

# Differential Requirements for $\alpha 4$ Integrins during Fetal and Adult Hematopoiesis

Alicia G. Arroyo, Joy T. Yang, Helen Rayburn, and Richard O. Hynes

Howard Hughes Medical Institute  
Center for Cancer Research and Department of Biology  
Massachusetts Institute of Technology  
Cambridge, Massachusetts 02139

## Summary

Mice chimeric for the expression of  $\alpha 4$  integrins were used to dissect the roles of these receptors in development and traffic of lymphoid and myeloid cells. During fetal life, T cell development is  $\alpha 4$  independent, but after birth further production of T cells becomes  $\alpha 4$  dependent. Precursors for both T and B cells require  $\alpha 4$  integrins for normal development within the bone marrow. In contrast, monocytes and natural killer cells can develop normally without  $\alpha 4$  integrins. Thus, there are lymphocyte-specific, developmentally regulated requirements for  $\alpha 4$  integrins in hematopoiesis in the bone marrow. We also show that  $\alpha 4$  integrins are essential for T cell homing to Peyer's patches, but not to other secondary lymphoid organs, including spleen, lymph nodes, and intestinal epithelium.

## Introduction

Hematopoiesis, the process by which pluripotent precursor cells undergo differentiation into different blood cell lineages, requires diverse cell migration pathways and takes place in several organs (such as the yolk sac and fetal liver) during embryonic life and mainly in the bone marrow in the adult (for review see Morrison et al., 1995; Weissman, 1994). These migrations are believed to rely on specific adhesion receptors. The adhesive interactions of hematopoietic progenitors with bone marrow stromal cells and with the extracellular matrix (ECM) have also been shown to play a key role in their normal development and function (Gordon et al., 1987), but the details of these interactions remain unclear.

Integrins are a widespread family of heterodimeric ( $\alpha\beta$ ) transmembrane glycoproteins that can function as cell-ECM or cell-cell adhesion receptors (for review see Hynes, 1992).  $\alpha 4$  integrins are particularly interesting owing to their involvement in various developmental and physiological processes (Yang et al., 1995; see below). The two  $\alpha 4$  integrins ( $\alpha 4\beta 1$  and  $\alpha 4\beta 7$ ) bind to an alternatively spliced segment of fibronectin (V25 or CS1) and to the counterreceptors, vascular cell adhesion molecule 1 (VCAM-1) and mucosal addressin cell adhesion molecule 1 (MAdCAM-1), and  $\alpha 4$  integrins have recently been reported to recognize the  $\alpha 4$  chain itself (Altevogt et al., 1995).  $\alpha 4$  integrins have been suggested to play roles in hematopoiesis (Williams et al., 1991), lymphocyte homing (Holzmann and Weissman, 1989), recruitment of leukocytes to inflammatory foci (Yednock et al., 1992), and metastasis (Qian et al., 1994).

Previous reports have suggested an involvement of

$\alpha 4$  integrins in hematopoiesis at several different levels, including the development of hematopoietic precursors, lymphoid and myeloid cells.  $\alpha 4$  integrins are widely expressed on different cell types, including hematopoietic progenitors, lymphocytes, natural killer (NK) cells, monocytes, eosinophils, and basophils (for review see Hemler, 1990; Sánchez-Madrid and Corbí, 1992). Members of the  $\beta 1$  subfamily, including  $\alpha 4\beta 1$  and  $\alpha 5\beta 1$  and their counterreceptors, have been implicated in the attachment of hematopoietic precursors to bone marrow stromal cells and ECM in *in vitro* experiments (Kerst et al., 1993; Levesque et al., 1995; Miyake et al., 1991b, 1992; Simmons et al., 1992; Teixidó et al., 1992). *In vivo* experiments with antibodies have suggested a role for the common  $\beta 1$  chain of integrins in migration of precursors to the spleen (Williams et al., 1991), and no colonization of fetal liver by hematopoietic precursors is observed in the absence of  $\beta 1$  integrins (Hirsch et al., 1996). A role for the  $\alpha 4\beta 1$  receptor in the attachment and homing of hematopoietic progenitors to the bone marrow in primates and mice has also been proposed (Papayannopoulou and Nakamoto, 1993; Papayannopoulou et al., 1995).

A stroma-dependent phase has been described in the first stages of B cell development in the bone marrow and in the fetal liver during embryonic life (Gisler et al., 1987; Hardy et al., 1991; Strasser et al., 1989). *In vitro* studies have suggested roles for  $\alpha 4\beta 1$  in murine B cell differentiation (Miyake et al., 1991a) and in terminal differentiation of mature B cells from the bone marrow (Roldán et al., 1992). Concerning T cell development, T cell precursors migrate from the yolk sac and fetal liver to the thymus, where they develop. After birth, the bone marrow supplies the thymus with a constant input of precursors for normal maintenance of the T lymphocyte population (Jotereau et al., 1987; Scollay et al., 1986). Previous reports have shown that the expression of  $\alpha 4$  integrins is differentially regulated in developing thymocytes, and a fibronectin receptor has been implicated in maturation of double-negative cells in the thymus *in vitro* (Salomon et al., 1994; Sawada et al., 1992; Utsumi et al., 1991). It has also been reported that myelopoiesis can be retarded *in vitro* by anti- $\alpha 4$  antibodies (Miyake et al., 1991a).

$\alpha 4$  integrins have also been implicated in regulation of leukocyte traffic. Studies with antibodies have suggested that the  $\alpha 4\beta 7$  receptor plays an important role during the migration of lymphocytes to mucosal-associated lymphoid tissues and intestine by recognizing its counterreceptor MAdCAM-1 (Hamann et al., 1994; Holzmann and Weissman, 1989) and in the recruitment of leukocytes to inflammatory foci (Yednock et al., 1992).

The studies reviewed above clearly suggest that  $\alpha 4$  integrins could play important roles in the development and functions of lymphoid and myeloid cells. However, the majority of the results concern *in vitro* systems, and the functions of these integrins, particularly during development *in vivo*, remain poorly defined. To study the *in vivo* roles of the adhesion receptors  $\alpha 4\beta 1$  and  $\alpha 4\beta 7$ , we knocked out the gene encoding the  $\alpha 4$  chain in mice.

That mutation is embryonic lethal (Yang et al., 1995), so for the analysis of hematopoietic development we generated somatic chimeric mice by injecting  $\alpha 4^{-/-}$  embryonic stem (ES) cells into  $\alpha 4^{+/+}$  blastocysts. In this paper, we report the use of such chimeras to define the roles of  $\alpha 4$  integrins in lymphopoiesis, myelopoiesis, and lymphocyte traffic.

**Results**

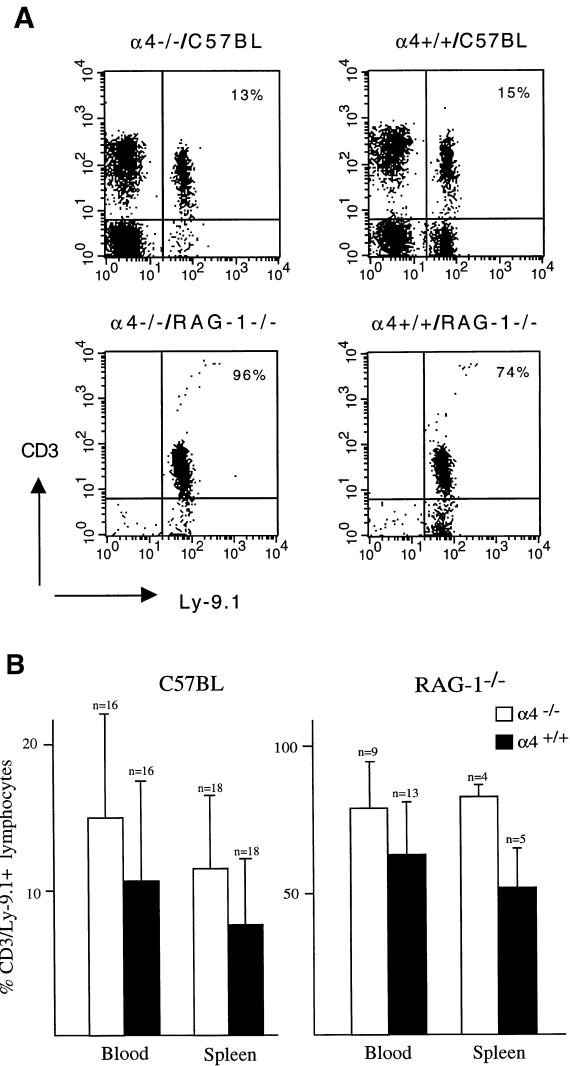
**A Switch in the Requirements for  $\alpha 4$  Integrins during T Cell Development**

The *in vivo* role of the  $\alpha 4$  integrins in hematopoietic differentiation was studied by generation of  $\alpha 4$  null and  $\alpha 4$  control chimeric mice by injection of  $\alpha 4^{-/-}$  or  $\alpha 4^{+/+}$  ES cells into either C57BL or RAG-1- or RAG-2-deficient blastocysts. These chimeras will be referred to as  $\alpha 4$  null or  $\alpha 4$  control/C57BL or RAG-1 or RAG-2 chimeras. Since RAG-1- and RAG-2-deficient mice do not develop any mature lymphocytes, RAG-1 and RAG-2 chimeras are particularly good models to evaluate the involvement of a specific gene in lymphocyte development (Chen et al., 1993; Mombaerts et al., 1992; Shinkai et al., 1992).

Lymphocytes from different organs were analyzed by flow cytometry using the marker Ly-9.1, which specifically stains the lymphocytes derived from the ES cells (129Sv strain) and not those derived from the blastocyst (C57BL strain) (Ledbetter et al., 1979). Using this approach, normal percentages of ES-derived T cells (CD3/Ly-9.1 positive) were observed in the peripheral blood and spleen of adult  $\alpha 4$  null chimeric mice (>4 weeks old) as compared with control chimeras in either C57BL or RAG-1 (Figures 1A and 1B). These T cells were confirmed to be  $\alpha 4$  null by flow cytometry and polymerase chain reaction (PCR) analysis (data not shown). The CD4/CD8 ratio and T cell receptor  $\alpha\beta$  (TCR $\alpha\beta$ ) or TCR $\gamma\delta$  expression displayed by these  $\alpha 4$  null T cells were normal, and they appeared to respond properly upon activation with different stimuli as assessed by expression of activation markers as well as proliferation rate (data not shown). Thus, development of various T cell subsets can proceed in the complete absence of  $\alpha 4$  integrins on the surfaces of these cells and their precursors.

Interestingly, when the thymi from  $\alpha 4$  null chimeric mice were analyzed, we observed a gradual decrease in  $\alpha 4$  null thymocytes after the birth of the animals. Thus, in  $\alpha 4$  null/RAG-1 or  $\alpha 4$  null/RAG-2 chimeric mice older than 3 weeks, very few CD4/CD8 double-positive cells remained, the thymus became progressively atrophic, and after 1 month only double-negative cells derived from the RAG-1 $^{-/-}$  or RAG-2 $^{-/-}$  blastocysts were observed compared with control mice, as assessed by flow cytometry (Figure 2) and histological analysis (Figures 3A and 3B). Since the wave of bone marrow-derived precursors is expected after birth, these data suggest that, in the absence of  $\alpha 4$  integrins, there is a defect in the input of T cell precursors from the bone marrow to the thymus.

To elucidate further the step of blockade (before, during, or after the bone marrow stage), reconstitution experiments were performed. Bone marrow from the  $\alpha 4$  null/RAG-1 chimeras injected into the blood of sublethally irradiated RAG-1 $^{-/-}$  recipients was able to reconstitute the peripheral T cell population in the short term



**Figure 1. T Lymphocytes Lacking  $\alpha 4$  Integrins Can Develop Normally**

(A) Blood from  $\alpha 4$  null and control chimeric mice was stained with anti-CD3 and anti-Ly-9.1 monoclonal antibodies, and lymphocytes were analyzed by flow cytometry. Note that the ES-derived T cell population (Ly-9.1 $^{+}$ /CD3 $^{+}$ ) is similar in both null and control chimeras. The Ly-9.1 $^{+}$ /CD3 $^{-}$  population present in  $\alpha 4$  control chimeras are B cells not seen in  $\alpha 4$  null chimeras (see text). This leads to the lower total percentages of Ly-9.1 $^{+}$ /CD3 $^{+}$  cells in control chimeras. (B) Lymphocytes from blood or spleen of chimeric mice (>30% coat color chimerism in C57BL or RAG-1) were stained and analyzed by flow cytometry as in (A). Mean and standard deviation are shown. Again, note that the percentages of T cells derived from  $\alpha 4^{+/+}$  ES cells are lower because in this case they also contribute to the B cell population (see text).

(Figure 4). This result means that the bone marrow contains  $\alpha 4$  null precursors able to migrate and differentiate in the thymus. No long-term reconstitution was observed, but secondary bone marrow transplantation from the  $\alpha 4$  null reconstituted mice into nonirradiated RAG-1 $^{-/-}$  recipients again gave rise to peripheral T lymphocytes, showing, first, that proper migration of precursors into the bone marrow can take place in the absence of  $\alpha 4$  integrins but, second, that development of  $\alpha 4$  null

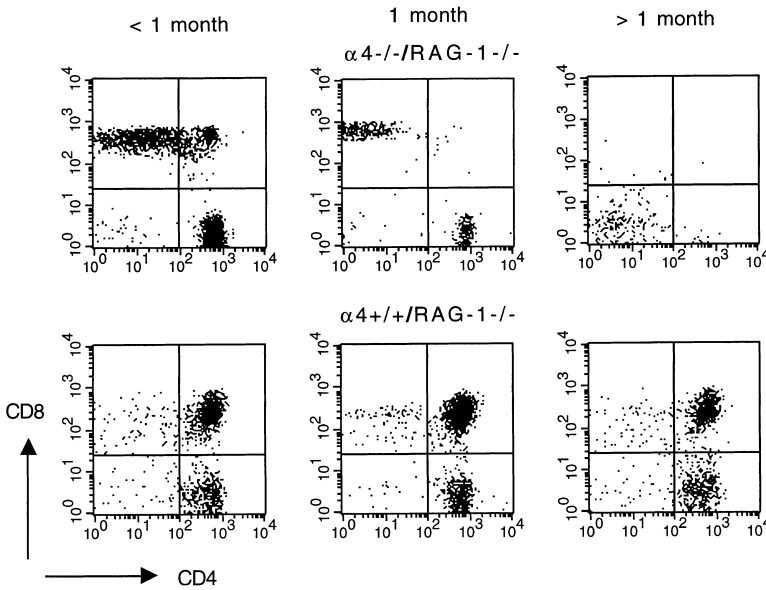


Figure 2. T Cell Development Is Blocked in the Thymi of  $\alpha 4$  Null Chimeric Mice Older than 3 Weeks

Thymi from  $\alpha 4$  null/RAG-1 and control/RAG-1 chimeric mice of different ages were analyzed for the expression of CD4 and CD8. Note that, while  $10^4$  cells were analyzed for control chimeras and for null chimeras less than 1 month old, the thymi of older chimeras are atrophic and fewer cells were analyzed (less than 500 cells). The important point is that the ratio of the different populations (double negative, double positive, and single positive) changes during the life of the null chimeras but does not vary in the control animals. After 1 month, only double-negative cells remain in the thymi of the null chimeras, and they are derived from the RAG-1<sup>-/-</sup> blastocysts. The number of thymocytes was on average  $3 \times 10^6$  in null versus  $100 \times 10^6$  in control chimeric mice older than 1 month.

T cell precursors to the point at which they can exit the bone marrow and migrate to the thymus does not occur (data not shown). Altogether, these results point to a problem within the bone marrow environment rather than in the seeding of the bone marrow with precursors or in the migration of T cell precursors into, or differentiation within, the thymus during adult life in the  $\alpha 4$  null chimeras.

These data clearly show that  $\alpha 4$  integrins are not essential for the in vivo development of mature T cells in the thymus during embryonic life, although they play a key role in the maintenance of normal T cell homeostasis during adult life, probably by regulating the normal development of T cell precursors in the adult bone marrow.

#### $\alpha 4$ Null T Lymphocytes Cannot Migrate to Peyer's Patches

The specific localization of  $\alpha 4$  null T lymphocytes (homing) was studied by the analysis of different secondary lymphoid organs from chimeric mice. As shown in Figure 5, very few  $\alpha 4$  null T lymphocytes were detected in Peyer's patches (gut mucosal-associated lymphoid tissue) of  $\alpha 4$  null/C57BL mice, and, remarkably, no Peyer's patches could be found in the  $\alpha 4$  null/RAG-1 chimeras. The levels of other adhesion receptors potentially involved in that migration pathway, such as L-selectin and CD44 in the  $\alpha 4$  null T cells, were comparable with controls (data not shown). In contrast, percentages of  $\alpha 4$  null T lymphocytes comparable with those seen in control animals were observed in the lymph nodes (mesenteric and inguinal) as well as within the intraepithelial compartment of the intestine of  $\alpha 4$  null chimeras (Figures 5A and 5B). The sizes of the lymph nodes were similar in  $\alpha 4$  null/RAG-1 and control/RAG-1 chimeric mice, and the histological analysis of these organs demonstrated a similar structure, although no germinal centers (B cell areas) were apparent in the  $\alpha 4$  null chimeras (data not shown; see below).

These data clearly demonstrate that  $\alpha 4$  integrins are essential for the in vivo homing of T lymphocytes to

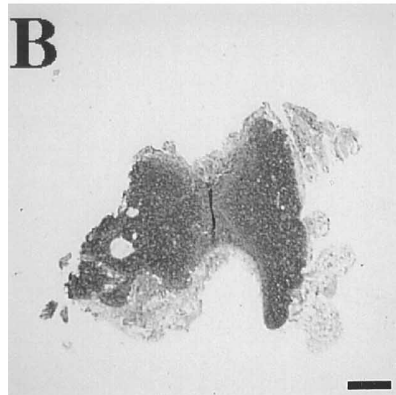
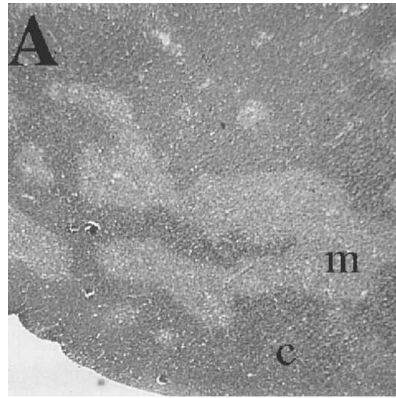
Peyer's patches but do not play an important role in the migration to lymph nodes and intestinal epithelium.

#### B Cell Maturation Is Severely Compromised in $\alpha 4$ Null Chimeric Mice

As shown in Figure 6, the number of B lymphocytes derived from  $\alpha 4$  null ES cells, assessed by the double expression of CD45R(B220) and Ly-9.1 markers, was greatly decreased in the blood of  $\alpha 4$  null chimeric mice compared with a normal ES-derived B cell chimeric population in control animals both in C57BL and RAG-1 chimeras. The percentage of  $\alpha 4$  null B cells present in C57BL chimeric mice was a little higher in animals younger than 3 weeks (Figure 6B), suggesting that some B cell development could have taken place during embryonic life in the absence of  $\alpha 4$  integrins. The reduced B cell population was mature, since it expressed surface immunoglobulin M (IgM), and the levels of serum IgM in the null chimeras were comparable with controls (data not shown). Similar results were obtained when other lymphoid organs such as spleen and lymph nodes were analyzed (Figure 6A; data not shown). In fact, spleens from  $\alpha 4$  null/RAG-1 chimeric mice were smaller than controls, and histology revealed an altered architecture showing a marked defect in the B cell-specific areas as compared with control animals (data not shown).

A different subset of B cells (B-1 cells) present mainly in the peritoneal cavity in the adult and expressing specific markers such as CD5 was also analyzed (Kantor and Herzenberg, 1993). Very few CD45R/CD5<sup>+</sup> cells (4.2% in  $\alpha 4$  null/RAG-1 chimeras versus 22.3% in controls [ $n = 4$ ]) were present in the peritoneal cavity of  $\alpha 4$  null chimeras (Figure 6A), suggesting a defect in B-1 cell migration to, or proliferation or survival in, the peritoneal cavity in the absence of  $\alpha 4$  integrins.

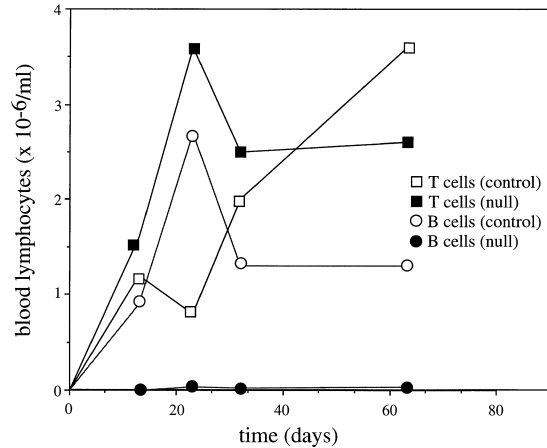
The bone marrow obtained from the different chimeric mice was also analyzed. A greatly reduced number of CD45R<sup>+</sup> cells was found in the bone marrow of  $\alpha 4$  null chimeric mice compared with controls (Figures 7A and 7D). Since the RAG-1<sup>-/-</sup> mice are on a mixed genetic



**Figure 3. Thymi from  $\alpha 4$  Null/RAG-1 or RAG-2 Chimeric Mice Become Progressively Atrophic**

Histological analysis of thymi from  $\alpha 4$  null/RAG-2 chimeric mice was performed at different timepoints (1 week in [A] and 2 months in [B]). Note that in  $\alpha 4$  null chimeras older than 2 months (B) the normal structure of the thymus (A) with cortical (c) and medullary (m) areas is not observed. Moreover, the thymi from those null chimeras are much smaller and atrophic. The appearance of thymi from  $\alpha 4$  control chimeras was similar to that shown in (A) at any timepoint analyzed (data not shown). Scale bar is 200  $\mu$ m.

background, we could not use the Ly-9.1 marker to distinguish whether those few B cells were immature pro-B cells normally present in the RAG-1-deficient mice or whether they derived from the  $\alpha 4^{-/-}$  ES cells. However, most of those CD45R<sup>+</sup> cells in the  $\alpha 4$  null/RAG-1 chimeras were positive for  $\alpha 4$  expression, demonstrating that they derived from the RAG-1 blastocyst. Moreover, a few  $\alpha 4$  null/RAG-2 chimeric mice analyzed by specific markers showed almost no  $\alpha 4$  null/CD45R<sup>+</sup> cells (data not shown). These results show that, in the absence of  $\alpha 4$  integrins, B cell development is blocked at an earlier stage than that observed in RAG-1- or RAG-2-deficient mice, i.e., before pro-B cell stage. To investigate this point further, we also stained this CD45R<sup>+</sup> population for markers differentially expressed along the B cell development pathway, including CD43 and surface IgM. In fact, most were immature pro-B cells (CD45R<sup>dull</sup>/CD43<sup>+</sup>) derived mainly from the RAG-1<sup>-/-</sup> blastocysts (Figures 7B and 7D). Pre-B cells (CD45R<sup>high</sup>/CD43<sup>-</sup>) and mature B cells (CD45R<sup>+</sup>/IgM<sup>+</sup>) were almost absent in  $\alpha 4$  null chimeras as compared with the control



**Figure 4. Bone Marrow from  $\alpha 4$  Null Chimeras Is Able to Reconstitute the Peripheral T Cell Compartment of Sublethally Irradiated Recipients**

Bone marrow cells from  $\alpha 4$  null/RAG-1 or control/RAG-1 chimeric mice were injected into irradiated RAG-1<sup>-/-</sup> recipients. The presence of reconstituted T and B cell lineages was determined at different timepoints by flow cytometry analysis of blood lymphocytes stained for CD3 and CD45R expression, respectively. Each point represents the mean of four mice transplanted. Standard deviation was 10%–15% for each point. Note that  $\alpha 4$  null bone marrow is able to reconstitute a normal number of T lymphocytes in the periphery, although it fails to reconstitute the B cell population (see text for further details about the B cell defect). Control bone marrow reconstitutes both populations. Thymi from  $\alpha 4$  null reconstituted mice were normal 1 month after injection, but became atrophic after 2 months (data not shown). PBS-injected mice did not show any T or B blood lymphocytes (data not shown).

mice (Figures 7B–7D), demonstrating a severe failure in *in vivo* B cell development in the absence of  $\alpha 4$  integrins. Furthermore, bone marrow from  $\alpha 4$  null chimeras failed to reconstitute the B cell compartment of irradiated recipients carrying wild-type bone marrow stroma (see Figure 4), confirming the *in vivo* inability of B cell precursors to develop properly in the bone marrow when they do not express  $\alpha 4$  integrins after birth. In contrast, preliminary data showing the ability of fetal liver from  $\alpha 4$  null chimeras to reconstitute a normal B cell population suggest that B cell development from fetal precursors can take place without  $\alpha 4$  integrins (data not shown).

Altogether, these data clearly demonstrate that the  $\alpha 4$  integrin adhesion receptors are essential for normal *in vivo* B lymphocyte differentiation after birth and that a severe defect at the B cell precursor level occurs in the adult bone marrow in the absence of  $\alpha 4$  integrin expression.

#### Monocytes and NK Cells Can Undergo Complete Development without Expressing $\alpha 4$ Integrins

The presence of monocytes derived from  $\alpha 4$  null precursors was assessed by testing for the double expression of the monocyte marker Mac-1 (CD11b/CD18) as well as the  $\alpha 4$  integrin chain in peripheral blood. A subset of Mac-1<sup>+</sup>/ $\alpha 4$ -negative cells was found in the peripheral blood of  $\alpha 4$  null chimeric mice, while no  $\alpha 4$ -negative cells within the monocyte Mac-1<sup>+</sup> population could be detected in the control animals (Figure 8). The double

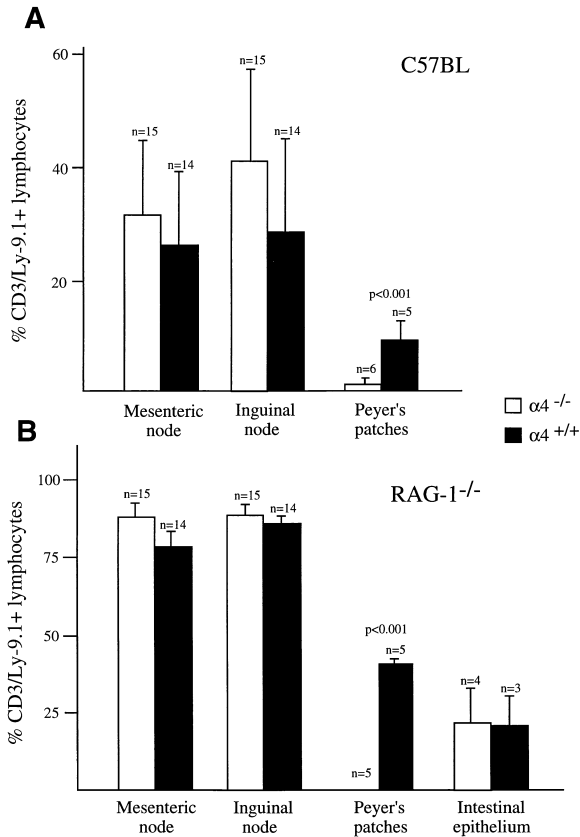


Figure 5.  $\alpha 4$  Null T Lymphocytes Are Unable to Migrate to Peyer's Patches

Lymphocytes from different lymphoid tissues and intestine of the different chimeras were stained for CD3 and Ly-9.1 expression and analyzed by flow cytometry. Mean and standard deviation are shown. Note that no  $\alpha 4$  null T cells are found in Peyer's patches. The differences in lymphocyte populations from mesenteric and inguinal nodes and intestine are not significant.

expression of CD44 (to stain monoblastic and monocytic cells) and  $\alpha 4$  was also analyzed in a monocytic population (gated by size and complexity) from the bone marrow of these animals. A proportion of CD44<sup>+</sup>/ $\alpha 4$ -negative cells was present in  $\alpha 4$  null chimeric mice, but no  $\alpha 4$ -negative cells were found within the monocytic CD44<sup>+</sup> population in control animals (Figure 8). There was no correlation between the percentage of  $\alpha 4$  null monocytes and the age of the mice, suggesting no defect in monocytic precursors lacking  $\alpha 4$  integrins during adult life.

NK cell development was also investigated. Specific markers for this lineage such as NK-1.1 are not expressed in NK cells from the 129Sv strain. Therefore, we used the CD8 $\alpha^+$ /CD3<sup>-</sup> phenotype in a population gated by size and complexity to identify NK cells in the blood of adult chimeric mice (>9 months old). A larger population of  $\alpha 4$ -negative NK cells was present in the  $\alpha 4$  null/C57BL chimeras compared with controls (21.6% in  $\alpha 4$  null versus 7.1% in  $\alpha 4$  control chimeras [n = 8]).

These data demonstrate that monocytes and NK cells do not require  $\alpha 4$  integrin receptors to become mature cells able to circulate in peripheral blood.

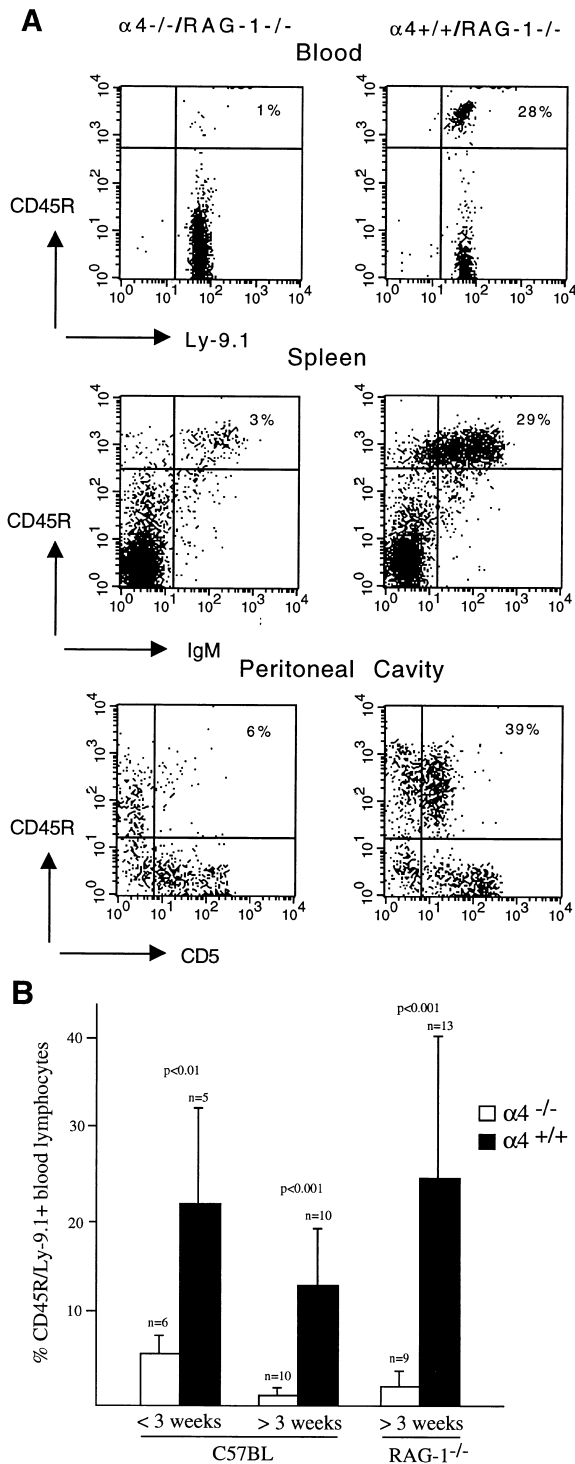
## Discussion

Here we have addressed several questions about the roles of  $\alpha 4$  integrin adhesion receptors during in vivo hematopoiesis. We find a lymphocyte-specific defect in the absence of  $\alpha 4$  integrins, since monocyte and NK cell development are not detectably affected. Furthermore, we show that  $\alpha 4$  integrins are not essential in the migration and cellular interactions that occur during embryonic lymphocyte development, but, interestingly, we detect a developmental switch in the requirements for  $\alpha 4$  integrins, since after birth no further T or B cell development occurs in the bone marrow in the absence of  $\alpha 4$  integrins. Moreover,  $\alpha 4$  integrins are completely required for lymphocyte homing to Peyer's patches (Figure 9).

### T Cell Development

T cell precursors originate in the yolk sac and fetal liver and migrate into the thymus to differentiate into single-positive CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes. After birth, the bone marrow constitutes the only organ where the T cell precursors proliferate to supply the thymus with a constant input of progenitors for normal maintenance of the T cell population (Jotereau et al., 1987; Scollay et al., 1986). In  $\alpha 4$  null/RAG-1 chimeras where no  $\alpha 4^{+/+}$  lymphocytes are present, phenotypically and functionally normal T lymphocytes can develop without any  $\alpha 4$  integrin receptors (Figure 1). It is possible that subtle functional defects could occur in these  $\alpha 4$  null T cells, since they are developed from embryonic precursors and a more restricted TCR repertoire with fewer N-nucleotide insertions would be expected (Lafaille et al., 1989). Thus,  $\alpha 4$  integrins are not essential for T cell precursors to migrate to the thymus or for T cell precursors to interact with the thymic epithelium or ECM to become mature T lymphocytes. While  $\alpha 6\beta 1$  plays a role in pro-T cell homing to the thymus (Ruiz et al., 1995), and E-cadherin and likely CD81(TAPA-1) function in thymocyte-thymic epithelium interactions (Boismenu et al., 1996; Lee et al., 1994),  $\alpha 4$  integrins do not appear to play an essential role in any of these processes.

However, it is important to note the switch in the requirements for  $\alpha 4$  integrins during T cell development. While embryonic precursor interactions with the micro-environment do not require these receptors, after birth  $\alpha 4$  integrins become essential for further T cell development, since no  $\alpha 4$  null thymocytes were detected in the thymus of chimeric mice older than 3 weeks (Figures 2 and 3). These data suggest several different points at which T cell precursor development could be blocked after birth: migration of precursors from the fetal liver to the bone marrow, defective differentiation in the bone marrow, exit from the bone marrow, or migration to or differentiation of the precursors in the thymus. To determine which of these steps is  $\alpha 4$  integrin dependent, we performed reconstitution experiments. A short-term reconstitution of the T cell population was obtained when bone marrow from  $\alpha 4$  null chimeras was injected into the blood of irradiated or nonirradiated recipients (Figure 4; data not shown). These data rule out a failure of precursors to migrate to or differentiate in the thymus,



**Figure 6. B Cell Development Is Severely Compromised in  $\alpha 4$  Null Chimeric Mice**

(A) Lymphocytes from blood, spleen, and peritoneal cavity from  $\alpha 4$  null/RAG-1 or control/RAG-1 chimeric mice were analyzed for the expression of different B cell markers and Ly-9.1. Note that very few conventional B cells and B-1 cells are present in blood and spleen and in the peritoneal cavity, respectively, of  $\alpha 4$  null chimeras as compared with controls.

(B) C57BL or RAG-1 chimeric mice (>30% of coat color chimerism) were analyzed. Lymphocytes from blood were stained and analyzed

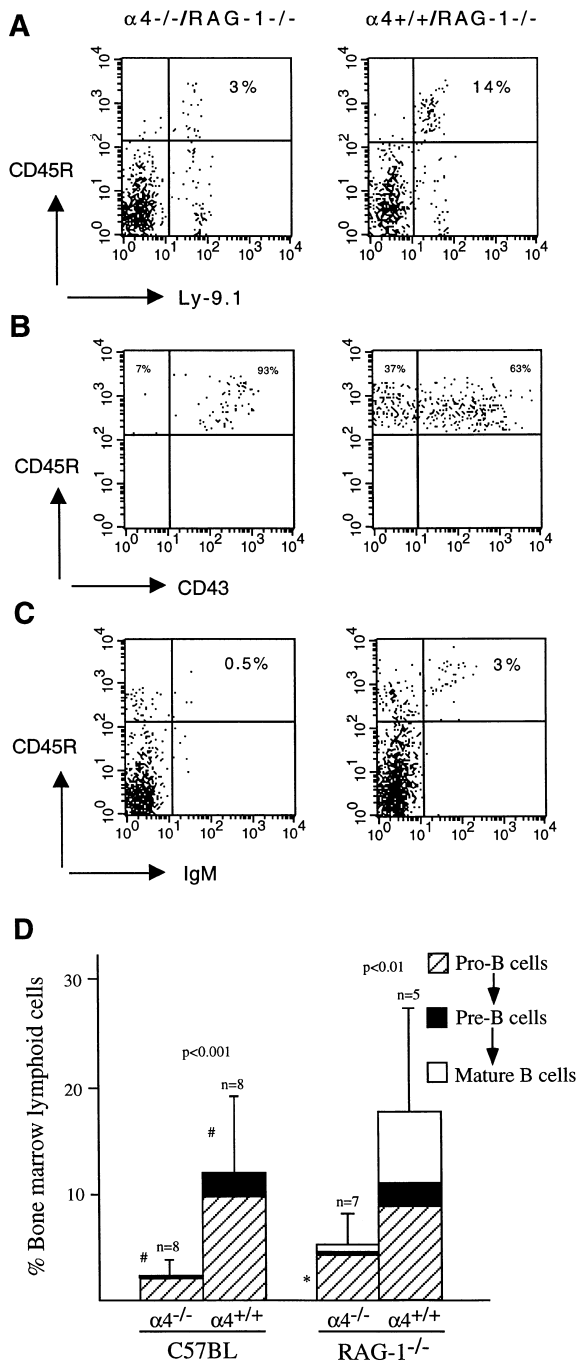
as well as a defect in migration of precursors from the fetal liver to the bone marrow, since T cell precursor activity is present in the bone marrow from  $\alpha 4$  null chimeric mice. We have not yet elucidated the exact nature of the defect, but it seems to be at the bone marrow level. A defect in the exit per se of T cell progenitors from the bone marrow in the absence of  $\alpha 4$  integrins could be possible; however, in this case an accumulation of precursors would be expected but is not observed. It is more likely that the  $\alpha 4$  null T cell progenitors cannot migrate from the bone marrow either because they do not survive long enough or because they fail to reach the maturation state required to exit the bone marrow. Altogether, the data point to a defect in the specific interactions between progenitors for T cells and the bone marrow in the absence of  $\alpha 4$  integrins, as will be discussed later.

**B Cell Development**

It was previously reported that there is a stroma-dependent phase in the first stages of B cell development in the bone marrow and in the fetal liver at the embryonic stage (Gisler et al., 1987; Hardy et al., 1991; Strasser et al., 1989). Although some in vitro studies suggested a role for  $\alpha 4$  integrins during B lymphopoiesis (Miyake et al., 1991a), little was known about the in vivo functions of these receptors in this process. In this report, we show that  $\alpha 4$  integrins are essential for complete maturation of B cells in vivo, since very few B cells are generated and development is largely blocked before the pro-B cell stage in the absence of  $\alpha 4$  integrins (Figures 6 and 7). These receptors may mediate key interactions between the progenitors and the bone marrow stromal cells or the ECM (or both). These interactions might be important only in the bone marrow during adult life, as the few  $\alpha 4$  null B cells detectable could have been developed during embryonic life from fetal liver precursors. This idea is supported by the higher number of B cells found in  $\alpha 4$  null chimeras younger than 3 weeks and the presence of B cell reconstitution in contrast with the absence of such reconstitution in fetal liver versus bone marrow transplant experiments (Figure 4; data not shown).

Why are these  $\alpha 4$ -mediated interactions essential for B cell development in the bone marrow? There are several possibilities. First, it is possible that the progenitors require an active signal for survival and that this signal could be provided by  $\alpha 4$  integrin-mediated interactions with the stromal cells or the ECM. Integrin-mediated interactions seem to be essential for cell survival (Boudreau et al., 1995; Ruoslahti and Reed, 1994). In this regard, a role for  $\alpha 4$  integrin-VCAM-1 interactions in the survival of activated B cells in the germinal centers has been reported (Koopman et al., 1994), and it has also

by flow cytometry. The mean and standard deviation are shown. Greatly reduced numbers of CD45R<sup>+</sup> B lymphocytes were observed in the  $\alpha 4$  null chimeras. Similar percentages of IgM<sup>+</sup> cells were found within the B cell population of the different chimeras, and similar results were obtained when lymphocytes from spleen were analyzed (data not shown). The number of cells in the spleen was on average  $67 \times 10^6$  in null/RAG-1 versus  $122 \times 10^6$  in control/RAG-1 chimeric mice.



**Figure 7. B Cell Precursors Fail to Mature in the Bone Marrow when They Lack  $\alpha 4$  Integrins**

(A–C) Bone marrow from  $\alpha 4$  null/RAG-1 or control/RAG-1 chimeric mice was obtained and analyzed for the expression of markers differentially expressed during B cell development. Note that very few CD45R<sup>+</sup> cells are Ly-9.1<sup>+</sup> in the  $\alpha 4$  null chimeras, and these are mostly pro-B cells (CD45R<sup>dim</sup>/CD43<sup>+</sup>) derived from the RAG-1<sup>-/-</sup> blastocyst. No mature B cells (CD45R<sup>+</sup>/IgM<sup>+</sup>) are present in the  $\alpha 4$  null chimeras. In (A) and (C), total bone marrow populations are analyzed. In (B), gated populations positive for expression of Ly-9.1 and CD45R are analyzed.

(D) Cells from bone marrow of chimeric mice were obtained, stained for different markers, and analyzed by flow cytometry. The total number of bone marrow cells from two femora was on average  $84 \times 10^6$  in  $\alpha 4$  null/RAG-1 versus  $69 \times 10^6$  in control/RAG-1 chimeric

been shown that bone marrow fibroblasts can rescue plasma cells from apoptosis (Merville et al., 1996). This interesting possibility deserves further investigation. A second possibility is that  $\alpha 4$  integrin–ligand interactions can trigger an active signal for synthesis of cytokines necessary in B cell development such as interleukin-7 (IL-7). It is known that progenitor–stroma interactions can induce the secretion of soluble growth factors (Sudo et al., 1989), although a recent report has shown that the stimulation of tyrosine kinases and IL-6 production on human bone marrow stromal cells induced by contact with B lymphocytes seems to be  $\alpha 4$  independent (Jarvis and LeBien, 1995). A third possibility is that  $\alpha 4$ -mediated interactions are necessary to trigger directly intracellular signals essential for B cell development. In fact, mice deficient for several genes involved in signal transduction, such as Syk and Jak3 tyrosine kinase genes, display a similar phenotype, with B cell development greatly impaired (Cheng et al., 1995; Nosaka et al., 1995; Russell et al., 1995; Thomis et al., 1995; Turner et al., 1995).

Finally, the B-1 cell lineage defect observed in the absence of  $\alpha 4$  integrins could be due to  $\alpha 4$ -mediated interactions playing a role in the migration of these cells into, or their survival or proliferation within, the peritoneal cavity. In fact, a role for peritoneal stromal cells in the survival of surface IgM<sup>+</sup> peritoneal B cells has been reported (Hardin et al., 1995). Another possibility is that the bone marrow may play a role in the maintenance of the B-1 cell population in the peritoneum, and since there is a defect in early B cell precursor development, the B-1 lineage would also be affected.

#### Bone Marrow Lymphopoiesis

Altogether, the data reported here concerning T and B cell development suggest that there may be a similar or common defect in both lineages and a switch in the dependence on  $\alpha 4$  integrins during lymphopoiesis. Thus, lymphopoiesis seems to be independent of  $\alpha 4$  integrins during embryonic life, but starts to be dependent on  $\alpha 4$  integrins when progenitors move to the bone marrow after birth. It is possible that, in the absence of  $\alpha 4$  integrins, fewer progenitor cells migrate to the bone marrow, as a recent report has suggested (Papayannopoulou et al., 1995). However, even a decreased number of progenitors should be able to reconstitute a normal lymphoid population, if the cells proliferate properly. This switch in the requirements for  $\alpha 4$  integrins indicates that there is a strict regulation in time or space (or both) of  $\alpha 4$ -mediated functions during lymphocyte development. This switch could occur within a single lineage of

mice. The percentages, with mean and standard deviation, of the different B cell populations are shown. Note that only pro-B cells (CD45R<sup>dim</sup>/CD43<sup>+</sup>) and almost no pre-B (CD45R<sup>high</sup>/CD43<sup>-</sup>) or mature B cells (CD45R<sup>+</sup>/IgM<sup>+</sup>) are detected in the  $\alpha 4$  null chimeras. Number signs indicate that the percentage of mature B cells in C57BL chimeras was not determined. The asterisk indicates that most of these pro-B cells were derived from the RAG-1<sup>-/-</sup> blastocysts, since they are positive for  $\alpha 4$  expression. This means that the real number of CD45R<sup>+</sup> cells derived from the  $\alpha 4$  null ES cells is even lower. In fact, a few  $\alpha 4$  null/RAG-2 chimeras analyzed with specific markers showed almost no  $\alpha 4$  null/CD45R<sup>+</sup> cells.

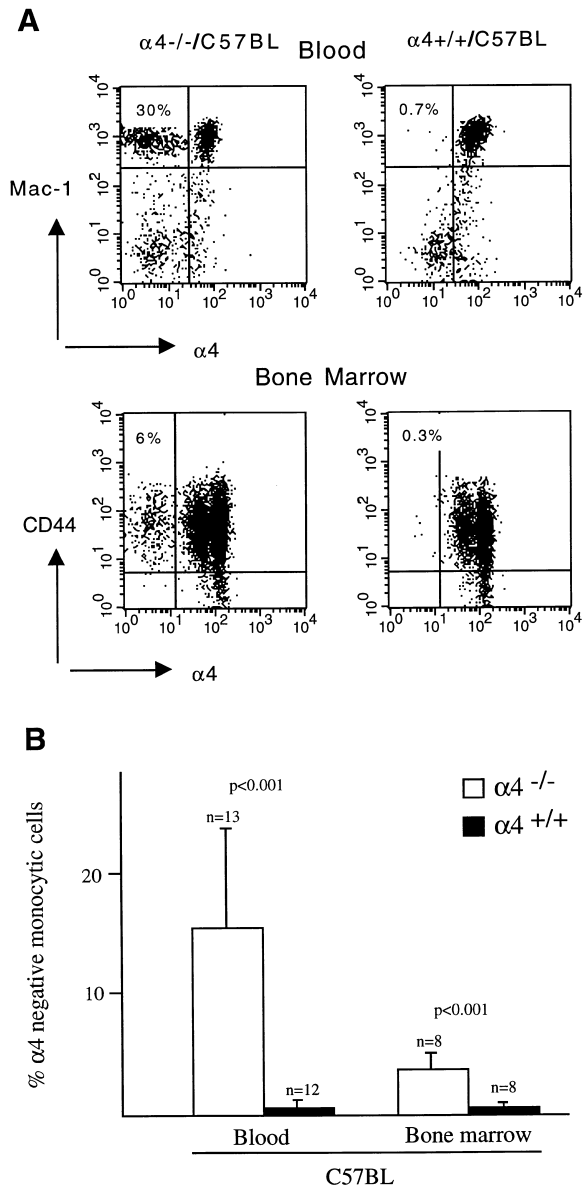


Figure 8. Monocytes Develop Normally without Expressing  $\alpha 4$  Integrins

(A) Monocytes from blood and monocyte precursors from bone marrow were analyzed for the expression of specific markers and  $\alpha 4$  integrins. Note that a subset of the monocytic population in both blood and bone marrow is negative for  $\alpha 4$  expression in  $\alpha 4$  null chimeras, in contrast with the absence of  $\alpha 4$ -negative cells in control animals.

(B) Cells from blood and bone marrow of chimeric mice were stained and analyzed for  $\alpha 4$  integrins as well as Mac-1 or CD44 expression. Mean and standard deviation are shown. Note that a population of  $\alpha 4$ -negative monocytes is present in  $\alpha 4$  null chimeras but absent in controls.

precursors as they develop or between separate fetal and adult lineages. It might be possible that  $\alpha 4$  integrins are not expressed by fetal hematopoietic precursors, although expression of these receptors in the yolk sac and fetal liver of murine embryos has been shown (Shepard et al., 1994), or that other receptors play the main

role in attachment of lymphoid precursors to the stroma during embryonic development.

The consequences of such a common defect in T and B cell precursor functions in the adult in the absence of  $\alpha 4$  integrins would be very different because of the different kinetics that govern T and B cell development. Since T cell development takes place in the thymus mainly during fetal life and, as shown in this report,  $\alpha 4$  seems not to play an essential role in hematopoietic fetal migration and differentiation pathways, it is possible to find relatively normal percentages of  $\alpha 4$  null T cells in the adult mice analyzed. However, because few B cells develop before birth and the large expansion of B cell development occurs normally in the first few months after birth, we detect in this case a severe defect in the B cell population lacking  $\alpha 4$  integrins. The defect might be occurring at a point between the pluripotent stem cell and the stage of a common precursor for T and B cells in the bone marrow, since only the lymphoid lineages (T and B) and neither monocytes nor NK cells are affected in the adult chimeric mice. Another possibility is that committed T and B cell progenitors have the same  $\alpha 4$  integrin requirements and differ in this respect from NK cell and myeloid precursors. Whichever is the case, it is clear that  $\alpha 4$  integrin-mediated interactions between early T and B cell progenitors and the stromal environment in the bone marrow are regulating key events required for normal development of such precursors. If such a critical point is skipped in embryonic development, for instance because other adhesion receptors are involved or the microenvironment or the requirements for signaling are different, then the precursors differentiate normally. In fact, it is well known that there are many switches concerning differentially regulated gene expression and the ability of precursors to differentiate, for example, during the development of the immune system in embryonic versus adult life (Li et al., 1993; for review see Weissman, 1994).

As pointed out before,  $\alpha 4$ -mediated interactions might be regulating the proliferation, survival, differentiation, signal transduction pathways, or synthesis of soluble growth factors required for normal development of lymphoid precursors. Interestingly, mice deficient for genes involved in regulation of cell death and cell growth (*bcl-2* and *c-abl*, respectively) show normal development of the immune system at birth, but defects start to appear after a few weeks, pointing to different regulation and requirements for these genes during embryonic and adult life, as is the case for  $\alpha 4$ -mediated interactions (Nakayama et al., 1993; Schwartzberg et al., 1991; Tybulewicz et al., 1991; Veis et al., 1993). Mice deficient for another gene involved in inhibition of apoptosis, *bcl-x*, also show a defect in the immature hematopoietic precursors (Motoyama et al., 1995). It will be interesting to investigate further the lymphoid-specific signals likely mediated by  $\alpha 4$  integrin interactions.

In summary, it was already known that bone marrow stromal cells support normal development of hematopoietic precursors (for review see Dorshkind, 1990; Kincaid et al., 1989). In this report, we show that  $\alpha 4$  integrins are key receptors mediating those interactions in vivo, since lymphoid development is completely blocked in the bone marrow at a very early stage in the absence



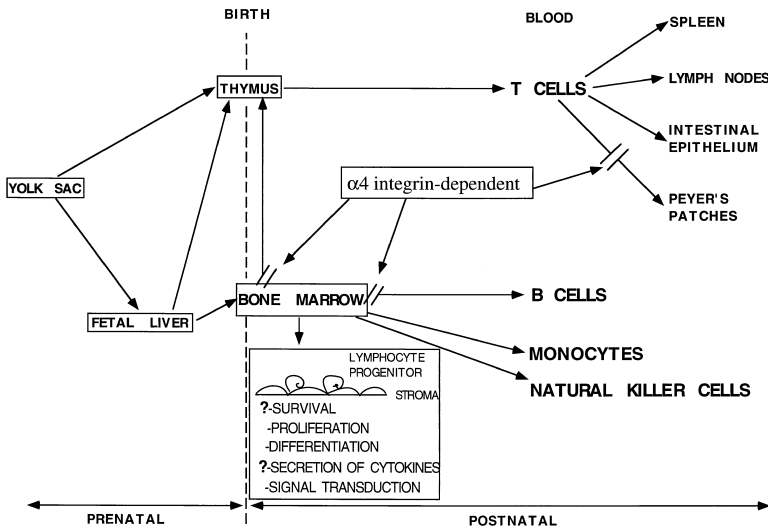


Figure 9.  $\alpha 4$  Integrins Play Essential Roles during In Vivo Lymphoid Development and Traffic

Normal pathways of development and traffic of different leukocyte lineages before and after birth are shown. The analysis of  $\alpha 4$  null chimeric mice has revealed important steps in which  $\alpha 4$  integrins play essential roles.  $\alpha 4$  integrins do not appear to play essential roles during embryonic lymphoid development (neither in migration pathways from the yolk sac to the fetal liver and to the thymus or bone marrow nor in the interactions of the progenitors with the microenvironment in these hematopoietic organs). After birth,  $\alpha 4$  integrins become essential for interactions between T and B cell progenitors and the bone marrow stromal environment producing a complete blockade in B cell development and a cessation of the T cell development that occurred normally in the thymus during embryonic life. In the box are shown several different processes that these interactions

might be regulating.  $\alpha 4$  integrins are also essential for migration of T cells to Peyer's patches, but not to other lymphoid organs. In contrast, NK cells and monocytes lacking  $\alpha 4$  integrins can develop normally without expressing  $\alpha 4$  integrins. The number of B-1 cells is also greatly diminished in the absence of  $\alpha 4$  integrins, although it is not yet clear at which step  $\alpha 4$  integrins are required.

of  $\alpha 4$  integrins. Regarding the specific integrins and counterreceptors likely involved in these essential  $\alpha 4$  integrin-mediated interactions, it is worth mentioning that  $\beta 7$  knockout mice, recently obtained, do not show any defects in T or B cell development (Wagner et al., submitted), pointing to the recognition of fibronectin or VCAM-1 (or both) by the  $\alpha 4\beta 1$  heterodimer as being responsible for the defect observed at the bone marrow level in our  $\alpha 4$  null chimeras.

#### Monocyte and NK Cell Development

The hematopoietic defect observed in the absence of  $\alpha 4$  integrins seems to be lymphocyte specific, since no defect in monocyte or NK cell development could be detected when the precursors do not express  $\alpha 4$  integrins (Figure 8). These data are somewhat in contrast with a report in which anti- $\alpha 4$  monoclonal antibodies were able to retard myelopoiesis in vitro (Miyake et al., 1991a). Nevertheless, our data indicate that monocytes do not need to express  $\alpha 4$  integrins to develop normally, although a delay in their development would be difficult to detect. A role for a soluble factor secreted as a consequence of  $\alpha 4$  integrin-mediated interactions of the wild-type monocytes present in the chimeric mice also cannot be ruled out at this point. The different requirements for  $\alpha 4$  integrins between NK and T or B cell progenitors suggest that these lineages are in fact different and they separate early during ontogeny.

#### T Cell Homing

After maturation in the thymus, T lymphocytes exit to the peripheral blood and lymph where they recirculate and, by recognizing specific addressins, migrate or localize in the different secondary lymphoid organs (for review see Springer, 1994). A role for the  $\alpha 4$  integrin subunit associated with the  $\beta 7$  chain and recognizing the ligand MAdCAM-1 in T lymphocyte migration to the mucosal-associated lymphoid tissue (Peyer's patches)

and intestine has previously been reported, although other receptors such as L-selectin have also been suggested to play a role (Hamann et al., 1994). In the migration of T lymphocytes to peripheral nodes, other adhesion receptors have been implicated, including L-selectin and CD44. Here we show that in vivo  $\alpha 4$  integrins are absolutely required for the migration of T lymphocytes to Peyer's patches (Figure 5), regardless of the presence of other adhesion receptors such as L-selectin. However, no decrease in migration of gut intraepithelial lymphocytes was detected in the absence of  $\alpha 4$ , suggesting that other receptors play the major role in that process. Interestingly,  $\beta 7$  knockout mice (Wagner et al., submitted) show a decreased but not zero number of lymphocytes in the Peyer's patches and intestine. Together with our data, this suggests that the  $\alpha 4\beta 1$  heterodimer may also play a minor role in homing to Peyer's patches and that the integrin  $\alpha E\beta 7$  plays the main role in migration to the intestinal epithelium.

In summary, this report provides insights into the in vivo roles of the two  $\alpha 4$  integrin adhesion receptors during murine lymphopoiesis, monocyte development, and lymphocyte homing (Figure 9). Interestingly,  $\alpha 4$ -mediated events involved in lymphopoiesis are finely regulated in time or space (or both), since there is a switch in their requirements from prenatal to postnatal life. The data presented here reveal the importance of cellular adhesion events in regulating essential processes in lymphocyte differentiation and traffic.

#### Experimental Procedures

##### Chimeric Mice

Double knockout (i.e., homozygous null)  $\alpha 4$  ES cells were obtained from heterozygous ES cell clones (Yang et al., 1995) by selection in high concentrations (1–1.5 mg/ml) of G418 (Mortensen et al., 1992). Chimeric mice were generated by injection of ES cells into blastocysts as previously described (George and Hynes, 1994). Other details of the generation and characterization of these  $\alpha 4^{-/-}$  ES cells

and chimeric mice will be reported elsewhere (J. T. Y. et al., unpublished data). Two independent  $\alpha 4$  null clones (4D47 and 4D20), wild-type (clone D3), or heterozygous  $\alpha 4^{+/-}$  (clone 182) ES cells were plated on feeders 2 days before the injection in DME medium plus 20% FCS,  $1 \times$  nonessential amino acids (GIBCO BRL), 0.1 mM  $\beta$ -mercaptoethanol, L-glutamine, and LIF (ESGRO from GIBCO BRL). The ES cells are derived from the 129Sv strain (agouti coat color). Blastocysts were isolated at 3.5 days postcoitum (dpc) from hormone-stimulated females (either C57BL strain or RAG-1- or RAG-2-deficient mice on mixed C57BL  $\times$  129Sv background, but with black coat color [a/a]). Approximately 15 ES cells ( $\alpha 4$  null or wild type) were injected into each blastocyst, and the blastocysts were transferred into the uteri of pseudopregnant recipients 2.5 dpc. When the litters were born, the chimerism was estimated by coat color. In this report, we show the results obtained injecting D3 wild-type ES cells as controls, but similar data were obtained when heterozygous  $\alpha 4^{+/-}$  ES cells were used. Mice were kept in the Massachusetts Institute of Technology animal facility under standard conditions. RAG-1- and RAG-2-deficient mice were provided by Drs. J. Lafaille and S. Tonegawa and by J. Chen, respectively.

#### Flow Cytometry Analysis

The following monoclonal antibodies from Pharmingen were used for flow cytometry staining: PE-conjugated anti-mouse CD3 $\epsilon$  (145-2C11), PE-conjugated anti-mouse CD45R/B220 (RA3-6B2), FITC-conjugated anti-mouse Ly-9.1 (Lgp100, 30C7), PE-conjugated anti-mouse CD4 (L3T4, RM4-5), CyC-conjugated anti-mouse CD8 $\alpha$  (Ly-2, 53-6.7), FITC-conjugated anti-mouse CD5 (Ly-1, 53-7.3), biotinylated anti-mouse CD43 (leukosialin, S7), and CyC-conjugated streptavidin, PE-conjugated anti-mouse CD44 (Pgp-1, Ly-24 or H-CAM, IM7), and FITC- and PE-conjugated anti-mouse CD49d (integrin  $\alpha 4$  chain, R1-2). PE-conjugated anti-mouse Mac1 (CD11b/CD18, Ly-40, M1/70) from Boehringer Mannheim was also used. Whole blood or single cell suspensions from different lymphoid organs obtained by routine techniques were incubated with purified anti-mouse CD32/CD16 (Pharmingen) to block binding via Fc receptors and with an appropriate dilution of the different antibodies at room temperature or 4°C, respectively. Red blood cells were lysed using lysis solution (Becton Dickinson). The samples were washed twice with PBS and resuspended in PBS. Dead cells in lymphoid organ samples were excluded by propidium iodide staining. The samples and the data were analyzed in a FACScan using CellQuest software (Becton Dickinson).

#### PCR Analysis

Lymphocytes from the spleens of  $\alpha 4$  null/C57BL chimeras were sorted in a FACStar Plus (Becton Dickinson) cell sorter for the expression of the  $\alpha 4$  chain and Ly-9.1 markers. Cells were lysed in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20, and 2 mg/ml proteinase K. DNA was subjected to PCR using  $\alpha 4$ -specific primers as well as primers for the *neo<sup>r</sup>* gene (Yang et al., 1995). Reactions were run as described by the manufacturer of Taq polymerase (Perkin-Elmer Cetus) under the following conditions: 72°C for 10 min and then 40 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 4 min.

#### Histological Analysis

Tissue specimens were 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) with an Axiophot microscope (Carl Zeiss).

#### Reconstitution of Irradiated Mice

RAG-1-deficient mice (6–12 weeks old) were used as recipients for bone marrow transplantation experiments. Those mice were nonirradiated or sublethally irradiated (400 rads of  $\gamma$ -rays) 4 hr before the injection. Cells from bone marrow of  $\alpha 4$  null or  $\alpha 4$  control/RAG-1 chimeras were obtained and resuspended in sterile PBS. Approximately  $10^7$  cells were injected intravenously (retro-orbitally) in each irradiated recipient. The presence of T and B lymphocyte lineages in

the blood and other organs was determined by staining for specific T or B cell markers by flow cytometry analysis at different timepoints.

#### Statistical Analysis

Data from the  $\alpha 4$  null and control chimeric mice were analyzed and compared for statistically significant differences using the Student's *t* test.

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