Lack of α4 integrin expression in stem cells restricts competitive function and self-renewal activity

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TU was involved in genotyping and evaluation of all transplant experiments by genomic PCR.
TP directed the overall study and wrote the manuscript.
ABSTRACT

Alpha4 integrin or VLA4 (CD49d/CD29) is a multitask molecule with wide expression within and outside the hematopoietic system. Because targeted ablation of \( \alpha_4 \) integrin leads to embryonic lethality, to study its effects on adult hematopoiesis, we used animals with conditional excision of \( \alpha_4 \) integrin (\( \alpha_4^{\Delta/\Delta} \)) in hematopoietic cells. In such animals, we previously documented weakened bone marrow retention of progenitor cells during homeostasis and impaired homing and short-term engraftment post-transplantation. In the present study we show that long-term repopulating cells lacking \( \alpha_4 \) integrins display a competitive disadvantage in hemopoietic reconstitution compared to normal competitors. Although initial dominance of \( \alpha_4^+ \) competitors is due to their better homing and proliferative expansion early post-transplantation, a progressive decline in contribution of \( \alpha_4^{\Delta/\Delta} \) hematopoiesis is compatible neither with normal homing nor normal function of \( \alpha_4^{\Delta/\Delta} \) HSCs in post-homing hematopoiesis. In the absence of \( \alpha_4^+ \) competitor cells, \( \alpha_4^{\Delta/\Delta} \) HSCs can establish long-term hematopoiesis in primary recipients, however, some resurgence of host hematopoiesis is evident and it becomes dominant in 2\(^{\circ} \) transplants, so that no survivors with exclusively \( \alpha_4^{\Delta/\Delta} \) cells are seen in 3\(^{\circ} \) transplants. Collectively our data provide compelling evidence that under regenerative stress \( \alpha_4 \) integrin assumes a greater importance than for maintenance of steady-state hematopoiesis.
INTRODUCTION

The α4 integrin (CD49d) or VLA4, the heterodimer of α4 and β1 integrin (CD49d/CD29) is widely expressed within and outside the hematopoietic system and exerts important functional control over many physiologic, as well as pathologic or neoplastic processes. Among hemopoietic cells, α4 integrin is expressed in a constitutively active stage in primitive cells and in an inactive state in several classes of mature cells. It exercises a decisive influence on the migration and recruitment patterns of mature cells, especially lymphocytes, to several tissues, whereas within bone marrow (BM), it plays a critical role in the interactions of hematopoietic cells with microenvironmental cells and their matrix. Because of its ability to serve not only as adhesion receptor, but to execute bi-directional signaling (outside-in and inside-out), and to interact with cytokines/chemokines and other integrins expressed by hematopoietic cells or microenvironmental cells, α4 integrin is uniquely poised to influence interactions of hemopoietic cells with their environment.

Early studies of α4 integrin on hematopoiesis relied on the use of anti-functional antibodies, both in vitro and in vivo. Targeted ablation of α4 integrin caused embryonic lethality unrelated to hematopoietic effects, but studies in chimeric mice showed modest effects on fetal liver hematopoiesis and no contribution to adult hematopoiesis beyond the first month of postnatal life.

To study the effects of α4 integrin on adult hematopoiesis, we engineered conditional knockout mice in which the α4 alleles can be disrupted upon treatment of adult animals with interferon or its inducer poly(I)-poly(C). Adult animals with
conditional excision of $\alpha_4$ integrin maintain a quantitatively normal hematopoiesis at homeostasis, but display alterations in biodistribution of progenitor cells with sustained elevations in circulation and in the spleen. Such an effect is consequent to their compromised retention within the BM in the absence of $\alpha_4$ integrin an effect reminiscent of the mobilization seen in vivo with anti-$\alpha_4$ antibodies\textsuperscript{11}. A counterpart of this effect is a partial impairment in BM homing and in short-term engraftment\textsuperscript{15}. In order to test the ability of $\alpha_4$ deficient cells for long-term, durable engraftment in irradiated recipients and the self-renewal properties of $\alpha_4^{\Delta/\Delta}$ HSCs, in the present study we carried out a series of transplantation experiments with or without competitor cells. Our results suggest that absence of $\alpha_4$ integrin in hematopoietic cells greatly compromises their competitive ability for hemopoietic reconstitution and their self-renewal capacity in serial transplantations.

MATERIALS AND METHODS

Mice

$\text{Mx.cre}^+ \alpha_4^{ff}$ and $\text{Mx.cre}^+ \alpha_4^{\Delta/\Delta}$ mice were generated in our laboratory as described previously\textsuperscript{15}. Three injections of poly(I)-poly(C) every other day were used for ablation of adult animals, whereas in neonates, two injections were used during the first week of postnatal life. Alpha4 ablation was tested two weeks later. Ablated animals are referred to as $\alpha_4^{\Delta/\Delta}$ and the animals with $>5\% \alpha_4^+$ cells in their peripheral blood were not included in experiments. Mice were bred and maintained in the specific pathogen free facility at the University of Washington in accordance with the Institutional Animal Care Use Committee guidelines.
Antibodies, cell staining and FACS analysis.

Cells from mouse tissues (bone marrow, peripheral blood or spleen) were stained using the following directly conjugated antibodies from BDBiosciences (San Diego, CA): B220 (RA3-682), CD3 (17A2), CD4 (RM4-5), CD8 (53-6.7), CD45 (30F-11), TER119, Mac-1 (M1/70), Gr-1 (RB6-8C5), CD117 (c-kit, 2B8), Sca-1 (D7), CD29 (β1, Ha2/5), anti-CD48 (HM48-1), CD49e (α5, 5H10-27 [MFR5]), anti-CD150 and anti-α4β7 (DATK32), directly conjugated CD150 (SLAM, IPO-3) purchased from eBioscience (San Diego, CA) and directly conjugated CD49d (anti-α4, PS/2) antibody was purchased from Southern Biotechnology Associates, Birmingham, AL. The cocktail of antibodies used for staining of Lineage-committed cells included: CD3 (17A2), Mac-1, TER119, Gr-1, B220. A FACSCalibur (BD Biosciences) and the CELLQuest software were used for cell analysis.

Proliferation assessment.

Assessment of proliferative status of BM cells of α4Δ/Δ and of controls (α4/f/f) was done using a single injection of BrDU (250 mg/kg body weight) and analyzing the cells 3 hours later, as described16. BrDU staining was carried out according to the manufacturer’s instructions, and was combined with anti-CD45-PE or anti-kit-APC.

Competitive repopulation.

Pooled BM cells from α4Δ/Δ mice (=Test cells) were mixed in different proportions (250,000-750,000) with α4/f/f/cre– cells (250,000) (=Competitor cells) and transplanted by i.v. injection (0.5-1.0 × 10⁶) into lethally irradiated (1200 cGy at a dose rate of >100
cGy/min) WT recipients using a cesium source. In addition to already ablated $\alpha 4^{\Delta/\Delta}$, $\alpha 4^{+/+}$ cre+ BM cells before ablation were competed (1:1) with $\alpha 4^{+/+}$ Mx.cre+ cells. Recipients of the latter pool of BM were ablated [by poly(I)-poly(C)] 4 weeks post transplantation. BM and PB cells of all recipients were evaluated at several points post transplantation by FACS, CFU-C cultures in the presence or absence of G418, and by genomic analysis (PCR). At chosen times post transplantation, selected 1° recipients served as donors for 2° transplants which were similarly analyzed 3-6 months later.

**Transplantation of $\alpha 4$-deficient BM or PB.**

Bone marrow cells (1-5 × 10^6 cells/recipient) from $\alpha 4$ ablated mice were infused into lethally irradiated recipients (1150 cGy whole body irradiation with a $^{137}$Cs source). Both splenectomized and non-splenectomized animals were used as recipients. Recipients were analyzed at 2, 10, 16, and 56 weeks later and serial transplantations (2° and 3°) were carried out from selected 1° and 2° recipient ($\alpha 4^{+/+}$, or $\alpha 4^{\Delta/\Delta}$) animals. Evaluation of hematopoietic reconstitution was carried out by FACS for different cell lineages, by hematopoietic cell quantitation in BM, PB and spleen, and through CFU-C assays with our without G418. Presence of “floxed” (f), “deleted” (Δ) or “WT” alleles were used in genomic analysis to assess donor cell contribution. Peripheral blood was also used for transplantation (0.3 mL from $\alpha 4^{\Delta/\Delta}$ or $\alpha 4^{+/+}$ animals). Recipients were analyzed 4 months later, using the approaches described above. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC).
**Clonogenic Progenitor Assays.**

Colony-Forming-Unit-in Culture (CFU-C) assays were performed using a methylcellulose mixture (Methocult™ GF, Stem Cell Technologies, Vancouver, BC), as described previously\(^{15}\). BM or PB cells were inoculated for culture, and colonies were identified morphologically in culture plates as erythroid (BFUe), or granulocyte/macrophage (CFU-GM) or CFU-Mixed. Whenever appropriate, G418 was added to cultures to test for the presence of the neo-allele in \(\alpha4\)-unexcised cells (\(\alpha4^{fl}\)), providing complementary evidence to that from genomic PCR analysis.

**Genomic PCR.** Genomic DNA was isolated using a Gentra Kit (Gentra Systems, Minneapolis, MN). The following primer combinations were used for PCR: \(\alpha4\) gene-specific primers: F1 (5'-CCACCTGGTGTATGAAAGC-3'), F2 (5' CGGGATCAGAAAGAATCCAAA-3'), and R1 (5'-CTGGCATGGGTTAAAATTG-3'); this primer combination allows discrimination between WT, “deleted” (\(\Delta\)), and floxed (f) alleles; neo-specific primers: sense (5'-GCACGCAGGTTCTCCGGC-3'), anti-sense (5'-GTCCTGATAGCGGTCCGCC-3'); cre-specific primers; sense (5'-CATTGAGCCAGCTAACCAT-3'), anti-sense (5'-TAAGCAATCCCCAGAAATGC-3').

**Statistical Analysis.** Data shown are means ± standard error of the mean (s.e.m.). Statistical analyses were performed by using a Student \(t\)-test.
RESULTS

Normal immunophenotypic profile of $\alpha 4^{\Delta/\Delta}$ stem cells

Previous studies in mice, in which excisions of $\alpha 4$ integrin in hematopoietic cells occurred early or late in their post-natal life, have shown normal quantitative hematopoiesis and normal lineage distribution at homeostasis. (Proportions of kit+ cells in BM, or of Gr-1+, Mac-1+, CD3+ and TER119+ were similar to those of normal cre(-) mice\textsuperscript{15}). A modest deviation in the frequency of B-cells (B220+) between bone marrow (BM) and PB was seen, as well as significant and sustained augmentations in circulating progenitor (CFU-C) cells of all classes\textsuperscript{15}, as documented by clonal growth in vitro. To test whether the immunophenotypic profile of $\alpha 4^{\Delta/\Delta}$ HSCs several months after $\alpha 4$-integrin excision is also quantitatively preserved, we assessed the proportion in BM of Lin-/Sca-1+/kit- (LSK) cells that contain an enriched population of HSCs. As seen in Fig. 1A, we found no differences in the proportions of either Lin-/kit+, or Lin-/Sca-1+/kit+ (LSK) cells between $\alpha 4^{\Delta/\Delta}$ and controls. More importantly, these populations were virtually negative for $\alpha 4$ expression (Fig. 1B). Recently, an improved immunophenotype was described for stem cells (CD150+/CD48\textsuperscript{−}) and we tested proportions of these cells between WT and $\alpha 4^{\Delta/\Delta}$ mice. No quantitative differences between controls and $\alpha 4^{\Delta/\Delta}$ BM cells were evident (CD150+/CD48+: 4.09% in controls and 6.6% in $\alpha 4^{\Delta/\Delta}$; CD150+/CD48\textsuperscript{−} were 0.014% and 0.018%, respectively, and all CD150+CD48 cells were $\alpha 4$ negative (Fig. 1C). In addition to the immunophenotypic profile, we tested the proliferative status of kit+ cells in BM of +/+ animals (n=4) and of $\alpha 4^{\Delta/\Delta}$ (n=8, before and after splenectomy). No significant differences in BrDU labeling were seen among the groups (BrDU+: 24.2±3.6% vs. 29.7±2.0% among CD45+ cells respectively, p=0.24).
Figure 1. FACS analysis of BM cells from α4-deficient or control mice Fig. 1A. Note the absence of α4 expression and the left shift in β1 integrin expression of BM cells from α4Δ/Δ mice compared to controls (four left panels). Solid lines represent isotype control antibody. The four panels on the right of Fig. 1A depict proportions of Lineage⁻/Sca-1⁺/kit⁺ (LSK) cells containing an enriched population of stem cells. Gated Lineage⁻/kit⁺ cells (square on upper left) were tested for Sca-1 positivity (four right panels). No differences are seen between the α4Δ/Δ (neonatally ablated and tested at 14 weeks) and control mice (14 weeks old), suggesting normal representation of LSK cells in α4 deficient mice. Furthermore, kit⁺ cells did not express α integrin (Fig. 1B). In Fig. 1C, BM cells were labeled with anti-CD150 PE and anti-CD48 FITC. Among >2×10⁵ cells analyzed, the proportion of CD150⁺ CD48⁻ cells were 0.014% and 0.02% (2 expts.) in control and 0.18% and 0.03% in neonatally ablated α4Δ/Δ animals of the same age (4-5 months). No α4⁺ cells were seen among CD150⁺ CD48⁻ cells from α4Δ/Δ BM (data not shown). The proportion of CD150⁺ CD48⁺ was 4.09% in controls and 6.62% in α4Δ/Δ. It is of interest that all kit⁺ cells were CD48⁺ in both sets of animals.
Figure 2. Competitive repopulation with pooled $\alpha^{4+/-}$ (Competitor cells) and $\alpha^{4\Delta/\Delta}$ (Test cells) BM cells. (A) Proportion of $\alpha^4+$ cells in the pooled BM sample used for transplantation is depicted in the left panel and proportion of kit+/Kit+ cells is shown in the right panel. (B) Blood samples from transplanted recipients were assessed 16-42 weeks post transplantation for the presence of $\alpha^4+$ cells (■) or $\alpha^4-$ (□). At all times tested, the proportion of $\alpha^4+$ cells was 2:1 in PB (compared to input ratio of 1:1). (C) Comparison of PB and BM positivity (right panels) for $\alpha^4$ integrin in transplanted mice sacrificed at 30 (n=4) or 42 weeks (n=5) post transplantation (PB: black bars, BM from the same group of mice: hatched bars). Reconstitution by $\alpha^4+$ cells was higher in BM compared to concurrently tested PB cells (total cells or kit+ cells, left panels), suggesting ongoing replacement of hematopoiesis by $\alpha^4+$ competitor cells. Error bars represent standard error of the mean.
Competitive Repopulation with $\alpha 4^{\Delta+/+}$ and $\alpha 4^{\Delta/}$ cells

To test whether the phenotypically defined $\alpha 4^{\Delta/}$ HSCs are endowed with a normal functional capacity for long term reconstitution of lethally irradiated recipients, we performed competitive repopulation experiments\textsuperscript{17}. Initially we mixed equal numbers of BM nucleated cells from $\alpha 4^{\Delta/}$ mice (ablated several months previously) and from BM of $cre^-\alpha 4^{ff}$ mice (1:1 test to competitor ratio). Equal mixing was verified by immunophenotyping of pool populations and proportions of kit+ cells (Fig. 2A). This 1:1 donor pool of cells was injected ($0.5 + 0.5 \times 10^6$ cells/recipient) into a cohort of ten lethally irradiated WT recipients. In this setting, hematopoiesis by $\alpha 4^{\Delta/}$, $\alpha 4^{ff}$ or $\alpha 4$ WT cells can be genetically distinguished. To assess long term donor contributions to hematopoiesis, PB from recipient mice was sampled at 16, 30 and 42 weeks post transplantation. Blood was analyzed by FACS (proportion of $\alpha 4^+$ cells) and for progenitor content in the presence or absence of G418 for scoring neo-resistant colonies ($\alpha 4^{ff}$ derived), or neo-sensitive ($\alpha 4^{\Delta/}$ or WT derived). The proportion of $\alpha 4^+$ cells (among CD45+ cells) at all times tested was between 65-75%. Proportions of Gr-1+ cells reflecting recent contributions by BM progenitors were also similar. One rather moribund animal was sacrificed earlier at 12 weeks and showed 52.68% $\alpha 4^+$ in PB (the expected contribution); however, in the BM he had 71.65% $\alpha 4^+$ cells. All the kit+ in the BM were $\alpha 4^+$, as was the great majority of Gr-1+, Mac-1+, TER119+ and B220+ cells (data not shown). This suggested that PB data may not accurately reflect BM hematopoiesis and that we may have an ongoing contribution by $\alpha 4^+$ at the expense
of α4(−) cells. This concept was tested at 30 weeks by sacrificing four mice. All mice showed further increase in PB contribution by α4+ cells, but this was not statistically significant from earlier times. However, BM analysis from all four mice disclosed 97.45±0.1% α4(+) cells compared to 77.49±4.2% in PB (p=0.02), suggesting complete replacement by α4(+) hematopoiesis. All CFU-C in vitro were G418 resistant, suggesting contribution by α4<sup>f/f</sup> competitor cells rather than host stem cell resurgence. Finally, at 42 weeks post transplantation, five more mice were euthanized for analysis. Their PB cells were 73.14±11.8% α4+, but the BM had 96.95±1.65% α4+ cells. Again, a similar picture emerged to the one seen earlier at 30 weeks. These data suggested not only an inferior competition by α4<sup>Δ/Δ</sup> cells starting early post-transplant, but also a progressive replacement of hematopoiesis by α4(+) cells long after hematopoietic reconstitution is stably established. They also validate the α4<sup>f/f</sup> competitor cells as functionally intact. To uncover the presence of any silent α4<sup>Δ/Δ</sup> HSCs that had not contributed to hematopoiesis in the 1<sup>st</sup> recipients, at 30 and 42 weeks post-transplantation, 1 × 10<sup>6</sup> pooled BM cells from 4 mice (97.4% α4+) were given to each of 14 recipients and BM pooled cells from 2 mice [90% α4(+)] were given to each of 10 recipients. The first cohort of recipients was tested at 27 weeks (11 mice) and the second at 13 weeks post (8 mice). All lineages within BM from both sets of animals showed α4+ hematopoiesis (>98%) in BM, PB and spleen.
Figure 3. Competitive repopulation experiments with 1:1 or 3:1 $\alpha 4^{\Delta/\Delta}$ (=Test cells) to $\alpha 4^{+/+}$ (=Competitive) cell ratio. (A) FACS profiles of pooled 1:1 BM or 3:1 BM samples showing proportions of $\alpha 4^-$ (grey) vs. $\alpha 4^+$ (black) cells. (CFU-C ratio of T:C cells was 6:1 in the latter.) (B) PB cell evaluation for $\alpha 4$ positivity shows an early and progressive reconstitution by $\alpha 4^+$ Competitor cells from 6-36 weeks post transplantation ($p=0.04$, grey: $%\alpha 4^-$, black: $%\alpha 4^+$). (C) Genomic PCR in 14 surviving mice (7 from the 1:1 and 7 from 3:1) from competitive repopulation at 36 weeks. Note the predominant presence of floxed ($f$) allele (=Competitor) compared to deleted ($\Delta$) one and a minor presence of WT allele from hosts (likely contaminating CD45$^-$ cells). Predominant
reconstitution by $\alpha 4^{flf}$ (=Competitors) was also corroborated by CFU-C cultures, showing mostly G418 resistant colonies ($\alpha 4^{flf}$ are neo+) from competitor cells. Only two mice (#1 from 1:1 and #2 from 3:1) show appreciable number of ($\Delta$) allele corresponding to higher proportions of $\alpha 4^-$ cells. Error bars represent standard error of the mean.

To further investigate the kinetics of the apparent competitive disadvantage of $\alpha 4^{\Delta/\Delta}$ cells and to overcome a putative partial homing defect for $\alpha 4^{\Delta/\Delta}$ HSC (LTRC), similar to the one documented for progenitor cells (CFU-C)\textsuperscript{15}, we repeated the competitive repopulation experiments. Keeping the number of competitor cells constant, we used 1:1 and 3:1 (6:1 by CFU-C ratio) of test $\alpha 4^{\Delta/\Delta}$ vs. $\alpha 4^{+/+}$ competitor cells (Fig. 3A). These cells were transplanted in splenectomized recipients to avoid contributions of $\alpha 4^{\Delta/\Delta}$ hematopoiesis by the spleen, which avidly sequesters $\alpha 4^{\Delta/\Delta}$ cells. Furthermore, we wanted to test whether contributions by the spleen could account for the differences between PB and BM seen previously, instead of evoking the postulate of progressive decline of $\alpha 4^{\Delta/\Delta}$ hematopoiesis. By 6 weeks, $\alpha 4^+$ blood cells were above 60% in all recipients receiving either 1:1 or 3:1 donor cells (74.76±2.4% for 1:1 and 60.65±3.6% for 3:1). By 24 weeks (168 days) $\alpha 4^+$ cells were 79±7.0% for the 1:1 and 80.01±4.9% for the 3:1 donor cohort. Thus, in accord with our earlier data, there was a progressive decline in the contribution by $\alpha 4^{\Delta/\Delta}$ cells from 6 to 36 weeks ($p=0.04$ for the 3:1 group of recipients), Fig. 3B. At the time of sacrifice (36 weeks), 92.55±11.5% of CFU-C from the BM of all recipients of 1:1 ratio were G418 resistant (i.e., $\alpha 4^{flf}$ derived), whereas in PB were 83.6±18.4%, and in the recipients of 3:1 donor pool, they were 95.5±2.4% and 80.84±6.0%, respectively. Moreover, DNA analysis of BM cells from all these recipients (Fig. 3C) confirmed the derivation of the great majority of multilineage hematopoiesis by $\alpha 4^{flf}$ competitor cells (Fig. 4).
Figure 4. Expression of α4+ cells (shown in black columns) among cells from different lineages in [(A) BM, (B) lymph nodes (LN), and (C) thymus] of mice transplanted with 3:1 (Test:Competitive) cell ratio and tested at 36 weeks post transplantation. (Grey columns show the proportion of α4− cells.) Proportions of α4+ cells (black columns) in all tested tissues post transplantation show only minor deviations from data in normal, non-transplanted tissues (i.e., normal BM has 95-99% α4+ cells), suggesting multilineage engraftment.

These data secured the fact that α4ΔΔ cells are out-competed by α4+ cells early during hematopoietic reconstitution and that any remaining contribution by α4ΔΔ cells is dwindling thereafter. Since the competitive advantage of α4+/+ cells was shown for the
first few weeks post-transplantation, the most likely interpretation is the superior homing of short term engrafting $\alpha 4^{+/+}$ cells. To eliminate homing differences in competitive repopulation, we decided to transplant pools composed of equal numbers of $\alpha 4^{+/+}/\text{cre}+$ BM cells and $\alpha 4^{+/+}/\text{cre}+$ cells that had not been ablated. This pool (1:1 ratio) consisted of 96.5% $\alpha 4+$ cells among kit+ at the time of transplantation. Upon hematopoietic reconstitution at ~4 weeks, we planned to induce $\alpha 4$-ablation in recipient animals (by poly(I)-poly(C)) injections). Thus, at 4 weeks, we tested the blood of recipient animals and, to our surprise, instead of the expected ~95% $\alpha 4$ positivity, we had only 75.4±1.9% in the entire group of 10 recipients. A proportion of 25% $\alpha 4$-negative cells in PB can only be explained by a partial $\alpha 4$-gene ablation occurring at post-transplantation period, presumably because of increased levels of endogenous Interferon. Nevertheless, we ablated the animals, hoping to uncover additional contribution by $\alpha 4^{+/+}$ cells and tested them again ~3 months later (67 days post ablation). At that time, PB had 82.4±1.4% $\alpha 4+$ cells, not significantly different from earlier determinations. These experiments suggested that no significant populations of unablated $\alpha 4^{+/+}$ existed before our ablation, presumable because they were out-competed earlier by normal cells. Technical issues of non-ablation [related to poly(I)-poly(C)) use] were excluded by successful ablation in other concurrent control animals. To solidify contributions of $\alpha 4^{+/+}$ competitor cells, 2° transplantations were carried out at the time of sacrifice. 2° recipients (n=5) analyzed 4 months later showed 86.9±0.9% $\alpha 4+$ cells in their BM, 78.5±1.8% in the spleen and 80.7±3.8% in PB.
Repopulation by $\alpha 4^{\Delta/\Delta}$ bone marrow or peripheral blood cells.

To test whether $\alpha 4^{\Delta/\Delta}$ bone marrow cells can establish long-term reconstitution in the absence of competitor cells, we transplanted mice with either $\alpha 4^{\Delta/\Delta}$ or with a similar number of $\alpha 4^{+/+}$ bone marrow cells per recipient (Table 1). Engraftment was assessed at 2, 10, 16 and 56 weeks later. To overcome homing problems and to test the role of the spleen in $\alpha 4^{\Delta/\Delta}$ short term hematopoiesis, we also used splenectomized recipients (1 and $5 \times 10^6$ $\alpha 4^{\Delta/\Delta}$ cells per recipient). The data were compared to $1 \times 10^6$ $\alpha 4^{+/+}$ concurrent control transplants. As seen in Table 1, short-term engraftment by $\alpha 4^{\Delta/\Delta}$ cells at 2 weeks was <6% and <20% of that seen in recipients of control BM splenectomized or not, respectively. Thus, engraftment in the absence of the spleen, where many $\alpha 4^{\Delta/\Delta}$ cells home and subsequently differentiate, is more severely impaired. Of interest is the peripheral blood leukocytosis in $\alpha 4^{\Delta/\Delta}$ recipients, which does not reflect higher engraftment, but only the increased release of $\alpha 4^{\Delta/\Delta}$ cells in circulation.
Table 1. Transplantation of BM α4ΔΔ cells to lethally irradiated α4+/+ recipients

### Non-splenectomized Recipients

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<th>Wks Post Tx</th>
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| Splenectomized Recipients |
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</table>

\*n=number of animals

Frequency of BM CFU-C in the recipients of 1 x 10^6 α4ΔΔ cells was lower than controls (P<.05). In the 5.0 x 10^6 α4ΔΔ recipients, despite their higher cellularity in BM, the frequency of CFU-C was lower than +/- controls, but not statistically significant.

**P<.02

In the long term donor reconstitution experiments, by 10-16 weeks bone marrow cellularity and progenitor content recovered to levels seen in concurrent controls (Table 1). This recovery had the hallmarks of α4ΔΔ hematopoiesis before transplantation: increase in progenitors in circulation and in spleen. However, detailed evaluation of 3 mice at 10 weeks showed that kit+/α4+ were 46.4% in one of them, and in their spleens...
all three animals showed a significant increase in total $\alpha 4^+$ cells (24.5%, 11.2% and 9.6%) and among kit+ cells (31.3%, 37.6% and 55.7%). Observations in the cohort of splenectomized recipients given the same number of $\alpha 4^{\Delta/\Delta}$ cells and evaluated at 10 weeks post-transplantation also showed an increase in $\alpha 4^+$ cells (7 recipients, 16.3±3.5%) and no increase in circulating progenitors compared to non-splenectomized recipients (118±36.9/mL vs. 564±46.4, Table 1).

In contrast to data at 10-16 weeks (Table 1), hematopoiesis in the 3 mice surviving longer (56 weeks) was not maintained at levels seen at 10-16 weeks and was not increased further (i.e., by BM cell and progenitor numbers) with age, as seen in non-transplanted mice$^{15}$. [Total progenitor (CFU-C) content in BM, spleen and PB was 815,789±54,705 in 50-week old $\alpha 4^{+/+}$ animals (n=4), and 1,363,601±144,885 in non-transplanted $\alpha 4^{\Delta/\Delta}$ animals (n=4) of the same age. The 3 survivors had 195,487±79,380 total CFU-C.] Unfortunately, at this time, transplanted controls were not available for meaningful comparisons.

Table 2. Secondary transplants (tested 4 months post) from primary recipients of $\alpha 4^{\Delta/\Delta}$ BM cells (at 10 weeks post 1° transplantation).

<table>
<thead>
<tr>
<th>Donor cells 0.5 x 10^6</th>
<th>Recips. #</th>
<th>Peripheral Blood</th>
<th>Bone Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WBC, x 10^9/μL</td>
<td>CFU-C /mL</td>
</tr>
<tr>
<td>$\alpha 4^{+/+}$ (n=3)</td>
<td>5</td>
<td>2.15±0.08</td>
<td>20±5.8</td>
</tr>
<tr>
<td>$\alpha 4^{\Delta/\Delta}$ (n=2)</td>
<td>5</td>
<td>2.29±0.36</td>
<td>30±7.0</td>
</tr>
<tr>
<td>$[\alpha 4^{+/\alpha 4^-}]$ (n=1)</td>
<td>5</td>
<td>2.17±0.47</td>
<td>10±4.1</td>
</tr>
</tbody>
</table>

*P<.05
**BM sample with increased numbers of CD45-negative cells
To assess the self-renewal capacity of α4^Δ/Δ HSCs, we carried out 2° transplantation experiments, both at 10 weeks, i.e. at the recovery phase, and at 56 weeks post-transplantation. As indicated above, one of the 3 mice evaluated at 10 weeks had ~50% α4+ cells in circulation, whereas the other two had mostly α4^Δ/Δ cells in circulation (Table 2 and Fig. 5). The latter two mice served as donors for five 2° recipients (1 × 10^6/recipient) and the one pseudo-chimeric mouse served as a donor for another five recipients. α4^+/+ cells from control 1° recipients were also transplanted in five 2° hosts. All recipients were evaluated about 4 months later. The data are shown in Table 2. At that time, a further increase in α4+ hematopoiesis is seen and inferior BM CFU-C content in the recipients of α4^Δ/Δ cells, compared to concurrent BM controls. The data collectively suggest that α4^Δ/Δ donor cells from 1° recipients with phenotypically restored hematopoiesis have an inferior subsequent reconstitution capacity, as indicated by emergence of surviving host hematopoietic cells and by comparing them with concurrent controls. At 56 weeks the 3 surviving mice served as donors for ten 2° recipients (Fig.5). Evaluation of nine of these recipients 26 weeks later showed that 5 had 33.6±10.5% α4+ cells in BM and the other four had 6.8±1.9% α4+ cells. Each of the latter four mice with mostly α4^Δ/Δ cells served as donors for five 3° recipients (20 total, Fig. 5A). 25 weeks later, there was only a 30% survival in this group (Fig.5B). The 6 surviving mice had 66.2±14.1% α4+ cells in blood. This represented host hematopoiesis (absence of neo in 4 mice [#1-4, Fig. 5C], indicating WT cells), whereas presence of neo in the other two (#5-6, Fig. 5C) reflected the recipient genotype (α4^fl) and not
unablated $\alpha4^{+/}$ donor cells. Taken together, our data indicate failing HSC self renewal activity in the absence of $\alpha4$ integrin.

**Figure 5.** Transplantation of $\alpha4^-$ BM or $\alpha4^-$ PB cells in lethally irradiated recipients. (A) Transplantation outcomes of mice given $\alpha4^{+/}$ BM donor cells sacrificed at 10 or 56 weeks post transplantation (see Table 1 for additional data.). $2^\circ$ transplantations (see also Table 2) were done at 10 or 56 weeks post $1^\circ$ transplantation and were tested 16 and 26 weeks post $2^\circ$ transplantation (white mice: <10% $\alpha4^+$ cells; grey mice: >10% $\alpha4^+$ cells; black mice: dead). Note the tendency in all $2^\circ$ transplant
recipients for reconstitution by surviving α4+ cells from hosts (grey mice). In 3° transplants, there was only 30% survival (B) and the 6 surviving mice (#1-6 in Fig. 5C) were reconstituted mostly (mice #1-4) by host cells that were neo-negative (=WT). Two mice (#5, 6) showing neo+ in (C) were not reconstituted by unablated α4<sup>fl</sup> cells, but by host cells, since these recipients were f/f/cre(-)/neo+ mice.

In addition to BM, PB cells from α4<sup>Δ/Δ</sup> mice were transplanted into lethally irradiated recipients to test for LTRCs in the circulation of these mice. For this purpose, blood from α4<sup>+/+</sup> or α4<sup>Δ/Δ</sup> mice was given to 10 irradiated recipients from each group (0.3 mL/recipient). By day 30 all control recipients died, however, 3 of the recipients of α4<sup>Δ/Δ</sup> survived beyond 90 days (Fig. 6). These recipients were sacrificed six months later for evaluation and showed greatly diminished hematopoiesis (i.e., decreased CFU-C content in BM, no increase of progenitors in circulation or in spleen, data not shown) similar to features seen long term from BM α4<sup>Δ/Δ</sup> cells. These results unequivocally suggest that the number of long term repopulating cells is increased in circulation of α4<sup>Δ/Δ</sup> animals compared to control animals, since despite their putative homing defect, they can establish long term hematopoiesis in 1° recipients.

Figure 6. Survival of mice transplanted with 0.3 ml PB from α4<sup>+/+</sup> or α4<sup>-</sup> mice. All recipients of α4<sup>+/+</sup> blood died early, whereas one third of α4<sup>-</sup> PB recipients survived long term.
DISCUSSION

Our previous studies in mice with α4 integrin gene ablation during adult life have shown no quantitative abnormalities in hematopoiesis during homeostasis for more than a year\textsuperscript{15}. However, there were aberrations in biodistribution of hematopoietic progenitors, which remained elevated in peripheral blood and were sequestered in the spleen. Furthermore, $\alpha 4^{\Delta/\Delta}$ BM cells had a partially impaired homing when given to irradiated recipients, confirming many prior studies using anti-functional $\alpha 4$ antibodies\textsuperscript{18}. In addition, there was a significant deficiency in short term engraftment during the first few weeks post-transplantation, suggesting impairment in expansion of hematopoiesis under stress, in agreement with independent studies of post 5FU-recovery\textsuperscript{15} and engraftment studies with anti-$\alpha 4$ treated cells delivered intra-femorally\textsuperscript{19}. As the role of $\alpha 4$ integrins in the establishment and maintenance of long-term hematopoiesis was not clear from prior data, the present studies were undertaken.

Alpha4 deficient donor cells display a competitive disadvantage in repopulation experiments.

All experiments in which $\alpha 4^{\Delta/\Delta}$ cells were transplanted together with $\alpha 4^{+/+}$ cells (at a ratio of 1:1 in two experiments and 3:1 test:competitor cells in one experiment), showed an early advantage of $\alpha 4^{+/+}$ competitor cells over $\alpha 4^{\Delta/\Delta}$ test cells. Such an early competitive advantage likely relies at least in part, on the better homing of $\alpha 4^{+/+}$ short term repopulating cells (CFU-C) compared to $\alpha 4^{\Delta/\Delta}$ ones, as documented in earlier studies\textsuperscript{15}. In addition to the inferior homing, impairment in the early expansion of $\alpha 4^{\Delta/\Delta}$ stem and progenitor cells post homing, also documented previously and further
supported by post 5-FU recovery data, could certainly compound the homing deficit. However, if the competitive disadvantage is consequent only to the impaired homing and early expansion of α4ΔΔ donor cells, one would have expected a stable contribution to hematopoiesis thereafter, as occurs with normally homing Rac2−/− CFU-C cells20, or SCL conditionally ablated cells21, or even a progressive contribution by α4ΔΔ LTRCs, if these did not have any homing/retention defect. Instead, what was seen was a progressive contribution by α4+/+ competitor cells, as indicated by peripheral blood evaluations comparing early and late times post-transplantation (Fig. 2,3) and by differences between bone marrow and peripheral blood at the time of sacrifice. Such a trend seen in all three experiments is neither compatible with a normal HSC homing, nor with functionally normal HSC behavior, at least in the presence of normal competitor cells. An analogous late decline, albeit of a smaller magnitude, was seen in chimera created with DBA/2 and C57BL/6 mice, and was attributed to genetic differences in stem cell renewal activity between the two murine strains22. Furthermore, the competitive repopulation outcome is reminiscent of data with chimeric mice (from α4−/− ES cells), in which there was no contribution to hematopoiesis by α4−/− cells beyond the first month of postnatal life13,14. These chimeric data and our data with adult cells emphasize therefore the competitive disadvantage of α4-deficient HSCs in the presence of normal competitors. A similar competitive disadvantage for adult HSCs was seen in chimeras with Tie2−/−23 and Sca-1−/−24 cells, whereas SCLΔΔ adult cells were competent in contrast to fetal ones25, emphasizing regulatory differences between fetal and adult stem cells. It is important to point out that in the case of α4 or Tie2 deficient cells, there were no documented adverse effects on proliferation or differentiation of isolated
hematopoietic cells, that could complicate evaluation of engraftment rates seen in other competitive repopulations (i.e., of PU.1\(^{26}\), or Rac deficient cells\(^{27}\)). It was of interest that on rare occasions in the competitive repopulating experiments (see Fig. 3C) there was a long-term contribution by \(\alpha 4^{\Delta/\Delta}\) donor cells. Such a result raised the question of whether indeed donor \(\alpha 4^{\Delta/\Delta}\) can establish and support on their own long-term hematopoiesis in irradiated hosts. This issue was further explored by transplantations of bone marrow or peripheral blood from \(\alpha 4^{\Delta/\Delta}\) mice.

**Donor \(\alpha 4^{\Delta/\Delta}\) stem cells can establish long term hematopoiesis, but subsequent transplantability is quickly lost.**

Serial observations in murine recipients of \(\alpha 4^{\Delta/\Delta}\) donor BM cells show that there is an initial delay in reconstitution, with eventual recovery between 10-16 weeks post-transplantation (Table 1), signaled by an increase in the number of circulating progenitors, similar to that seen in steady state \(\alpha 4^{\Delta/\Delta}\) hematopoiesis. However, the few mice from 1\(^{o}\) transplants surviving long-term (56 weeks) showed a picture of greatly diminished hematopoiesis. Of interest, there were signs of resurgence of host hematopoiesis in all late survivors (10-56 weeks, Table 1). More insight regarding the self-renewal of \(\alpha 4^{\Delta/\Delta}\) stem cells was gained by serial transplantation experiments. In spite of the phenotypic recovery at 10 weeks, recipients of these recovered cells (in 2\(^{o}\) transplants) had a significant deficit in progenitor content in their bone marrow, compared to concurrent controls, and a sizeable component (~\(\frac{1}{3}\)) of host hematopoiesis. Similarly, 2\(^{o}\) transplant experiments from \(\alpha 4^{\Delta/\Delta}\) donor cells at 56 weeks showed an increasing contribution by residual host hematopoietic cells (15/19 mice)
several months later. Emergence of hematopoiesis by non-ablated $\alpha 4^{ff}$ stem cells was excluded by DNA analysis. Nevertheless, 4 out of 19 secondary recipients displayed $\alpha 4$-deficient hematopoiesis in peripheral blood. 3° transplants using these phenotypically deficient cells as donor cells, however, relied on recovery of host hematopoiesis for their survival (30% survival). Dependence on host surviving cells late in transplantation is a hallmark of a reduced number of engrafting donor cells in serial transplantation experiments. We did not encounter any contribution to hematopoiesis by non-ablated $\alpha 4^{ff}$ cells in this setting, as seen in other examples with Mx. cre mediated excision (PU.1 or TEL).

These results collectively suggest that the number of $\alpha 4^{\Delta/\Delta}$ stem cells capable for engraftment was low in 2° transplants and virtually absent in 3° transplants. In other words, the expected recovery, or self-renewal of HSC function several months post-transplantation, did not occur. Certainly some dilution of functional stem cells in the 2° and 3° transplants is expected and may be compounded by their homing defect. However, since the latter, at least for progenitor cells is only a partial defect, it may not easily account for the early exhaustion, or the virtual absence of $\alpha 4^{\Delta/\Delta}$ HSCs in the inocula of most 2° and in all 3° transplants. Diminished survival is not expected until after the 5th serial transplantation in this mouse strain transplanted with similar inocula. 

Thus, homing defects alone cannot readily explain the outcome unless a drastically reduced number of stem cells is also present, or the homing and retention deficiencies of the transplanted $\alpha 4^{\Delta/\Delta}$ HSCs is more extreme than the one seen with progenitor cells. More likely, we believe, is the inability of adult $\alpha 4^{\Delta/\Delta}$ HSCs to balance
self renewal vs. downstream differentiation especially under stress, either because of an intrinsic defect of $\alpha^4\Delta^\lambda$ HSCs to respond to signals from the bone marrow microenvironment under stress, or from their location away from short range signals emanating from the stem cell "niche". Future experiments may shed light on these issues.

**Is there any role of $\alpha_4$ integrin at the stem cell niche?**

Parameters regulating the function of stem cells and their ability to self-renew are currently under intense investigation. Emerging information suggests that stem cell renewal and/or survival is governed by a complex interplay between signals from stem cells and those emanating from the stem cell "niche". Several molecules actively transcribed by stem cells are shown to be important for their survival (i.e. BCL2, MCL-1, TEL, ATM), whereas other molecules interfacing with the cell cycling machinery are important for their proliferation, directly or indirectly, and for increasing the balance of self-renewal vs. differentiation (HoxB4, Gfi-1, Bmi1, HoxA-9, Notch-1/Wnt, NF-Ya, p21, SHIP-1, Myc). In addition to stem cell intrinsic signals, extrinsic signals from the stem cell "niche" are of critical importance. If these signals act at short range, they would require intimate contact of stem cells with cells or matrix at the "niche", but what secures this contact has not been worked out in detail. Recently a couple of adhesion pathways have been described (N-Cadherin/β-catenin, Tie2/Ang-1) influencing HSC retention at the niche, especially by osteoblasts, a critical structural "niche" component. Such a close interaction between stem cells and osteoblasts towards hypoxic areas of bone marrow (like the endosteal surface) maintains stem cell
quiescence by insulating HSCs from regional proliferative influence and protects their survival from outside injury or insults.

It is of interest that the adhesion through the Tie2 expressed by stem cells and of Ang-1 expressed by osteoblasts was inhibited by β1 and α4 integrins\textsuperscript{23,45}. Thus, the latter can presumably influence stem cell contact at the "niche" downstream of the Tie2/Ang-1 molecular pathway. Furthermore, osteoblasts express an abundance of osteopontin\textsuperscript{48} and the latter is an important ligand for α4 integrins\textsuperscript{49,50}. Additional adhesive pathways controlled by Myc expression were also postulated to affect HSC/niche interactions\textsuperscript{44}. Loss or overexpression of Myc in HSC led to increase or decrease respectively of adhesion molecules involved in HSC –niche interaction (44). However, in these studies mainly β2 integrins were involved and no preferential maintenance of Myc\textsuperscript{−/−} cells at endosteal sites was documented. Whatever the nature of adhesion molecules involved it is presently unclear whether they function only as adhesion receptors or participate in signaling cascades required to retain cells at the niche. Moreover, it is presently unsettled whether osteoblasts lining the endosteal areas of bone marrow comprise the only unique stem cell " niches" important for preservation of stem cell function. Such a concept, for example, will not explain the survival and function of HSCs in extramedullary sites. Indeed, despite the recent resurgence of evidence favoring the endosteal niche definition, new evidence suggests that sinusoidal endothelial cells create a "niche" for HSCs that sustains a substantial fraction of the HSC pool\textsuperscript{51}. This concept is further bolstered by the fact that there are no frequency differences in HSCs among bones with different ratios of endosteal to central bone marrow, both when examined by modern approaches\textsuperscript{52} or by decades-old
approach. Furthermore, it is unclear whether geographic differences in the
distribution of stem cells reported in normal, non-conditioned bone marrow by old or by
newer studies are abolished or disturbed in response to injury (i.e. post lethal irradiation).

Whatever the anatomic location of stem cell "niche", perivascular or endosteal, a
close contact likely protects the survival of stem cells and assures their quiescence. It is
theoretically possible that an integrin, i.e., α4, may be one of the participating molecules
securing the close contact of stem cells with cells at the niche, but further studies are
needed to approach this issue.

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REFERENCES


Lack of $\alpha_4$ integrin expression in stem cells restricts competitive function and self-renewal activity

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