Constitutive Activation of Integrin α4β1 Defines a Unique Stage of Human Thymocyte Development

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Summary
Our understanding of thymocyte development and of the positive and negative selection events involved in shaping the repertoire of mature T lymphocytes has been greatly facilitated by the use of transgenic and gene knockout animals. Much less is known about the factors that control the homing and population of the thymus by T cell precursors and the subsequent migration of developing thymocytes through the thymic architecture. As the integrins represent a candidate group of cell surface receptors that may regulate thymocyte development, we have analyzed the expression and function of α4β1 and α5β1 on human thymocytes. A major portion of double positive (CD4+CD8+) human thymocytes express α4β1 in a constitutively active form and adhere to fibronectin and vascular cell adhesion molecule 1. α4β1 expression is similar on adherent and nonadherent populations, thus, activity reflects the receptor state and not simple expression. The adherent cells are immature, expressing high levels of CD4/CD8 and low levels of CD3 and CD69. In contrast, nonadherent cells possess the phenotype of thymocytes after positive selection, expressing intermediate levels of CD4 and/or CD8 and high levels of CD3 and CD69. The adherent population fails to respond to activation with anti-CD3 and fibronectin, whereas nonadherents exhibit an r proliferation. Differential regulation of α4β1 and α5β1 receptors may provide a mechanism controlling cellular traffic, differentiation, and positive selection of thymocytes.

The normal development of mature T lymphocytes requires the successful completion of a complex maturation process that begins with CD4−CD8−CD3− cells in the thymus (1−4). These precursors rearrange their TCR genes and express low levels of TCR-α/β with the CD3 complex, CD4 and CD8 (5, 6). The next stage of development involves positive selection of those CD4+CD8+ double positive (DP)1 cells which express TCR capable of recognizing antigens in the context of self-MHC molecules. Positive selection of DPs expressing MHC class II-restricted TCRs involves an accessory signal delivered by CD4 engagement whereas selection of class I-restricted TCRs involves CD8 (7). Although a large fraction of the DPs die without undergoing positive selection, when positive selection is successful, the DPs increase their expression of the TCR/CD3 complex, the CD69 antigen (8, 9), and progressively decrease either CD4 or CD8 (10, 11). Subsequently, negative selection results in the deletion of autoreactive thymocytes (12). Although T cell maturation in the thymus can be followed by changes in the expression of multiple cell surface antigens, surprisingly little is known about the factors that control the homing and population of the thymus by T cell precursors and the subsequent migration of developing thymocytes through the thymic architecture from cortex to medulla during which the critical signals for selection and maturation are delivered. It is likely that these events are regulated at different stages of thymocyte development by manipulating the activity of specific cell surface receptors. In turn, these receptors direct and facilitate thymocyte interactions with various components of the thymic stroma including cortical and medullary epithelium, antigen presenting or dendritic cells, and the extracellular matrix (ECM) proteins of the interstitium.

One candidate group of cell surface receptors that may con-

1 Abbreviations used in this paper: AD, adherent; DN, double negative; DP, double positive; ECM, extracellular matrix; FN, fibronectin; ICAM, intercellular adhesion molecule; MFI, mean fluorescence intensity; NAD, nonadherent; SP, single positive; UNS, unseparated; VCAM-1, vascular cell adhesion molecule 1.
The function of the integrins in cell–cell and cell–ECM interactions can be changed by regulating their level of cell surface expression (13, 25). However, cells can also modulate the binding properties of the integrins without changing their level of expression (17, 26). On T lymphocytes, αLβ2 is not constitutively avid for ICAM-1, but is converted to a high avidity state after cell activation by phorbol ester or by stimulation of the TCR (27). Similarly, cell activation is required to upregulate the activity of αβ1, α5β1, and α6β1 on resting mature peripheral T cells (20). A number of mechanisms have been identified that may control how an integrin is transcribed and directed movement of peripheral blood T lymphocytes on extracellular matrix proteins including collagen, fibronectin (FN), and laminin (16, 17). Specifically, αβ1 is a receptor for FN and α6β1 is a receptor for laminin (16, 18–20). Moreover, αβ1 can bind to vascular cell adhesion molecule 1 (VCAM-1), a member of the Ig gene superfamily (21–22). Thymocytes also uniformly express high levels of the leukocyte-specific β2 chain integrin, αLβ2 (15, 16, 23), often called LFA-1. The ligands for αLβ2 are integral membrane proteins of the Ig gene superfamily intercellular adhesion molecules (ICAM) 1, 2, and 3 (13). ICAM-1 is expressed by thymic epithelial cells and its expression can be upregulated by a number of inflammatory cytokines (e.g. IL-1, TNF, and IFN-γ) (24).

Our understanding of thymocyte development has largely been derived from murine model systems and has been greatly facilitated by the use of transgenic and knockout animals (7, 50). However, a detailed investigation of the expression and function of integrins in murine T cell development has not been possible because of the lack of appropriate reagents. In this paper, we have analyzed both the expression and function of αβ1 and αβ5 on human thymocytes. We demonstrate that human DP thymocytes express high levels of αβ1 and that a major portion (40–50%) of the DP cells exhibit constitutive activation of αβ1 as measured by adhesion to both FN and VCAM-1. The constitutively adherent (AD) DP thymocytes expressed higher levels of CD4/CD8 and lower levels of CD3 and CD69 compared with the nonadherent (NAD) DP, which is consistent with the phenotype of less mature thymocytes. The NADs expressed similar levels of αβ1, relatively lower levels of CD4/CD8, higher levels of CD3 and CD69, and were also enriched for the CD4hi CD8lo and CD4loCD8hi “transition” cells that define the phenotype of DP thymocytes that have undergone or are in the process of undergoing positive selection (10, 11). Stimulation of the ADs with the combination of TCR cross-linking and plate-bound FN failed to induce T cell proliferation, whereas the NADs proliferated vigorously to this combination. Although the NADs continued to express high levels of αβ1, their proliferative response was mediated predominantly by αβ5. In sum, our results demonstrate that a unique population of immature DP human thymocytes can be defined and separated by expression of an activated form of the β1 integrin, αβ1. These DP cells appear poised at a stage just before positive selection, a process that results in the loss of αβ1 activity without significant change in its surface ex-
pression concomitant with marked changes in the expression of CD3, CD4/CD8, and CD69. Models for the integration of these new findings into current paradigms for thymic T cell development will be discussed.

Materials and Methods

Antibodies. The following antibodies were used: TS2/7 anti-\(\alpha\beta 1\) (T Cell Diagnostics, Cambridge, MA), G9 anti-\(\alpha 2\beta 1\) (Amac, Inc., Westbrook, ME), P1B5 anti-\(\alpha 3\beta 1\), P1B5 anti-\(\alpha 5\beta 1\) (GIBCO BRL, Gaithersburg, MD), HP2/1 anti-\(\alpha 4\beta 1\) (Amac, Inc.), L25 anti-\(\alpha 4\beta 1\) (Becton Dickinson, San Jose, CA), P1D6 anti-\(\alpha 5\beta 1\) (GIBCO BRL), SAM1 anti-\(\alpha 5\beta 1\) (Amac, Inc.), GoH3 anti-\(\alpha 6\beta 1\) (Amac, Inc.), mAb16 anti-\(\alpha 5\beta 1\) and mAb13 anti-\(\beta 1\) (generous gifts of Drs. K. Yamada and S. Akiyama, National Institutes of Health), Leu 23 (L78) anti-CD69 (Becton Dickinson), OKT3 anti-CD3 (American Type Culture Collection, [ATCC], Rockville, MD), 13B8.2 anti-CD-4/CD40 biotin or PE (Amac, Inc.), B9.11 FITC control (Amac, Inc.), mouse IgG1 and IgG2 isotype controls (Amac, Inc.), 18.5 IgG2 isotype control (ATCC), and purified mouse IgG (Sigma Chemical Co., St. Louis, MO).

Cytophotometric Analysis. Flow cytometry was performed on a FACSScan® (Becton Dickinson) and analyzed with Lysis II software. Three-color detection involved FITC, PE, and Tricolor (Caltag Laboratories, South San Francisco, CA) excited at 488 nm. List mode data were collected on 4 x 10⁴ cells and data is expressed as log mean fluorescence intensity (MFI).

Fresly prepared thymocytes (10⁴ cells/ aliquot) in HBS/3% FCS/0.1% NaAzide were incubated with 2 μg per tube of purified mAb or 1:100 dilution of ascites for 45 min at 4°C. Next, cells were incubated with 50 μl per tube of a 1:300 dilution of PE-labeled goat F(ab)² anti-mouse affinity-purified antibody (Tago Immunologicals, Burlingame, CA). Unbound secondary antibody sites were then blocked by 20 μg per tube of purified mouse IgG (Sigma Chemical Co.). For 4 min followed by addition of FITC-labeled mouse anti-human CD8 and biotin-conjugated mouse anti-human CD4 (Amac, Inc.) for 45 min. The final step was addition of SA-Tricolor (Caltag Laboratories) for 30 min. At least two isotype-matched control antibodies were used. Compensation and quadrant parameters were set with both controls using PE-labeled anti-mouse as well as directly labeled FITC and biotin-conjugated control antibodies with SA-Tricolor. Nonviable cells were excluded by incubation with propidium iodide and by setting an additional gate at the extreme range of FL3.

Cell Separation, Adhesion, and Proliferation. Fresh thymus fragments were obtained during cardiac surgery on children (aged 1 mo to 3 yr) with congenital valvular malformations. Thymocytes were prepared by physical disruption of tissue over a 60-mesh stainless steel screen and separated by ficoll-diatrizoate gradient (Organon Teknika, Durham, NC). Separation by adhesion was done with 100 x 20 mm plates (Costar Corp., Cambridge, MA) coated by 10 μg/ml of purified human FN (Calbiochem-Novabiochem Corp., La Jolla, CA) for 4 h at 37°C, blocked with 1% heat denatured BSA, and then incubated with thymocytes in serum-free media (AIM-V, GIBCO BRL) for 1 h at 37°C. After three washes the adherent cells were removed with gentle agitation and EDTA (1:5,000 Versene, GIBCO BRL). Separation into CD4⁺ and CD8⁻ subsets was done by two rounds of negative selection with either anti-CD4 or anti-CD8 magnetic beads (Dynal, Inc., Great Neck, NY) to a purity of 98% by flow cytometry.

FN-adhesion assays were performed in triplicate in 96-well microtiter plates (Dynatech Labs., Inc., Chantilly, VA) coated with human FN at 4°C overnight and blocked with 2.5% BSA (17). ¹⁸⁵Cr-labeled cells (5 x 10⁵/well) at 37°C were added in Medium 199 (GIBCO BRL) with 0.5% BSA and incubated for 1 h at 37°C. Antibody blocking studies were done at 1-2 μg/ml of mAb though antibody titrations from 0.1-10 μg/ml were also done with similar results (data not shown). Adhesion to rVCAM-1 was done in 48-well microtiter plates (Costar Corp.) coated overnight at 4°C with 2.8 μg/ml purified rVCAM-1 (modified from reference 51). After blocking with 2.5% BSA, the ¹⁸⁵Cr-labeled cells were added and incubated for 90 min at 37°C. Data for all adhesion assays are expressed as percent specific adhesion after subtraction of the BSA control.

Cell proliferation assays were performed in triplicate in 96-well microtiter plates (Costar Corp.) coated with anti-CD3 antibody overnight followed by FN for 4 h at 37°C. Thymocytes at 2 x 10⁵ cells/well were cultured in serum-free media for 4 d, pulsed with [³H]Tdr (1 μCi/well) for the final 12 h, harvested onto glass filter paper, and counted by liquid scintillation. In assays testing for mAb inhibition of proliferation, the plates were also blocked with 1% heat denatured BSA and the media was supplemented with 20 μg/ml of purified polyclonal mouse IgG to exclude any artifact from introduction of Ig protein to the cultures in serum-free media. For mitogen activation the combination of PMA (10 ng/ml) and ionomycin (200 ng/ml) or PHA (5 μg/ml), alone, were added from serum-free stock solutions.

Results

Expression of β1 Integrins on Human Thymocyte Subsets. As an initial approach to determining a role for the β1 integrins during thymocyte maturation, we used three-color flow cytometry to study the levels of expression of these integrins on the major thymocyte subsets (Fig. 1). αβ1 and α2β1 were expressed at very low but consistently detectable levels by the DN and SPs. The levels of expression of α3β1, α5β1, and α6β1 were very similar and were higher on the SPs, particularly the CD4⁺ cells, than on the DPs. The very small DN population could be divided into two distinct populations that were positive or negative for these three integrins. These results are not consistent with previous reports on human thymus in which the expression of α3β1 and α5β1 were described as negligible and the levels of α1β1 and α2β1 were not detectable (14–16). However, some of these studies were performed on frozen sections, cryopreserved thymocytes, or thymocytes that had been incubated in culture media overnight. When we analyzed frozen thawed thymocytes, we only detected α4β1 and α6β1 at levels substantially lower than those expressed on the fresh populations.

Nonetheless, the integrin most prominently expressed on human thymocytes was α4β1; DP cells expressed the highest level, whereas the CD8 SPs expressed <50% the level of that found on the DPs. The CD4 SPs demonstrated two distinct populations. When gated separately, the majority population had an α4β1 level similar to that of the CD8 SPs. The DN cells, comprising <3% of human thymocytes, could be divided into an α4β1 negative subpopulation and a population that expressed a level of α4β1 similar to that of the SPs. About 3% of human thymocytes are γδ T cells, many of which will express the DN phenotype. We have determined that the expression of
α4β1 and α5β1 by thymic γ/δ T cells is at least equivalent to the mature SP α/β thymocytes (data not shown). Thus, it is likely that the integrin-positive population of DNs is substantially comprised of γ/δ T cells.

A Subpopulation of Thymocytes Demonstrates Constitutive Adhesion to FN Mediated Predominantly by α4β1. The α3β1 and α6β1 integrins are receptors for the ECM protein, laminin, whereas α3β1, α4β1, and α5β1 are receptors for FN as well as other ligands (13). These integrins on peripheral T lymphocytes exist in a low affinity state and cell activation is required to upregulate their affinity of binding (20, 42). Because of their expression on the majority of the human thymocytes, we next evaluated the capacity of human thymocytes to adhere to FN or laminin-coated plates. Surprisingly, approximately half of the thymocytes adhered to plate-bound FN in the absence of TCR-mediated or phorbol ester activation (Fig. 2 A). The adherent thymocytes represented almost entirely DPs (only 2% SPs) with relatively high mean CD4 and CD8 expression as compared with the NADs that were significantly enriched for the SPs and intermediate CD4/CD8-

![Figure 1. Flow cytometry of human thymocytes. Three-color flow cytometry on human thymocytes consistently revealed the CD4/CD8 profile depicted in the top frame (n = 6). 2 x 10^5 thymocytes were analyzed for each frame and gates were set after setting compensation first with a directly labeled FITC control (FL1: CD8), a biotin-labeled control plus SA-Tricolor (FL3: CD4) and three isotype-matched mouse antibody controls with anti-mouse PE-labeled antibody (FL2: integrin). Histograms represent the log mean fluorescent intensities of integrin expression within the individual CD4/CD8 quadrants: DN, DP, CD4SP, and CD8 SP. The anti-α4 antibody clone was HP2/1 and the anti-α5 was SAM 1.]

![Figure 2. Thymocyte adhesion to FN and VCAM-1. Freshly prepared thymocytes were ^51Cr-labeled and incubated on plates coated with either FN (A and C) or VCAM-1 (B) with or without addition of the specified mAbs or peptides. (A and B) Thymocytes plated without any activation; (C) Thymocytes preactivated with PMA (10 ng/ml) for 30 min before plating. All FN and VCAM-1 plates were also blocked with 2.5% BSA before the adhesion step. The background binding to plates coated only with BSA (~7-10%) was subtracted and only percent specific adhesion is depicted here. (*) Values too low to graph.]

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The range of constitutive FN adhesion has been from 45 to 55% in six similar experiments (expressed as percent specific adhesion). Very low constitutive adhesion to laminin was observed (~10%, data not shown). Adhesion was completely inhibited by the addition of either anti-α4 or anti-β1 (Fig. 2 A). The latter result excludes the possibility that an alternative α4 receptor such as α4β7 is involved (52). The addition of anti-α5 mAb (SAM 1 or MAB 16) had no effect on constitutive adhesion nor did addition of anti-α1, 2, or 3 mAbs (data not shown). The α5β1 integrin recognizes an RGDS site contained in the cell-binding region of FN (53), whereas α4β1 utilizes an alternative CS-1 peptide located in the heparin-binding region (18, 34, 43, 54). Further evidence that α4β1 was the predominant integrin mediating constitutive adhesion of human thymocytes to FN was obtained by demonstrating that soluble CS-1, but not RGD peptide, could inhibit constitutive adhesion (Fig. 2 A). RGE and a “scrambled” sequence version (CS-1s) of CS-1 served as negative peptide controls. We also evaluated the binding of nonstimulated human thymocytes to VCAM-1. Approximately 40% of the human thymocytes demonstrated constitutive adhesion to purified recombinant VCAM-1-coated plates (Fig. 2 B). This adhesion was partially inhibited by anti-α5 and completely by anti-β1 mAbs. In contrast, α4β1-mediated adhesion of peripheral blood T cells to VCAM-1 is activation dependent (55).

To determine the role of activation which is so critical in the adhesion of peripheral T cells, we repeated these experiments with PMA-activated thymocytes. Approximately 75–85% of the thymocytes adhered to FN monolayers after activation (Fig. 2 C) and the AD cells included the majority of SPs. Activation-dependent adhesion was inhibited by >50% by anti-α4 (HP2/1) and 100% by anti-β1 mAbs. Whereas anti-α5 mAb (SAM 1) had no effect alone, the combination of anti-α5 and anti-α4 mAbs completely inhibited adhesion. This last result indicates that some component of PMA-activated adhesion is also mediated by α5β1 in collaboration with α4β1. The failure of the CS-1 peptide to inhibit the activation-dependent adhesion (Fig. 2 C) is likely secondary to the involvement of α5β1 in the binding or to the relatively poor ability of this reagent to inhibit α4β1-mediated binding. Nonetheless, the much higher levels of α4β1 receptor expression as compared with α5β1 (Fig. 1 and see Fig. 4) and the fact that anti-α5 alone had no inhibitory effect on adhesion of naive or PMA-activated cells suggests that the predominant FN receptor on human thymocytes is α4β1. This is in contrast to the mature peripheral T lymphocytes in which the predominant FN receptor is an activation-dependent α5β1.

**Constitutive Adhesion to FN Is a Property of a Subpopulation of Less Mature, CD31~176 hi Thymocytes.**

Our observation that 50% of thymocytes from multiple donors constitutively adhered to FN strongly suggested that only a subpopulation of thymocytes was capable of adhesion. Therefore, we separated thymocytes into AD and NAD populations by constitutive adhesion to FN-coated plates and characterized their surface antigen phenotype (Fig. 3). The ADs were essentially all DP (97%, Fig. 3 A) with no enrichment for DNs as reported for murine thymocytes (48, 49). Although the NADs were also predominantly DP (74%), the NAD population was enriched for both SP CD4 and CD8 cells. More importantly, when the DP populations were compared by gating on the entire DP quadrant, the mean levels of CD8 and particularly CD4 expression on the ADs were significantly higher than on the NADs.
higher than on the NADs (see MFIs, Fig. 3 A). The lower mean levels of CD4 and CD8 expression by the DP NADs were, in part, secondary to an enrichment of DP transition cells which express intermediate levels of staining for either CD4 or CD8. These CD4hiCD8lo and CD4hiCD8hi transition cells resemble the "streak" cells recently described in class II–deficient transgenic mice as defining an intermediate level of maturation before mature SPs (11). The cell gates shown in Fig. 3 B were chosen to exclude the transition cells and determine the expression of either CD4 or CD8 by only the CD4hiCD8lo (DPhi) subpopulation. These results are expressed as histograms in Fig. 3 C. It is striking that the MFI of CD4 is still lower on the DPhi NADs (open area) as compared with the DPhi ADs (shaded area) whereas the levels of CD8 expression are equivalent. Thus, even the true DPhi cells can be separated into two overlapping populations after adhesion to FN as defined by levels of CD4 expression. These results also suggest that CD4 expression may be more rapidly downmodulated after positive selection which is supported by the large CD4hiCD8hi population evident in the DP NADs (Fig. 3 A). In this context it is interesting to note that developing human thymocytes have been reported to express CD4 before CD8 in the transition from DN to DP (56) and that a population of early murine thymocyte precursors are also CD4+ (57).

Additional evidence in favor of the hypothesis that constitutive adhesion to FN defines an early maturation stage of DP thymocyte development was obtained by comparison of the levels of CD3 and CD69 on the DP FN ADs and NADs (Fig. 4). In both murine and human models of thymocyte development, the increased expression of these two markers correlates with a more advanced developmental stage for DPs

(8, 9). The DP NADs expressed a higher number of cells with intermediate to high CD3 expression and CD69-positive cells than the DP ADs (Fig. 4, open areas). Some of the increase in the levels of expression of CD3 and CD69 was correlated with an increased percentage of the CD4hiCD8lo and CD4hiCD8hi transition cells in the NAD population. However, increased levels of CD3 and CD69 expression were also observed when we gated exclusively on the CD4hiCD8hi population (Fig. 4, shaded areas). Thus, the DP population of maturing human thymocytes can be separated by the property of constitutive adhesion to FN and this separation correlates with maturation level by differential expression of CD3, CD69, CD4, and CD8. We also determined the surface phenotype of the thymocytes separated by constitutive adhesion to plate-bound recombinant VCAM-1. Similar to the FN ADs and NADs, the VCAM-1 ADs were almost all DPs and demonstrated fewer intermediate to high CD3- and CD69-positive cells as compared with the NADs (data not shown).

One possible explanation for the separation of thymocytes into FN AD and NAD populations is a differential level of expression for α4β1. However, in multiple experiments, the DP FN ADs exhibited only a slightly higher level of expression of α4β1 when compared with the NADs. This was true whether the entire DP population was analyzed as depicted in Fig. 4 (open areas; mean channel 128 vs. 109) or when only the DPhi were gated (shaded areas; mean channel 111 vs. 83). Similarly, the DP ADs and NADs expressed comparable levels of α5β1, though the NADs were more heterogeneous in expression. The expression of α5β1 in Fig. 4 is somewhat less than that shown for the unseparated DPs in Fig. 1 but this was due to donor variation and a lower FL2 channel gain setting in this experiment and not to any difference in affinity of the anti-α5β1 antibodies used (e.g., SAM1 vs. P1D6). It is consistent that the expression of α4β1 is also somewhat less, and these results were confirmed in multiple similar experiments. The expression of the integrin αLB2 (LFA-1) was the same on the ADs and NADs (data not shown).

Only FN NADs Proliferate when Stimulated with Anti-CD3 and FN. Proliferation of peripheral blood T cells can be induced by relatively high concentrations of plate-bound anti-CD3 antibody or by suboptimal concentrations of anti-CD3 if a costimulatory signal is provided by plate-bound ECM proteins such as FN (19, 42, 43). In contrast to peripheral blood T cells, unseparated thymocytes or purified CD4 and CD8 SPs failed to proliferate when stimulated with plate-bound anti-CD3 over a wide range of concentrations (data not shown). However, when FN was coimmobilized with anti-CD3, the unseparated and the purified CD4 and CD8 SP populations demonstrated significant proliferative responses (Fig. 5 A). Moreover, the proliferative response of the CD4 SP population to intact FN was much greater than that of the unseparated thymocytes and the purified CD8 SPs. Proliferation was dependent on concomitant TCR cross-linking by anti-CD3 because plate-bound FN without anti-CD3 antibody failed to induce proliferation (data not shown). The addition of PMA did not supplant the requirement for TCR
cross-linking despite the fact that PMA stimulation significantly increased thymocyte binding to FN including a population using the α5β1 receptor (Fig. 2 C). Flow cytometry of thymocytes activated by the combination of anti-CD3 and FN was performed at initiation of culture and at 96 h. This demonstrated that the proliferating cells increased their CD3 and CD69 expression, increased the relative number of both CD4 and CD8 SPs, yet still demonstrated at least 50% DPs, particularly of the intermediate CD4/CD8 subset (data not shown). Thus, the phenotype of the input population does change during the culture period which is consistent with the observations that the majority of proliferation can be attributed to the more mature SPs.

The proliferative responses of all the subsets were markedly inhibited by the addition of anti-α5 (SAM 1 or MAB16), but not by anti-α4 mAb (Fig. 5 B). These results were confirmed with separated CD4 and CD8 SPs. However, the combination of both mAbs did result in higher inhibition at limiting concentrations of each antibody, suggesting that α4β1 may supply some collaborative signal for activation in concert with the critical α5β1-derived signal. Nonetheless, only the RGDS-containing 120-kD FN fragment (α5β1 specific), but not the CS-1-containing 40-kD fragment (α4β1 specific), could provide the costimulation required for measurable thymocyte proliferation (Fig. 5 A). Thus, thymocyte proliferation to TCR cross-linking and FN is predominantly α5β1 dependent.

Thymocytes separated on the basis of constitutive adhesion to FN were also tested for their ability to be stimulated by plate-bound anti-CD3 and FN (Fig. 6). The FN ADs failed to proliferate, whereas the FN NADs showed an enhanced proliferative response relative to the unseparated cells. However, both the FN ADs and NADs proliferated equally well when triggered with the combination of PMA and ionophore (Fig. 6). Thus, although the ADs express a constitutively functional form of α4β1 that can bind FN, they have not reached the maturational stage of the NADs where they can be triggered via their TCR even in the presence of FN as a costimulus. In contrast, the NADs which include all the SPs and do not demonstrate constitutive activation of α4β1, do have the capability of TCR-mediated activation of their α5β1 receptors, such that these cells can proliferate to the combination of plate-bound OKT3 and FN.

Discussion

We have identified a unique subset of immature DP human thymocytes defined by their expression of a constitutively activated form of the β1 integrin, α4β1, which mediates the binding of these cells to both FN and VCAM-1. This subset of DPs is characterized by high levels of CD4 and CD8 expression but low to intermediate levels of CD3 and CD69. In parallel, we have described a more mature population of

| MAb µg/ml | UNS | CD4+ | CD8+ | No ECM |
|-----------|-----|------|------|--------|
| 0         | 250000 | 200000 | 150000 | 50000 |
| 0.001     | 220000 | 180000 | 140000 | 30000 |
| 0.01      | 190000 | 150000 | 110000 | 20000 |
| 1         | 160000 | 120000 | 80000  | 5000  |

Figure 5. Proliferation to plate-bound anti-CD3 and FN. (A) The proliferation ([3H]Tdr incorporation) of unseparated (UNS) and purified CD4+ or CD8+ thymocytes after a 72-h culture in plates coated with both anti-CD3 and intact FN or the FN fragments: 120-kD (RGDS site) and 40-kD (CS-1 site). Cells cultured with anti-CD3 and no FN (No ECM line) gave <10,000 cpm backgrounds. The results are representative of six similar experiments. All proliferation assays were performed in serum-free media. (B) The dose titrations for antibody blocking of unseparated thymocytes proliferating to anti-CD3 and FN, though similar results for mAb inhibition were also obtained with purified CD4+ and CD8+ cells. The mAb control was clone 18.5 (IgG2), anti-αβ5 was SAM 1 (similar results obtained with MAB 16) and anti-α4 was HP2/1. Proliferation without any added mAb was ~60,000 cpm.

| MAb µg/ml | UNS | ADs | NADs |
|-----------|-----|-----|------|
| 0.001     | 320000 | 280000 | 240000 |
| 0.01      | 280000 | 240000 | 200000 |
| 1         | 240000 | 200000 | 160000 |

Figure 6. Proliferation of FN ADs and NADs. UNS, AD, or NAD thymocytes were cultured in serum-free media for 72 h on plates coated with anti-CD3 and FN (FN) or with PMA/ionomycin (PMA/ION). The proliferation of cells (PH]Tdr incorporation) in media, alone (data not shown), or with anti-CD3 and no FN (No ECM) was <1,000 cpm. (*) Values too low to graph.
DP thymocytes that have lost the constitutive function of α4β1 without a significant change in its surface expression and that express lower levels of CD4 and/or CD8 and higher levels of CD3 and CD69. In addition, we have demonstrated that the FN NAD population which contains SP thymocytes has acquired the ability to proliferate to TCR cross-linking, but only if a costimulatory signal is provided by plate-bound FN or the RGDS-containing 120-kD fragment of FN. Costimulation is mediated primarily by a second FN receptor, α5β1. These studies demonstrate that integrin expression and function mark specific stages in human thymocyte development and suggest a direct involvement of regulated integrin function in thymic T cell development.

Our results appear to be markedly different from those previously reported for both the expression and function of α4β1 on murine thymocytes (48, 49). The expression of α4β1 decreased significantly as murine thymocytes progressed from the DN to the DP stage and constitutive binding of murine thymocytes to FN was primarily, but not exclusively, a property of DN thymic precursors. Thus, it appeared that the high absolute levels of α4β1 expression on DN murine thymocytes determined their ability to adhere to FN. However, it is difficult to directly compare these results with our studies as the differential expressions of α4β1, CD4, CD8, CD3, and CD69 were not examined for the murine DP cells adherent and nonadherent to FN. It does appear that the majority of DN murine thymocytes express α4β1 in a constitutively active state and that a similar population of cells is not present in human thymus. Thus, activation of α4β1 may also be important in the differentiation of murine thymocytes, but its role may be restricted to an earlier stage of thymocyte development.

A relationship between the constitutive function of an integrin receptor and maturation stage has been previously observed in both lymphoid (58) and nonlymphoid cells (59). For example, human peripheral blood B lymphocytes resemble peripheral T lymphocytes in that they express high levels of α4β1, but are unable to bind to the CS-1 site in FN unless activated (60). In contrast, human bone marrow–derived B cell precursors demonstrated α4β1-mediated constitutive binding to VCAM-1 on the surface of bone marrow–derived fibroblasts (61). Similarly, early CFU spleen cells obtained from mice expressed α4β1 and could be separated from bone marrow mixture by constitutive adherence to the CS-1 peptide site of FN (62). Pretreatment of bone marrow cells with anti-α4β1 antibodies blocked subsequent colony formation in the spleen as well as medullary hematopoiesis. Similarly, anti-α4β1 has been shown to block lymphopoiesis and to retard myelopoiesis in long-term cultures of mouse bone marrow (63). Collectively, these studies provide strong evidence for the presence of constitutively active α4β1 on a stem cell population of the B lineage in both human and murine bone marrow.

Our observations on the developmental regulation of α4β1 on human thymocytes in many respects resembles the type of regulation reported for several different β1 integrins (α2, α3, and α5β1) on human keratinocytes (59, 64). Terminal differentiation of keratinocytes in culture is accompanied by a loss of adhesiveness to several ECM proteins including type IV collagen and FN (64). More importantly, the downmodulation of α5β1-mediated adhesion to FN can be separated into an initial stage when the receptor loses its ability to bind to FN and a second stage at which the receptor is lost from the cell surface (40). Although the absolute level of β1 integrin expression correlates well with later stages of keratinocyte differentiation, the early transition from stem cells to "transit amplifying cells," is accompanied by the same modest decrease in β1 integrin expression (59, 65), but absolute loss in function, that we have observed in the human DP thymocyte population for α4β1. A major difference between the differentiation of skin cells and thymocytes appears to be that the loss of integrin function is followed by a complete loss of β1 integrin expression during terminal differentiation of keratinocytes whereas there is only a modest decrease in expression of α4β1 and a parallel increase in expression of α5β1 in mature SP thymocytes.

The α4β1 integrin recognizes distinct binding sites on FN and VCAM-1 (21, 22) and, under suboptimal conditions for adhesion the binding of α4β1 to the CS-1 peptide and VCAM-1, may be differentially regulated (66). VCAM-1 is expressed by cytokine-activated endothelial cells (67, 68) and is an important determinant of leukocyte adhesion and function during inflammation. Therefore, the adherence of DP thymocytes to VCAM-1 may have important implications for thymocyte development. VCAM-1 is expressed on some nonendothelial stromal elements in the bone marrow (69), and thus it is possible that VCAM-1 expressed on stromal or epithelial elements in the thymus may be the true ligand for α4β1 on thymocytes. An activation-dependent upregulation of VCAM-1 on thymic endothelium or epithelium may occur and involve cytokine production which has been described in certain thymic epithelial cultures (70). VCAM-1 has been found on dendritic cells in the germinal centers of lymph nodes (71). It is possible that VCAM-1 is expressed on dendritic cells in the thymus and may therefore play a role in the signaling events involved in negative selection.

In addition to the role of α4β1 in mediating the adhesion of the less mature DP thymocytes, it should be emphasized that the induction of a proliferative response in the more mature FN NAD population was completely dependent on a costimulatory signal mediated via α5β1. Furthermore, in contrast to peripheral blood T cells which can be induced to proliferate by stimulation with high concentrations of immobilized anti-CD3, even the most mature SP thymocytes required costimulation by plate-bound FN at all concentrations of anti-CD3. Thus, α5β1 may play a critical role in T cell differentiation by regulating the proliferative expansion of certain maturing thymocyte clones. In contrast, the major function of α4β1 appears to be the regulation of cellular traffic for the immature DPs.

How can we integrate these new observations with human thymocytes into the current paradigms of thymocyte development that have derived from murine models? The objective of thymocyte maturation is the development of a stable
peripheral T cell repertoire responding to antigenic challenges in the context of self-MHC restricting elements. Although we have not as yet directly proven that the FN-ADs differentiate into FN-NAD, we propose that differential regulation of the expression and function of α4β1 and α5β1 provides a mechanism by which thymocytes move from the cortex to the medulla and interact with both thymic epithelium and bone marrow-derived APCs, presumably at distinct sites and stages of maturation. Our results are most consistent with the possibility that α4β1-mediated adhesion of the immature DPs to FN and/or VCAM-1 expressed on thymic epithelial surfaces or to ECM in close proximity facilitates positive selection. Successful positive selection is followed by a substantial increase in TCR/CD3 and CD69 expression with a parallel reduction in either CD4 or CD8 expression. These DP intermediate or transition cells are enriched in the FN NAD population and are the precursors of the mature SPs. Loss of constitutive α4β1 activity after positive selection would allow the maturing DPs to move into the medulla where engagement with marrow-derived APCs or medullary epithelium may occur. Upregulation of TCR/CD3 expression by these maturing cells also marks the capacity to respond to TCR cross-linking with proliferation if costimulation is provided by FN via α5β1 facilitating the clonal expansion of mature thymocytes after negative selection in preparation for their transport to the periphery.

We have used the term “constitutive” to describe the activated phenotype of α4β1 and to reflect the experimental observation that the immature DP population demonstrates adhesion to plate-bound FN or VCAM-1 without any activation step. We can suggest two models to explain this constitutive activity and its significance in thymocyte maturation (Fig. 7). It is possible that immature thymocytes during maturation from the DN to the DP stage express a constitutively activated form of α4β1 because they do not possess the regulatory elements in the cell membrane or cytoplasm (28, 72, 73) necessary to inhibit the activated conformation (Fig. 7, MODEL I). The binding of α4β1 would facilitate the interaction of the TCR with its ligand, presumably expressed on the surface of cortical epithelium, and lead to positive selection and the upregulation of TCR/CD3 and CD69 expression. Successful positive selection would be followed by a signal to deactivate the integrin and allow the maturing T cell to disengage from the cortical epithelium. If positive selection was unsuccessful the cell would remain fixed in the cortex by α4β1 and apoptosis. Alternatively, the earliest DP precursors

Figure 7. Two models for the role of α4β1 in positive selection. (Model I) The early DP thymocytes express a constitutively active form of α4β1 and use this integrin to bind FN expressed on or near the surface of cortical epithelium. By stable adhesion to FN the α4β1 facilitates the interaction of the few TCRs expressed with MHC/ligand. If successful positive selection occurs, the α4β1 receptor switches to the inactive form and the cell detaches as a more mature TCR hiCD69 hi DP cell prepared for migration and/or negative selection. (Model II) The early DP thymocytes express an inactive form of α4β1. A positive interaction between the TCR and MHC/ligand on the cortical epithelium permits activation of α4β1 but is insufficient to allow full cell activation. The activated α4β1 stabilizes the DP cell on the cortical epithelium permitting full activation and completing the positive selection stage. Again, after full activation, the α4β1 receptor changes to the inactive form and the cell detaches to permit the next stage in maturation.
may express α4β1 in the inactive state similar to PBLs (Fig. 7, MODEL II). DP cells whose TCR had sufficient affinity to bind to self-MHC for positive selection would use a preliminary but unstable encounter to activate α4β1 in a manner analogous to the TCR-mediated activation of mature peripheral T cells. Activation of α4β1 would then permit a stable interaction with self-MHC on the epithelial surface and allow the maturing thymocyte to receive the microenvironment-derived signals required to complete positive selection. Failure to activate α4β1 by positive selection would prevent delivery of these maturation signals and the cell would apoptosis. Completion of maturation would be marked by increases in TCR/CD3 and CD69 expression and the subsequent loss of α4β1 activity would be consistent with the transient nature of integrin receptor activation described in several model systems (13, 17). This model would account for the presence of some immature DP TCR/CD3+ cells in the FN NAD population whose TCR lacked sufficient affinity for positive selection and which were therefore destined to die.

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