with \(\alpha 4\) integrin–deficient cells, may be of consequence for their proliferative expansion short term\(^{13}\) and/or their self-renewal in serial transplantations.\(^{35}\) A partial impairment in endosteal placement of normal cells given to recipients lacking membrane-bound KL (Sli/Sli mice) was previously published in nonirradiated recipients.\(^{36}\) We found that after irradiation the endosteal migration of normal donor cells to the Sli/Sli recipients was moderately impaired in these mice (Figures 4,6). However, short-term engraftment was drastically reduced,\(^{16,36}\) implying that other cues from the microenvironment may have a dominant influence on cell proliferation that follows homing. These data suggest that even with endosteal placement (Figure 6) the response to membrane-bound KL is dominant for the regenerative process in these recipients.

It has long been observed that early regeneration of hematopoiesis following irradiation\(^{23,24,37}\) begins at or near the endosteum. This challenges the prevailing view of the importance of endosteum in preservation of quiescence\(^{26,38}\) and/or the need for disengagement or dislodgment of HSCs from the endosteal niche toward the central vascular niche for their proliferation.\(^{26,39,40}\) Thus one could propose that the endosteal niche environment, although fostering HSC quiescence under steady-state conditions, shifts the balance toward proliferation/differentiation in response to irradiation or chemotherapy. This constitutes a dynamic adaptation of the niche to meet stress demands by producing factors that both attract and favor proliferative expansion of cells located there. In line with this conclusion are data recently reported suggesting a reciprocal
functional interaction between hematopoietic cells and niche cells for hematopoietic regeneration. Such an interaction is greatly favored by their preferential positioning (in quantitative terms) in trabecular bone.

In summary, our data brought into light several considerations to be taken into account when donor cell microanatomic distribution is compared in irradiated versus nonirradiated hosts. Although it could be argued that our data with Lin-kit cells may not represent stringently enriched populations of HSCs, our conclusions relevant to their intramarrow microanatomic partitioning are not dissimilar to those of highly purified cells. Furthermore, if trabecular bone is where the action is, then the issue of preferential endosteal placement may be mostly semantic, as in these areas, cells are never far away from bone or blood vessels. Although α4 integrin was frequently included in previous models and reviews of endosteal niche, there was no direct testing of its role previously. Our present experiments show that these cells do not display the expected distribution to endosteal regions in irradiated hosts. In agreement with our in vitro observations, these data suggest that α4 integrin is a critical component for cell migration toward factors elaborated by irradiated endosteal cells, likely including SDF-1. Similar data were encountered with PTX-treated donor cells, presumably due to similar mechanisms. It is important to note that it is only in the postirradiation BM environment that impaired patterns of microanatomic distribution were uncovered with mutant cells and that these may impact on a durable hematopoietic reconstitution. Our data do not resolve the issue of whether cellular contact between HSCs and endosteal cells (perivascular, endosteal, or endosteal osteoblasts) is required for formation of “stem cell synapse” and whether α4 integrin and its putative ligands at endosteum (ie, osteopontin or vascular cell adhesion molecule 1) are involved in this process. Further experiments with mutant donor cells or mutant microenvironment using other more detailed in vivo imaging approaches will shed light on these issues.

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Authorship

Contribution: Y.J. was involved in all technical aspects of the experiments described; H.B. performed experiments and cowrote the paper; T.U. and K.C. performed experiments; and T.P. designed and evaluated the experiments and wrote the paper.

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On the adaptation of endosteal stem cell niche function in response to stress

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