Combinatorial and distinct roles of $\alpha_5$ and $\alpha_4$ integrins in stress erythropoiesis in mice

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To delineate the role of specific members of $\beta_1$ integrins in stress erythropoiesis in the adult, we compared the response to phenylhydrazine stress in 3 genetically deficient models. The survival of $\beta_1^-$-conditionally deficient mice after phenylhydrazine is severely compromised because of their inability to mount a successful life saving splenic erythroid response, a phenotype reproduced in $\beta_1^{-/-}$ reconstituted animals. The response of bone marrow to phenylhydrazine-induced stress was, unlike that of spleen, appropriate in terms of progenitor cell expansion and mobilization to peripheral blood although late differentiation defects qualitatively similar to those in spleen were present in bone marrow. In contrast to $\beta_1^-$-deficient mice, $\alpha_4^{+/+}$ mice showed only a kinetic delay in recovery and similar to $\beta_1^{+/+}$, terminal maturation defects in both bone marrow and spleen, which were not present in VCAM-1-/- mice. Convergence of information from these comparative studies lends new insight to the distinct in vivo roles of $\alpha_5$ and $\alpha_4$ integrins in erythroid stress, suggesting that the presence of mainly $\alpha_5\beta_1$ integrin in all hematopoietic progenitor cells interacting with splenic microenvironmental ligands/cells is instrumental for their survival and accumulation during hemoletic stress, whereas presence of $\alpha_5$, or of both $\alpha_5$ and $\alpha_4$, is important for completion of terminal maturation steps. (Blood. 2011;117(3):975-985)

Introduction

During normal hematopoiesis, cells committed to specific lineages complete all their differentiation steps within hematopoietic tissues, and only the most mature cells are released into circulation. Thus, cues provided by the microenvironment (ME) in bone marrow (BM) or spleen, either through ligand/receptor interactions or through secreted cytokines and/or engagement of signaling molecules, are critical for lineage-committed cells and their differentiated descendants to complete their differentiation program. This interaction is uniquely exemplified in studies of erythroid lineage maturation. Close association of erythroid cells with cellular components of the ME seems to influence their differentiation; and among them, the macrophage (MØ) has received the most attention. Through physical contact with erythroid cells, MØs create a functionally interactive unit, the erythroblastic island (EI), which appears to exert major influence on later stages of erythroid differentiation/maturation. The EI was first described by Bessis et al,1 but its functional influence was only recently analyzed by in vitro and in vivo approaches. Several proteins on the surface of MØs in partnership with proteins on erythroid cells appear to mediate these effects.2-4 Furthermore, secreted MØ proteins, like Gas6, seem to interact with their cognate receptors present in erythroid cells enhancing integrin-dependent binding.7 Whether these proteins have a redundant function or work in complexes cooperating with each other, or become functional at different stages of differentiation and under different conditions in vivo, is presently unclear.

In addition to erythroid cell/MØ interactions within the EI, direct interactions of erythroid cells with extracellular matrix components, like fibronectin, have been implicated in the regulation of erythropoiesis. The presence of fibronectin counter-receptors on erythroid cells, like members of the beta1 integrins ($\alpha_5\beta_1$ and $\alpha_4\beta_1$), is thought to mediate these interactions. Both $\alpha_4$ and $\alpha_5$ integrins are widely expressed in hematopoietic cells and have been implicated in several functional aspects, such as proliferation, survival, maturation of erythroid cells, or in homing and proliferation of hematopoietic progenitor cells.9-15 However, in vitro and in vivo data have not been consistent, so that the exact role of these 2 integrins has remained inconclusive. For example, adhesion to fibronectin is dependent on both $\alpha_5\beta_1$ and $\alpha_4\beta_1$, and it was found to influence stem cell homing of hematopoietic progenitors to BM or spleen, but the results have been controversial for $\alpha_5$ integrin.8,11,14 Other in vitro experiments comparing effects of $\alpha_5$ and $\alpha_4$ have shown that only $\alpha_4\beta_1$, not $\alpha_5\beta_1$, influences proliferation and protection from apoptosis of erythroid cells,10 whereas opposite conclusions were reached by others,12 or effects were selective for BM and not spleen.18 Yet, genetic deletion of all $\beta_1$ integrins showed no effects on erythroid differentiation, only effects on colonization of hematopoietic tissues during development (fetal liver, spleen, and BM)19and no effects on baseline or stress erythropoiesis in $\beta_1$-conditionally deleted adult animals.20 Thus, the $\beta_1$ genetic data are in contrast with differentiation defects described for fetal erythroblasts in $\alpha_4\beta_1$ knockout mutants21 and in $\alpha_4$ chimeras,22 or with impairment of stress hematopoiesis/erythropoiesis in adult animals with $\alpha_5$ conditional deletion.23 These diverse and controversial data have been difficult to reconcile and prompted us to reexamine the phenotype of $\beta_1$ conditionally deleted mice and compare them to $\alpha_5$ or VCAM-1 deleted mice under conditions of stress.

Our data dissecting the influence exerted by $\alpha_5$ versus $\alpha_4$ integrins in response to erythroid stress provide novel information on the role of $\beta_1$ integrins in erythropoiesis. Their effects are distinctly manifested in different hematopoietic environments (ie, BM vs spleen) and seem to be
directed at discreet stages of erythroid differentiation. Further, transplantation experiments have indicated that the great majority of the effects seen in β<sub>1</sub>-<sup>−/−</sup> animals are, by and large, hematopoietic cell autonomous, but as yet undefined effects from a β<sub>1</sub>-deficient ME may provide contributory function.

**Methods**

**Mice and treatment**

MxCre<sup>+</sup>/β<sub>1</sub><sup>−/−</sup> mice were obtained in our laboratory by breeding β<sub>1</sub><sup>−/−</sup> mice<sup>24</sup> with MxCre<sup>+</sup> transgenic mice (both from The Jackson Laboratory). β<sub>1</sub> integrin deletion was induced by 6 intraperitoneal injections of polyriboniosinic acid/polyribocytidylic acid (Sigma-Aldrich), and mice were used at least 4 weeks later. Polyriboniosinic acid/polyribocytidylic acid-treated Cre-negative littermates served as controls. α<sub>4</sub> integrin-deficient mice were generated in our laboratory.<sup>23,25</sup> VCA-1-deficient mice were described previously.<sup>26</sup>

To induce acute anemia, mice were injected with phenylhydrazine (PHZ; Sigma-Aldrich) at 60 mg/kg intraperitoneally on 2 consecutive days. Peripheral blood (PB) parameters were determined using a HEMAVET<sup>950</sup> (Drew Scientific). To study the role of macrophages, mice received a single intravenous injection of 200 μL of clodronate-loaded or empty liposomes. Animals were housed at the University of Washington Comparative Medicine Specific Pathogen-Free Vivarium, with irradiated chow and autoclaved water ad libitum. All procedures were done according to protocols approved by the University of Washington Institutional Animal Care and Use Committee.

**Transplantation**

B6 × 129.F1 mice (The Jackson Laboratory) were used as recipients. Mice were lethally irradiated (1150 cGy) and injected intravenously with 0.25 × 10<sup>6</sup> (for colony-forming unit-spleen [CFU-S]) or 10 × 10<sup>6</sup> β<sub>1</sub><sup>−/−</sup> or α<sub>4</sub><sup>−/−</sup> BM cells for long-term repopulation. In the latter, after complete reconstitution of hematopoiesis, acute hemolytic anemia (PHZ) was induced. Mice reconstituted with wild-type β<sub>1</sub><sup>+</sup> or α<sub>4</sub><sup>+</sup> BM cells served as controls.

**FACS analysis**

To study integrin expression in cells from PB, BM, and spleen at steady state or after PHZ treatment, we used the following antibodies: CD44, CD45, CD6, F4/80 (AbD Serotec), and α<sub>5</sub> (Southern Biotechnology), CD71, c-kit (LSK) cells in the femurs of mice. 

**CFU-C assay**

The committed progenitors of all lineages, colony-forming unit-colony (CFU-C), were assessed in methylcellulose cultures. For "stress" burst-forming units-erythroid (BFU-E) in spleen, the cells were cultured in the presence of erythropoietin (Epo) only.<sup>27,29</sup> All colony types were counted at day 7, except for CFU-E, which were counted on day 3.

**Homing in spleens of nonirradiated recipients**

Nonirradiated wild-type (α<sub>4</sub><sup>+/+</sup>, β<sub>1</sub><sup>+/+</sup>) mice were injected with 20 × 10<sup>6</sup> control (α<sub>4</sub><sup>−/−</sup>, β<sub>1</sub><sup>−/−</sup>) or β<sub>1</sub><sup>−/−</sup> BM cells and were killed 24 hours later (supplemental Figure 3, available on the Blood Web site; see the Supplemental Materials link at the top of the online article).
CFU-S assay

Information on CFU-S assay is contained in Figure 5.

Adhesion to spleen sections in vitro

Information on adhesion to spleen sections in vitro is contained in supplemental Figure 4.

Immunohistochemistry

Bones and spleens fixed with 4% paraformaldehyde were treated as previously described.29,30 Frozen sections were labeled with anti-CD31 (MEC, 13.3) and anti-CD29 (9EG7, BD Biosciences, and MB1.2, Chemicon/Millipore), anti–bone morphogenetic protein 4 (BMP4; ab 39973, Abcam), anti-CD68 (FA-11, AbD Serotec), and F4/80 (CI:A3-1, Fitzgerald Industries International) and visualized with 2° Alexa 594–conjugated fluorochromes (Invitrogen).

BMP4 quantitative reverse-transcribed polymerase chain reaction

Total RNA was isolated from the spleens of control and /H9252/H11545/H9252/H11006/H9004/H9004/mice treated with PHZ (d4) and reverse-transcribed using random primers. Obtained cDNA was used in quantitative polymerase chain reaction with primer sets for BMP4 (F-ctcccaagaatcatggactg, R-aaagcagagctctcactggt) and b-actin (F-atcctcaccctgaagtaccc, R-atttcccgctcggccgtggt).

Results

Genetic models

MxCre+/β1Δα (β1 Δα) mice. Generation of these mice has been previously described,24 although a detailed evaluation of their erythropoiesis and expression of different β1 heterodimers in hematopoietic tissues have not been presented. Partial expression data were also presented in a similar β1 conditional model.31 Ablation of β1 in BM cells was more than 95% (95.5% ± 0.84%, n = 16). Specific β1 integrin heterodimer expression is shown in Figure 1A. Drastic reduction in ααβ1 expression is seen, whereas other dimers, like α4 or α6, are well expressed, probably because of their association with alternate β1 partners (ααβ1 or α6β3). Overall dramatic differences in the 2 most abundant integrins, α4

Figure 2. Stress-induced erythroid differentiation is impaired in β1Δα mice. (A) Survival curve (ctrl, n = 25; β1Δα, n = 16), splenic weight (ctrl, n = 25; β1Δα, n = 6), and splenic cellularity (ctrl, n = 25; β1Δα, n = 6). (B) Erythroid differentiation in BM and spleen. FACS profiles of CD71/TER119-labeled splenic cells (left panels in BM and spleen), and CD44/side scatter analysis of TER119+ cells from days 0 and 6 after PHZ (right panels in BM and spleen). Note the failure of β1Δα erythroid cells to advance to later maturation stages (ie, from R1 to R2 in the CD71/TER119 profiles after RBC lysis). CD44/side scatter analysis of TER119+ cells shows discreet stages of progressive maturation (from I-V) before PHZ but a significant overlap after PHZ treatment in both sets of animals. (C) Progenitors (total CFU-C, left panel; BFU-E/stress BFU-E, middle panel; and CFU-E, right panel) in spleen before and after PHZ treatment in control and β1Δα-deficient mice. (Stress BFU-E are shown as a proportion of total BFU-E.) Note the significant reduction of all progenitors in spleen after PHZ treatment in β1Δα mice (day 6).
and α4, were seen in BM hematopoietic cells of these mice. Integrin α3 was virtually absent in all BM cells, but α4 was expressed at high levels, with a significant overexpression of α4β1.32 Cellularity in hematopoietic tissues (BM, spleen) was normal to increased in β1 mice (Figure 1B-C left panel). Significant differences in progenitor content were present in both BM and PB; however, no significant differences were recorded in the spleen (Figure 1C right panel). Hematocrit and hemoglobin levels were not statistically different from controls, but platelets were higher in β1 mice (Figure 1B-C left panel). Significant differences in progenitor and progenitor cell content were carried out after PHZ, detailed studies on spleen cell populations, differentiation of erythroid cells, and progenitor cell content were carried out (Figures 2-4). TER119+ erythroid cells were analyzed by FACS using coexpression with CD7135 or CD44.36 Immature cells, especially R1 (Figures 2B, 3), outnumbered the more mature types (R1:R2 ratio at day 6 was 1:8 in controls vs 1:0.4 in β1 mice) and all subsets of erythroid cells in spleen were diminished compared with controls (Figure 4 top panels; supplemental Figure 2). Not only were erythroid cells very low in β1 mice, but also progenitor cells of all classes (Figure 2C). Specifically BFU-E, “stress” BFU-E and CFU-E were all drastically diminished (Figure 2C). Importantly, there was an increased proportion of apoptotic cells in Erythroid response and survival of β1,Δα mice is critically impaired after PHZ stress

Both β1,Δα and control mice received 2 injections of PHZ and were studied at days 4 and 6 after the first injection. All control mice survived the hemolytic challenge, but half of the β1,Δα mice died by day 6 (Figure 2A). Hematocrit levels reached a nadir in control animals 2 days after the last injection (day 4) and began to recover from that point on. β1,Δα mice had similar initial and nadir hematocrit levels, but in contrast to controls, hematocrit levels continued to drop (supplemental Figure 1). Dramatic differences between controls and β1,Δα mice were seen when splenic weights and cellularities were compared at days 4 and 6 (Figure 2A).

To further explore differences between control and β1,Δα mice after PHZ, detailed studies on spleen cell populations, differentiation of erythroid cells, and progenitor cell content were carried out (Figures 2-4). TER119+ erythroid cells were analyzed by FACS using coexpression with CD7135 or CD44.36 Immature cells, especially R1 (Figures 2B, 3), outnumbered the more mature types (R1:R2 ratio at day 6 was 1:8 in controls vs 1:0.4 in β1,Δα) and all subsets of erythroid cells in spleen were diminished compared with controls (Figure 4 top panels; supplemental Figure 2). Not only were erythroid cells very low in β1,Δα spleen, but also progenitor cells of all classes (Figure 2C). Specifically BFU-E, “stress” BFU-E and CFU-E were all drastically diminished (Figure 2C). Importantly, there was an increased proportion of apoptotic cells in
total splenocytes and in CD71+/TER119+ cells by activated caspase-3 evaluation (Figure 5A-B).

In contrast to spleen, BM revealed a different picture in which both cellularity and total CFU-C content were no different between controls and β1ΔΔ mice (Figure 4). However, erythroid cells, albeit sufficient in numbers, exhibited a deficiency toward more mature cell numbers, so that, as in spleen, immature erythroid cells outnumbered their mature counterparts, suggesting a differentiation/maturation deficit (Figure 4 upper and middle right panels; supplemental Figure 2). The proportion of active caspase-3 cells in spleen was significantly increased over controls but to a lesser degree in BM (Figure 5B). Of interest, there was also an increase in circulating progenitor cells after PHZ in both sets of animals, but β1ΔΔ-deficient mice had much higher numbers, including BFU-E and CFU-E, suggesting no impairment in generating BM progenitors in β1ΔΔ mice (Table 1).

Inability of β1ΔΔ mice to respond to hemolytic stress is largely cell autonomous

To test whether the lack of splenic response to PHZ challenge was dependent on environmental cells in the spleen or only on the absence of β1 integrin in hematopoietic cells, we compared responses of nontransplanted β1ΔΔ mice to irradiated wild-type recipient mice reconstituted with β1ΔΔ donor cells. Transplanted controls were wild-type mice reconstituted with normal cells. Approximately 8 weeks after transplantation, after verification that the phenotype of PB cells was as expected (ie, in either β1+/ or β1ΔΔ; data not shown), all transplanted mice were subjected to PHZ challenge. As seen in Figure 4, the spleens of mice reconstituted with β1ΔΔ cells not only failed to respond as the transplanted controls, but the same pattern of differentiation failure as in nontransplanted β1ΔΔ mice was seen (Figure 4 right top panel).
Table 1. PB progenitors (total CFU-C/mL PB) before and after PHZ challenge

<table>
<thead>
<tr>
<th>Animal</th>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>85.7 ± 22.4 (n = 10)</td>
<td>572.3 ± 103.0 (n = 11)</td>
<td>344.8 ± 66.1 (n = 20)</td>
</tr>
<tr>
<td>β1,α</td>
<td>1742 ± 358 (n = 17)</td>
<td>2985 ± 1302 (n = 8)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>β1,αTx</td>
<td>1405 ± 219 (n = 10)</td>
<td>14.997 ± 6117 (n = 5)</td>
<td>2877 ± 1047 (n = 5)</td>
</tr>
<tr>
<td>α4</td>
<td>1226 ± 12 (n = 7)</td>
<td>11.308 ± 1455 (n = 3)</td>
<td>6812 ± 1252 (n = 6)</td>
</tr>
</tbody>
</table>

P values are versus controls.

Counts are available on 2 moribund mice (377 ± 131 CFU-C/mL PB) and another mouse (> 2000 CFU-C/mL PB).

Control mice transplanted with control cells had 2505 ± 807 CFU-C/mL PB; n = 7 at day 4 after PHZ.

Some notable differences were present: the splenic weight (122 ± 12 g in nontransplanted β1,α, n = 6 vs 168 ± 15 g in wild-type recipient mice reconstituted with β1,α, donor cells, n = 5) and total number of R1 cells in spleen were higher in β1,α reconstituted versus nontransplanted β1,α mice (Figure 4 upper panel). However, the erythroid cells in the recipients of β1,α, cells, in both BM and spleen, showed the same differentiation defect as the nontransplanted β1,α mice (Figure 4 upper and middle panels). These data suggest that recipients of β1,α cells do generate a few more TER119+ cells than the nontransplanted mice but display the same overall phenotypic response.

The modest quantitative differences between mice reconstituted with β1,α cells and nontransplanted β1,α mice would suggest an ameliorating effect of the normal splenic environment in the former
group. Ideally, the reverse transplantation (ie, normal $\beta_1^{\Delta\Delta}$ cells into $\beta_1^{\Delta\Delta}$ recipients) would test this issue. However, this type of experiment is not practically feasible because of the poor health (nonhematopoietically related) of $\beta_1^{\Delta\Delta}$ animals exacerbated by the toxicity of irradiation. Instead, we used CFU-C in nonirradiated normal spleens (Figure 5C; supplemental Figure 3), which tests the ability of $\beta_1^{\Delta\Delta}$ cells to settle in the nonirradiated environment of a normal spleen. In the second approach, the read-out of $\beta_1^{\Delta\Delta}$-derived CFU-S (size and colony number) in the irradiated normal spleen tests the ability of normally lodged $\beta_1^{\Delta\Delta}$ progenitors to form CFU-S. The data from these experiments are presented in Figure 5D (bottom panels). It is evident from these experiments that the transient homing of $\beta_1^{\Delta\Delta}$ progenitor cells to spleen is not impaired, but their subsequent development into CFU-S is impaired, suggesting a postlodgment defect in the expansion of $\beta_1^{\Delta\Delta}$ progenitors in a normal splenic environment. Assuming a similar stress environment after irradiation and after PHZ, the data would suggest that $\beta_1^{\Delta\Delta}$ cells cannot efficiently expand and differentiate in the erythroid stress environment of the normal spleen, consistent with the picture in $\beta_1^{\Delta\Delta}$ transplanted mice. To provide an insight on the erythroid maturation defect, we tested the ability of both control and $\beta_1^{\Delta\Delta}$ TER119$^+$ cells taken from spleens on day 6 after PHZ to adhere to control or $\beta_1^{\Delta\Delta}$ spleen frozen sections. Normal erythroblasts adhere less to $\beta_1^{\Delta\Delta}$ than to normal spleen (294 ± 23 adhered cells and 641 ± 122 adhered cells, respectively, $P < .05$) and $\beta_1^{\Delta\Delta}$ erythroblasts adhered, as expected, less to normal spleen (supplemental Figure 4B). If adhesion is a requisite step for erythroid maturation, our data would suggest that the splenic environment or its altered architecture after PHZ in $\beta_1^{\Delta\Delta}$ mice may fail to provide an additional support mechanism for proper red cell maturation. However, to what extent these in vitro adhesion-dependent data together with in vitro EI formation using $\beta_1^{\Delta\Delta}$ or control Møs for attachment of erythroid cells (supplemental Figure 4C-D) reflects the in vivo picture is unclear.

### Comparison of PHZ responses in $\beta_1^{\Delta\Delta}$, $\alpha_4^{\Delta\Delta}$, and VCAM-1$^-\Delta$ mouse models

Both $\alpha_\beta_1$ and $\alpha_\beta_1\beta_1$ integrins have been implicated in proliferation of erythroid cells, and $\alpha_4$ integrin particularly has been considered a participant in EI interactions. To characterize the in vivo effects of these integrins in adult hematopoiesis and avoid the use of antibody treatment, we compared the response to stress erythropoiesis among the 3 genetic models: $\beta_1^{\Delta\Delta}$ mice with a deficiency primarily of $\alpha_\beta_1$ in erythroid cells, $\alpha_4^{\Delta\Delta}$ mice lacking both $\alpha_\beta_1\beta_1$ and $\alpha_\beta_2$ (the latter being overexpressed in $\beta_1^{\Delta\Delta}$ mice), and VCAM-1$^-\Delta$ mice lacking the major ligand of $\alpha_4$ integrin in ME cells. Survival and detailed spleen and BM responses were assessed in these models in a similar way as described earlier for $\beta_1^{\Delta\Delta}$ mice.

Whereas occasional death was seen in $\alpha_4^{\Delta\Delta}$ mice after PHZ treatment, 90% of animals tested survived and responded to PHZ stress (supplemental Figure 1). A transient deficit in progenitor content was seen at day 4 in both BM and spleens of $\alpha_4^{\Delta\Delta}$ compared with controls, but there was a quick recovery at day 6 and beyond (Figure 6). By day 6 after PHZ treatment, their response in spleen was normal in cellularity, progenitor content, and total erythroid cells compared with their controls, unlike the response of $\beta_1^{\Delta\Delta}$ mice (Figure 6). However, a defect in maturation profile was evident in both spleen and BM of these mice (Figure 6A-B right panels). This was probably responsible for their delayed hematocrit response to PHZ (supplemental Figure 1) and alluded to in our earlier publication. No differentiation defect was seen in the spleen or BM of VCAM-1$^-\Delta$ mice, in contrast to the 2 other models (Figure 6C).

Overall, $\alpha_4^{\Delta\Delta}$ mice share the terminal maturation defects present in BM and spleen with $\beta_1^{\Delta\Delta}$ mice. The data are supportive of the concept that $\alpha_4$ exerts a critical influence on the accumulation and survival of hematopoietic progenitors in spleen (ie, on the Epo-dependent stage of erythroid cell differentiation), whereas $\alpha_4$ alone or together with $\alpha_4$ in $\beta_1^{\Delta\Delta}$ mice influences the Epo-independent maturation stages of erythropoiesis.

### $\beta_1^{\Delta\Delta}$ integrin ablation in nonhematopoietic cells

Dramatic differences in erythroid stress responses are seen in the spleen versus BM of both $\beta_1^{\Delta\Delta}$ mice and mice transplanted with $\beta_1^{\Delta\Delta}$ cells. To explain some of these differences, we tested whether differences in nonhematopoietic cell $\beta_1$ ablation in these 2 tissues may be responsible for this outcome. Three candidate populations (fibroblasts, endothelial cells, and resident Møs) were tested for $\beta_1$ ablation after polyriboinosinic acid/polyribocytidylic acid treatment. Efficient ablation in Møs, but only partial ablation in fibroblastic cells, was found (supplemental Figure 5). The data for cells in spleen were similar.

To examine the expression of $\beta_1$ integrin in endothelial cells in situ both in BM and spleen, we labeled them with anti-$\beta_1$ and with 9EG7, a monoclonal antibody reacting only with activated $\alpha_\beta_1$. As seen in Figure 7, endothelial cells in BM and spleen were similarly labeled in control and $\beta_1^{\Delta\Delta}$ tissues. Megakaryocytes were
labeled only in controls, not in β1/3/α, but were labeled in all mice with anti-CD31 (data not shown). Thus, among the 3 populations tested, we found no evidence for better ablation of β1 integrin in spleen versus BM to explain differences in response. Despite phenotypic similarities, however, it is possible that cells may behave differently in the 2 environments.

To further understand the mechanisms responsible for observed impairment in stress erythropoiesis in spleen, we assessed the expression of BMP4, a signaling molecule implicated in stress erythropoiesis and produced by stromal and hematopoietic cells. By quantitative reverse-transcribed polymerase chain reaction (data not shown) and as supported by immunofluorescent staining, no gross differences in BMP4 expression were found in the spleens of β1/3/α versus control mice (Figure 7I-J). Furthermore, we assessed the role of Mφs by treating mice with clodronate (supplemental Figure 6). Although the response of clodronate-treated animals to PHZ challenge was severely compromised (supplemental Figure 6 top panels), the interpretation of these experiments is confounded by the effects of clodronate treatment in spleen beyond Mφ purging (supplemental Figure 6 bottom panels).

Discussion

Studies using several genetic mutant mice have shown impaired responses to erythroid stress, although erythropoiesis is fairly normal at homeostasis (ie, ICAM, Gas-6, and Stat5a,b Rac1/2). Furthermore, in several models of stress erythropoiesis, impairment of response is observed in both BM and spleen or only in one of these tissues. For example, in the absence of the glucocorticoid receptor, erythropoiesis is compromised both in BM and spleen. Yet, in other models (ie, in Rb null mice), prominent changes are seen only in the spleen. In contrast to Rb, the absence of Rac1/Rac2 signals leads to defective early erythropoiesis in BM, but in the spleen erythropoiesis is completed successfully, as the splenic environment apparently circumvents the need of Rac1/Rac2 in early erythropoiesis.

Our results with β1/3/α mice have some features in common to previously described mutant mice, but others appear to be unique to our model. Similar to Rb null mice or the TRalpha null mice and FAK−/− mice and in sharp contrast to Rac1/Rac2-deficient...
defects in β1 integrin mice are prominent only in the spleen, leading to failure to survive stress (Figure 2). Precise mechanistic differences between BM and spleen responses have not been explored in most of the cases, although several insights have been obtained. For example, it was previously shown that convergence of Sonic hedgehog (Shh), BMP4, and stem cell factor-dependent signaling in spleen is necessary for the development and expansion of BMP4-responsive stress erythroid progenitors.27,28 Thus, Smoαβ1 mice,28 with absence of Shh signaling, do not recover from a second PHZ treatment,28 resulting from failed splenic response.

In our β1αβ1 mice, the generation of progenitor cells by BM and their egress to PB, which is necessary for subsequent colonization of the spleen as it occurs in normal animals, was not impaired (Figure 4; Table 1). But why did these progenitors not accumulate in β1αβ1 spleens? To explain these data, we entertained the following mechanistic scenarios.

An attractive possibility is that progenitors generated in BM and delivered to circulation, as shown by their increase in PB after PHZ (Table 1), cannot home and/or cannot be firmly retained in the spleen if they lack β1 integrin. Such homing/retention properties may be dependent primarily on the presence of αβ2 because in the other 2 related mutants (ie, the α5β1 or VCAM-1αβ1 mice in which fibronectin/α5 interactions are present), homing of progenitors to spleen is not impaired.23 Consistent with this concept, previous data with fetal liver β1 knockout cells have shown failure of BM and splenic colonization by fetal liver β1 null cells45; and in another study, there was a small decrease in homing to irradiated spleen with anti-α5 integrin antibody-treated donor cells.15 However, our present data, showing no significant impairment of splenic homing in nonirradiated recipients given adult ablabeled β1 cells (Figure 5; supplemental Figure 1), appear inconsistent with these data. In this context, it is important to emphasize that homing in some of the previous studies was evaluated by successful engraftment (colonization), whereas in our studies we have dissociated homing/lodgment events from subsequent expansion/engraftment events with β1αβ1 cells. Despite the normal homing, we found that the development of CFU-S-S was significantly impaired in β1αβ1 mice as shown by the reduced number and size of CFU-S (Figure 5; supplemental Figure 1), suggesting impairment in survival and/or expansion of homed progenitors. Thus, the absence of β1 signaling in the context of fibronectin-dependent interactions in the spleen has a profound impact on progenitor cell survival and proliferation. Activation of signaling pathways intersecting β1 integrin signaling46 may promote expression of unique sets of genes responsible for cell proliferation/survival, and our data in β1-deficient PHZ-treated spleens are consistent with this thesis.

The connection of β1 or αβ1 signaling with survival and/or proliferation is supported by a host of previous data:

1. Fibronectin, and not VCAM-1, is the preferential ligand for cycling cells, and αβ1 integrin is up-regulated in cycling cells, in contrast to α5, which is not different in cycling versus noncycling cells.47
2. In vivo treatment of mice with C274, the RGD-dependent recombinant fibronectin, reduced splenic progenitor growth (HPP-CFC).12 By contrast, treatment with CH296 fibronectin containing all binding sites, including the CS-1 VL4A4 binding site, did not decrease splenic progenitors.
3. Donor cells treated with a rabbit anti-β1 antibody resulted in reduced numbers of CFU-S in irradiated normal spleen.58
4. Wv mutant mice, which have an impaired splenic response to stress after PHZ or after transplantation, are not able to activate α5.12
5. Impairment of β1-dependent FAK in conditional mutant mice led to a picture of PHZ response similar to our β1αβ1 mice with drastically reduced splenic response and decreased proliferation and survival of progenitor cells.44
6. αβ1 integrin influenced Shh signaling in intestinal epithelia proliferation, and the phenotypic changes of β1; deletion in intestinal epithelial cells were strikingly similar to mice with defective Shh expression or signaling. In aggregate, these findings, together with our own, support the concept of impaired survival and proliferation of αβ1-deficient progenitors in the stress splenic environment.

But why would an intrinsic defect like β1-deficiency lead to impaired interactions selectively within the splenic environment? It is possible that in the spleen, in contrast to BM, retention and subsequent development of erythroid cells are mainly an Arg-Gly-Asp (RGD)-dependent process (RGD being the best known recognition sequence in fibronectin for αβ1 integrin). This process will fail in β1αβ1 mice in which erythroid cells lack αβ1 integrin. However, in BM other redundant signals on erythroid cells (ie, CD44, αβ3, and β1 integrin) can interact with environmental ligands unique to BM (ie, tenasin-C, laminin species, osteopontin, thrombospondin, syndecan-4, or heparan sulfate proteoglycans),46,49 and usurp or substitute for the RGD effects on developing erythroid cells. Furthermore, beyond RGD-dependent effects, it is possible that other ME cells or matrix in BM display distinct functional behavior compared with spleen. Such a functional difference could be attributed to differences in the efficiency of the ablation of β1 integrin between BM versus splenic stromal cells. We have obtained no evidence that cultured fibroblasts are better ablated in BM versus the spleen, whereas endothelial cells in both BM and spleen are, like normal cells, positive for β1 and9EG7, an antibody that reacts with the activated form of β1; (Figure 7), suggesting no effective β1 ablation. The latter data are consistent with difficulties previously encountered with ablation of β1 integrin in adult endothelial cells.50 By contrast, Mφs or F4/80+ cells in the BM and the great majority of those in the spleen are ablabeled for β1 integrin. However, despite phenotypic appearances and depending on the signals they receive from their MEs, it is possible that the Mφs are functionally different between these 2 tissues as previously shown for other tissues.51 Nevertheless, it is doubtful that only the functional impairment of β1αβ1 macrophages can explain the severe phenotype in β1αβ1 spleens, as all classes of progenitors were affected. Furthermore, the functional role of Mφs in proliferation/maturation/enucleation of erythroid cells has not been fully elucidated, and controversial information is currently available regarding the molecular interactions between erythroid cells and Mφs (ie, ICAM-4/αβ1).52 Although many of the maturational defects have been cell-intrinsic (glucocorticoid receptor, thyroid receptor, β1 integrin), the specific interactions of these structures with ME cells or their effects on apoptosis pathways specifically activated in spleen in erythroid stress have been unclear.

Although progenitor defects in spleen quickly recovered in PHZ-treated α5β1 mice, defects on terminally differentiated cells were present in both α5- and β1-deficient animals. Because the differentiation defects were similar in α5β1 and β1αβ1 mice, it is possible that interactions of both α5 and α5 through fibronectin or the interaction of α5 with both fibronectin and Mφs are needed for efficient terminal erythroid differentiation. It is tempting to speculate that the effect in β1αβ1 mice is a combination of absent α5 together with hypofunctional α5, compromising adhesive interactions with both fibronectin and Mφs in spleen. The presence of
αβ2 did not seem to provide any protective effect in βεΔA mice. It is of interest that, despite prior implications of VCAM-1,5 there was absence of differentiation defects in VCAM-1Δε mice in our experiments (Figure 6) that were consistent with experiments in fetal liver, in which the effects of VCAM-1 were dissociated from those of αεβ2.33 Moreover, using K562 cells as a model, it was shown that prolonged engagement to fibronectin through VLA3 (αεβ2) triggered the induction of αε in K562 cells (normally lacking α4) and the up-regulated αε influenced erythroid differentiation by activating p38, an effect blocked by the RGD peptide.11 Thus, the weight of evidence from previous and present data seems to support the notion that, at least in adults, αεβ2 interactions (with fibronectin or other partners) may be important for influencing terminal erythroid differentiation, probably within the context of E1, and may act in parallel or in cooperation with αε integrins (interacting with ICAM4 and VLA4 on erythroid cells).3 However, further studies are needed to clarify some of these issues.

Transplanted mice reconstituted with βεΔA cells had a somewhat better erythroid response (Figure 4), suggesting that the stroma in the spleens of transplanted mice may have contributed to this. A cellular candidate of host origin that might have made a difference in transplanted animals is the F4/80+ Mφs in the spleen. Although the total number of F4/80+ cells was not significantly different in nontransplanted versus transplanted animals, the proportion of F4/80+/F4/80+ cells was higher (23% vs < 10% in βεΔA) in transplanted animals, indicating incomplete reconstitution of Mφ population by donor cells. Thus, the presence of F4/80+ Mφs (ie, αεβ2) could engage βεΔA erythroid cells through ICAM4 or through other undefined ways to promote erythroid expansion and terminal maturation. Intrinsic functional defects in βεΔA Mφs are also possible. In this context, it was recently noted that, using Lysozyme M-mediated adhesion of βε in Mφs, βε integrin is important for Mφ maturation, as F-actin assembly in these cells was impaired through diminished Rac expression.54 A similar defect in EMP null Mφs led to failure of enucleation in erythroid cells.5

In conclusion, our data evaluating stress erythropoiesis in 3 genetically deficient animals (βε conditional, αε and VCAM-1 with Tie-2Cre-mediated ablation) redefine the functional role of αε and αε integrins in this process and their distinctive interactions in BM versus spleen.

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Authorship

Correction: T.U. performed experiments and wrote relevant parts of the manuscript; Y.J., S.P., and B.N. performed experiments; and T.P. designed the experiments, evaluated data, and wrote the manuscript.

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Combinatorial and distinct roles of $\alpha_5$ and $\alpha_4$ integrins in stress erythropoiesis in mice

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