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Stromal Cells Provide the Matrix for Migration of Early Lymphoid Progenitors Through the Thymic Cortex[1]

Susan E. Prockop,* Sharina Palencia,* Christina M. Ryan,[2]* Kristie Gordon,* Daniel Gray,[‡] and Howard T. Petrie[3]*[†]

During steady state lymphopoiesis in the postnatal thymus, migration of precursors outward from the deep cortex toward the capsule is required for normal differentiation. Such migration requires, at a minimum, expression of adhesive receptors on the migrating lymphoid cells, as well as a stable matrix of their ligands persisting throughout the region of migration. In this study, we address the nature of this adhesive matrix. Although some precursor stages bound efficiently to extracellular matrix ligands, a specific requirement for the cell surface ligand VCAM-1 was also found. In situ analysis revealed that early precursors are found in intimate contact with a matrix formed by stromal cells in the cortex, a proportion of which expresses VCAM-1. In vivo administration of an anti-VCAM-1 Ab resulted in decreased thymic size and altered distribution of early precursors within the cortex. These results indicate that precursors migrating outward through the cortex may use a cellular, rather than extracellular, matrix for adhesion, and suggest that the VCAM-1$^+$ subset of cortical stroma may play a crucial role in supporting the migration of early precursors in the steady state thymus. *The Journal of Immunology,* **2002, 169: 4354–4361.**

Like other cells of hemopoietic origin, T lymphocytes must be produced throughout life to replace cells lost to senescence, trauma, and other causes. This process occurs in the thymus and involves several distinct phases. The initial phase involves recruitment into the thymus of marrow-derived progenitors that circulate in the bloodstream (reviewed in Ref. 1). It is possible that some of these thymic seeding progenitors are already committed to the lymphoid lineages, because there is a population of cells in bone marrow that has increased propensity to generate lymphoid cells (2). However, it is clear from a number of studies that the earliest precursors found within the thymus can give rise to numerous lymphoid and nonlymphoid lineages (for examples, see Refs. 3–10), suggesting that not all cells that home to the thymus are lymphoid committed (reviewed in Ref. 11). During a period of intrathymic residence that spans ~14 days (reviewed in Ref. 12), these multilineage progenitors are induced to undergo a series of differentiative events that lead to T lineage commitment (13). In addition, a great deal of proliferative expansion occurs, such that each progenitor entering the thymus gives rise to approximately one million immature progeny (12), thus generating the cells that are subsequently subjected to TCR-mediated screening.

In general, lineage commitment and proliferation are not cell autonomous processes, but rather occur in response to combinations of signals (morphogens, growth factors, hormones, etc.) that progenitor cells receive from the external microenvironment. The nature of signals that drive lineage commitment and proliferation in early intrathymic progenitors remains largely unknown. A few such signals have been identified in the form of Notch-1 (10, 14, 15), c-*kit* (16, 17), and the IL-7R (18), although the exact functions (i.e., differentiation, proliferation, survival) in some cases remain controversial. Regardless, it seems unlikely that this relatively small number of signals is adequate to explain the complex sequence of events that lead to production of a large number of T lineage progeny from a relatively few undifferentiated progenitors. To evaluate what other signals might be involved, we began from the assumption that early precursors receive specific signals from their external microenvironment that lead to T lineage commitment and/or proliferative expansion. This led us to define a stratified pattern of precursor distribution in the thymic cortex (19), wherein the earliest progenitors (CD4/8 double-negative stage 1, DN1)[4] are in the deep cortex, the next stage (DN2) in the midcortex, and the last stage (DN3) predominates in the outer third of the cortex. Transition to the early CD4/8 double-positive (pre-DP) stage, together with the most obvious wave of proliferation (20, 21), correlates with localization to the subcapsular zone (22). This is followed by a reversal in the polarity of migration, with movement of expanded DP progeny progressively deeper into the cortex toward the medulla (23).

There are two distinct categories of functions that are implicated by the behaviors described above. The first is the stratified distribution of signals that induce sequential stages of lineage commitment and proliferation in the thymic cortex. The second, and the topic of the present study, is a mechanism for moving cells between them. Directional migration of cells within tissues indicates the presence of a number of biochemical requirements. Among these are adhesion receptors on the migrating cells, as well as a stable matrix of their ligands or counterreceptors to provide the traction for cell movement. In this study, we characterize the presence of adhesion receptors on lymphoid cells traveling outward through the cortex, as well as their ability to adhere to matrix

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[4] Abbreviations used in this paper: DN, double negative; DAPI, 4′,6′-diamidino-2-phenylindole; DP, double positive; ECM, extracellular matrix; FN, fibronectin; LN, laminin.

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ligands predicted by these receptors. Our data suggest that migrating lymphoid progenitors use a cellular, rather than extracellular, matrix for adhesion and migration. Combined functional and in situ analysis suggests that this cellular matrix consists of a subset of cytokeratin$^+$ cortical stromal cells that express the α_4 integrin ligand VCAM-1. Together, our findings demonstrate a close association of early lymphoid progenitors in the thymus with the stromal elements on which their differentiation depends. Furthermore, in addition to revealing unreported heterogeneity among cytokeratin$^+$ stromal cells of the thymic cortex, these studies suggest that early lymphoid progenitors and other, more mature lymphoid cells that occupy the same cortical space may nonetheless interact with very distinct stromal cell types.

Materials and Methods

Cells and Abs

Precursor thymocytes were prepared by sorting of lineage-negative thymocytes isolated from 4- to 8-wk-old C57BL/6 mice, as previously described (21). Thymic stromal cells were isolated as previously described (24). The cloned thymic stromal cell line 100-4 (25) was grown in DMEM supplemented with 10% FBS, 2 mM glutamine, and 5×10^{-5} M 2-ME. Purified anti-integrin Abs were clone 9C10 (anti-α_4), clone 5H10-27 (anti-α_5), clone GoH3 (anti-α_6), clone 9EG7 (anti-β_1), clone 346-11A (anti-β_4), and clone M293 (anti-β_7), all from BD PharMingen (San Diego, CA). Secondary Ab used for integrin detection was biotinylated goat F(ab′)$_2$ anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), followed by PE-streptavidin (Molecular Probes, Eugene, OR). Anti-cytokeratin Ab was clone C-11 conjugated to FITC (Sigma-Aldrich, St. Louis, MO), recognizing cytokeratins 4, 5, 6, 8, 10, 13, and 18. Abs used to define cortical stromal cells by flow cytometry were M 5/114.15.2 and 6C3, as previously described (26). Anti-VCAM-1 Ab used for in situ analysis was clone 429 conjugated to biotin (BD PharMingen). Anti-VCAM-1 Ab used for in vitro blocking was clone M/K2.7. Abs used to identify precursor thymocytes in situ were clones PC-61 (anti-CD25) or ACK-2 (CD117) coupled to biotin. Detection of VCAM-1 or CD117 by immunohistochemistry or immunofluorescent microscopy was performed using tyramide signal amplification (NEN, Boston, MA).

Assay for adhesion to purified extracellular matrix (ECM) ligands

Murine fibronectin (FN) and laminin 1 (LN1) were purchased from Life Technologies (Carlsbad, CA). Ninety-six-well trays (Nunc 473768; Nalge Nunc International, Rochester, NY) were coated with ECM proteins by overnight incubation at 4°C with 1 (FN) or 5 μg (LN1) purified protein in 100 μl PBS. These concentrations were determined by measurement of optimal binding efficiency using unsorted DN thymocytes (i.e., lineage-depleted thymocytes). Blocking of excess protein binding was performed by incubating wells in a solution of heat-inactivated BSA (1% in PBS) for 1 h at room temperature. Purified DN precursors were added in PBS/BSA and allowed to settle for 30 min at 4°C. In any given experiment, the wells contained identical numbers of cells for each developmental stage, although the absolute number of cells varied between experiments, ranging from $4–5 \times 10^5$ cells/well. Plates were then incubated at 37°C for 30 min, followed by repeated washes in PBS/BSA. After a final wash in PBS, cells were fixed using 4% formaldehyde for 30 min at room temperature, followed by washing in PBS, treatment with 20% methanol in water (10 min at room temperature), and staining with 2% crystal violet. A single field at the center of each well was photographed, and the digital image was analyzed using the colony count function of Quantity One software (Bio-Rad, Hercules, CA). For an estimation of total cells in the well before washing, a phase-contrast image of the center of the well was used.

VCAM-1 adhesion assay

The 100-4 cells were grown in eight-well glass slide chambers (Lab-Tek; Nalge Nunc International), as described above. Purified T cell precursors ($4–5 \times 10^5$/well) were added in DMEM supplemented as described above, followed by incubation at 37°C for 1 h. The glass slide was removed from the chambers and washed by stirring in a beaker containing PBS. Fixation was then performed as described above, followed by staining in Harris' Modified Hematoxylin (Fisher Scientific, Pittsburgh, PA) and mounting. For blocking experiments, anti-VCAM-1 Ab (5 μg/ml) was added to the adherent monolayers 30 min before addition of lymphoid cells, and left in the medium during subsequent incubation.

Flow cytometry and microscopy

Thymic sections (4 μM) were prepared by cryosectioning after embedding in OCT. Immunohistochemical detection was performed using the VectaStain ABC kit (Vector Laboratories, Burlingame, CA). Staining for immunofluorescent microscopy was performed as previously described (19). Tissue counterstains were hematoxylin (immunohistochemistry) or 4′,6′-diamidino-2-phenylindole (DAPI; immunofluorescent microscopy). Digital microscopy was performed using an Olympus (Melville, NY) BX-50 microscope equipped with a mercury light source. Flow cytometric analysis was performed using a three-laser LSR cytometer (BD Biosciences, San Jose, CA).

Anti-VCAM-1 administration in vivo

Animals were injected daily for 5 days with 100 μg/day mAb recognizing VCAM-1 (clone MK-2.7) or control (nonspecific) Ab. Following this, animals were euthanized and the thymus was removed carefully. One lobe was frozen immediately for immunohistochemical and/or immunofluorescent analysis, while a single cell suspension was prepared from the other lobe. The latter was used for determination of total cellularity (by hemacytometer counting) as well as for phenotypic analysis of developmental stage by flow cytometry.

Results

Use of integrin expression profiles on intrathymic progenitors to predict adhesive substrates for cell migration

Our previous work has shown that early intrathymic precursors migrate outward through the cortex before differentiating into CD4$^+$8$^+$ cells (19). In an effort to understand the mechanisms of transcortical migration, we sought to analyze the expression of integrins by defined stages of intrathymic differentiation. Expression of integrins is implicit in cell migration through tissues, and although the mere presence of an integrin does not necessarily imply functional activity, it does indicate potential for adhesion to the corresponding ligand. It should be noted that while a number of studies have evaluated integrin expression on thymocytes, especially fetal progenitors or total DN cells, integrin expression relative to defined stages of differentiation in the postnatal progenitors has not been characterized, nor has binding to the corresponding ligands. Given the recent description of stratified regions through which progenitors migrate during differentiation (19), these were the goals of the experiments described in this work.

A number of integrins were not found at any appreciable level on DN cells, including α_1, α_2, α_3, and α_v. The latter is particularly informative, because it eliminates potential involvement of β_3-, β_5-, β_6-, and β_8-containing heterodimers in the precursor migration process. Likewise, the involvement of β_2 integrins has been largely ruled out by gene-targeting experiments (27), thus leaving β_1-, β_4-, and β_7-containing heterodimers as the major areas of interest. Analysis of these integrins and their α partners reveals substantial heterogeneity among the various stages of differentiation, as illustrated in Fig. 1. For instance, all DN stages express α_4 integrin, although DN1 express it in a bimodal pattern, and at levels that are lower than either DN2 or DN3. Likewise, α_5 integrin is expressed by all DN stages, although a bimodal distribution is again noted, this time in DN2, but not DN1 or DN3 cells. Thus, although these two integrins are consistently expressed on DN cells, they are not uniformly expressed; the relevance of this finding is addressed by other experiments in this manuscript, and in *Discussion*. Expression of integrin α_6 is fairly uniform on all DN cells, as is that of β_1. However, heterogeneity is again revealed by analysis of both β_4 integrin, which is up-regulated upon transition from DN1 to DN2, and β_7 integrin, which appears to be largely specific for DN2 cells. Thus, the functional heterodimers that can be expressed by different stages of intrathymic precursor differentiation are identifiable, and can be used to predict the potential ligands for adhesion at each stage of transcortical migration. The

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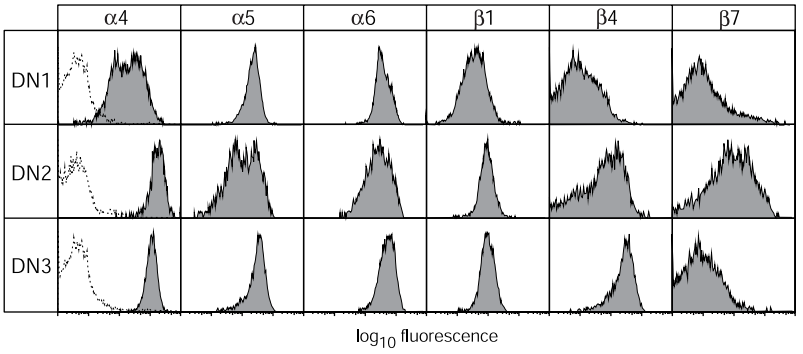

FIGURE 1. Analysis of integrin expression profiles to predict ligands for adhesion during DN precursor migration. Lineage-depleted thymocyte suspensions were stained with CD24, CD25, and CD44 Abs to reveal the various $CD4^-8^-$ stages of differentiation, as well as a panel of Abs recognizing various integrins. α_1, α_2, α_3, and α_v integrins were not found with appreciable frequency on any precursor population (not shown). However, α_4, α_5, α_6, β_1, β_4, and β_7 were expressed, as shown. In some cases (e.g., β_4 and β_7), expression fluctuated dramatically at certain developmental stages, indicating the potential for specific adhesion requirements at these stages. Possible integrin heterodimer combinations and relevant ligands for each stage are summarized in Table I. Hatched lines show the relative staining intensity of an isotype-matched nonspecific control Ab.

major predicted ligands, as summarized in Table I, were the ECM proteins FN, LN1, and LN5, as well as VCAM-1, which is generally implicated in cell-cell rather than cell-matrix interactions. The ability of integrin heterodimers on DN precursors to bind these ligands was subsequently tested, as described in the next section.

Analysis of precursor binding to ECM components

To confirm the integrin expression data shown in Fig. 1, and to further characterize the nature of the matrix for transcortical migration of lymphoid precursors, static adhesion to purified ECM proteins was performed (Fig. 2). Although static adhesion does not allow measurement of absolute affinity for a given ligand, it can be used to determine relative affinity of cells expressing a given set of receptors. Adhesion to LN5 was not tested, because it is found only in the basal layers of the thymic capsule and thymic blood vessels (28, 29), neither of which are primary sites for DN localization in the cortex (19). For the remaining ECM ligands, namely FN and LN1, 96-well tissue culture trays were coated with optimal levels of purified ECM proteins (see *Materials and Methods*), and equal numbers of cells at each stage were added. Following incubation and washing, relative levels of binding were determined. Absolute quantitation of such assays is complicated by an accumulation of cells at the edge of the well, in which the hydrodynamic forces of washing are greatly reduced. Consequently, counting was restricted to a single microscopic field at the center of the well. The number of cells in this field was quantitated using the colony-counting function of Quantity One software (Bio-Rad) and compared with the total cells present before washing (see *Materials and Methods*). Several observations were made using this assay. First, the most efficient binding was of DN3 cells to FN, with 70–80% of cells bound on average (numerous experiments were performed, but not all DN populations were examined in all experiments). DN1 also bound efficiently to FN, with a similar proportion of cells bound (60–70%). However, despite expressing multiple FN receptors (Table I), DN2 cells did not bind with appreciable frequency to FN (10–15% of cells bound). For all populations, the frequency of binding to LN1 was less than that of FN, although appreciable numbers of cells still bound at both the DN1 and DN3 stages (30–35%). However, binding of DN2 cells to LN1, although significant by the Student's *t* test for paired samples, was only trivially higher than binding to BSA alone. Together, these findings suggest that DN1 and DN3 cells express

Table I. *Integrins expressed on early intrathymic precursors, and potential ligands*

Stage	α Integrins	β Integrins	Potential Dimers	Major Ligands
DN1	α_4[a], α_5, α_6	β_1[b]	$\alpha_4\beta_1$	FN, VCAM-1
			$\alpha_5\beta_1$	FN, LN1
			$\alpha_6\beta_1$	LN1
DN2	α_4, α_5[a], α_6	β_1, β_4[a], β_7	$\alpha_4\beta_1$	FN, VCAM-1
			$\alpha_4\beta_7$	FN, VCAM-1
			$\alpha_5\beta_1$	FN, LN1
			$\alpha_6\beta_1$	LN1
			$\alpha_6\beta_4$	LN5
DN3	α_4, α_5, α_6	β_1, β_4	$\alpha_4\beta_1$	FN, VCAM-1
			$\alpha_5\beta_1$	FN, LN1
			$\alpha_6\beta_1$	LN1
			$\alpha_6\beta_4$	LN5

[a] Bimodal expression.
[b] Low level expression.

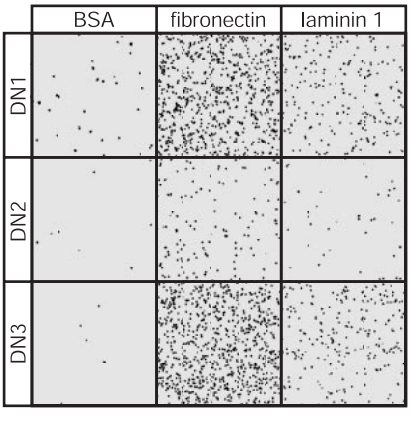

FIGURE 2. Adhesion of precursor thymocytes to ECM ligands predicted by integrin expression profiles. Equal numbers of cells were added to wells coated with FN or LN1, as indicated. After incubation and washing, bound cells were stained with crystal violet and the wells were photographed. Nonspecific binding to plates coated with BSA only was insignificant. Cells at the DN1 and DN3 stages bound with high frequency to FN (60–80% of cells bound), and somewhat less efficiently to LN1 (~30% of cells bound). However, DN2 thymocytes did not bind efficiently to either of these ECM components, despite expressing multiple relevant receptors (see Table I), indicating that DN2 cells have adhesion requirements that differ substantially from their precursors and progeny, and that may not include ECM ligands.

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active FN receptors and, to a lesser extent, LN1 receptors, while DN2 cells do not bind with high affinity or frequency to either of these, despite expressing the appropriate receptors.

Analysis of precursor binding to cellular matrix components in the thymic cortex

Our previous studies suggest that DN2 cells must be highly migratory, because they span the midcortical regions between DN1 and DN3 cells (19). Directional cell migration requires a matrix for cell adhesion, yet the results shown in Fig. 2 suggest that DN2 do not bind efficiently to ECM components for which they bear receptors. DN2 cells also bear multiple receptors for VCAM-1 (Fig. 1 and Table I), a counterreceptor that is generally found in cell surface form and mediates cell-cell interactions. VCAM-1 has been shown to be expressed in human thymus by both nonhemopoietic stroma (30) and macrophages (31). Interestingly, the stromal cells expressing VCAM-1 in the former case created a reticular lattice in the cortex. To evaluate the possibility that a cellular matrix composed of VCAM-1$^+$ cells might form a substrate for cell migration across the thymic cortex, we first analyzed expression of VCAM-1 in the mouse thymus (Fig. 3). Similar to previous findings on the human thymus, we find that VCAM-1 is expressed on reticular cells that form a radially aligned matrix in the thymic cortex, while expression in the medulla is restricted to scattered cells with macrophage-like morphology, or to vascular elements. The nature of various VCAM-1$^+$ cells is further revealed by dual staining using an Ab recognizing cytokeratins (Fig. 3). Numerous phenotypes can be observed, including VCAM-1$^+$ keratin$^-$ cells with macrophage/dendritic morphology (located mainly in the medulla), and a significant number of VCAM-1$^-$ keratin$^+$ stromal cells in the cortex. However, throughout the cortex there is also a subset of keratin$^+$ cells that is VCAM-1$^+$. This finding is confirmed by flow cytometric analysis, which shows that approximately one-third of cortical stromal cells (MHC-II$^+$, 6C3$^+$; see *Materials and Methods*) are also VCAM-1$^+$ (Fig. 3). Together, the data presented in Fig. 3 reveal that VCAM-1 is expressed in the thymus on a subset of stromal cells that form a reticular matrix in the cortex. Especially given the lack of DN2 binding to ECM components, this raised the possibility that stromal cells, and in particular VCAM-1$^+$ stromal cells, might represent the substrate for precursor migration outward through the cortex, as evaluated below.

If a stromal matrix does provide the substrate for precursor migration across the cortex, then precursors and stromal cells should be in direct contact with each other. The data in Fig. 4 show that this is, in fact, the case. An Ab recognizing CD117 (c-*kit*) was used to identify early intrathymic precursors (DN1 and DN2) found in the inner and midcortex (19), together with an Ab recognizing cytokeratins, to identify the most abundant stromal cells in the cortex. The vast majority of CD117$^+$ cells were found to be in direct contact with cytokeratin$^+$ stromal cells, in support of the above hypothesis. It should be noted that in any single plane, there were always a few (one or two) precursors that did not appear to be in contact with a stromal cell, suggesting the possibility that not all lymphoid precursors remain in contact with the stromal matrix at all times. However, it is quite possible that these few cells may have been associated with a stromal cell that was above or below the plane of the tissue section being examined. In any case, the majority of precursor cells in any given plane are found to be directly in contact with a stromal cell. The intimate nature of this interaction is further revealed by high magnification views at various depths in a single tissue section (Fig. 4, *b* and *c*). Lymphoid precursors are not only in contact with stromal cells, but are virtually surrounded by reticular processes from the stromal cells that they contact. These data not only support the hypothesis that stromal cells may provide a matrix for migration of early precursors outward through the cortex, but also show that intimate lymphostromal contacts are formed during this process, providing a basis for reciprocal signals that may influence differentiation, proliferation, and/or survival of either cell type, as predicted by the findings of others (for examples, see Refs. 32–34).

To further evaluate the role of the VCAM-1$^+$ subset of cytokeratin$^+$ stroma in the above process, we first sought to demonstrate a correlation between early precursors (i.e., c-*kit*$^+$ cells) and VCAM-1$^+$ cells in the cortex. Unfortunately, detection of c-*kit* and VCAM-1 in

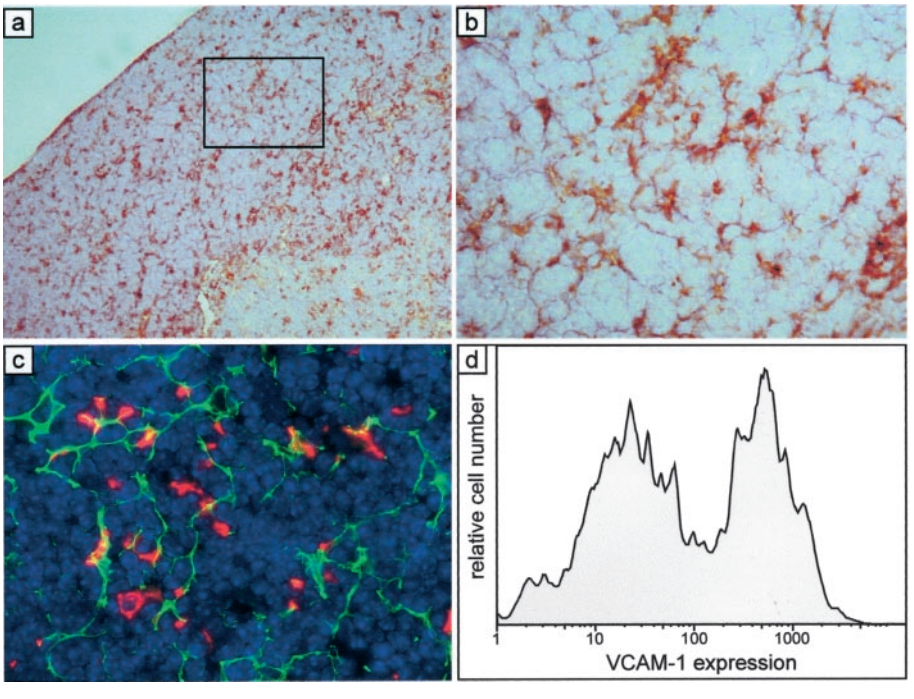

FIGURE 3. VCAM expression on a subset of the cytokeratin$^+$ stromal cell matrix of the cortex. *a*, Shows immunohistochemical detection of VCAM-1 staining (brown) on a transverse section of thymus; staining is mainly found in a reticular pattern in the cortex, as well as in vascular regions of both the medulla and cortex. *b*, Shows a higher magnification view of the area of the cortex indicated by the box in *a*. In *c*, it is shown that VCAM-1 expression (red) in the cortex corresponds to a subset of cells that also express cytokeratins (green). Flow cytometric analysis of VCAM-1 expression on cortical stromal cells (MHC-II$^+$, 6C3$^+$; see text) confirms that approximately one-third to one-half of such cells express VCAM-1 (*d*). Counterstains were hematoxylin (*a* and *b*) or DAPI (*c*). Original magnifications: *a* = ×100; *b* and *c* = ×400.

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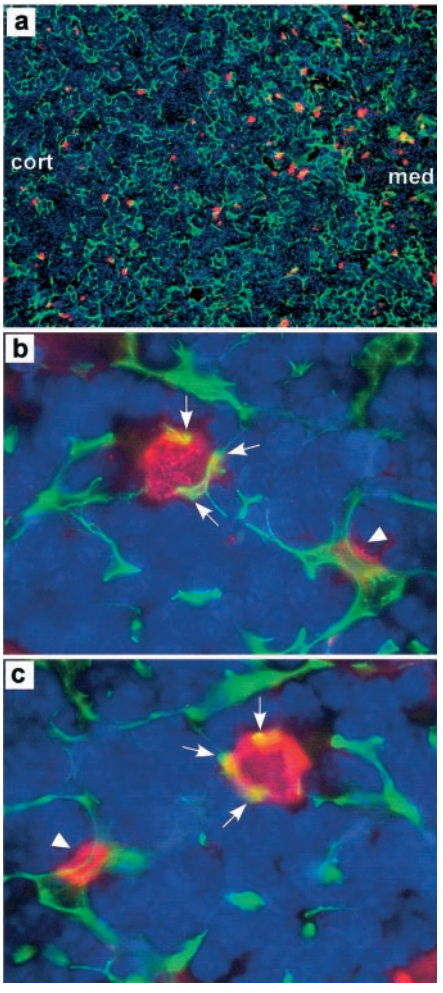

FIGURE 4. Colocalization studies reveal intimate association of early lymphopoietic precursors with the cytokeratin$^+$ stromal matrix of the cortex. *a*, Shows a lower power view (original magnification, ×100) of a transverse section of thymus, including midcortical regions and a portion of central medulla, as indicated. CD117$^+$ cells (red) are nearly always found in direct contact with cytokeratin$^+$ cells (green) that form a reticular matrix in the cortex. In *b*, a higher power view of another section is shown (original magnification, ×1000). A view from the reverse orientation is shown in *c*; the plane of focus in *b* and *c* differs by ~2 μM. The arrows around the most centrally located CD117$^+$ cell indicate a cytokeratin$^+$ cell seen from above in *b*, and through the center of the CD117$^+$ cell in *c*; note that the processes from the cytokeratin$^+$ cell virtually envelop the CD117$^+$ precursor. A similar relationship can be seen in a second precursor-stromal pair in this field (marked by an arrowhead), albeit at a different tissue depth.

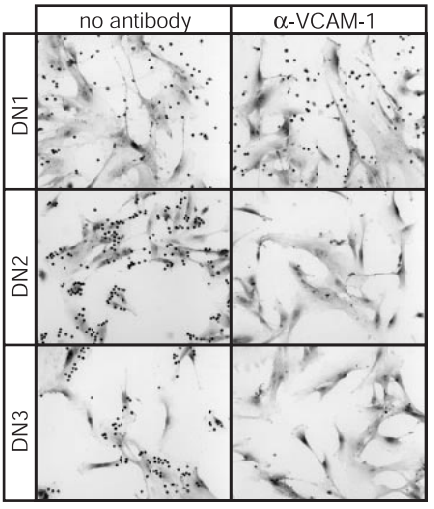

FIGURE 5. Adhesion of precursor thymocytes to stromal cells via VCAM-1. The ability of DN precursors to adhere to VCAM-1 expressed by thymic stromal cells was tested by a coincubation assay. Equal numbers of purified precursors were added to each well, followed by incubation, washing, fixation, and staining with hematoxylin. Thymocytes appear as small, darkly stained dots, while thymic stromal cells are large, reticular, and less darkly stained. DN1 thymocytes adhered in a relatively nonspecific manner to stromal cell cultures, as evidenced by binding to noncellular regions of the plastic substrate; such binding could not be blocked with an anti-VCAM-1 Ab. However, DN2 and DN3 precursors adhered specifically to the VCAM-1$^+$ stromal cells, but not to the surrounding plastic. This adhesion specifically involved VCAM-1, because it could be blocked almost completely by pretreatment of the stromal culture with an anti-VCAM-1 Ab.

the thymus is difficult, requiring some form of enzymatic amplification (either peroxidase alone or peroxidase/tyramide; see *Materials and Methods*) to be detected. It is not clear whether this results from low Ag levels on target cells, or whether the Abs and/or Ab conjugates are of low affinity, although it is worth noting that multiple clones and multiple direct conjugates have been used with similar results. Consequently, a functional assay for interaction of early precursors was used, in which binding to a cloned VCAM-1$^+$ thymic stromal line (100-4, the gift of A. Farr, Seattle, WA) was tested (Fig. 5). In this assay, DN1 cells bound in a relatively nonspecific manner to both the stromal cells and the plastic substrate; consequently, the ability of DN1 cells to bind specifically to VCAM-1 cannot be reliably determined. However, both DN2 and DN3 cells bound specifically to these VCAM-1$^+$ cells in a manner that could be blocked almost completely by preincubation with anti-VCAM-1. This finding is most revealing in relation to the DN2 subset, which had poor affinity for ECM ligands under similar assay conditions (Fig. 2). Because the anatomic location of DN2 cells is intermediate between that of DN1 (inner cortex) and DN3 (outer cortex), the reliance of this stage on cell surface-expressed VCAM-1 for adhesion strongly implicates requirement for a cellular, rather than extracellular, matrix for transmigration of early precursors between different cortical microenvironments.

In vivo effects of anti-VCAM-1 administration on the thymus

The data presented to this point show that early lymphoid precursors directly interact with stromal cells during migration outward through the cortex, and strongly implicate adhesion to this stromal matrix via a VCAM-1-dependent mechanism. To confirm the relevance of this data, an in vivo assay was highly desirable. Several factors complicate such analysis. First, disruption of lymphoid receptors for VCAM-1 results in an inability of bone marrow precursors to intravasate and travel to the thymus (35), making interference with α_4 integrins problematic. Second, it is possible that VCAM-1 may be involved not just in the intrathymic migration process, but in the interaction of blood-borne progenitors with vascular endothelium in thymic blood vessels, and thus in entry of early precursors into the thymus. Inhibition of entry into the thymus and inhibition of early precursor differentiation inside the thymus could have similar phenotypes, i.e., an overall reduction in thymocyte number, thus making it difficult to unconditionally distinguish between these two potential roles for VCAM-1.

With this caveat in mind, we performed such experiments, using in vivo administration of a mAb against VCAM-1 for 5 consecutive days in mice. Overall, the size of the thymus was reduced by approximately one-third, both in terms of cross sectional area (Fig. 6) and cell number (Table II), in animals receiving anti-VCAM-1

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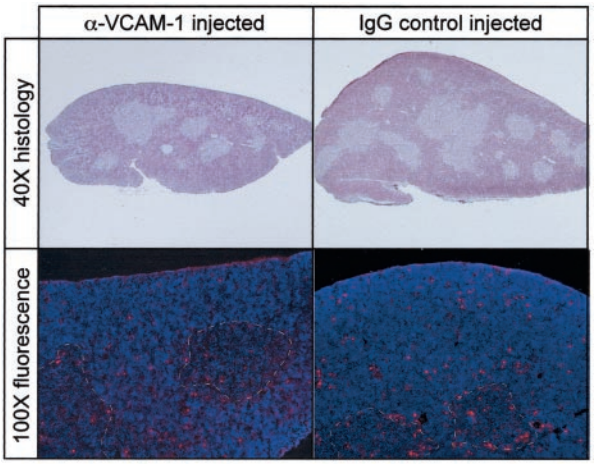

FIGURE 6. Reduced thymic size and altered precursor distribution and frequency resulting from anti-VCAM-1 administration in vivo. The *top two panels* show hematoxylin staining (original magnification, ×40) of transverse sections of thymus from a mouse treated for 5 consecutive days with an anti-VCAM-1 Ab (*left*) or a control Ab (*right*). In each case, sections were selected as those having the greatest cross sectional area. Anti-VCAM-1-treated thymuses were smaller, had fewer cells (see Table II), and have a reduced cell density per unit area. The *bottom panels* show higher power views (original magnification, ×100) of a portion of the thymus from mice treated similarly, indicating the presence of $CD117^+$ precursors (red). $CD117^+$ precursors were less frequent and more centrally located in mice treated with anti-VCAM-1 Ab, compared with controls. Dashed lines in immunofluorescent images represent the boundaries of medullary regions; counterstain is DAPI (blue).

Ab vs a nonspecific control Ab. The density of cells in the anti-VCAM-1-injected thymus was different from that of controls, with the cortex assuming a less densely packed appearance (i.e., more space between cells). The frequency and anatomic distribution of early ($CD117^+$) precursors were also influenced, with fewer cells being present overall, and markedly fewer cells in the mid- to outer cortex of anti-VCAM-1-injected mice. Overall, the proportion of total $CD4^-8^-$ cells did not differ substantially in thymuses from anti-VCAM-1 injected vs controls (Table II). Likewise, the proportion of individual DN subsets, including the $CD117^+$ stages (DN1 and DN2), was not dramatically affected (data not shown). This result is to be expected given the relatively short period of VCAM-1 administration (5 days) compared with the total life span of DN cells (~2 wk; see Ref. 12), especially because the transit time through the $CD117^+$ stages is at least 10 days. Thus, 5 days of anti-VCAM-1 administration can effectively block migration to the outer cortex without substantially changing the number of precursor cells that are present. Administration of Ab for longer periods incurs the risk of anti-Ig immune responses in either VCAM-1- or nonspecific Ab-treated mice. Nonetheless, 5 days of anti-VCAM-1 Ab had clear effects on both thymic size and precursor distribution. The fact that early progenitors were biased toward the inner cortex in anti-VCAM-1-treated animals further suggests that VCAM-1 must play a role in intrathymic migration in addition to any potential role in precursor entry; effects on precursor entry alone should result in an accumulation of precursors in the outer, rather than the inner, cortex. Thus, our findings are consistent with a requirement for VCAM-1 in the intrathymic migration of early progenitors, although an additional role in precursor entry cannot be excluded by these studies.

Table II. *Effects of in vivo administration of anti-VCAM-1 Ab*[a]

Treatment Type	Total Cells[b]	% of Cells in Phenotype		
		$CD4^-8^-$	$CD4^+8^+$	Mature
Anti-VCAM-1	110 ± 27	5 ± 3	88 ± 9	6 ± 4
IgG control	146 ± 37	6 ± 1	86 ± 2	8 ± 2

[a] Mean ± SD for three to six experiments.
[b] Total of $\times 10^{-6}$ per lobe of the thymus.

Discussion

In this study, we used integrin expression profiles (Fig. 1 and Table I) to predict the mechanical requirements for migration of immature precursors from the deep cortex to the capsule. Although deep cortical (DN1) and outer cortical (DN3) stages were capable of efficient binding to ECM ligands predicted by integrin expression profiles, DN2 cells that span the midcortex were not. This led us to evaluate binding to alternative matrix ligands, specifically the cell surface-expressed integrin counterreceptor VCAM-1. Functional and in situ studies revealed that lymphoid progenitors migrating outward through the cortex were found in intimate contact with a radially aligned stromal cell matrix (Fig. 4), a subset of which was $VCAM\text{-}1^+$ (Fig. 3). Although technical limitations prevented us from performing direct colocalization studies of VCAM-1 and early precursors in situ, the capacity for these cells to interact in a VCAM-1-specific manner was demonstrated by in vitro binding (Fig. 5). Furthermore, administration of an anti-VCAM-1 Ab in vivo resulted in reduced thymic size and cortical density, as well as a reduction in the presence of early progenitors in the outer cortex (Fig. 6). This phenotype is consistent with a role for $VCAM\text{-}1^+$ stromal cells in providing the adhesive matrix for intrathymic precursor migration, although, as mentioned earlier (see *Results*), VCAM-1 could also play a role in the entry of precursors into the thymus. Our data conclusively show that early lymphoid progenitors form intimate contacts with a matrix composed of cortical stromal cells (Fig. 4); that they express receptors for VCAM-1 (Fig. 1 and Table I), which is found on components of this cellular matrix (Fig. 3); and that they bind specifically to VCAM-1 on cloned cortical stromal cells in vitro (Fig. 5). Together with supportive results from in vivo studies (Fig. 6 and Table II), these findings provide direct evidence for intimate interactions between stromal cells and early lymphoid progenitors during their migration across the cortex, and further suggest that VCAM-1 and α_4 integrins (respectively) are probably responsible for this interaction. Although inducible deletion of VCAM-1 has been reported to have no obvious effect on the distribution of thymic subsets (36), deletion was performed in the immediate postnatal period, when the embryonic wave of thymic precursor differentiation predominates (reviewed in Ref. 1). Thus, a requirement for VCAM-1 in either the extravasation or transcortical migration of progenitors in the steady state thymus may not be obvious from such experiments. Furthermore, the overall size of the thymus in gene-targeted animals was not reported, and consequently the results of those studies may be completely consistent with those obtained by us using in vivo Ab administration (Fig. 6 and Table II).

It should be noted that stromal cells in the thymic cortex are scattered (Fig. 4), such that not every cortical lymphocyte (which are mostly DP cells) can be in contact with a stromal cell at any one time. Consequently, the finding that early progenitors do remain in contact with stromal cells during their migration across the cortex may reveal important principles about the differentiation process, as follows. Stromal cells are generally believed to be responsible for establishing the thymic microenvironment (reviewed in Refs. 37 and 38), and consequently, for inducing the steady state production of mature T lymphocytes from uncommitted progenitors. Our previous

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work has shown that although stromal cells from various cortical regions may be morphologically indistinct, they can be functionally differentiated, and establish a series of stratified microenvironments in which distinct stages of early precursor differentiation occur (19). Many of the signals that induce cellular differentiation, including those known to be important for intrathymic differentiation, such as Notch and c-*kit* ligands (see above), exist as cell surface proteins. Thus, successful differentiation of early lymphoid progenitors may require direct, sequential interactions with stromal cell-expressed ligands in each cortical region (for an example, see Ref. 22). Interstitial migration, i.e., migration along an ECM, could leave many such interactions to chance, while migration along a matrix of the very cells that generate these signals would ensure the efficiency of required interactions. This does not mean that the cortical stromal matrix exists merely to support DN migration and differentiation, because DP cells undoubtedly have a requirement for stromal cells as well. In fact, given that VCAM-1 expression reveals clear heterogeneity among cytokeratin$^+$ cortical stroma (Fig. 3), it is conceivable that DN and DP cells may use quite different stromal cell types, despite being present in the same cortical regions. Likewise, the ECM plays a required role in the differentiation, proliferation, and/or survival of lymphoid progenitors (39), either by direct signaling to the progenitors themselves, or through the organization of other stromal cells in their corresponding microenvironments.

A requirement for cell migration in progenitor differentiation is not unique to the steady state thymus. Of course, migration into and/or away from different microenvironments is a well-known paradigm of differentiation in the embryo. In addition, progenitor migration between different extracellular microenvironments is an integral component of steady state differentiation in a variety of postnatal tissues, including the epidermis (40), intestinal crypts (41), germ cells (42, 43), and even bone marrow (44, 45). Frequently, the divergence of independent cell fates, i.e., commitment to one lineage vs another, or self-renewal vs differentiation, is intricately linked to the position of a given stem/progenitor cell relative to other cells in its microenvironment. It is likely that progenitors in the thymus undergo a similar process, because they not only migrate, but make numerous cell fate choices as well, including (for instance) divergence of α/β vs γ/δ lineages, or the decision to differentiate or remain (albeit temporarily) in an undifferentiated state (46). The signals that regulate this asymmetry in the thymus are largely unknown. However, it is worth noting that integrin expression analysis reveals heterogeneity among otherwise homogenous DN1 and DN2 subsets, in which many such cell fate decisions occur (see Fig. 1). It is interesting to speculate that such changes in integrin expression may, in fact, be linked to asymmetry of cell fates, as it is in differentiating epidermis (see Ref. 40). However, not only are integrins nonhomogenously expressed on DN cells, but their ligands are nonhomogenously distributed in the thymus (this study, and Refs. 28, 29, and 47). Further structural and biochemical mapping of specific regions in which asymmetric cell fate decisions take place thus represents an important next step in deciphering the signals for steady state T cell production in the postnatal thymus.

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