

α 4 Integrins Regulate the Proliferation/Differentiation Balance of Multilineage Hematopoietic Progenitors In Vivo

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Summary

We investigated roles of α 4 integrins during hematopoiesis using mutant and chimeric mice. Yolk sac erythropoiesis and migration of hematopoietic progenitors to fetal liver, spleen, and bone marrow can occur without α 4 integrins. Although terminal differentiation of these progenitors is possible without α 4 integrins, these receptors are essential to maintain normal hematopoiesis in fetal liver, spleen, and bone marrow microenvironments. Moreover, α 4-deficient erythroid progenitors and pre-B cells neither transmigrate beneath the stroma nor expand properly in vitro. In contrast, α 4-null cells migrate and differentiate efficiently into T lymphocytes within the thymus. In summary, α 4 integrins are essential for normal development of all hematopoietic lineages in fetal liver, bone marrow, and spleen, likely by regulating the proliferation/differentiation balance of hematopoietic progenitors.

Introduction

Hematopoiesis requires continuous migration, proliferation, and differentiation of pluripotent progenitors into the different blood cell lineages (Ogawa, 1993; Morrison et al., 1995). Colonization of all hematopoietic organs occurs early in development from a single intraembryonic source, the paraaortic splanchnopleura and its derivative, the aorta-gonad-mesonephros (AGM) region, where multipotent progenitors are detected before embryonic day 8.5 (E8.5) (Delassus and Cumano, 1996). In mouse, primitive nucleated erythrocytes develop in the yolk sac before that stage. After E10, definitive pluripotent progenitors are found in the fetal liver, thymus, bone marrow, and spleen, but active hematopoiesis begins at different times, depending on the development of each hematopoietic environment. In spite of a common precursor for primitive erythropoiesis and definitive hematopoiesis (Kennedy et al., 1997), it is clear that the requirements for their development are different (reviewed by Shivdasani and Orkin, 1996). After birth, erythropoiesis, myelopoiesis, B lymphopoiesis, and generation of T lymphocyte progenitors take place in the bone

marrow, although erythropoiesis and myelopoiesis can also occur in the spleen in mouse.

Interactions with the microenvironment play important roles during hematopoiesis (Dorshkind, 1990). α 4 integrins mediate binding to a specific region (CS-1) on fibronectin (FN), to VCAM-1, and to MAdCAM-1 (Hynes, 1992; Wagner and Müller, 1998). α 4 integrins are developmentally regulated during hematopoiesis; they are expressed on early immature multipotent progenitors, but expression is downregulated during erythrocyte and neutrophil differentiation (Hemler and Lobb, 1995). Moreover, α 4 integrins can mediate adhesion of hematopoietic progenitors to stromal cells likely through binding to VCAM-1 (Verfaillie et al., 1991; Williams et al., 1991; Papayannopoulou et al., 1995). α 4 integrins might also regulate migration, homing, and proliferation of hematopoietic progenitors (Papayannopoulou and Nakamoto, 1993; Coulombel et al., 1997).

α 4 integrins have been shown to participate in erythrocyte development (reviewed by Hanspal, 1997). α 4 and α 5 integrins are expressed on early erythroid progenitors, being downregulated during erythrocyte differentiation together with the loss of attachment to fibronectin (Vuillet-Gaugler et al., 1990). Antibodies against α 4 or α 5 integrins block the attachment of erythroid precursors to FN, and anti- α 4 antibodies block migration and differentiation of progenitors in vitro (Yanai et al., 1994; Goltry and Patel, 1997). The injection of anti- α 4 antibodies intrautero in mice has also implicated α 4 integrins during erythropoiesis in vivo (Hamamura et al., 1996).

α 4 integrins also seem to participate in regulating the development of other hematopoietic lineages. In vitro studies with antibodies have shown inhibition of lymphoid colonies, blockade of B cell differentiation, and delay in myelopoiesis (Miyake et al., 1991; Ryan et al., 1992). Roles for α 4 integrins at different stages of T cell development in the thymus have also been suggested (Crisa et al., 1996).

Despite these studies, the detailed functions of α 4 integrins during hematopoiesis in vivo remain unclear. Previous characterization of α 4-deficient chimeric mice (Arroyo et al., 1996) showed that α 4 integrins are essential for postnatal lymphoid development in the bone marrow and suggested that embryonic requirements for α 4 integrins during hematopoiesis might be different. The present study extends the analysis to other lineages and to earlier stages of development. The results reveal a key role for α 4 integrins during hematopoietic development and underscore the functions of α 4 integrins in regulating the proliferation/differentiation balance of hematopoietic progenitors in vivo.

Results

α 4 Integrins Are Not Essential for Erythropoiesis in the Yolk Sac

Yolk sac hematopoiesis was analyzed in yolk sacs from α 4-null embryos at E9 to E12. No major differences in histology or in total yield of cells were observed between

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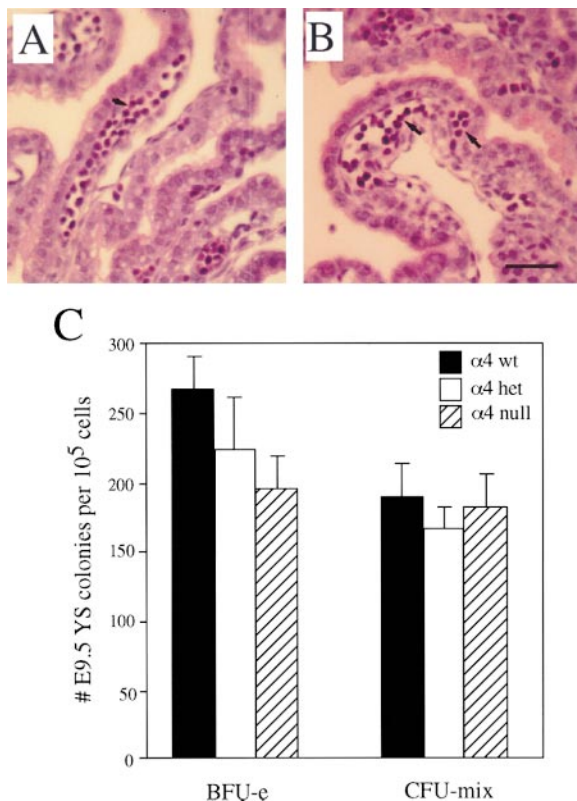


Figure 1. $\alpha 4$ Integrins Are Not Essential for Yolk Sac Erythropoiesis In Vivo

(A and B) Histology of yolk sacs from wild-type (A) and $\alpha 4$ -null embryos (B) at E11.5 stage shows no major differences in blood island distribution and composition (arrows). At least five embryos of each genotype were analyzed. Bar, 166 μ m.

(C) Yolk sac cells from wild-type, heterozygous, and $\alpha 4$ -null embryos at E9.5 stage were used for colony assays in the presence of different cytokines. Assays were run in duplicate. No significant differences (BFU-e $p = 0.18$ and CFU-mix $p = 0.36$) were found in the absolute yield of colonies. The total number of yolk sac cells was similar in embryos from the different genotypes. The mean and SD of two to three littermates of each genotype are shown. One experiment out of two is presented.

yolk sacs from null and wild-type, suggesting that erythropoiesis occurs normally in the absence of $\alpha 4$ integrins (Figures 1A and 1B). Cytospins from $\alpha 4$ -null and wild-type embryos at E11.5 displayed a similar pattern of nucleated red blood cells (data not shown).

Colony assays on yolk sac cells from wild-type, heterozygous, and $\alpha 4$ -null embryos at E9.5 showed no statistically significant differences in the number or morphology of colonies obtained (Figure 1C), and $\alpha 4$ -null yolk sac cells gave rise to both erythroid and myeloid lineages (data not shown). Therefore, $\alpha 4$ integrins are not essential for yolk sac erythropoiesis in vivo.

$\alpha 4$ Integrins Play an Important Role in Erythropoiesis in the Fetal Liver

Definitive hematopoiesis starts in the fetal liver at E10. Since most $\alpha 4$ -null embryos die after E12 (Yang et al., 1995), fetal liver erythropoiesis was studied before that stage. Fetal livers from $\alpha 4$ -null embryos at E10 did not show any significant differences from wild-type (Yang et al., 1995; data not shown). However, after E11, fetal

livers from otherwise well developed $\alpha 4$ -null embryos were consistently smaller, paler, and more fragile than their wild-type and heterozygous counterparts (Figure 2A). The number of nucleated fetal liver cells in $\alpha 4$ -null embryos was significantly lower at E11.5 and E12 compared with wild-type and heterozygotes (Figure 2B). This suggests a defect in erythropoiesis, since, at that stage, erythroid cells account for most of the liver cells. Cytospins from $\alpha 4$ -null fetal livers at E12 showed a higher ratio of proerythroblasts to erythroblasts ($\alpha 4$ -null = 2.2 and wt = 0.6; $n = 3$, $p = 0.002$) as well as decreased cellularity and smaller cellular aggregates compared with wild-type samples (Figure 2C). However, nonnucleated erythrocytes were observed in the blood and fetal livers of $\alpha 4$ -null embryos (data not shown), indicating that erythroid terminal differentiation in the fetal liver can occur in the absence of $\alpha 4$ integrins, albeit less efficiently. To test whether the defect observed might be due to impairment in erythroid progenitor activity, colony assays were performed. Comparable relative numbers of CFU-e colonies were obtained with E11.5 and E12 fetal liver cells from wild-type, heterozygous, and $\alpha 4$ -null embryos (Figure 2D), suggesting no defects at the progenitor level.

To investigate later stages of development, circumventing early lethality due to the $\alpha 4$ -null mutation, chimeric embryos ($\alpha 4^{-/-}$ ES cells injected into C57BL or RAG-2 $^{-/-}$ blastocysts) were analyzed. Terminal differentiation of erythrocytes was assessed by analyzing β -globin isoforms derived from the donor (Hbb^d) and recipient cells (Hbb^s) in the chimeras (Whitney, 1978). $\alpha 4$ -deficient cells differentiate into mature red blood cells (Hbb^d) at all stages of embryonic development (Figure 3A). The percentage contribution of $\alpha 4$ -null ES cells to the mature erythroid population is, however, lower than that of wild-type ES cells in control chimeras, and it decreases gradually from E14 to E18 (Figures 3A and 3B).

The defect observed in erythroid development could be due either to a defect in erythroid progenitor cells or to a defect in the fetal liver microenvironment. Fetal liver cells of chimeric embryos at E14 were analyzed by flow cytometry, staining for expression of $\alpha 4$ integrins and Ter-119, a specific erythroid marker (Figures 3B and 3C). Since expression of $\alpha 4$ integrins is regulated during erythroid development, being positive at early stages and downregulated later, we observed only a small population of Ter-119⁺ $\alpha 4^{-}$ cells, corresponding to late normoblasts in control chimeras (Figures 3B and 3C). However, significantly higher percentages of Ter-119⁺ $\alpha 4^{-}$ cells are present in fetal livers from $\alpha 4$ -null chimeric embryos at E14, E16, and E18 compared with controls (Figures 3B and 3C). Cytospins showed that a majority of these Ter-119⁺ $\alpha 4^{-}$ cells were early erythroid cells (data not shown). Therefore, $\alpha 4$ -deficient cells contribute to the early erythroid population at all stages of embryonic development but are unable to differentiate efficiently into mature erythrocytes.

Colony assays in the presence of G418 (to select for $\alpha 4$ -null-derived colonies) were also performed. The percentage of G418-resistant fetal liver $\alpha 4$ -null BFU-e colonies at E18 was proportional to the percentage of $\alpha 4$ -null cells that contributed to other tissues in the chimera, estimated by GPI analysis (Figure 3D). These results showed no significant reduction of progenitors at late stages of embryonic development. Thus, in contrast to

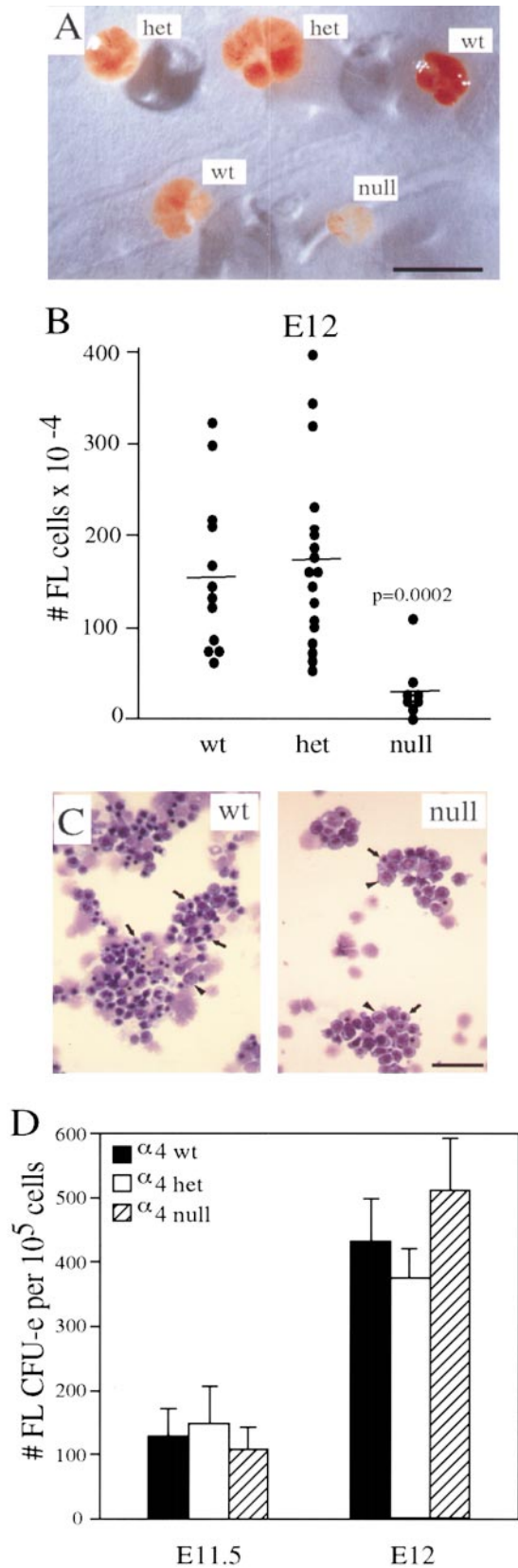


Figure 2. Fetal Livers Are Smaller in the Absence of $\alpha 4$ Integrins Due to Impairment in Erythroid Development

(A) Fetal livers from E12 $\alpha 4$ -null embryos are smaller and paler than their wild-type and heterozygous littermates. Bar, 2 mm.

a defect in the ability of $\alpha 4$ -null progenitor cells to mature into erythrocytes in vivo (Figure 3A), BFU-e colony assays showed that $\alpha 4$ -null progenitor cells were able to differentiate and form colonies in vitro. These results suggest that the in vivo defects observed are not due to defects in the $\alpha 4$ -null erythroid progenitor cells per se but more likely depend on interactions of these cells with the microenvironment.

Myeloid and B Lymphoid Progenitors Require $\alpha 4$ Integrins for Efficient Development in the Fetal Liver

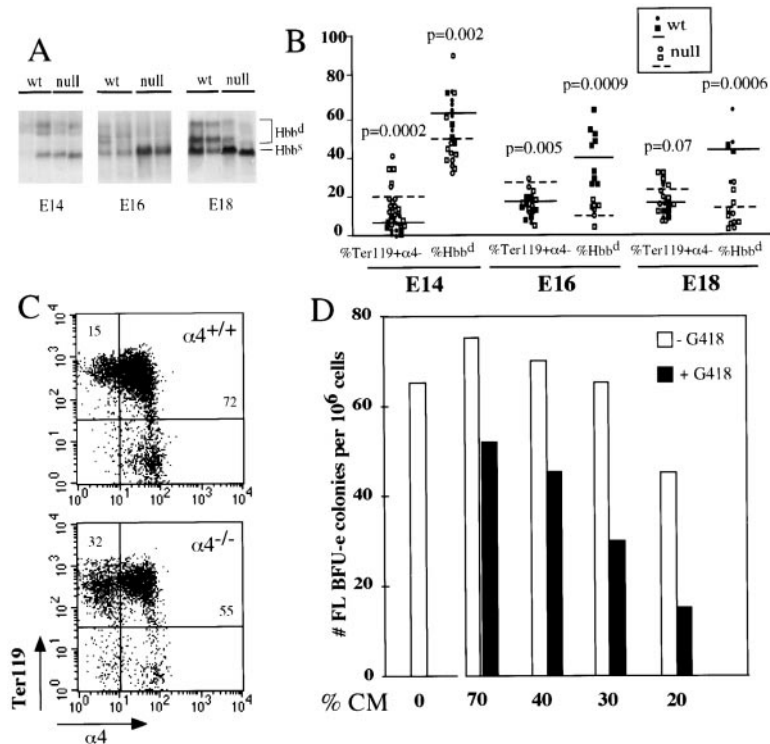
The development of other hematopoietic lineages in the absence of $\alpha 4$ integrins was analyzed. Cytospin preparations showed myeloid cells in fetal livers from $\alpha 4$ -null embryos at E12 (Figure 4A). E12 $\alpha 4$ -null fetal liver cells give rise to erythroid and myeloid lineages including macrophages, granulocytes, and sparse megakaryocytes in CFU-mix colony assays (data not shown) and to pre-B lymphoid cell lines indicating that myeloid and B lymphoid progenitors are present in the fetal livers in the absence of $\alpha 4$ integrins. Differentiation of these progenitors in vivo was assessed by flow cytometry with the myeloid markers Gr-1 (granulocyte) and Mac-1 (myeloid) and the B cell marker B220 versus the strain-specific markers Ly-9.1 or Ly-5.2 in chimeric embryos. Myeloid and B lymphoid differentiation is possible in the absence of $\alpha 4$ integrins since Ly-5.2⁺ $\alpha 4$ -null-derived Mac-1, Gr-1, and B220-positive cells were all detected in the fetal livers of $\alpha 4$ -null chimeric embryos. However, the percentage of $\alpha 4$ -deficient cells contributing to myeloid and B lymphoid lineages is significantly lower than the controls (Figure 4B), suggesting a requirement for $\alpha 4$ integrins to maintain normal myeloid and B lymphoid differentiation in the fetal liver.

The population of progenitors was also analyzed at E18. CFU-mix colony assays in the presence of G418 show percentages of $\alpha 4$ -deficient resistant colonies roughly proportional to the contribution in other tissues estimated by GPI analysis, suggesting no defect at the progenitor level (Figure 4C). Moreover, the colonies contained mature erythrocytes, macrophages, polymorphonuclear cells, and megakaryocytes, indicating that these progenitors can complete terminal differentiation in vitro. The contribution of $\alpha 4$ -null ES cells to the colonies was better than to the mature myeloid population in vivo

(B) The number of cells is significantly lower in fetal livers from $\alpha 4$ -null embryos at E12 ($p = 0.0002$) compared with wild-type and heterozygous littermates. Similar results were observed at E11.5 ($p = 0.002$; data not shown).

(C) Cytospin preparations of $\alpha 4$ -null fetal liver cells show (1) the presence of erythroid cells at different stages of differentiation with an increase in the ratio between immature proerythroblasts (arrowheads) and maturing erythroblasts (arrows) compared with wild-type littermates; (2) a decrease in cellularity; and (3) smaller size of the cellular aggregates. Bar, 166 μ m.

(D) Fetal liver cells from wild-type, heterozygous, and $\alpha 4$ -null E11.5 and E12 embryos were used for erythroid colony assays. Experiments were run in duplicate. No significant differences ($p = 0.32$) are found in the relative number of CFU-e colonies obtained. The mean and SD of two embryos of each genotype from the same litter are represented. One experiment out of three is shown. $\alpha 4$ -null cells also gave rise to BFU-e colonies although no accurate quantitation was possible (data not shown).



(D) The presence of erythroid progenitors at E18 was tested by BFU-e colony assays with fetal liver cells from $\alpha 4$ -null chimeric embryos in the presence of 1.5 mg/ml G418. SD within duplicates was less than 10%. One experiment out of two is shown. Fetal liver cells from wild-type chimeras did not survive in the presence of G418.

at E18 (Figure 4B), suggesting that the in vivo defects depend on interactions with the microenvironment. It is also possible to obtain G418-resistant pre-B cells from fetal livers of $\alpha 4$ -null chimeric embryos at E18, suggesting that the defect observed in B lymphoid development at late stages is not due to a deficit of progenitors (data not shown). All these data demonstrate that $\alpha 4$ -deficient myeloid and B lymphoid progenitors are able to complete terminal differentiation in vivo and in vitro but their capacity to develop in vivo is impaired when they lack $\alpha 4$ integrins.

$\alpha 4$ Integrins Are Not Essential for T Lymphocyte Development in the Thymus during Embryonic Life

At E10.5, hematopoietic progenitors migrate to the thymus, where they differentiate into mature T lymphocytes (Jotereau et al., 1987). T lymphocyte development was analyzed by flow cytometry of thymus from chimeric embryos. Staining with T cell (Thy-1 and CD4) and strain-specific (Ly-9.1 or Ly-5.2) markers showed good numbers of $\alpha 4$ -null-derived thymocytes, indicating that $\alpha 4$ -deficient cells can migrate and differentiate efficiently into T lymphocytes within the thymic microenvironment at all embryonic stages analyzed (Figures 5A and 5B). No major differences were found in $CD4^- CD8^-$, $CD4^+ CD8^+$, $CD4^+ CD8^-$, and $CD4^- CD8^+$ subsets or in the histology of the thymus when $\alpha 4$ -null chimeric embryos were compared with controls (data not shown). At E14, the thymi of $\alpha 4$ -null of chimeras seemed to be consistently larger than controls. This observation, together with the significantly higher percentage of $CD4^+$ cells derived from

Figure 3. Erythroid Progenitors Are Present in the Fetal Liver in the Absence of $\alpha 4$ Integrins but They Require $\alpha 4$ Integrins for Efficient Differentiation In Vivo

(A) Terminal erythroid differentiation in chimeric mice was assessed by analysis of β -globin isoforms (Hbb^d and Hbb^s) characteristic of 129Sv (ES cells) or C57BL (recipient blastocysts), respectively. Differentiation of erythrocytes is possible in the absence of $\alpha 4$ integrins at E14 but decays at later stages (E16 and E18) compared with control mice. Comparison of embryos with similar percentage of chimerism estimated by GPI is shown at each stage (E14 = 85 and 30%; E16 = 65%; E18 = 75%).

(B) Analysis of $Ter119^+ \alpha 4^-$ expression in fetal liver cells and the percentage of β -globin isoforms in blood from wild-type and $\alpha 4$ -null chimeric embryos at different stages of development. Chimeric embryos in C57BL (circles) and $RAG-2^{-/-}$ (squares) were analyzed. Only embryos with >30% chimerism were analyzed. There was no significant difference in the percent of chimerism between $\alpha 4$ -null and wild-type chimeric embryos (Figure 4C). (C) Flow cytometry analysis of fetal liver cells for the expression of the erythroid marker Ter-119 and $\alpha 4$ integrins. Note that the percentage of $Ter119^+ \alpha 4^-$ cells is significantly higher in $\alpha 4$ -null chimeras than in controls at E14; 90% chimeric embryos are shown. Bar, 166 μ m.

$\alpha 4$ -null progenitors at E16 (Figure 5B), suggests an advantage for T cell differentiation in the absence of $\alpha 4$ integrins at early stages of embryonic development.

Definitive Hematopoiesis in Spleen and Bone Marrow Is Severely Compromised in the Absence of $\alpha 4$ Integrins

Hematopoietic progenitors colonize the bone marrow and spleen at E12–13, although active hematopoiesis is not detected until E15 (Delassus and Cumano, 1996). Previous data showed a defect in postnatal lymphopoiesis in the bone marrow in the absence of $\alpha 4$ integrins and suggested different requirements for $\alpha 4$ integrins in prenatal versus postnatal hematopoiesis (Arroyo et al., 1996). We therefore explored hematopoietic development in the bone marrow and spleen around the critical point of birth.

Multipotent progenitors are present in BM and spleen of $\alpha 4$ -null chimeric embryos at E18 assessed by in vitro colony assays (data not shown). Differentiation of these progenitors in vivo was analyzed by flow cytometry with markers for myeloid (Gr-1) and B lymphoid (B220) lineages. As shown in Figure 6A, $\alpha 4$ -deficient cells from spleens of chimeric embryos at E18 contribute to $Gr-1^+$ myeloid and $B220^+$ B lymphoid population at percentages close to, although significantly lower than, control wild-type chimeras (average percent contribution [$n = 8$]: $\alpha 4$ -null/Gr-1 = 11.2 and wt/Gr-1 = 31.4, $p = 0.01$; $\alpha 4$ -null/B220 = 15.7 and wt/B220 = 38.4, $p = 0.02$). Similar results were observed in the bone marrow (average percent contribution [$n = 6$]: $\alpha 4$ -null/Gr-1 = 20 and wt/Gr-1 = 41.5, $p = 0.03$; $\alpha 4$ -null/B220 = 14.3 and

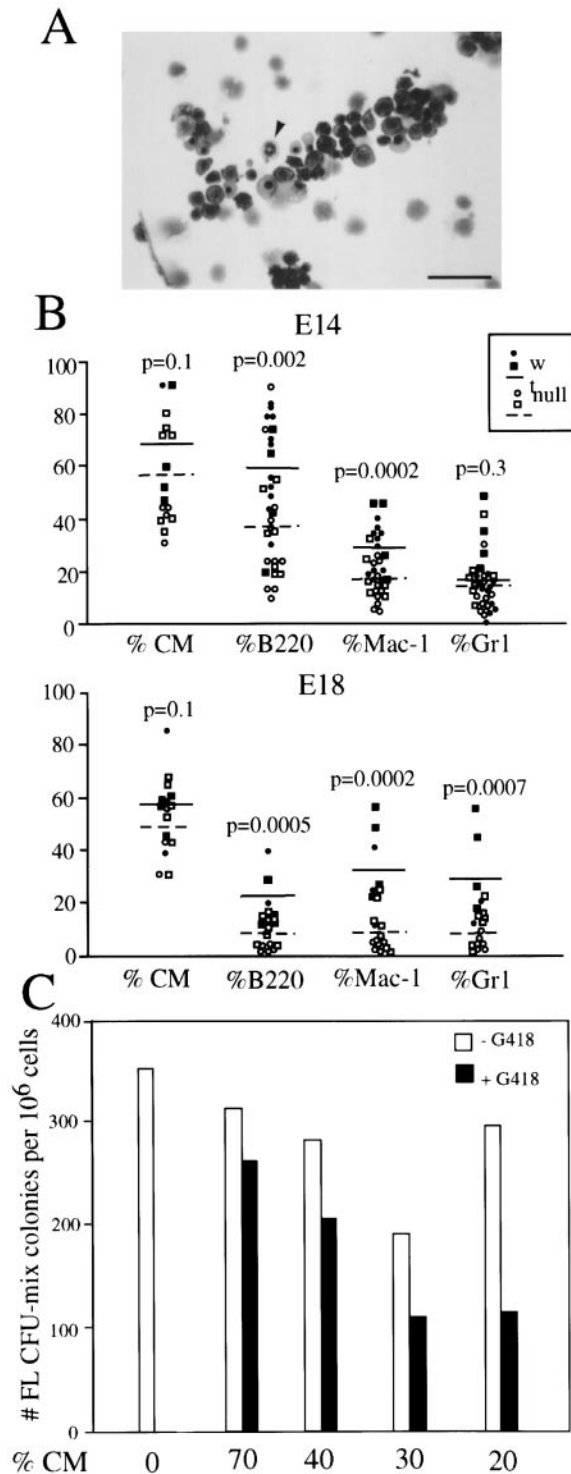


Figure 4. Myeloid and B Lymphoid Progenitors Are Present in the Fetal Liver in the Absence of $\alpha 4$ Integrins, but They Require $\alpha 4$ Integrins for Efficient Differentiation In Vivo

(A) Histological analysis of fetal livers from $\alpha 4$ -null embryos at E12 shows the presence of mature myeloid forms (arrowhead). Bar, 166 μ mB).

(B) Summary of analyses of the contribution of wild-type and $\alpha 4$ -deficient cells to myeloid (Mac-1 and Gr-1) and B lymphoid (B220) lineages in fetal liver assessed by flow cytometry. Chimeric embryos in C57BL (circles) and RAG-2^{-/-} (squares) were analyzed. The percentage contribution of ES cells to the total specific population is

wt/B220 = 24, $p = 0.1$) (data not shown). These results suggest that $\alpha 4$ -deficient progenitors can migrate to the spleen and bone marrow microenvironments and complete differentiation.

The roles of $\alpha 4$ integrins in erythropoiesis at perinatal and postnatal stages were also studied. β -globin analysis shows that $\alpha 4$ -null cells in chimeric mice younger than 1 week can develop into mature erythrocytes (4/6) although the percentage contribution is lower than in control chimeras (Figure 6B). However, 0/38 $\alpha 4$ -null chimeric mice older than 1 week showed any $\alpha 4$ -deficient mature erythrocytes compared with wild-type (15/21), $\alpha 5$ -null (4/6), and αv -null (4/4) control chimeric mice (Figure 6B; data not shown). These data demonstrate that $\alpha 4$ integrin-mediated interactions are essential for normal erythroid development in the bone marrow and spleen microenvironments, since a complete blockade of postnatal erythropoiesis occurs in vivo in the absence of these receptors. However, in vitro colony assays in the presence of G418 show that bone marrow and spleen from 3-month-old $\alpha 4$ -null chimeras (40%–50% chimerism) contained up to 25% resistant BFU-e and CFU-mix colonies, suggesting that the failure in erythroid development was not due to a complete deficit in progenitors but to a defect at later steps of differentiation.

Similar results were obtained when myeloid and B lymphoid development were studied at perinatal and postnatal stages. Figure 6C shows that $\alpha 4$ -null chimeras younger than 1 month have a detectable population of maturing myeloid and B lymphoid cells in the bone marrow. The percentages are, however, significantly lower than the controls. In contrast, in $\alpha 4$ -null chimeras older than 1 month, almost no B lymphoid cells and few myeloid cells are developing in the bone marrow, and subsequently the numbers of mature cells in blood and spleen are low in contrast to control chimeric mice (Figure 6C; Arroyo et al. 1996; data not shown).

These data indicate that $\alpha 4$ integrins are essential for postnatal multilineage hematopoietic development in vivo, since erythroid, myeloid, and B lymphoid development are severely compromised in the bone marrow and spleen in the absence of these receptors.

$\alpha 4$ Integrins Are Essential for Transmigration and Proliferation of Hematopoietic Progenitors In Vitro

To elucidate the mechanisms responsible for the defects observed in the absence of $\alpha 4$ integrins, two in vitro approaches were used.

First, in vitro erythropoiesis assays were performed. As shown in Figure 7A, fetal liver $\alpha 4$ -null cells attach to bone marrow stroma at day 1 like controls. However, $\alpha 4$ -null primary colonies gave only tiny reddish colonies

represented. Every symbol represents an individual mouse. The percentage of chimerism of the embryos was estimated by GPI analysis of the tail and was not statistically different between controls and $\alpha 4$ -null chimeras; only mice with >30% chimerism were analyzed.

(C) The presence of mixed progenitors at E18 was tested by CFU-mix colony assays with fetal liver cells from $\alpha 4$ -null chimeric embryos in the presence of 1.5 mg/ml G418. SD within duplicates was less than 10%. One experiment out of two is shown. Fetal liver cells from wild-type chimeras did not survive in the presence of G418.

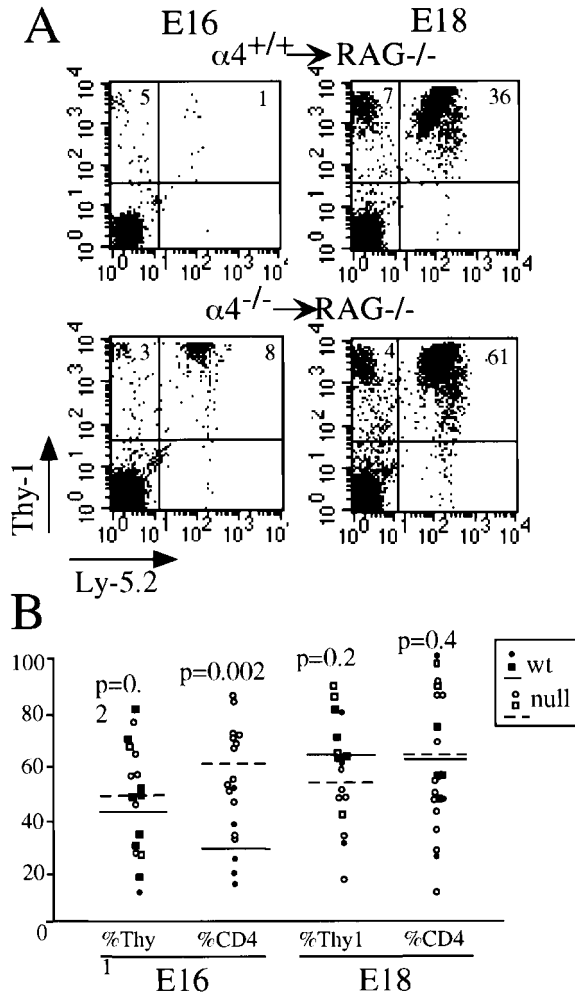


Figure 5. $\alpha 4$ -Deficient Cells Differentiate Normally into T Lymphocytes in the Thymus during Embryonic Development
(A) Staining of thymocytes from control and $\alpha 4$ -null chimeric embryos with Thy-1 (T cell-specific) versus Ly-5.2 (strain-specific) is shown at E16 and E18 stages of development. Embryos of similar percentage of chimerism (40% at E16 and 55% at E18) are compared.
(B) Summary of the contributions of wild-type and $\alpha 4$ -null cells to T lymphocyte population (Thy-1⁺ or CD4⁺) at E16 and E18. The percentage contribution to the total population is represented. Every symbol represents an individual mouse. The percentage of chimerism of the embryos was estimated by GPI analysis of the tail; only mice with >30% chimerism were analyzed. Averages of the different groups and statistical significance are shown.

by day 4, and no cells were observed beneath the stroma, whereas wild-type cells transmigrate beneath the stroma, expand, and develop into large, red erythroid colonies. Thus, $\alpha 4$ -null erythroid progenitors cultured under conditions resembling in vivo interactions with stroma can complete terminal differentiation (reddish colonies), but the yield of mature erythrocytes is greatly diminished due to a defect in transmigration and proliferation.

Second, IL-7-dependent pre-B lymphoid cell lines were derived from fetal livers from wild-type and $\alpha 4$ -null E12 embryos. Flow cytometry of wild-type and $\alpha 4$ -null cell

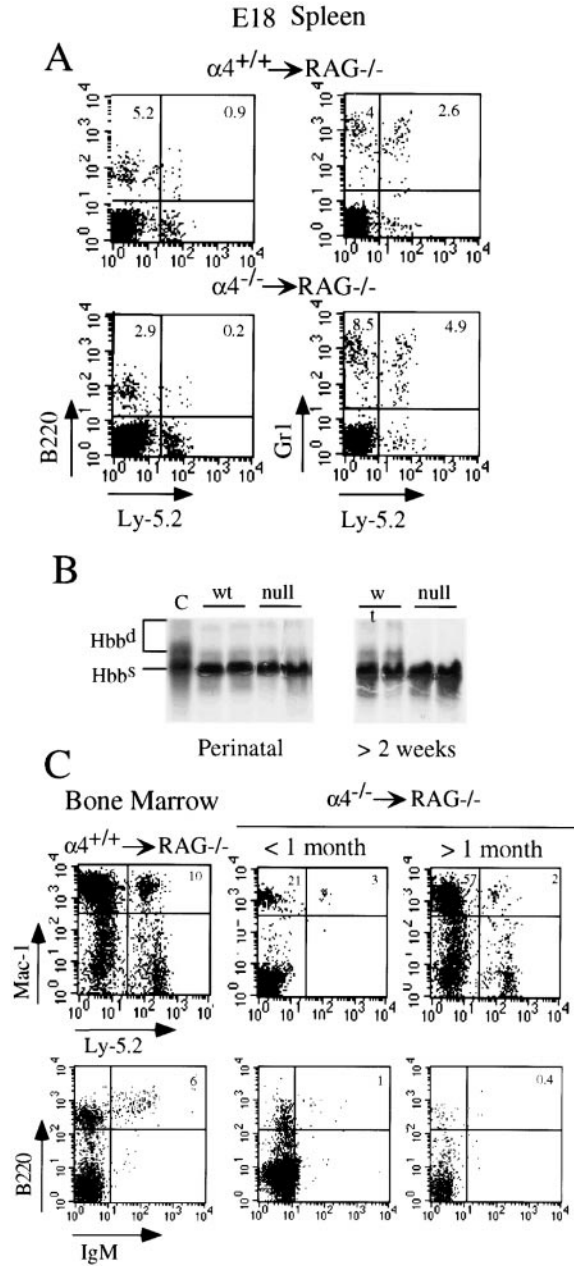


Figure 6. $\alpha 4$ -Null Definitive Progenitors Migrate to Spleen and Bone Marrow, but They Fail to Contribute to Erythroid, Myeloid, and Lymphoid Lineages after Perinatal Stage
(A) Flow cytometry of spleen cells at E18 for myeloid (Gr-1) and B lymphoid (B220) lineages versus the strain-specific marker Ly-5.2. Note that there is good contribution of $\alpha 4$ -deficient cells to these lineages. Embryos of about 55% chimerism are shown.
(B) β -globin analysis of blood from chimeric mice at perinatal and adult stage. Although some contribution is observed at perinatal stage, no contribution of $\alpha 4$ -null cells is detected in older chimeras.
(C) Flow cytometry of bone marrow cells from chimeric mice with the myeloid (Mac-1) versus strain-specific (Ly-5.2) and B lymphoid (B220 and IgM) markers shows a low but consistent perinatal contribution of $\alpha 4$ -null cells. However, 1 month after birth the contribution of $\alpha 4$ -deficient cells decreases and becomes almost undetectable compared with controls. Mice of about 70% chimerism are shown.

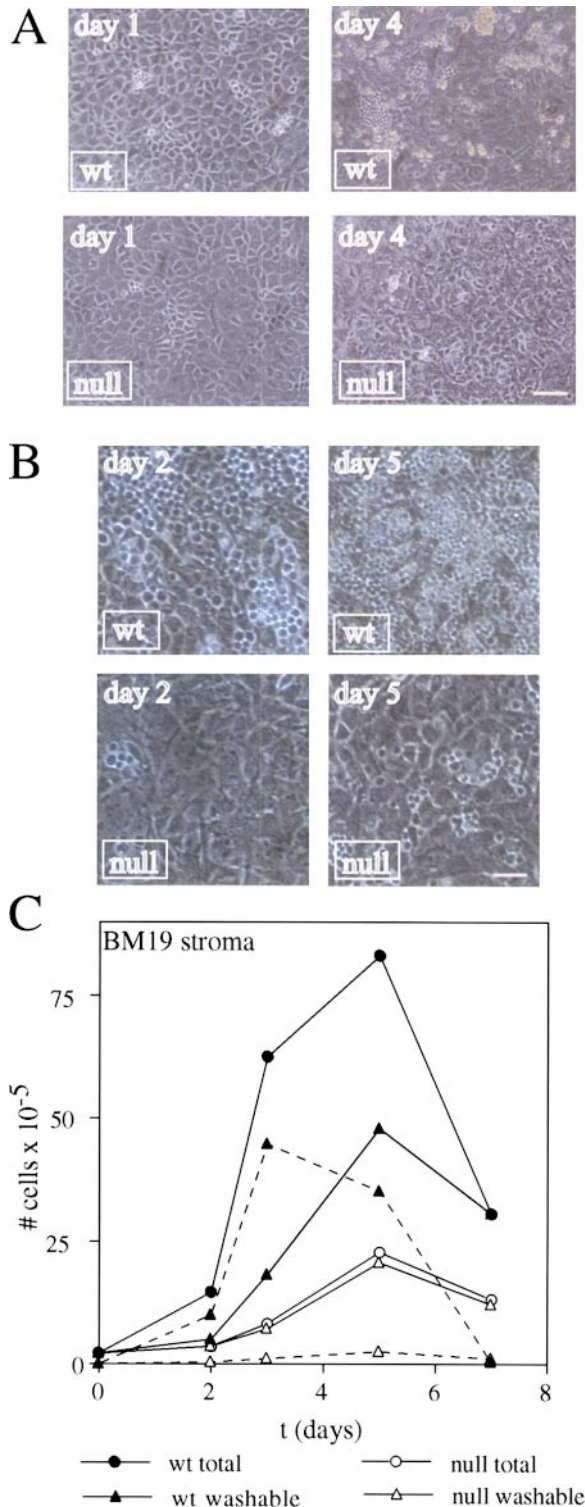


Figure 7. Erythroid and B Lymphoid Progenitors Fail to Transmigrate and Proliferate in the Absence of $\alpha 4$ Integrins

(A) In vitro erythropoiesis assays performed with fetal liver cells from wild-type and $\alpha 4$ -null embryos at E12. At day 1, small groups of erythroid progenitors attached to the stroma are observed in both wild-type and null cultures. After 4 days, $\alpha 4$ -null cells give rise only to tiny reddish colonies and no cells are observed underneath the stroma in contrast to wild-type cells that differentiate into larger and redder colonies with plenty of cells lying underneath. Each

lines showed that most cells expressed the pan-B cell marker B220 and low levels of IgM but they differed in $\alpha 4$ integrin expression (data not shown). Kinetics of attachment, transmigration, and proliferation of these cell lines showed that $\alpha 4$ -deficient cells were very inefficient in attaching and transmigrating beneath the bone marrow stroma (Figure 7B), and they proliferated at a lower rate than wild-type (Figure 7C). The kinetics of transmigration and cytopspin preparations showing poor mitotic activity in transmigrated $\alpha 4$ -null cells compared with wild-type suggest that both processes, transmigration and proliferation, are affected by the absence of $\alpha 4$ integrins. Moreover, the efficiency in obtaining pre-B cell lines from $\alpha 4$ -null fetal livers was consistently much lower, and it was not possible to obtain clones from $\alpha 4$ -null cell lines by limiting dilution because the cells failed to expand (data not shown). From these in vitro data, we conclude that $\alpha 4$ -null cells from erythroid and B lymphoid lineages have hampered ability for transmigration through the stroma, and this may result in lower rates of cellular proliferation.

Discussion

In this report, analyses of $\alpha 4$ -null mutant and chimeric mice during embryonic development have shown that (1) $\alpha 4$ integrins are not essential for erythropoiesis in the yolk sac; (2) multipotent definitive progenitors can migrate to fetal liver, thymus, bone marrow, and spleen in the absence of $\alpha 4$ integrins; (3) terminal differentiation of erythroid, myeloid, and lymphoid lineages can occur in the absence of $\alpha 4$ integrins, albeit inefficiently; and (4) $\alpha 4$ integrins are essential for maintenance of efficient development of multilineage progenitors in the fetal liver, bone marrow, and spleen microenvironments, probably by regulating transmigration and proliferation of progenitors.

$\alpha 4$ Integrins and Erythroid Development

Several reports had suggested roles for $\alpha 4$ integrins during erythropoiesis (Hanspal, 1997). We have shown that erythropoiesis in the yolk sac can occur in the absence of $\alpha 4$ integrins, indicating that $\alpha 4$ integrins are not essential for commitment and terminal differentiation of erythroid progenitors. Moreover, $\alpha 4$ -null erythroid progenitors can migrate to fetal liver, bone marrow, and

experiment was run in triplicate. One experiment out of two is shown. Bar, 330 μm .

(B and C) Pre-B lymphoid cell lines were derived from E12 fetal livers from wild-type and $\alpha 4$ -null embryos in the presence of IL-7. Pre-B cell lines were plated on the bone marrow stromal cell line CBR-BM, L#19 and their transmigration (B) and proliferation (C) behavior recorded after washing cells in suspension. As shown, $\alpha 4$ -null pre-B cells migrate underneath the stroma very poorly in contrast with wild-type cells. Proliferation on bone marrow stroma was also tested; pre-B cells were separated into two categories, washable, i.e., cells in suspension and cells weakly attached to the stroma, and nonwashable, cells located underneath the stroma and the few strongly attached to the stroma. Results show that $\alpha 4$ -null pre-B cells transmigrate very poorly and also they do not proliferate as well as wild-type. Experiments were run in duplicate, and SD was less than 5%. The mean is represented. One experiment out of three similar ones is shown. Bar, 166 μm .

spleen as demonstrated by *in vitro* colony assays and flow cytometry. In these microenvironments, the progenitors can differentiate without $\alpha 4$ integrins, but only low yields of mature erythrocytes are obtained, with the defect being more evident in bone marrow, and spleen. These *in vivo* data point to a defect in proper expansion of erythroid populations. This is supported by *in vitro* data showing defective transmigration and proliferation of erythroid progenitors in the absence of $\alpha 4$ integrins. Interestingly, the expression pattern of $\alpha 4$ integrins, being expressed on early erythroid progenitors (BFU-e and CFU-e) and downregulated later, conforms with the stages at which high rate of proliferation occurs. These results provide an explanation for *in vitro* data showing an inhibition of erythroid development by anti- $\alpha 4$ integrin antibodies (Yanai et al., 1994) and *in vivo* data showing perinatal anemia in pups from mice injected with antibodies against $\alpha 4$ integrins (Hamamura et al., 1996).

Macrophage-erythroid progenitor interactions in erythroblastic islands may be mediated by $\alpha 4$ integrin-VCAM-1 (Sadahira et al., 1995). In the absence of $\alpha 4$ integrins, erythroblastic island formation would be defective, and this might explain the defects in proliferation, the smaller size of the cellular aggregates observed in $\alpha 4$ -null fetal livers, and the fragility of $\alpha 4$ -null fetal livers compared with wild-types. It is possible that FN also participates in these interactions, since it has been reported that anti-VCAM-1 antibodies blocked only partially (Yanai et al., 1994). Antibodies against $\alpha 4$ integrins can block attachment, migration, and differentiation on stroma or FN of erythroid progenitors *in vitro* in contrast to anti- $\alpha 5$ integrin antibodies that block only attachment (Yanai et al., 1994; Goltry and Patel, 1997). Our data confirm that $\alpha 4$ -null erythroid progenitors cannot transmigrate through stroma and differentiate efficiently although attachment seems to be less affected, likely due to $\alpha 5$ integrins. Interestingly, no defects in erythroid development are found in $\alpha 5$ -null chimeric mice, suggesting that *in vivo* roles for $\alpha 4$ and $\alpha 5$ integrins are in fact distinct during erythroid development (Arroyo et al., unpublished data).

Finally, we have shown that erythroid development is impaired in some microenvironments (fetal liver, bone marrow, and spleen) but not others (yolk sac). It has recently been shown how the environment can provide the specific signals for determining the fate of hematopoietic progenitors (Geiger et al., 1998). Erythrocytes originating in the yolk sac are nucleated in contrast to nonnucleated definitive erythrocytes, and interactions with the stroma might regulate the enucleation process, suggesting that adhesion receptors might be differentially required (Hanspal, 1997). It is also known that several growth signal pathways act in synergy with integrins (Clark and Brugge, 1995). Thus, synergism with other proteins such as Epo, c-kit, c-myb, and TEL, whose requirements are differentially regulated in yolk sac versus fetal liver hematopoiesis, might also account for the observed differences in $\alpha 4$ integrin dependence.

$\alpha 4$ Integrins in Myeloid and Lymphoid Development

In vitro experiments with antibodies had suggested important roles for $\alpha 4$ integrins during lymphoid and myeloid development (Miyake et al., 1991; Ryan et al.,

1992). However, little was known about the *in vivo* roles of $\alpha 4$ integrins in the development of these lineages. Intrauterine injection of antibodies against $\alpha 4$ integrins revealed only a minor decrease in the number of myeloid and lymphoid colonies at birth (Hamamura et al., 1996).

We report here that terminal differentiation of T and B lymphocytes, monocytes, and granulocytes is possible in the absence of $\alpha 4$ integrins during fetal development in accord with our previous data (Arroyo et al., 1996). However, although $\alpha 4$ -deficient multipotent progenitors are detected in the fetal liver, bone marrow, and spleen at different stages of embryonic development, only small percentages of $\alpha 4$ -null progenitors complete successful differentiation, indicating that $\alpha 4$ integrins are required for efficient myeloid and B lymphoid development *in vivo*. As for the erythroid lineage, the defects in B lymphoid and myeloid development become more obvious after birth when bone marrow takes the major role in hematopoietic development. A low steady-state level of myeloid differentiation is consistently present in the bone marrow in the absence of $\alpha 4$ integrins, in contrast to B lymphoid and erythroid differentiation that is practically undetectable. These differences among lineages might be due to differences in compensatory mechanisms or differences in the renewal rate. This agrees with reports that anti- $\alpha 4$ integrin antibodies block B cell development but only delay myeloid development *in vitro* (Miyake et al., 1991) and with the previous analysis of adult $\alpha 4$ -null chimeric mice in which a certain population of $\alpha 4$ -deficient monocytes was present in blood (Arroyo et al., 1996).

Results obtained with pre-B cell lines show that $\alpha 4$ integrins are required for proper attachment, transmigration, and proliferation of B cell precursors likely by mediating progenitor-stroma interactions. The ligand involved in these processes seems to be FN rather than VCAM-1 since no major defects in hematopoiesis are found in VCAM-1-null mice (Friedrich et al., 1996b). Lymphocyte transmigration is directed by chemokines that can also activate integrin functions in lymphocytes. It is possible that the absence of $\alpha 4$ integrins decreases the responsiveness of lymphoid cells to chemokines, in particular to SDF-1/CXCR4 complex. The phenotypes of knockout mice for SDF-1 and its receptor CXCR4 with defects in B cell lymphopoiesis and bone marrow myelopoiesis (Nagasawa et al., 1996; Tachibana et al., 1998) recall the defects observed in the absence of $\alpha 4$ integrins. It would be interesting to investigate if $\alpha 4$ integrins are targets for chemokine hematopoietic functions *in vivo*. Modulation by SDF-1 of the VLA-4/VCAM-1 pathway in migration of progenitors through bone marrow endothelial cells has recently been reported (Imai et al., 1999).

Since the severity of the developmental defects differ in the diverse lineages analyzed (erythroid, myeloid, and B lymphoid), there might be partial compensation by other surface receptors. Since both $\alpha 4$ and $\alpha 5$ integrins have been implicated in transmigration of lymphoma cells (Miyake et al., 1992), it is possible that $\alpha 5$ integrins might compensate for part of the defect. $\alpha L\beta 2$ is expressed on erythroblasts and on B lymphocytes, and $\alpha L\beta 2$, $\alpha M\beta 2$, and $\alpha X\beta 2$ are on myeloid cells. This expression pattern might explain differential compensation.

Moreover, two new integrins, $\alpha D\beta 2$ and $\alpha 9\beta 1$, have recently been reported to bind VCAM-1, raising the possibility of compensation for binding to this ligand (Grayson et al., 1998; Taooka et al., 1999).

We had previously shown a switch in $\alpha 4$ integrin requirements for T lymphocyte development after birth (Arroyo et al., 1996). Here, we show that T cell progenitors migrate to the thymus and differentiate efficiently into all subsets of T lymphocytes during embryonic development. Some results even suggest that $\alpha 4$ -deficient T cell progenitors might have a certain advantage for T cell differentiation, but this point deserves further investigation. Our results do not confirm previous *in vitro* data on $\alpha 4$ integrin roles on thymocyte attachment and differentiation (Crisa et al., 1996). There might be different requirements *in vitro* and *in vivo* or compensation by other receptors *in vivo*. The interesting point is that $\alpha 4$ -deficient hematopoietic progenitors can proliferate and differentiate well in the thymic microenvironment but not in fetal liver, bone marrow, or spleen. This might be explained by regulation of transmigration of thymocytes by specific thymic chemokines (Vicari et al., 1997; Kim et al., 1998) or by different requirements for proliferation of thymocytes independent of transmigration.

A Model for Regulation of the Proliferation/Differentiation Balance of Hematopoietic Progenitors through $\alpha 4$ Integrins

$\alpha 4$ integrins had been suggested to play roles in migration, attachment, transmigration, proliferation, and differentiation of hematopoietic progenitors *in vitro* (reviewed by Coulombel et al., 1997). The complex phenotype we observe *in vivo* in the absence of $\alpha 4$ integrins suggests that one or more of these processes are impaired *in vivo*.

We have shown that $\alpha 4$ integrins are not essential for migration of progenitors to the different hematopoietic organs. This is in contrast with the defect in migration to fetal liver observed in the absence of $\beta 1$ integrins (Hirsch et al., 1996), suggesting that other $\beta 1$ heterodimers might be involved, and with reports suggesting differential roles for $\alpha 4$ integrins in lodgment of progenitors in bone marrow and spleen (Papayannopoulou et al., 1995). It is possible that $\alpha 4$ integrins play a role during those processes, but our data demonstrate that their functions are not essential and other receptors can substitute in their absence.

We report here that transmigration of progenitors through the stroma is impaired in the absence of $\alpha 4$ integrins and that progenitor cells lacking $\alpha 4$ integrins do not proliferate as efficiently as wild-type. Previous reports had suggested that $\alpha 4$ integrins could be involved in regulation of proliferation of progenitors (Moritz et al., 1994; Levesque et al., 1996), and it has recently been reported that fibronectin, particularly the CS-1 region that binds $\alpha 4$ integrins, facilitates and increases proliferation of hematopoietic progenitors (Schofield et al., 1998; Yokota et al., 1998). Synergism between integrins and growth factors such as c-kit has been reported (Papayannopoulou et al., 1998). It has also been shown that the protooncogenes c-myc and Ets family members act synergistically to regulate $\alpha 4$ gene promoter in hematopoietic cells (Postigo et al., 1997). The phenotypes

of c-myc mutant mice (Mucenski et al., 1991) and TEL-null chimeric mice (Wang et al., 1998) recall the defects observed in the absence of $\alpha 4$ integrins. Thus, $\alpha 4$ integrins might be acting as important *in vivo* targets for c-myc and/or TEL. The *in vivo* roles of $\alpha 4$ integrins in progenitor proliferation highlight the importance that these adhesion receptors might have in leukemogenesis (Schwartz, 1997).

We propose a working model for the roles of $\alpha 4$ integrins in regulating hematopoiesis. Hematopoietic progenitors normally seed and attach to the stroma. Weissman (1994) distinguishes three compartments within the stroma for self-renewal, expansion, and maturation of progenitors, and distinct elements of the stromal microenvironment controlling growth and differentiation of progenitors have recently been identified (Aiuti et al., 1998). Differential signaling in these stromal compartments might involve the secretion of soluble factors such as cytokines and chemokines as well as different stroma-progenitor cell interactions. The balance among all these signals (reviewed by Ogawa, 1993) and the competition for the niches among different progenitors would determine the fate of the various committed hematopoietic progenitors. In the expansion stroma, progenitors would transmigrate in response to chemokines, which might also facilitate this process by activating adhesion receptors such as integrins (Coulombel et al., 1997). In that niche, progenitors proliferate in contact with extracellular matrix proteins such as fibronectin and growth factors presented by the proteoglycans. After this expansion stage, progenitors continue differentiation into mature cells under the influence of different cytokines in the maturation compartment. In our model, $\alpha 4$ integrins would act as a retention signal for progenitors (likely at different stages depending on the lineage) to remain in the expansion compartment. In the absence of $\alpha 4$ integrins, two major steps would be impaired: first, the response to cytokines and chemokines and subsequent attachment and transmigration beneath the stroma, and second, proliferation mediated by contact with fibronectin and growth factors. As a consequence, $\alpha 4$ -deficient progenitors would detach prematurely, skipping the expansion phase, and shift toward differentiation. This might result in low numbers of progenitors involved in hematopoiesis and lower yields of mature cells. Defects in multilineage development may be more evident at postnatal stages because $\alpha 4$ integrins might be differentially required in bone marrow and spleen versus fetal liver environments or because the rate of production of hematopoietic cells is higher after birth, and defective competition and proliferation would become more evident. The differences in the severity of the defects in various lineages might be due to different stages of differentiation being affected depending on the lineage or to substitution by other receptors. It may be important to keep these defects in mind since anti- $\alpha 4$ integrin inhibitors are being used for therapy of chronic inflammatory diseases (Lobb and Hemler, 1994).

In summary, we describe how $\alpha 4$ integrin-mediated interactions are essential for normal expansion of hematopoietic progenitors *in vivo*. These data point to $\alpha 4$ integrins as major regulators of the proliferation/differentiation balance during hematopoiesis.

Experimental Procedures

Mice

$\alpha 4$ integrin mutant mice were generated (Yang et al., 1995), and chimeric mice were obtained by injection of $\alpha 4$ -null or wild-type ES cells into blastocysts from C57BL or RAG-2-deficient mice (Arroyo et al., 1996). Embryos were dissected, and either the yolk sac or the embryo was used for colony assays and genotyping by PCR (Yang et al., 1995).

Cells

CBR-BM, L#19 bone marrow stromal cell line (Friedrich et al., 1996a) was kindly provided by Dr. J. C. Gutiérrez-Ramos (Millenium). Cells were maintained in culture in Iscove's modified Dulbecco's medium (IMDM; GIBCO) plus 10% fetal bovine serum (FBS) and β -mercaptoethanol at the permissive temperature 33°C.

Hemoglobin Analysis

Blood was obtained from the umbilical cord of embryos or from the heart or eye in pups and adult mice in tubes containing EDTA. Hemoglobins were analyzed by cellulose acetate electrophoresis of hemolysates treated with cystamine (Whitney, 1978). This method permits the discrimination of the codominant mouse hemoglobin "single" (Hbb^s) allele present in C57BL mouse strain and "diffuse" (Hbb^d and Hbb^f) alleles present in 129Sv strain. The sensitivity of the assay is 1%–5% (data not shown). Densitometric analysis of the gels was performed using ID-multilane Scan of the IS-1000 Digital Imaging System (Alpha Innotech).

GPI Assays

Glucose-phosphate-isomerase assays were performed as described (Yang et al., 1996). In brief, samples were lysed by four cycles of freezing and thawing, and GPI isoforms were analyzed by cellulose acetate electrophoresis and detected by applying an agarose gel containing fructose-6-phosphate, NDP, MTT, PMS, and glucose-6-phosphate dehydrogenase (all reagents from Sigma) at room temperature. Pictures were taken and densitometry performed using the IS-1000 Digital Imaging System. This method permits the discrimination of the isoforms GPI-I^{aa} and GPI-I^{bb} present in 129Sv and C57BL strains, respectively.

Flow Cytometry and Sorting

The following monoclonal antibodies from PharMingen were used for flow cytometry: PE-conjugated anti-mouse CD90.2 (Thy-1.2, 53-2.1), PE-conjugated anti-mouse CD4 (L3T4, RM4-5), PE-conjugated anti-mouse CD45R/B220 (RA3-6B2), FITC-conjugated anti-mouse IgM (R6-60.2), PE-conjugated anti-mouse CD11b (Mac-1 α chain, M1/70), PE-conjugated anti-mouse Ly-6G (Gr-1, RB6-8C5), PE-conjugated anti-mouse TER-119, FITC-conjugated anti-mouse CD49d (integrin $\alpha 4$ chain, R1-2), FITC-conjugated anti-mouse CD45.2 (Ly-5.2, 104), and FITC-conjugated anti-mouse Ly-9.1 (30C7). Single cell suspensions were incubated with purified anti-mouse CD32/CD16 (PharMingen) to block Fc receptors and with an appropriate dilution of the different antibodies at 4°C. The samples were washed twice and resuspended in PBS. Dead cells were excluded by propidium iodide staining. Samples and data were analyzed in a FACScan using CellQuest software (Becton Dickinson). Single cell suspensions from fetal liver at different stages of development were stained for Ter-119 and $\alpha 4$ integrin, and sorting of Ter119⁺ $\alpha 4$ ⁻ cells was done excluding dead cells in a FACStar Plus (Becton Dickinson).

Cytospins and Histology

Single cell suspensions from fetal livers or from sorted populations were cytospun onto slides, air dried, and stained with Diff-Quick (Baxter). Tissue specimens were fixed overnight in 4% formaldehyde and embedded in paraffin. Blocks were cut, and slides were processed and stained with hematoxylin-eosin by routine techniques. Slides were examined and photographed (Ektachrome 160T film; Eastman Kodak) on an Axiophot microscope (Carl Zeiss).

Colony Assays

For yolk sac progenitor assays, embryos were used for genotyping. Yolk sacs were incubated in PBS with 20% FBS and 0.1% collagenase (Sigma) at 37°C for 1 hr. Cells were disaggregated by passage through a 22-gauge needle (Wang et al., 1998). The yield from each yolk sac was 1–5 $\times 10^4$ cells. Cells (5×10^3) of each yolk sac were plated in IMDM plus 0.9% α -methylcellulose (StemCell), 30% FBS, 1% BSA, 2 mM β -mercaptoethanol, and growth factors. For erythroid colonies (BFU-e), 2 units/ml erythropoietin (Epo) and 20 ng/ml recombinant c-kit ligand were added. For CFU-mix colonies, 2 units/ml erythropoietin (Epo), 20 ng/ml recombinant c-kit ligand, 12.5 ng/ml IL-3, and 12.5 ng/ml IL-6 were added. Epo was from Amgen and the rest of the cytokines from R&D Systems.

For fetal liver colony assays, single-cell suspensions from fetal livers at different stages were plated in the same medium as for yolk sacs. For erythroid colonies (CFU-e), 2 units/ml Epo were used.

For fetal liver colony assays from chimeric embryos, the same system was used but colonies were quantitated in the absence and presence of G418 (1.5 mg/ml). For BFU-e, GM-CSF instead of IL-6 was used.

Erythropoiesis In Vitro

CBR-BM, L#19 bone marrow stromal cell line was grown to form a monolayer in a 24-well plate (Falcon) at 33°C and then transferred to 37°C the night prior to the assay. Cocultivation of erythroid progenitor cells on stromal cells was performed as described (Yanai et al., 1994). Single cell preparations from E12 livers from wild-type or $\alpha 4$ -null embryos were suspended in IMDM with 30% heat-inactivated FBS, 0.4% methylcellulose, 1% BSA, 100 μ M/L 2-mercaptoethanol, and 0.1 U/mL Epo, and cultured on the stromal cells for 4 days.

Establishment of Pre-B Cell Lines

Establishment of pre-B cell lines was as described (Rolink et al., 1991). Fetal liver cells from E12 wild-type or $\alpha 4$ -null embryos were cultured at 5×10^4 to 2×10^5 /ml in IMDM plus 30% FBS and 2-mercaptoethanol on a confluent layer of CBR-BM, L#19 stromal cell line in the presence of 1 ng/ml IL-7 (R&D Systems). After 3–4 days, when the pre-B cells were grown to a semiconfluent layer on the stromal cells, they were transferred from 96-well plates to a new stromal cell layer in a 24-well plate and again to 6-well plates when they reached semiconfluency (3–4 days). Cells were fed every 3–4 days and transferred to new stromal cell layers when old stroma looked exhausted. Cloning of pre-B cell lines established under these conditions was done by limiting dilution on stromal cell layers in 96-well flat-bottomed plates. $\alpha 4$ -null pre-B cell lines could not be cloned by limiting dilution because, although growth of single cells were obtained, they were not able to expand.

Statistical Analysis

Data from mice of the different phenotypes were analyzed and compared for statistically significant differences using the Student's *t* test.

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