Cell Adhesion and Migration Are Regulated at Distinct Stages of Thymic T Cell Development: The Roles of Fibronectin, VLA4, and VLA5

By Laura Crisa,*Vincenzo Cirulli,‡ Mark H. Ellisman,§
Jennifer K. Ishii,* Mariano J. Elices,‖ and Daniel R. Salomon*

From the *Department of Molecular and Experimental Medicine and Department of Immunology,
The Scripps Research Institute, La Jolla, California 92037; ‡The Whittier Institute, Department of
Pediatrics and §National Center for Microscopy and Imaging Research, University of California, San
Diego, California 92093; and ‖Cytel Corporation, San Diego, California 92130

Summary

T cell development in the thymus requires the establishment of stable interactions with cell-selecting elements such as the cortical epithelium followed by a regulated movement of selected progenitors to the medulla. Cell adhesion and migration are mediated by integrins in a number of biological systems though little is known regarding their function in the thymus. We demonstrated previously that immature CD3^{lo}CD69^{lo} double positive human thymocytes adhere avidly to FN via the integrin, VLA4. We now demonstrate that the interaction of mature CD3^{hi}CD69^{hi} thymic subsets with FN triggers migration rather than firm adhesion. Migration requires the engagement of VLA4 in cooperation with VLA5 and both receptors regulate the persistence and directionality of movement. While migration capability is linked to maturation state, ligand concentration determines the efficiency of migration. In fact, FN and the alternatively spliced CS1 site are predominant in the thymic medulla, suggesting an instructive role of this ECM protein in vivo. Our studies identify a novel VLA4 and VLA5/FN-mediated pathway likely to be involved in regulating cell traffic between the cortex and medulla of the thymus. Moreover, the data provides evidence that VLA4 exists in at least two functional states at distinct stages of T cell development. While different states of VLA4 activation have been described on cell lines, this represents the first evidence supporting a biological significance for this integrin property.

Positioning of developing thymocytes within the thymic cortex and medulla as well as the regulation of their ability to move from one region to another are crucial to T cell differentiation. Thus a stable interaction with cortical epithelial cells facilitating the recognition of MHC/peptide complexes is a prerequisite for the positive selection of immature double positive T cells (1). In fact, there is evidence that positive selection within the cortex is limited by contact with these selecting elements (2, 3) underscoring the importance of migratory events leading T cell progenitors to the epithelium. Subsequent events of maturation, including negative selection, appear to occur in the medulla involving bone marrow–derived antigen-presenting cells as well as a phenotypically distinct medullary epithelium (4). Implicit in these compartment-specific events is the programmed detachment of developing thymocytes from the cortical epithelium and the regulated movement of those committed to the mature single positive phenotype to the medulla. While much has been learned about the function of the T cell receptor (TCR) and the costimulatory molecules, CD4 and CD8, these molecules cannot directly mediate stable cell–cell adhesion or migration. In contrast, the role of interactions mediated by integrin adhesion molecules expressed by thymocytes with extracellular matrix (ECM)[1] proteins such as fibronectin (FN) in thymocyte motility, has not been elucidated.

FN is a major structural component of the thymus (5, 6), but its function in thymic physiology is presently unknown. Nevertheless, extensive evidence supports a pivotal role of FN in cell adhesion and migration in other biological systems (7). FN has been shown to regulate the migration and accumulation of activated cells during acute and chronic inflammation (8, 9), wound healing (10), tissue regeneration (7), and tumor metastases (11). Likewise, during embryogenesis, FN provides the adhesive cues necessary to promote and guide the migration of neural crest and meso-

---

[1]Abbreviations used in this paper: CFN, cellular fibronectin; cx, cortex; ECM, extracellular matrix; FN, fibronectin; LC, lower chamber; m, medulla; UC, upper chamber; VN, vitronectin.

dermal cells over long distances (7, 12). Multiple cell binding sites have been characterized within the FN molecule, including the RGDS sequence, located within the central region of FN, and the CS1 sequence, at the carboxy-terminal end (13, 14). The primary cell surface receptors recognizing such sites are members of the integrin gene superfamily (15). Two integrin receptors capable of binding FN are expressed on human thymocytes: VLA5 ($\alpha5\beta1$, CD49e/CD29), recognizing the RGDS site (16), and VLA4 ($\alpha4\beta1$, CD49d/CD29) recognizing the CS1 site (17, 18). These integrins can mediate either stable adhesion or the more complex function of migration.

The adhesive or migratory response of cells to FN can be regulated by various mechanisms including changes in the local production of FN, alternative splicing of its cell binding sites and modulation of integrin expression or function. For example, the loss of FN production is a feature of some malignant cells (7, 11). Alternative splicing of the FN transcript may involve the CS1 site and generates a number of functionally different FN isoforms (19, 20). Production of FNs highly enriched for CS1 occurs during embryonal development suggesting a key role of this sequence in cell motility and/or growth (21). FN variants lacking CS1 fail to support migration of melanoma cells (22). In addition, preferential splicing and expression of this motif is present at the synovial endothelium of patients with rheumatoid arthritis (23) and the inhibition of binding to this sequence abrogates leukocyte cell adhesion and migration into local tissues (24, 25). Similarly, the addition of the type III repeats EIIIA and EIIIB by alternative splicing, distinguishes cellular forms of FN from plasma FN (26). Expression of these EIIIA+ and/or EIIIB+ forms of FN correlates with higher embryonal cell motility (27). Finally, adhesion can be modulated through changes in VLA4 and VLA5 expression or function (28, 29). In particular, an important feature of these integrins is that they may exist on a cell's surface in several states of activation with different avidities for FN (30–33) providing a strategy by which cells can rapidly modulate the strength of interactions with this ligand.

We have previously characterized the differential expression of several integrins on human thymic subsets and demonstrated that VLA4 is highly expressed on most thymocytes, whereas VLA5 is upregulated on single positive cells (34, 35). We demonstrated that VLA4 expressed by immature CD3^{lo}CD69^{lo} double positive thymocytes is constitutively activated as measured by its ability to mediate firm attachment to FN (34). In contrast, mature CD3^{hi}CD69^{hi} CD4 and CD8 single positive as well as the CD4$^+$CD8^{lo} transition subsets fail to adhere to this substrate. Interestingly, VLA4 expression is similar on both adherent and non-adherent populations, suggesting that the function of this receptor rather than its levels of expression is modulated at different stages of T cell maturation.

In the present study we hypothesized that the functional modulation of VLA4, resulting in a loss of firm adhesion at the transition between the double and single positive stage, may actually enable the migration of more mature thymocytes, thereby providing a mechanism by which posi-

tively selected thymocytes move from the cortex to the medulla. Therefore, we developed a migration assay to determine the ability of human thymocytes to migrate on FN in vitro. We demonstrate that CD3^{hi} thymic subsets display a high migratory activity on FN which is ligand concentration-dependent, whereas most CD3^{lo-int} double positive thymocytes are stationary. However, at relatively high concentrations of ligand we can demonstrate the recruitment of a CD3^{int}CD69^{hi} double positive population indicating that there are differences in migratory efficiency within the double positive subset that also correlate with activation or maturation stage. Interestingly, immunofluorescence microscopy revealed a differential distribution of both FN- and the CS1-binding site between the cortex and medulla of the thymus, suggesting that FN has an instructive role in the control of thymocyte motility in vivo imparted by the structural organization of this integrin ligand. Finally, we demonstrate that thymocyte migration is mediated by the cooperative engagement of both VLA4 and VLA5 integrins on the CS1 and RGDS sequences of FN and that information determining the speed and direction of migration can be signalled by these interactions. Based on these data we propose a new model integrating the regulation of thymocyte migration mediated by VLA4 and VLA5 on FN with the current knowledge of T cell development in the thymus.

Materials and Methods

Tissue and Cell Preparation. Fragments of human thymus were obtained from 1 month to 7-yr-old children undergoing elective cardiac surgery at the Children's Hospital (San Diego, CA). A single cell suspension of thymocytes was prepared by gently teasing the tissue on 60-μm stainless steel meshes into ice-cold serum-free HBSS. After washing, cells were resuspended in serum-free HBSS and viability determined by trypan blue exclusion. Only preparations with viability $\geq98\%$ were used.

Antibodies and Reagents. Monoclonal antibodies used for triple immunofluorescence and flow cytometry analysis of human thymocytes were: RPE-conjugated anti-CD4 (clone 13B8.2; Immunotech, Westbrook, ME), FITC-conjugated anti-CD8 (clone.B9.11; Immunotech), Cy-Chrome™-conjugated anti-CD3 (clone HIT3a; PharMingen, San Diego, CA), FITC-conjugated anti-CD69 (Becton Dickinson, San Jose, CA), RPE-conjugated anti-HLA A,B,C (clone G46-2.6, PharMingen), and matching, fluoro-conjugated, isotype controls. Monoclonal antibodies specific for human plasma FN (clone FN-15) or cellular FN (clone FN-3E2) were obtained as ascites from Sigma (St. Louis, MO). The anti-FN Mab clone 333 was a kind gift of Drs. S. Akiyama and K. Yamada. Antibodies used as secondary reagents were biotin-conjugated goat F(ab)$_2$ fragments specific for mouse IgG (Caltag, San Francisco, CA) or mouse IgM (Cappel, Durham, NC). FITC-conjugated streptavidin (SA) was obtained from GIBCO BRL (Gaithersberg, MD). Lissamine rhodamine-conjugated SA was from Jackson Laboratories (West Grove, PA). Purified anti-$\alpha5$ mAb (clone SAM1) was obtained from Immunotech. Anti-human $\alpha4$ mAb (clone HP2/1) was either purchased from Immunotech or was a kind gift of Dr. Sanchez-Madrid (Madrid, Spain). The anti-$\alpha4\beta7$ antibody, ACT-1, was a kind gift of Drs. Newman and Ringler (LeukoSite Inc., Cambridge, MA). Purified anti-human CD54 (ICAM-1) mAb (clone P5F5) blocking

the binding of ICAM-1 to its ligand LFA-1 as well as a mAb raised against the CS1 peptide sequence of FN (clone 90.45) were obtained from Cytel Corporation (San Diego, CA). A synthetic molecule resembling the CS1 sequence of FN (phenylacetic acid-Leu-Asp-Phe-d-Pro-amide, compound P96) and a scrambled version (phenylacetic acid-Asp-Leu-Phe-d-Pro-amide, compound P66) were also provided by Cytel. The IC_{50} (50% inhibition concentration) in vitro of the CS1 peptide for VLA-4–mediated adhesion to FN is 0.2–0.5 μM (9). Purified human plasma FN was obtained from Sigma. Purified human vitronectin (VN) was from Promega (Madison, WI). The 40- and 120-kD chymotryptic fragments of FN were obtained from GIBCO BRL.

Immunofluorescence and Flow Cytometry. Three-color immunofluorescence staining of human thymocytes was performed by incubating the cells in HBSS/2% FCS/0.05% NaN_3 in the presence of RPE, FITC and Cy-Chrome conjugated specific antibodies or species and isotype-matched controls for 30 min at 4°C. After washing, samples were analyzed on a FACScan® (Becton Dickinson, San Jose, CA) equipped with Lysis II software. A live cell gate was created by excluding cell debris and non-viable cells on forward angle and side light scatter and analysis performed on at least 10,000 events. The live gate was confirmed in some experiments by propidium iodide staining.

Cell Migration Assay. Cell migration assays were carried out in Transwell™ culture chambers (Costar 3415, Cambridge, MA) using the same ECM coating conditions and culture medium as described in our previous report (34). The 3-μm porous membrane separating the upper from the lower chamber of the Transwells™ was precoated (10 μg/ml in PBS) on both sides with VN, intact FN or chymotryptic fragments of FN overnight at 4°C. After rinsing with PBS, non-specific binding sites were blocked by incubation for 30 min at 37°C in PBS/1% heat-treated BSA (80°C, 20 min). Thymocytes prepared in serum-free HBSS were seeded in serum-free AIMV culture medium (GIBCO BRL) in the upper chamber of the Transwells™ at 3 × 10^6 cells/well and cultured at 37°C in a 5% CO_2 humidified incubator. At various time points, thymocytes retained in the upper chamber and those migrated to the lower chamber were harvested, counted and stained for flow cytometry. In the migration inhibition studies, thymocytes were pre-incubated for 15 min at 4°C in the presence of saturating concentrations of anti-VLA4, anti-VLA5, anti-ICAM-1 mAbs or control mouse IgG and added to the upper chamber of the Transwells™ without further washing. Alternatively, thymocytes were incubated in the presence of the VLA-4 specific P96 blocking peptide or P66 control peptide. Blocking antibodies and peptides were present at a final concentration of 10 μg/ml during culture. Results were expressed as percent of migrating cells in the control cultures. Statistical analysis was performed using the Wilcoxon test (StatView; Abacus Concepts Inc., Berkeley, CA). In the experiments assessing the proliferative status of migrating and non-migrating thymocytes, triplicate cultures were pulsed for 8 h with 2 μCi/well of [³H]thymidine (New England Nuclear, Dupont, Wilmington, DE). Cells were then harvested onto glass fiber filters using the cell harvester 96 Mach III (Tomtec Inc, Orange, CT) and counted in a scintillation counter (model 1450 Microbeta; Wallac Oy, Turku, Finland).
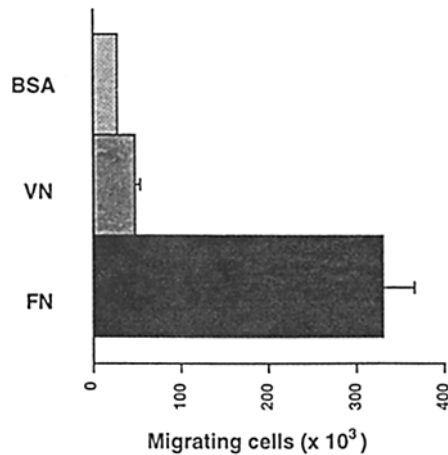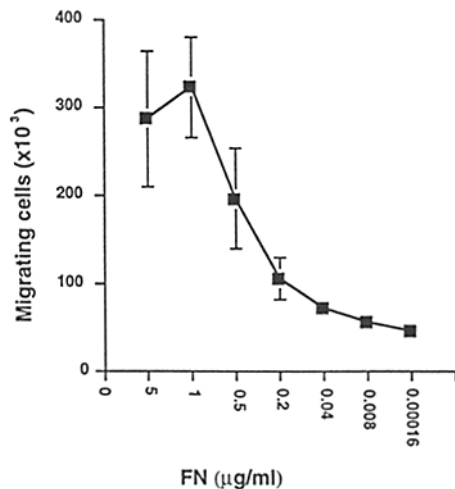
Immunofluorescence Microscopy. 8-μm frozen thymic sections were air-dried for 2 h at room temperature, fixed in absolute ethanol for 2 min at −20°C, and blocked overnight at 4°C in PBS/1% heat-inactivated BSA containing 20 μg/ml of purified goat Ig (Jackson Immunoresesearch Laboratories, West Grove, PA). Sections were incubated for 1 h at room temperature in the presence of

a 1:400 dilution of either anti-FN or anti-CFN ascites or 1 μg/ml of purified anti-CS1 mAb (IgM). Control slides were incubated in the presence of an anti-thyroglobulin ascites or normal mouse IgM (Dako, Glostrup, Denmark). After extensive washings in PBS/0.1% BSA/0.05% Tween 20, binding of primary mAbs was detected by sequential incubations with a biotin-conjugated goat anti–mouse IgG or goat anti–mouse IgM followed by SA-Lissamine Rhodamine. Slides were mounted in Gel-Mount (Biomeda, Foster City, CA) and analysed for specific fluorescence (Olympus Microscope, Tokyo, Japan).

Time-lapse Video Microscopy. Thymocytes were allowed to migrate for 8 h through Transwell™ membranes coated with FN (0.2 μg/ml) in medium containing either DiI or DiO fluorescent carbocyanins (1 μg/ml; Molecular Probes Inc., Eugene, OR). This treatment results in labeling of thymocytes with a red (DiI) or green fluorescence (DiO). At the end of the incubation, DiO-labeled thymocytes retained in the upper chamber and DiI-labeled cells which had migrated to the lower chamber were harvested. DiI- and DiO-labeled cells were mixed at a 1:2 ratio to account for the lower frequency of migrating cells present among thymocytes from the upper chamber. Cells were then resuspended in AIM V medium supplemented with 20 mM Hepes, seeded on top of a glass bottom microwell (Mat Tek Corporation, Ashland, MA) coated with FN (0.2 μg/ml) and cultured at 37°C on a temperature-controlled microscope stage. Cell migration was recorded at one frame/70 s for ~2.5 h with a Zeiss Axiovert M35 microscope equipped with a confocal attachment (MRC1024; BioRad, Hercules, CA) and all images were stored on optical disks. In the antibody inhibition experiments, the control mAb was added at the initiation of the first 2.5 h period. The blocking mAb (10 μg/ml) were then added to the same cells and migration observed for an additional 2.5 h. Cell migration tracks were measured using NIH Image (version 1.59b9, Bethesda, MD).

Results

Purified FN Supports Migration of Human Thymocytes. To assess whether FN can serve as an adhesive ligand for thymocyte migration we developed a Transwell assay system. Freshly isolated thymocytes were plated in the upper chamber onto the porous membrane coated with purified FN or control substrates. The number and phenotype of the cells migrating from the upper to the lower compartment was then determined by cell counting and flow cytometry analysis at various times of culture. Fig. 1 *A* compares thymocyte migration after 8 h of culture on membranes coated with 10 μg/ml of either FN, VN, or BSA. Previous experiments titrating the coating concentration of these proteins demonstrated that 5–10 μg/ml was optimal for migration (data not shown). FN supported an eight- to ninefold increase in the number of migrating thymocytes (~10% of the cells plated in the upper chamber) as compared with the migration on BSA or VN-coated membranes. In cultures continued up to 48 h, a greater number of thymocytes (up to 22% of the cells plated in the upper chamber) migrated to the lower chamber across FN-coated membranes. To exclude the possibility that cell proliferation during the 8-h assay contributed to the increased number of cells counted in the lower chamber after FN-mediated migration, [³H]thymidine incorporation assays
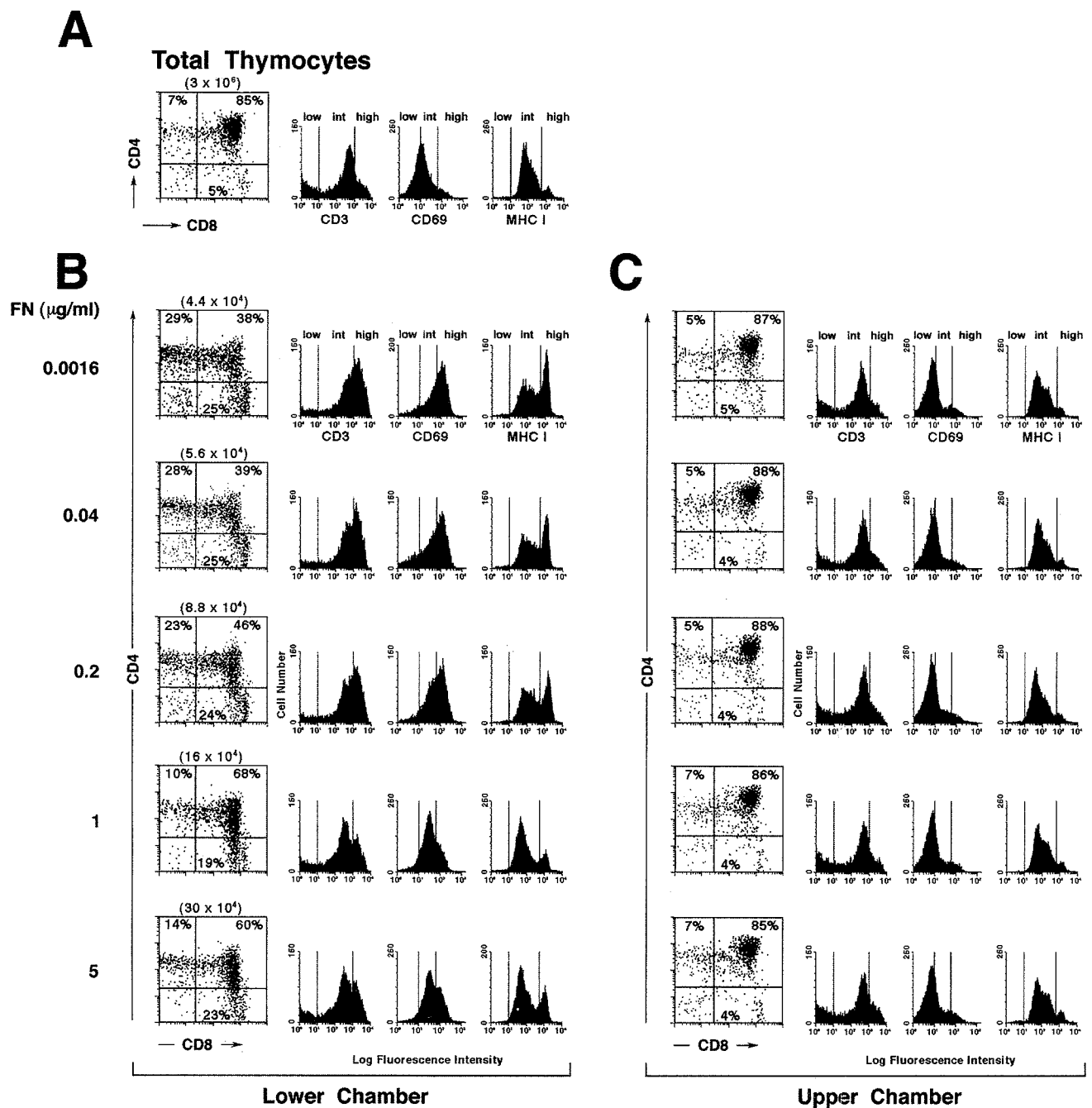
A

B

tions of FN. These data indicate that FN supports thymocyte migration in a concentration-dependent fashion.

Differential Migratory Capability of Double Positive and Single Positive Thymocytes as a Function of Ligand Concentration. Flow cytometric analysis of the migrating cells revealed that different thymic subsets migrated as a function of different concentrations of membrane-bound FN (Fig. 2). In these experiments all results are depicted for cells analyzed in the live gate which consistently represented >80% of the total cells. Thymocytes migrating across membranes coated with the lowest concentrations of FN (0.0016–0.04 μg/ml) were enriched for CD4 and CD8 single positive subsets as well as for the $CD4^+CD8^{lo}$ transition cells as compared to the unseparated thymocytes (Fig. 2, *B* vs *A*). Conversely, relatively few double positive cells, mostly $CD4^{lo}CD8^{lo}$, were seen among migrating thymocytes. Notably, $CD4^+CD8^+$ double positive thymocytes represent the major population remaining in the upper compartment of the Transwell™ (Fig. 2 *C*). Consistent with their more mature CD4/CD8 phenotype, migrating cells also expressed higher levels of CD3, CD69 and MHC class I as compared to those remaining in the upper chamber (Fig. 2, *B* vs *C*) or the unseparated thymocytes (Fig. 2 *A*). In terms of absolute numbers, thymocytes displaying a migratory activity on the lowest coating concentrations of FN represented ∼1–2% of the input population.

Membranes coated with relatively higher concentrations of FN (0.2–5 μg/ml) substantially enhanced the absolute numbers of migrating $CD3^{hi}$ single positive and $CD3^{hi}$ double positive thymocytes (Fig. 2 *B*). However, the recruitment of an increasing proportion of $CD3^{int}$ cells expressing intermediate levels of CD69 and MHC class I was also observed. Three-color immunostaining for CD4, CD8 and CD69 or MHC I followed by selective gating on such $CD69^{int}$ and MHC I^{int} cells demonstrated that they were primarily double positive cells (data not shown). In these cultures, migrating cells represented 2.9–10% of the original input population. Nonetheless, we calculated that the number of migrating single positive cells was 30–40% of the single positive cells in the input population, whereas the total of migrating double positive thymocytes represented only 5–7% of the input double positive population. Most thymocytes retained in the upper chamber of the Transwells™ were $CD3^{lo-int}CD4^+CD8^+$ and expressed very low levels of CD69 (Fig. 2 *C*). With the exception of the cells cultured on membranes coated with the highest concentration of FN (5 μg/ml), the migrating cells expressed slightly lower levels of VLA4 but higher levels of VLA5 as compared to the non-migrating subsets (Table 1). Finally, migration assays performed to assess the phenotype of migrating subsets as a function of different times in culture demonstrated that $CD3^{hi}$ thymocytes migrated at earlier time points than $CD3^{int}$ double positive cells. Furthermore, while ∼60% of single positive cells still remained in the upper chamber after 8 h, if we extended the incubation to 48 h essentially all the single positive cells migrated to the lower chamber (data not shown). These results also suggest a heterogeneity of migration efficiency in this mature population.

Figure 1. Fibronectin supports thymocyte migration. (*A*) Thymocytes (3 × 10⁶) were cultured in serum-free conditions in the upper chamber of Transwells™ whose porous membranes were pre-coated (10 μg/ml) with either FN or VN. The membranes of the control wells (*BSA*) were coated with the blocking solution, 1% heat-inactivated BSA. Number of cells which migrated across the membrane to the lower chamber of the Transwell™ within an 8-h culture period are shown. Bars represent mean ± SD of triplicate samples. FN but not VN supports thymocyte migration. (*B*) Thymocytes were cultured for 8 h on membranes pre-coated with the indicated concentrations of FN. Number of cells which migrated to the lower chamber within this time period are shown. Each point represents the mean ± SD of triplicate samples. Thymocytes migrate on FN in a ligand concentration-dependent fashion.

were performed. The data (*n* = 3) expressed as cpm per cell revealed the following: cultures of total thymocytes, mean ± SE = 6.8 (±0.3) × 10⁻³; upper chamber cells = 7.3 (±0.5) × 10⁻³; lower chamber cells = 5.9 (±2.0) × 10⁻³. Thus, the migrating thymocytes (lower chamber) are not significantly enriched for proliferating cells. As shown in Fig. 1 *B*, increasing numbers of thymocytes were recruited to migrate in response to higher coating concentra-

Figure 2. Differential migratory activity of single positive and double positive thymocytes on FN. Thymocytes (3×10^6) were cultured for 8 h in either uncoated plastic tissue culture wells or on Transwell™ membranes coated with 0.0016, 0.04, 0.2, 1, or 5 μg/ml of FN, respectively. At the end of the culture period, cells in the plastic wells (A), those which migrated to the lower chamber (B), and those which remained in the upper chamber of the Transwells™ (C), were harvested and analysed for the expression of CD4, CD8, CD3, CD69, and MHC class I by flow cytometry. Three-color immunofluorescence was performed using anti-CD4-RPE, anti-CD8-FITC, and anti-CD3-CyChrome mAbs. Levels of CD69 and MHC I were assessed using anti-CD69-FITC and anti-MHC class I-RPE mAbs. The CD4/CD8 dot plots relative to each experimental condition are shown and the percentage of single positive and double positive cells indicated in each quadrant. Quadrants were set to comprise the staining given by control antibodies in the left lower quadrant. Data represent the analysis of 10,000 events collected within a live gate comprising >80% of the total cells in all experimental conditions. The absolute number of viable cells recovered at the end of the culture period is given in parentheses on top of each dot plot. Histograms represent the fluorescence intensities of CD3, CD69 and MHC class I expression. The vertical bars within the histograms were set using the profiles of the Total Thymocytes (A) to define cell subpopulations with low, intermediate and high levels of antigen-specific fluorescence. The fluorescence intensity with the isotype-control antibodies is contained within the region defined as low.

219   Crisa et al.

Table 1. *Expression of VLA4 and VLA5 on Migratory and Non-migratory Thymocytes*

	FN (μg/ml)*	0.0016	0.04	0.2	1	5
VLA4	Upper chamber cells	294 ± 11	327 ± 0	316 ± 11	316 ± 11	310 ± 16
	Lower chamber cells	246 ± 26	249 ± 4	245 ± 0	255 ± 27	365 ± 13
VLA5	Upper chamber cells	27 ± 2	29 ± 0	25 ± 5	24 ± 0.5	30 ± 1.5
	Lower chamber cells	45 ± 3	45 ± 0.5	42 ± 5.5	40 ± 1.5	31 ± 2

Migratory (*lower chamber*) and non migratory (*upper chamber*) thymocytes cultured under conditions identical to the experiment depicted in Fig. 2, were harvested from the upper and lower chamber of the Transwells™ stained for the expression of VLA4 and VLA5 and analyzed by flow cytometry. Values are mean ± SD of medians of fluorescence intensity (log units). Median of fluorescence intensity in control samples was <10 log units.
*Coating concentrations of FN on Transwell's™ porous membranes.

The data demonstrate that a substantial fraction of the most mature CD3^{hi} single positive and double positive cells migrate on FN more efficiently than the relatively less mature CD3^{int} double positive cells, the latter subset being recruited only at higher coating concentrations of FN. In contrast, we could not demonstrate the migration of the CD3^{lo} cells under any condition tested. This observation suggests that thymocyte motility on FN is determined by the differentiation state of the cells. Moreover, migration triggered by cellular interactions with FN is further regulated by the amount of ligand present in the extracellular environment.

Distribution of FN, CFN and the CS1 Peptide Sequence of FN within the Human Thymus. Differences in the distribution of FN within tissues as well as the alternative splicing of certain adhesive sequences, particularly CS1, are mechanisms by which FN may regulate cell adhesion and migration in vivo. Double positive and single positive thymocytes, displaying distinct adhesive (34) and migratory capability on FN in vitro, are positioned in vivo within the cortex and the medulla of the thymus, respectively. Therefore, we examined the possibility that these two compartments of the thymus present a differential distribution and/or alternative splicing of endogenous FN. The expression of the plasma form of FN, its alternatively spliced isoform cellular FN (CFN) and the distribution of the CS1 peptide sequence, representing the primary FN binding site of VLA4, was determined by in situ immunofluorescence.

As shown in Fig. 3, staining of frozen sections of human thymus with plasma FN- or CFN-specific antibodies revealed a similar distribution of the two isoforms predominantly in the corticomedullary junction and the medulla (m). In the medulla, FN appears to be organized in either fibrillar structures or large irregular patches, the latter likely to comprise clusters of stromal cells. In contrast, relatively few FN-containing fibers are present in the cortex (cx). Interestingly, immunostaining for the CS1 peptide sequence revealed a unique pattern as compared to the distributions of either the plasma form of FN or CFN. CS1 staining highlights the medulla, sharply defining the boundary with the surrounding cortex. Moreover, the medullary CS1-specific
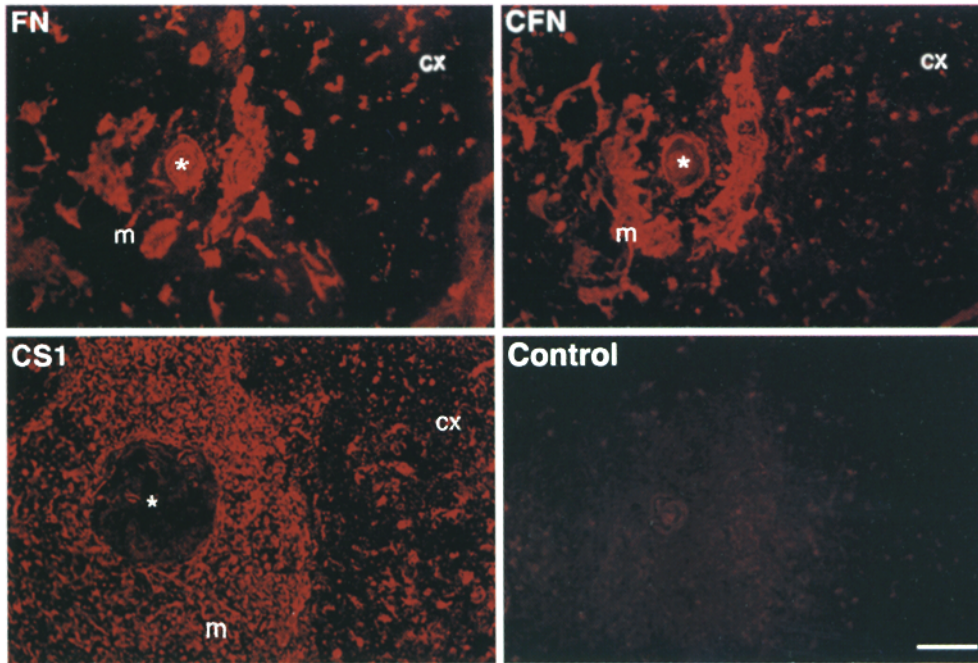
sequences are distributed in a reticular fashion which at higher magnification was confirmed to mark the pericellular matrix around thymocytes.

Thus the medulla of the human thymus provides resident thymocytes with a microenvironment highly enriched for FN and specifically the alternatively spliced CS1 binding site for VLA4. In contrast, the cortex of the thymus is relatively poor in FN and CS1 sequence. The concentration of FN regulates the migration of thymic subsets in vitro. Therefore, the differential expression of FN between the cortex and medulla suggests that FN may also regulate the migration of cortical and medullary thymocytes in vivo.
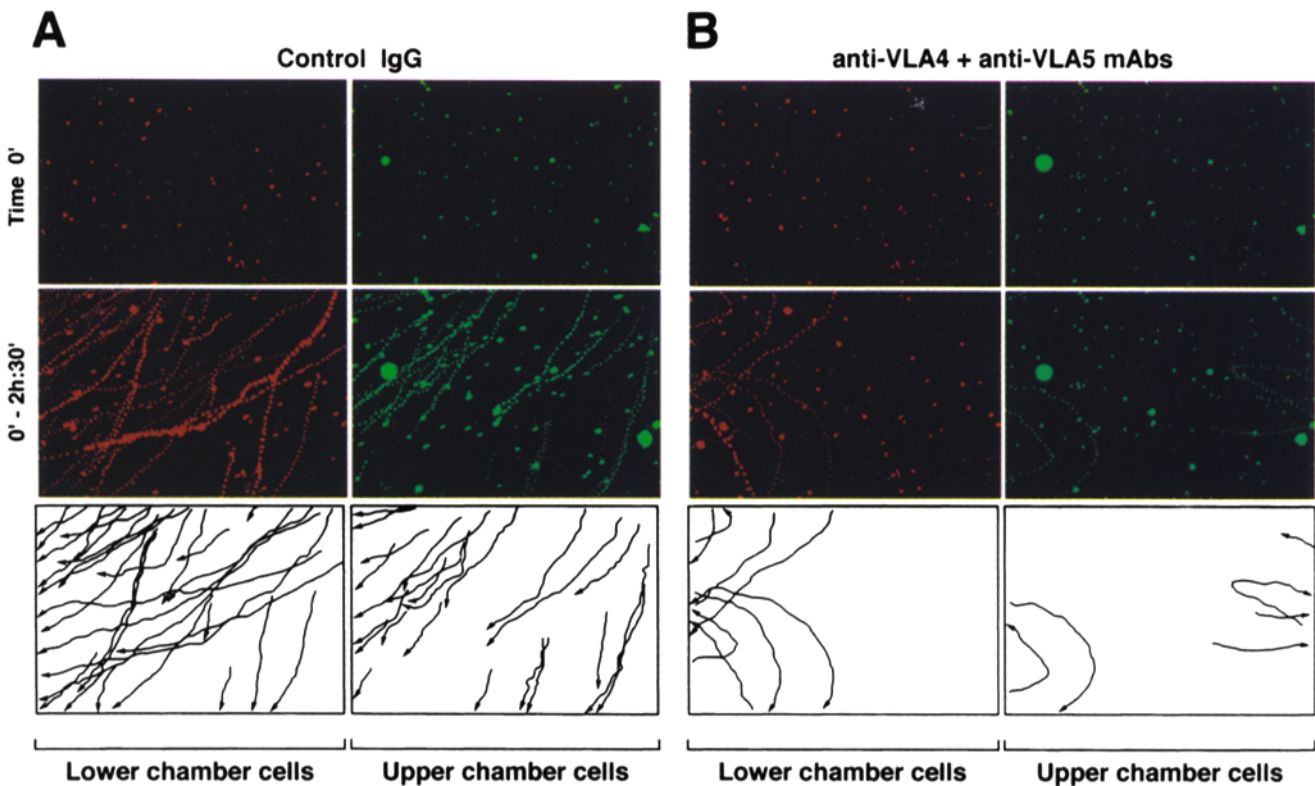
Migration of Thymocytes on FN Involves the Engagement of VLA4 and VLA5. The integrins VLA4 and VLA5 have been implicated in both the adhesion and migration of various cell types on FN (22, 36, 37). We have demonstrated that VLA4, but not VLA5, mediates firm adhesion of human thymocytes to FN (34). To investigate whether these integrins mediated thymocyte migration, we performed migration assays in the presence of blocking anti-α4 and anti-α5 antibodies or peptides. As shown in Fig. 4 *A*, thymocyte migration was significantly inhibited by blocking both VLA4 and VLA5, simultaneously (~75% inhibition as compared to control cultures, $P = 0.06$, $n = 4$). In contrast, blocking either VLA4 or VLA5 alone had little or no effect respectively. This result was confirmed by the inability of the α4β1-specific blocking peptide, P96, to inhibit migration (Fig. 4).

Thymocytes express high levels of the integrin, αLβ2 (LFA-1). The binding of this integrin to ICAM-1 has been involved in the adhesion and migration of peripheral blood T lymphocytes and T cell lines (38, 39). We tested the effects of blocking LFA-1/ICAM-1 interactions on thymocyte migration with an anti-ICAM-1 mAb. These experiments failed to demonstrate any inhibition (data not shown). Indeed, this treatment consistently increased thymocyte migration above that of the control cultures by ~25%. Though α4β7 is an alternative FN receptor (40) we observed only minimal thymocyte expression and no inhibition of migration with ACT1, a specific blocking mAb (data not shown).
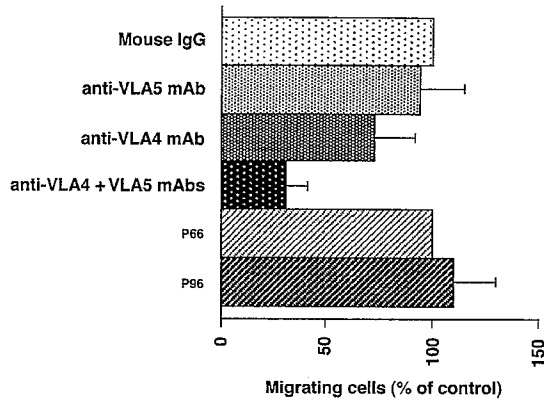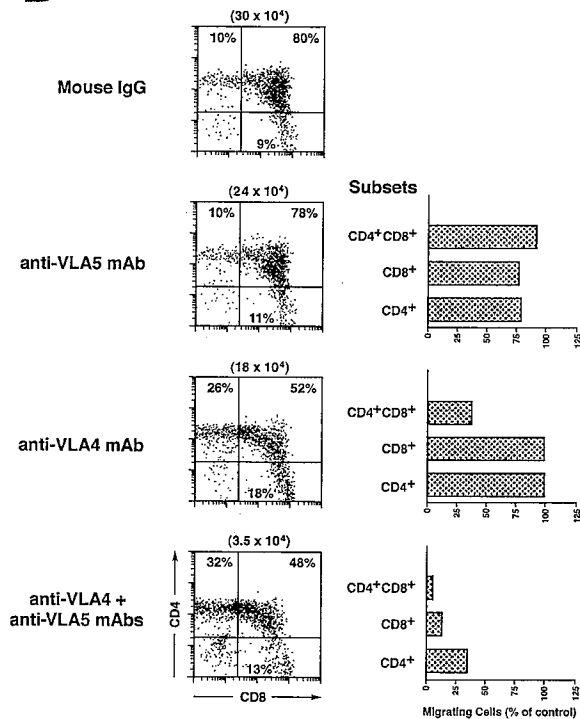
Figure 3. Distribution of FN, CFN and CS1 sequences within the human thymus. Frozen thymic sections were stained by indirect immunofluorescence using mAbs specific for plasma FN, cellular FN (*CFN*), and CS1. FN and CFN-specific immunoreactivity (*upper panels*) is predominantly detected in large patches within the medulla (*m*), whereas relatively less frequent positive structures are observed in the cortex (*cx*). CS1-specific staining (*lower left panel*) reveals a fine reticular pattern, strongly highlighting the medulla and sharply marking the cortico-medullary junction. Sparse CS1 positive areas are also observed in the cortex. The star identifies a Hassal's corpuscle. The lower right panel represents a section stained with a control IgM and secondary reagents. Similar background levels of fluorescence were observed using control IgGs (not shown). Bar, 22 μm.

Figure 6. Time-lapse video microscopy of thymocyte migration. Thymocytes migrating over a period of 8 h through Transwell™ membranes coated with 0.2 μg/ml of FN were labeled with the red fluorescent carbocyanine DiI, whereas thymocytes that remained in the upper chamber were labeled with the green fluorescent carbocyanine DiO. The two cell populations were then mixed at a 1:2 ratio and cultured on a glass microwell precoated with FN (0.2 μg/ml). Images were collected for both fluorochromes every 70 seconds for two consecutive periods of 2.5 h. The migratory activity of red- and green-labeled cell populations was first recorded in the presence of control mouse IgG (10 μg/ml) for 2.5 h (*A*). Anti-VLA4 and VLA5 mAbs (10 μg/ml) were then added to the well and cell migration recorded for additional 2.5 h (*B*). The migratory paths traced by individual thymocytes were obtained by projecting 130 images using the NIH Image software. In the presence of control IgG, cells from the lower chamber are observed to trace longer paths than those displayed by cells from the upper chamber. All migrating cells show a similar direction of movement. Addition of anti-VLA4 and anti-VLA5 mAbs arrests the migration of the majority of thymocytes. The few cells which are not inhibited display randomly oriented paths of migration.

A

Mouse IgG

anti-VLA5 mAb

anti-VLA4 mAb

anti-VLA4 + VLA5 mAbs

P66

P96

0   50   100   150

Migrating cells (% of control)

B

(30 x 10⁴)

Mouse IgG
10%   80%
9%

(24 x 10⁴)   **Subsets**

anti-VLA5 mAb
10%   78%   CD4⁺CD8⁺

CD8⁺

11%   CD4⁺

0   25   50   75   100 125

(18 x 10⁴)

anti-VLA4 mAb
26%   52%   CD4⁺CD8⁺

CD8⁺

18%   CD4⁺

0   25   50   75   100 125

(3.5 x 10⁴)

anti-VLA4 +
anti-VLA5 mAbs
32%   48%   CD4⁺CD8⁺

CD8⁺

13%   CD4⁺

0   25   50   75   100 125

CD8 →

Migrating Cells (% of control)

Figure 4. Thymocyte migration on intact FN involves engagement of the integrins VLA4 and VLA5. (A) Cooperatve signaling by VLA4 and VLA5 mediates thymocyte migration. Thymocytes were allowed to migrate across porous Transwell™ membranes coated with FN (10 μg/ml) in the presence of 10 μg/ml of the indicated antibodies or peptides. Cells migrating to the lower chamber of the Transwells™ by 8 h were harvested and counted. Results are expressed as percentage of migrating cells in the control experimental conditions (e.g., 100 × [no. of cells migrating in the presence of specific blocking Abs or peptide/no. of cells migrating in the presence of control mouse IgG or peptide]). Bars represent the mean ± SEM of four experiments. (B) Characterization of thymic subsets migrating in the presence or absence of anti-VLA4 and/or anti-VLA5 mAbs. Thymocytes were allowed to migrate across Transwell™ membranes coated with FN (10 μg/ml) in the presence of control mouse IgG or anti-integrin mAbs. After 8 h, thymocytes which migrated to the lower chamber were harvested, counted and analyzed by flow cytometry. The expression of CD4 and CD8 is shown in the dot plots and percentages of single positive and double positive subsets are indicated in the appropriate quadrants. Total number of thymocytes migrating in the different experimental conditions are shown in parentheses on top of dot plots.

Fig. 4 B shows a FACS® analysis representative of five similar experiments comparing the CD4/CD8 phenotype of thymocytes migrating in the presence or absence of anti-α4 and anti-α5 mAbs. We found that blocking VLA4 alone has little or no effect on the migration of either CD4 or CD8 single positive thymocytes. On the other hand, the anti-α4 mAb inhibits the migration of double positive cells by ∼60%. Three color immunofluorescence revealed that this latter subset comprises thymocytes expressing intermediate levels of CD3 (data not shown). The anti-α5 mAb inhibited the migration of all three subsets by ∼20% (Fig. 4 B). In contrast, blocking both VLA4 and VLA5 inhibited the migration of the CD4 single positive by 64% and the CD8 single positive by 85%. This treatment also inhibited migration of ∼90% double positive cells (Fig. 4 B).
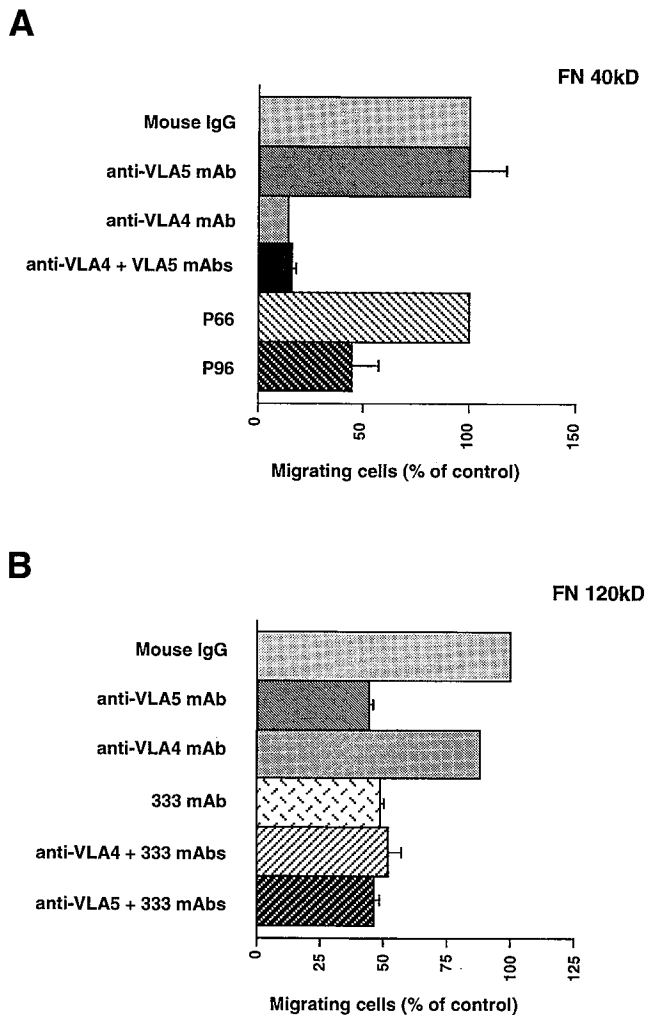
The results indicate that the VLA4 and VLA5 integrins play a major role in thymocyte migration on FN. In particular, the higher migratory efficiency of single positive thymocytes on this substrate appears to reflect the engagement and cooperative signalling of both receptors. Conversely, the double positive thymocytes contain two distinct populations defined by their use of VLA4 and VLA5. The motility of one population, expressing intermediate levels of CD3, is preferentially mediated by VLA4 alone, while a second population, comprising mostly CD3ʰⁱ cells, uses both integrins.

FN Binding Sites for VLA4 and VLA5-mediated Cell Migration. The medulla of the human thymus is highly enriched for FN relative to the cortex, particularly a form of FN containing the alternatively spliced CS1 site. However, other sites exist on FN which can be engaged by VLA4 (14, 41–43). To specifically determine the FN binding sites used by migrating thymocytes we tested two non-overlapping 40- and 120-kD chymotryptic fragments (13) for their ability to support migration. The 40-kD fragment of FN contains the CS1 peptide sequence for VLA4, but lacks the RGDS binding site for VLA5 (18). Conversely, the 120-kD fragment comprises only the RGDS binding site recognized by VLA5 (44).

As shown in Fig. 5 A, migration of thymocytes on the 40-kD fragment of FN was almost completely inhibited by anti-α4 mAb and not affected by anti-α5 mAb. The VLA4-specific blocking peptide, P96, but not the control P66, also inhibited migration, although less effectively. These differences in the inhibitory effect of blocking mAbs and peptides on β1 integrins are consistent with previous reports (45–47). Thymocyte migration on the 120-kD frag-

A

FN 40kD

Mouse IgG

anti-VLA5 mAb

anti-VLA4 mAb

anti-VLA4 + VLA5 mAbs

P66

P96

0   50   100   150

Migrating cells (% of control)

B

FN 120kD

Mouse IgG

anti-VLA5 mAb

anti-VLA4 mAb

333 mAb

anti-VLA4 + 333 mAbs

anti-VLA5 + 333 mAbs

0   25   50   75   100   125

Migrating cells (% of control)

Figure 5. Inhibition of thymocyte migration on the 40- (A) and 120-kD (B) chymotryptic fragments of FN. Thymocyte were allowed to migrate on Transwell™ membranes coated with the 40- or the 120-kD fragments of FN (10 μg/ml) containing the CS1 and RGDS binding sites, respectively. Migration assays were performed in the presence of 10 μg/ml of either control mouse IgG, anti-VLA5 and/or anti-VLA-4 mAbs, or peptides P66 and P96 (A). In addition, a mAb blocking the RGDS cell binding site of FN (mAb 333) was used in migration experiments performed on the 120-kD fragment (B). After 8 h, cells which migrated in the lower chamber were counted and data expressed as % of the cells migrating in control cultures. Bars represent the means ± SEM of four (A) and three (B) separate experiments.

ment was inhibited by ~50% using the anti-α5 but not the anti-α4 mAb (Fig. 5 B). In fact, when we coated the wells with an equimolar mixture of the 120- and 40-kD fragments we could demonstrate the nearly complete inhibition of migration as observed with the intact FN (data not shown). Migration was also inhibited by an anti-FN mAb, clone 333, which binds a site just proximal to the RGDS site and blocks cell binding (37). Thus, thymocytes appear to use the two primary binding sites on FN, CS1 for VLA4 and RGDS for VLA5. The partial inhibition of migration obtained by blocking VLA5 on the 120-kD fragment of

FN suggests that in the absence of a VLA4-mediated signal thymocyte migration may be mediated by an alternative mechanism.

Analysis of Thymocyte Migration by Time Lapse Video Microscopy. Time lapse video microscopy has several advantages for the investigation of cell migration. It permits the measurement of migration velocity for a single cell or subpopulations. In addition, it reveals the characteristics of migration with respect to direction, persistence of movement and the effects of blocking specific receptors. We separated thymocyte subpopulations demonstrating different migration activities using the Transwell™ migration assay. The more motile single positive and double positive thymocytes that segregate in the lower chamber were labeled with the red fluorescent dye DiI while the less motile double positive cells that remain in the upper chamber were labeled with the green fluorescent dye DiO. The two thymocyte subsets isolated by this means will be referred to as LC (lower chamber) and UC (upper chamber) cells. The two labeled subsets were then combined in a single FN coated well to study the migration of both subsets simultaneously.

Fig. 6 A shows the migration paths followed by individual LC (red) and UC (green) thymocytes over an observation period of 2.5 h. These results are representative of three experiments. Most LC thymocytes traced long paths across the entire field of observation. In contrast, a large number of UC cells made only limited movements around the point of initial binding to FN as evidenced by a blurring of the individual cell profiles as compared to the time 0 image. Although some UC cells were seen to migrate in this assay, they demonstrated shorter migration paths than those typical of the LC cells (mean distance covered within the field of observation ± SEM; UC cells = 224 μm ± 27; LC cells = 379 μm ± 41; $P = 0.005$). The persistence of cell movement is another characteristic of migrating cells. Video analysis of serial images, revealed that LC cells moved continuously, whereas UC cells were often pausing or arresting their movement during the observation period. This distinct behavior correlated with the significant difference in path lengths displayed by the two populations. Finally, determination of mean migration velocities revealed a value of 9.6 ± 1.8 μm/min for the LC cells and 7.2 ± 0.9 μm/min for the UC cells ($P = NS$).

A consistent observation in this series of experiments was that mobile cells of either subset maintain a similar and constant direction of movement (Fig. 6 A, bottom panel). To determine the effect of blocking the interaction with FN we added both anti-α4 and anti-α5 mAb to the cultures. These antibodies abruptly arrested the migration of the majority of thymocytes (Fig. 6 B). Interestingly, the few cells whose migration was not inhibited lose their original direction of movement and traced parabolic paths in random directions (Fig. 6 B, *bottom panels*).

Discussion

In the present study we demonstrate that the interaction of CD3^{hi} double positive and single positive cells with FN

signals migration of these more mature thymocytes. Furthermore, the amount of ligand regulates thymocyte motility such that at low concentrations of FN the migration of the most mature thymocytes is favored, whereas higher FN concentrations are required to promote the migration of less mature cells. The differential distribution of FN and CS1 binding sites that we document in the thymic medulla and cortex indicates that the expression and density of this ligand is in fact regulated in vivo, thereby providing a mechanism for controlling thymocyte migration between these two compartments. Finally, thymocyte migration involves engagement of both VLA4 and VLA5 to the CS1 and RGDS sequences of FN and both receptors are involved in maintaining the persistence and directionality of cell movement. Our results provide strong evidence for a FN/integrin-mediated pathway regulating thymocyte motility at the transition from the double positive to the single positive stage of maturation.

We have previously identified a unique population of immature CD3^{lo} double positive thymocytes characterized by firm adhesion to FN via VLA4 and proposed that VLA4/FN interactions regulate the positioning of immature thymocytes within the cortex (34). Indeed our present data demonstrate that thymocytes enriched for the more mature CD3^{hi} single positive and double positive cells migrate at high efficiency on FN, whereas most immature CD3^{lo} double positive cells appear to be stationary. Taken together, the adhesive and migratory characteristics of CD3^{lo} and CD3^{hi} thymic subsets suggest that cells which adhere avidly to FN (i.e., CD3^{lo} double positive cells) are unable to migrate, whereas those establishing low levels of adhesion to this substrate are motile. Human hematopoietic progenitors also demonstrate a selective interaction with FN such that the earliest colony-forming unit (CFU) cells are firmly adherent and lose this property with maturation (48). Similar results have been reported for bone marrow progenitors of the erythroid and B cell lineages (46, 49). However, these studies have only inferred that the biological significance of this loss of adhesion is the initiation of migration. Here we demonstrate that FN can provide both anchorage and migratory cues for thymic subsets at distinct stages of maturation. Thus, the regulation of both adhesion and migration to FN may be a conserved mechanism determining the position of developing progenitor cells and the subsequent release of their mature progeny from various hemopoietic compartments.

We observed that the migratory activity of thymocytes is influenced by the concentration of ligand. Compared to the migration observed on low concentrations of FN, higher concentrations of surface-bound FN induced a 10–15-fold increase in the number of migrating double positive and a 3–4-fold increase in single positive thymocytes. A similar site density–dependent efficiency of migration on FN has been documented for T lymphoblastoid cell lines (47), indicating that this phenomenon may regulate motility. Moreover, analysis of integrin expression comparing the migrating to the non-migrating cells (Table 1) demon-
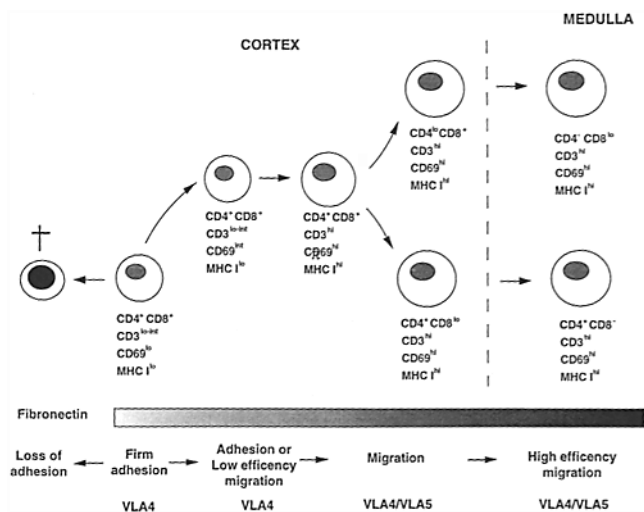
strates that cells migrating on relatively low concentrations of FN are enriched for single positives expressing only slightly less VLA4 than the non migrating population. In contrast, on the highest concentration of FN (5 µg/ml) a double positive population is recruited to migrate with higher levels of VLA4 than the non-migrating cells. Thus, receptor expression levels, alone, do not determine the differences in cell migration or adhesion. This is consistent with the current understanding that integrin function is the net effect of receptor expression level and receptor activity (30–33).

These data for migration on different concentrations of FN have two implications. First, they suggest that while the migratory capability of a given cell on FN depends on its maturation state, the amount of FN present in the microenvironment determines the efficiency of cell movement. In this context, capability refers to the intrinsic potential of a given cell to migrate, whereas efficiency represents the actual migration of the cell in response to a ligand. Second, migration determined by different concentrations of FN defines at least three functionally distinct double positive thymic populations: (a) CD3^{hi}CD69^{hi} migrating on low concentrations, (b) CD3^{int}CD69^{int} recruited by higher concentrations and (c) CD3^{lo-int}CD69^{lo} which do not migrate at all under these experimental conditions. Notably, double positive thymocytes which can be recruited to migrate by increasing amounts of FN, have the same intermediate levels of CD3 and MHC class I as the non-migratory double positive cells. However, they are distinguished by the higher levels of CD69. This result suggests that thymocytes may acquire the potential to migrate at a stage in double positive development marked by increased levels of expression of the activation marker CD69 (50). Overall, the data indicate that single positive and double positive thymic subsets, otherwise relatively homogeneous for phenotype, are in fact functionally heterogeneous populations with respect to their migratory activity.

The predominant distribution of FN in the medulla of the human thymus is consistent with previous reports (5, 51). We also present the novel finding that the medulla is highly enriched for an alternatively spliced form of FN containing the CS1 cell binding site. In light of our functional studies demonstrating the regulation of thymocyte migration by ligand concentrations and the requirement for binding to both the RGDS and the CS1 site, this pattern suggests that FN might in fact control the migratory efficiency of developing thymocytes in vivo. The lower abundance of FN and CS1 sites in the cortex would favor the stationary phenotype of immature double positive thymocytes preventing their premature movement to the medulla. Conversely, the high abundance of CS1-containing FN in the medulla may facilitate migration of the more mature single positive cells into this compartment and eventually export to the periphery. In addition, it may also provide a microenvironment favorable to the recirculation of peripheral T cells and entry of antigen-presenting cells into the medulla (4).

Although we have demonstrated that FN can support thymocyte migration, we do not exclude the possibility that other mechanisms regulate the movement of developing cells from one thymic compartment to another in vivo. VCAM-1 is an alternative ligand for VLA4 and supports adhesion with substantially higher avidity than FN (22, 52). We have recently demonstrated that VCAM-1 is expressed by thymic epithelial cells in the cortex and that this ligand mediates the adhesion of immature double positive thymocytes to thymic epithelial cells (Salomon, D.R., L. Crisa, C.F. Mojcik, J.K. Ishii, G. Klier, and E.M. Shevach, manuscript submitted for publication). These data suggest that the interaction between VLA4 and VCAM-1 may be an alternative adhesive pathway anchoring immature thymocytes within the cortex. On the other end, studies in rodents have determined that the residency of single positive thymocytes in the medulla may be quite long, ranging from 1 to 2 wk (53). During this time thymocytes acquire the ability to produce cytokines (54) and proliferate in response to antigen stimulation (55). Interactions holding thymocytes in the medulla are therefore crucial to the completion of these differentiation steps. Certain cytokines may influence lymphocyte motility (56). It has been shown that CD2/LFA-3 and LFA-1/ICAM-1 adhesive interactions may also be involved in thymocyte differentiation (57, 58). Interestingly, we observed that thymocyte migration on FN is enhanced by the addition of an antibody blocking ICAM-1. These results suggest that cell–cell interactions mediated by alternative pairs of adhesion molecules such as LFA-1 and ICAM-1 may be one molecular mechanism counter-balancing thymocyte motility.

The migration of CD4 and CD8 single positive thymocytes is mediated by a cooperative interaction between VLA4 and VLA5. In contrast, a substantial fraction (∼60%) of the mobile double positive thymocytes appears to use only VLA4 for migration. These double positive cells are relatively immature (e.g., CD3^{int}) and have the same phenotype as those which can be "recruited" to migrate on increasing amounts of FN. These data suggest that a functional modulation of VLA4 from a highly adhesive to a less adhesive state may be the earliest event initiating migration at the double positive stage. However, this mechanism may not be very efficient at promoting migration as it requires a high density of ligand. Ultimately, the high efficiency of migration exhibited by single positive thymocytes may reflect the upregulation of VLA5 at this later stage of development (34, 35 and Table 1) and the engagement of both VLA4 and VLA5. A similar cooperation of these integrins in mediating cell movement has been reported previously for highly motile neural crest cells (59). These studies further demonstrated that while binding to CS1 (presumably by VLA4) determines the speed of migration, binding to RGDS sequences by VLA5 is involved in maintaining the direction of migration (59), a result recently confirmed by studies using α5-transfected cells (60). In this regard, our analysis of thymocyte motility by time lapse video microscopy demonstrated that migrating thymocytes follow a per-

Figure 7. A model integrating the roles of fibronectin, VLA4 and VLA5 in thymocyte migration with the developmental changes occurring at the transition from the double positive to single positive stage of maturation.

sistent direction of movement which is disrupted by blocking VLA4 and VLA5. The data suggest that VLA4 and VLA5/FN interactions determine not only the occurrence of cell movement but also signal information for the persistence and directionality of migration.

We propose a model in which thymocyte adhesion and migration mediated by VLA4 and VLA5 is coordinated with development in the thymus (Fig. 7). (a) At the early CD3^{lo-int}CD69^{lo} double positive stage of maturation, thymocytes express a form of VLA4 that firmly anchors these cells to FN expressed on cortical epithelium. This binding may serve to stabilize TCR/MHC/co-receptor interactions during positive selection. (b) Successful positive selection signals thymocyte activation marked first by the upregulation of CD69. The functional state of VLA4 has changed on these cells to the extent that they could be recruited to migrate if exposed to high concentrations of FN containing the CS1 site. However, their location in the cortex, which is relatively poor in this form of FN, prevents migration at this stage. In parallel, the majority of double positive cells that are not selected at this stage progressively lose the capacity to adhere or migrate using VLA4 and undergo apoptosis. (c) Further maturation produces a double positive cell which is CD3^{hi}CD69^{hi}MHC I^{int-hi}. These cells begin to upregulate VLA5 expression and function (34, 35) which in collaboration with VLA4 initiates an efficient migration on the low concentrations of FN in the cortex. (d) At the late single positive stage of maturation, thymocytes have acquired the highest capacity to migrate by means of VLA4 and VLA5. The high concentration of FN and CS1 binding sites present in the medulla support this activity. While it is possible that single positive thymocytes are actively migrating in the medulla, it is more likely that other interactions contribute to hold thymocytes at this site and allow

their differentiation program to be completed. Nevertheless, their high migratory capability should allow them to efficiently exit the medulla when full maturation is finally achieved.

References

1. Jameson, S.C., K.A. Hogquist, and M.J. Bevan. 1995. Positive selection of thymocytes. *Ann. Rev. Immunol.* 13:93–126.
2. Huesmann, M., B. Scott, P. Kisielow, and H. von Boehmer. 1991. Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. *Cell.* 66:533–540.
3. Merkenschlager, M., C. Benoist, and D. Mathis. 1994. Evidence for a single-niche model of positive selection. *Proc. Natl. Acad. Sci. USA.* 91:11694–11698.
4. Rothenberg, E. 1992. The development of functionally responsive T cells. *Adv. Immunol.* 51:85–215.
5. Berrih, S., W. Savino, and S. Cohen. 1985. Extracellular matrix of the human thymus: Immunofluorescence studies on frozen sections and cultured epithelial cells. *J. Hystochem. Cytochem.* 33:655–664.
6. Boyd, R.L., C.L. Tucek, D.I. Godfrey, D.J. Izon, T.J. Wilson, N.J. Davidson, A.G.D. Bean, H.M. Ladyman, M.A. Ritter, and P. Hugo. 1993. The thymic microenvironment. *Immunol. Today.* 14:445–459.
7. Hynes, R.O. 1990. Fibronectins. Springer-Verlag, New York.
8. Springer, T.A. 1990. Adhesion receptors of the immune system. *Nature (Lond.).* 346:425–434.
9. Molossi, S., M. Elices, T. Arrhenius, R. Diaz, C. Coulber, and M. Rabinovitch. 1995. Blockade of very late activation antigen-4 integrin binding to fibronectin with connecting segment-1 peptide reduces accelerated coronary arteriopathy in rabbit cardiac allografts. *J. Clin. Invest.* 95:2601–2610.
10. Grinnel, F., and T.V. Phan. 1983. Deposition of fibronectin on material surfaces exposed to plasma: quantitative and biological studies. *J. Cell. Physiol.* 116:289–296.
11. McCarthy, J.B., M.L. Basara, S.L. Palm, D.F. Sas, and L.T. Furcht. 1985. The role of cell adhesion proteins-laminin and FN in the movement of malignant and metastatic cells. *Cancer Metastasis Rev.* 4:125–152.
12. Thiery, J.P., J.L. Duband, and A. Delouvee. 1982. Pathways and mechanisms of avian trunk neural crest cell migration and localization. *Dev. Biol.* 93:324–343.
13. Ruoslahti, E., E.G. Hayman, E. Engvall, W.C. Cothran, and W.T. Butler. 1981. Alignment of biologically active domains in the fibronectin molecules. *J. Biol. Chem.* 256:7277–7281.

14. Yamada, K.M. 1991. Adhesive recognition sequences. *J. Biol. Chem.* 266:12809–12812.
15. Ruoslahti, E. 1988. Fibronectin and its receptors. *Annu. Rev. Biochem.* 57:375–413.
16. Pytela, R., M.D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140 kD cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell.* 40:191–198.
17. Guan, J., and R.O. Hynes. 1990. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha4\beta1$. *Cell.* 60:53–61.
18. Wayner, E.A., A. Garcia-Pardo, M.J. Humphries, J.A. McDonald, and W.G. Carter. 1989. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain CS1 in plasma fibronectin. *J. Cell Biol.* 109:1321–1330.
19. Kornblihtt, A.R., K. Umezawa, K. Vibe-Pedersen, and F.E. Baralle. 1985. Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1755–1759.
20. FFrench-Constant, C. 1995. Alternative splicing of fibronectin. Many different proteins but few different functions. *Exp. Cell Res.* 221:261–271.
21. Boucaut, J., T. Darribere, T.J. Poole, H. Aoyama, K.M. Yamada, and J. Thiery. 1984. Biologically active synthetic peptides as probes of embryonic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest migration in avian embryos. *J. Cell Biol.* 99:1822–1830.
22. Mould, A.P., J.A. Askari, S.E. Craig, A.N. Garratt, J. Clements, and M.J. Humphries. 1994. Integrin $\alpha4\beta1$-mediated melanoma cell adhesion and migration on Vascular Cell Adhesion Molecule-1 (VCAM-1) and the alternatively spliced IIICS region of fibronectin. *J. Biol. Chem.* 269:27224–27230.
23. Elices, M.J., V. Tsai, D. Strahl, A.S. Goel, V. Tollefson, T. Arrhenius, E.A. Wayner, F.C.A. Gaeta, J.D. Fikes, and G. Firestein. 1994. Expression and functional significance of alternative spliced CS1 fibronectin in rheumathoid arthritis microvasculature. *J. Clin. Invest.* 93:405–416.

24. Wahl, S.M., J.B. Allen, K.L. Hines, T. Imamichi, A.M. Wahl, L.T. Furch, and J.B. McCarthty. 1994. Synthetic fibronectin peptides suppress arthritis in rats by interrupting leukocyte adhesion and recruitment. *J. Clin. Invest.* 94:655–659.

25. Ferguson, T.A., H. Mizutani, and T. Kupper. 1991. Two integrin-binding peptides abrogate T cell mediated immune responses in vivo. *Proc. Natl. Acad. Sci. USA.* 88:8072–8076.

26. Hershberger, R.P., and L.A. Culp. 1990. Cell-type-specific expression of alternatvely spliced human fibronectin IIICS mRNAs. *Mol. Cell. Biol.* 10:662–671.

27. Xia, P., and L.A. Culp. 1995. Adhesion activity in fibronectin's alternatively spliced domain ED_a (EIIIA): complementarity to plasma fibronectin function. *Exp. Cell Res.* 217:517–527.

28. Damsky, C.H., and Z. Werb. 1992. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr. Opin. Cell Biol.* 4:772–781.

29. Chan, B.M., P.D. Kassner, J.A. Schiro, H.R. Byers, T.S. Kupper, and M.E. Hemler. 1992. Distinct cellular functions mediated by different VLA integrin a subunit cytoplasmic domains. *Cell.* 68:1051–1060.

30. Hynes, R.O. 1992. Integrins: versatility, modulation, and signalling in cell adhesion. *Cell.* 69:11–25.

31. Faull, R.J., N.L. Kowach, J.M. Harlan, and M.H. Ginsberg. 1993. Affinity modulation of integrin $\alpha5\beta1$: regulation of the functional response by soluble fibronectin. *J. Cell Biol.* 121:155–162.

32. Masumoto, A. and M.E. Hemler. 1993. Multiple activation states of VLA-4. Mechanistic differences between adhesion to CS1/fibronectin and to vascular cell adhesion molecule-1. *J. Biol. Chem.* 268:228–234.

33. Yednock, T.A., C. Cannon, C. Vandevert, E.G. Goldbach, G. Shaw, D. Ellis, C. Liaw, L.C. Fritz, and L.I. Tanner. 1995. $\alpha4\beta1$ integrin-dependent cell adhesion is regulated by a low affinity receptor pool that is conformationally responsive to ligand. *J. Biol. Chem.* 270:28740–28745.

34. Salomon, D.R., C.F. Mojcik, A.C. Chang, S. Wadworth, D.H. Adams, J.E. Coligan, and E.M. Shevac. 1994. Constitutive activation of integrin $\alpha4\beta1$ defines a unique stage of human thymocyte development. *J. Exp. Med.* 179:1573–1584.

35. Mojcik, C.F., D.R. Salomon, A.C. Chang, and E.M. Shevach. 1995. Differential expression of integrins on human thymocyte subpopulations. *Blood.* 86:4206–4217.

36. Straus, A.H., W.G. Carter, E.A. Wayner, and S. Hakomory. 1989. Mechanisms of fibronectin-mediated cell migration: dependence or independence of cell migration susceptibility on RGDS-directed receptor (integrin). *Exp. Cell Res.* 183:126–139.

37. Nagai, T., N. Yamakawa, S. Aota, S.S. Yamada, S.K. Akiyama, K. Olden, and K.M. Yamada. 1991. Monoclonal antibody characterization of two distinct sites required for function of the central cell-binding domain of fibronectin in cell adhesion, cell migration and matrix assembly. *J. Cell. Biol.* 114:1295–1305.

38. Lawrence, M.B., and T.A. Springer. 1991. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell.* 65:859–873.

39. Kavanaugh, A.F., E. Lightfoot, P.E. Lipsky, and N. Oppenheimer-Marks. 1991. Role of CD11/CD18 in adhesion and transendothelial migration of T cells. Analysis utilizing CD18-deficient T cell clones. *J. Immunol.* 146:4149–4154.

40. Altevogt, P., M. Hubbe, M. Ruppert, J. Lohr, P. von Hoe-gen, M. Sammar, D.P. Andrew, L. McEvoy, M.J. Humphries, and E.C. Butcher. 1995. The $\alpha4$ integrin chain is a ligand for $\alpha4\beta7$ and $\alpha4\beta1$. *J. Exp. Med.* 182:345–355.

41. Iida, J., A.P.N. Skubitz, L.T. Furcht, E.A. Wayner, and J.B. McCarthy. 1992. Coordinate role for cell surface chondroitin sulfate proteoglycan and $\alpha4\beta1$ integrin in mediating melanoma cell adhesion to fibronectin. *J. Cell Biol.* 118:431–444.

42. Dedhar, S., and V. Gray. 1990. Isolation of a novel integrin receptor mediating Arg-Gly-Asp-directed cell adhesion to fibronectin and type I collagen of human neuroblastoma cells. Association of a novel $\beta1$-related subunit av. *J. Cell. Biol.* 110:2185–2193.

43. Vogel, B.E., G. Tarone, F.G. Giancotti, J. Gailit, and E. Ruoslahti. 1990. A novel fibronectin receptor with an unexpected sequence (av$\beta1$). *J. Biol. Chem.* 265:5934–5937.

44. Pierschbacher, M.D., E.G. Hayman, and E. Ruoslahti. 1981. Location of the cell attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. *Cell.* 26:259–267.

45. Yamada, K.M., D.W. Kennedy, S.S. Yamada, H. Gralnick, W. Chen, and S.K. Akiyama. 1990. Monoclonal antibody and synthetic peptide inhibitors of human tumor cell migration. *Cancer Res.* 50:4485–4496.

46. Yanai, N., C. Sekine, H. Yagita, and M. Obinata. 1994. Roles of integrin Very Late Activation Antigen-4 in stroma-dependent erythropoiesis. *Blood.* 83:2844–2850.

47. Chan, P., and A. Aruffo. 1993. VLA-4 integrin mediates lymphocyte migration on the inducible endothelial cell ligand VCAM-1 and the extracellular matrix ligand fibronectin. *J. Biol. Chem.* 268:24655–24664.

48. Verfaillie, C.M., J.B. McCarthy, and P.B. McGlave. 1991. Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clonogenic progenitors is accompanied by alterations in their interactions with fibronectin. *J. Exp. Med.* 174:693–703.

49. Roldan, E., A. Garcia-Pardo, and J.A. Briera. 1992. VLA-4-fibronectin interaction is required for the terminal differentiation of human bone marrow cells capable of spontaneous and high rate immunoglobulin secretion. *J. Exp. Med.* 175:1739–1747.

50. Bendelac, A., P. Matzinger, R.A. Seder, W.E. Paul, and R.H. Schwartz. 1992. Activation events during thymic selection. *J. Exp. Med.* 175:731–742.

51. Reza, J.N., and M.A. Ritter. 1995. Differential expression of adhesion molecules within the human thymus. *Dev. Immunol.* 4:55–60.

52. Makarem, R., P. Newham, J.A. Askari, L.J. Green, J. Clements, M. Edwards, M.J. Humphries, and A.P. Mould. 1994. Competitive binding of vascular cell adhesion molecule-1 and the HepII/IIICS domain of fibronectin to the integrin $\alpha4\beta1$. *J. Biol. Chem.* 269:4005–4011.

53. Scollay, R., and D.I. Godfrey. 1995. Thymic emigration: conveyor belts or lucky dips? *Immmunol. Today* 16:268–274.

54. Rothenberg, E.V., K.L. McGuire, and P.D. Boyer. 1988. Molecular indices of functional competence in developing T cells. *Immunol. Rev.* 104:29–50.

55. Chang, J.F., C.A. Thomas, and J.T. Kung. 1991. Mitogen-induced IL2 production and proliferation at defined stages of T helper cell development. *J. Immunol.* 147:860–865.

56. Levesque, J., D.I. Leavesley, S. Niutta, M. Vadas, and P.J. Simmons. 1995. Cytokines increase human hemopoietic cell adhesivness by activation of Very Late Antigen (VLA)-4 and VLA-5 integrins. *J. Exp. Med.* 181:1805–1815.

57. Carlow, D.A., N.S.C. van Oers, S. Teh, and H. Teh. 1992. Deletion of antigen-specific immature thymocytes by dendritic cells requires LFA-1/ICAM interactions. *J. Immunol.* 148:1595–1603.

58. Fine, J.S., and A.M. Kruisbeek. 1991. The role of LFA-1/ICAM-1 interactions during murine T lymphocyte development. *J. Immunol.* 147:2852–2859.

59. Dufour, S., J. Duband, M.J. Humphries, M. Obara, K.M. Yamada, and J. Thiery. 1988. Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2661–2671.

60. Bauer, J.S., C.L. Schreiner, F.G. Giancotti, E. Ruoslahti, and R.L. Juliano. 1992. Motility of fibronectin receptor deficient cells on fibronectin and vitronectin: collaborative interactions among integrins. *J. Cell. Biol.* 116:477–487.