

# Epithelial Cell-Specific Laminin 5 Is Required for Survival of Early Thymocytes<sup>1</sup>

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The gene *LamC2* encoding the  $\gamma_2$  chain of laminin 5, an epithelial cell-specific extracellular matrix protein, was identified in a PCR-based subtracted cDNA library from mouse thymic stromal cells. The mRNA existed in two alternative forms (5.1 and 2.4 kb). The full-length message was highly expressed in SCID thymus and in a nurse cell line, but not in other thymic epithelial cell lines, while the short form was more widely expressed. *In situ* hybridization and immunohistochemical staining revealed laminin 5 expression mostly in the subcapsular region of the adult thymus. Addition to fetal thymic organ cultures of a cell adhesion-blocking mAb to the  $\alpha_3$  chain of laminin 5 interrupted T cell development. There was a 40% reduction in the total yield of thymocytes, and the most profound decrease (75–90%) was seen in the CD25<sup>+</sup>CD44<sup>+</sup> and CD25<sup>+</sup>CD44<sup>-</sup> subsets of the CD4<sup>-</sup>CD8<sup>-</sup> double negative fraction. Most of the surviving double negative thymocytes expressed Sca-1, and there were significant increases in the number of cells with CD69 expression and in the fraction of annexin V-stained cells. None of these changes were observed with a nonblocking anti-laminin  $\alpha_3$  chain mAb. These results suggest that the interaction between double negative thymocytes and laminin 5 made by subcapsular epithelial cells is required for the survival and differentiation of mouse thymocytes. *The Journal of Immunology*, 2000, 165: 192–201.

Extracellular matrix (ECM)<sup>3</sup> molecules such as fibronectin, laminin, collagen, and glycosaminoglycans are important components of the thymic microenvironment (reviewed in Ref. 1). These secreted molecules are thought to support the growth and development of thymocytes and epithelial cells, as well as serve as an anchoring substratum to bring these two cell types together for functional interactions. In cell culture, these molecules have also been shown to play a role in cell migration by providing anchoring sites (2) with a specific release mechanism mediated by selective protease cleavage (3, 4). Thymocytes undergoing development need to interact with specific thymic stromal cells in a defined order. To achieve this in an efficient manner, it is necessary for them to travel to different areas of the thymus for the various stages of their development. Consistent with this idea, one observes that most double negative (DN) thymocytes are located in the subcapsular region of the thymus, while double positive (DP) thymocytes are found throughout the cortex, and single positive (SP) thymocytes are found in the medulla (5).

Accumulating evidence suggests that the laminin family of ECM proteins plays a key role in the local interactions between some thymocytes and stromal cells. These proteins are secreted by endothelial and epithelial cells, and they are found associated with basement membranes and hemidesmosomes (6, 7). In the thymus, certain isoforms of laminin have been localized to the subepithelial basement membrane of the capsule (8, 9) and to the medulla (10). These molecules are not found in the cortex, except in the basal laminae of large blood vessels (8, 9, 11). Laminins are recognized by integrin receptors, which are found on both T cells and epithelial cells (8, 11–16). Interestingly, only a subset of immature (heat stable Ag<sup>high</sup>) thymocytes binds to laminin *in vitro* (15, 17), and this subset also uniquely expresses the  $\alpha_6\beta_4$  integrin receptor (12). The binding *in vitro* is inhibited by Ab to both  $\alpha_3$  and  $\alpha_6$  integrins (15, 17). DP thymocytes do not bind to laminin *in vitro*, although they still express some  $\alpha_6$ -containing integrins (12). Furthermore, cortical epithelial cells do not express  $\alpha_3$  and  $\alpha_6$  integrins (11). These observations suggest that laminin plays an important role in early thymocyte development, but possibly not during later events such as positive selection. To date, however, there have been no functional studies to test this hypothesis.

We previously created a PCR-based subtractive cDNA library from isolated fetal thymic stromal cells to generate new molecular reagents to study mouse thymic stromal cells (18). One of the gene fragments we isolated, thymic stromal origin (TSO)-2D1, was chosen for further study based on its selective expression in a thymic epithelial cell line with the phenotype of a nurse cell. The sequence of TSO-2D1 showed strong homology to the human *LamC2* gene of laminin 5, formerly called B2t, (19), and near identity to the mouse *LamC2* (20), which encodes the  $\gamma_2$  chain of mouse laminin 5. Laminin 5 is a well-known epithelial cell-specific protein, previously called by several different names: epiligrin, kalinin, ladsin, and nicein, (6, 21, 22). It is comprised of three polypeptide chains,  $\alpha_3$ ,  $\beta_3$ , and  $\gamma_2$ , which are the products of different genes. The  $\beta_3$  and  $\gamma_2$  chains are unique components of laminin 5, while the  $\alpha_3$  chain is known to be used in two other laminin isoforms (6 and 7)

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<sup>3</sup> Abbreviations used in this paper: ECM, extracellular matrix; DN, double negative; DP, double positive; SP, single positive; TSO, thymic stromal origin; FTOC, fetal thymic organ culture; Sca-1, stem cell Ag 1; PI, propidium iodide.

(6). The  $\gamma_2$  chain is a homologue of the  $\gamma_1$  chain (formerly called B2) from which the N-terminal domain is missing and domains III, IV, and V are shortened (20). In the skin, laminin 5 forms the anchoring filaments that link epithelial cells to the basement membrane by bridging the  $\alpha_6\beta_4$  or  $\alpha_3\beta_1$  integrin receptors on the cells to other forms of laminin (1, 6, and 7) in the lamina densa layer of the membrane (7). Mutations in the human genes encoding each of the chains of laminin 5 can result in the junctional form of epidermolysis bullosa, a recessively inherited blistering skin disorder in which the epidermal layer separates from the dermal layer (23, 24). Interestingly, laminin 5 behaves as a multifunctional protein in that it can act under certain circumstances as a motility signal and at other times as an adhesive component (2, 3, 25). Thus, its expression in epithelial cells in the thymus raised the possibility that this protein might play some specific role in T cell development.

In this report, we localize the expression of the *LamC2* gene to the subcapsular region of the adult mouse thymus and show that a cell-adhesion blocking anti-laminin 5 mAb interferes with the development of T cells in fetal thymic organ culture (FTOC). The CD25<sup>+</sup>CD44<sup>+</sup> and CD25<sup>+</sup>CD44<sup>-</sup> DN thymocytes were severely reduced in number, and this reduction was associated with increased expression of stem cell Ag I (Sca-I) and CD69 as well as increased binding to annexin V on the remaining DN cells. These observations suggest that the thymocytes are dying early in their development and that interaction with laminin 5 is critical for their survival and differentiation.

## Materials and Methods

### Mice and cells

Fetal thymii of 14.5 day's gestation were obtained from timed matings of C57BL/6 (B6) mice (National Cancer Institute, Frederick, MD). The day of appearance of the vaginal plug was counted as day 0. C.B-17 mice bearing the SCID mutation were bred in our animal facility (National Institute of Allergy and Infectious Disease, Frederick, MD). SV40-transformed thymic epithelial cell lines from transgenic animals expressing the SV40 T Ag were the kind gift of Dr. Barbara Knowles (The Jackson Laboratory, Bar Harbor, ME). The five cell lines used were classified as follows: thymic subcapsular/nurse cell (427.1.86), cortico-reticular epithelial cell (1308.1.86), medullary dendritic cell (6.1.1), medullary epithelial cell (1307.6.1.7), and an unidentified medullary cell type (1307.1.11) (26). The characteristics of line 427.1.86 used to call it a nurse cell were the presence of tonofilaments, intermediate filaments, and desmosomal-like structures in electron micrographs, as well as low MHC class I expression and staining with a panel of thymic epithelial cell mAb, such as 3OH.12 and A2B5, specific for subcapsular cells. The fibroblast cell line, NIH 3T3, and the thymic lymphoma line, EL-4, were obtained from the American Type Culture Collection (Manassas, VA). Thymic stromal cells were prepared by trypsinizing the thymic rudiment of 2-deoxyguanosine-treated FTOC (27, 28) and plating the cells into petri dishes.

### Antibodies

The 2.4.G2 mAb (29) was either prepared by growing the hybridoma obtained from the American Type Culture Collection in a Bioreactor, Cell-Pharm 100 (Unisyn Technologies, Hopkinton, MA) and collecting the culture supernatant or prepared from ascites fluid after injecting an aliquot of the hybridoma cells into BALB/c *nu/nu* mice. Anti-human laminin 5 mouse mAb, blocking (P3H9-2, IgG1) and nonblocking (P3E4, IgG1), were obtained from Chemicon International (Temecula, CA). These monoclonals were generated by immunizing mice with cultured human keratinocytes and selecting for Ab reactive against affinity-purified laminin 5 (epiligrin) (21). They did not react with human fibronectin, placental laminin, or collagen types I and III. Both Ab were shown to react with the  $\alpha_3$  chain of laminin 5 by Western blotting (30), but only P3H9-2 blocked the adhesion of the HUT 78 T cell line to purified laminin 5 and to ECM formed by human foreskin keratinocytes (21).

### Isolation of the cDNA

A SCID thymus cDNA library (31) was constructed in a LambdaZapII vector (Stratagene, San Diego, CA) using mRNA isolated (mRNA purification Kit; Pharmacia, Washington, DC) from thymii of 4- to 6-wk-old C.B-17 SCID animals. The cDNA library was screened using a [<sup>32</sup>P]dATP-

labeled 1.0-kb fragment isolated from the PCR-based subtractive library described previously (31).

### In situ hybridization

Newborn and 4- to 6-wk-old thymus specimens from C57BL/6 mice were fixed in 4% paraformaldehyde solution and embedded in paraffin, and 5- $\mu$ m sections were prepared (American Histolab, Gaithersburg, MD). After deparaffinization, the sections were treated with proteinase K (10  $\mu$ g/ml) for 15 min at room temperature, and endogenous alkaline phosphatases were inactivated using 0.2 N HCl (32). For generation of the probes, *LamC2*-specific primers containing T3 and T7 initiation sequences were designed by examining a matrix comparison of the *LamC1* and *LamC2* nucleotide sequences to look for nonidentical regions. The forward primer, ImC2F1T3, extends from nucleotide 637 to nucleotide 660 and includes the T3 site: GCAATTAACCCTCACTAAAGGGCCGACTTCAGTGTCCACAAAATC. The backward primer, ImC2B5T7, extends from nucleotide 1217 to nucleotide 1194 and includes the T7 site: GTAATACGACTCAC TATAGGGCAATTGTCCCTTGTACCCAGCAGG. These primers only generate probes that have no homology to any other genes as determined by basic local alignment search tool (BLAST) search (33) and detect only the larger message of the *LamC2* gene. RNA probes were generated using an in vitro transcription system (Boehringer Mannheim, Indianapolis, IN). T3 RNA polymerase was used to generate the sense probe and T7 RNA polymerase was used to generate the anti-sense probe. Hybridizations with the probes were performed using 50% formamide, 10 mM Tris-HCl, pH 7.6, 200  $\mu$ g/ml tRNA, 1 $\times$  Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, and 1 mM EDTA at 50°C for 16 h in a humidified chamber. Slides were washed with 2 $\times$  SSC containing 50% formamide at 55°C for 30 min and then rinsed at 37°C for 10 min in TNE (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA). Nonhybridized transcripts were digested with 20  $\mu$ g/ml RNase A (Sigma, St. Louis, MO) in TNE at 37°C for 30 min. The slides were washed in TNE at 37°C for 10 min, then once with 2 $\times$  SSC at 50°C for 20 min, and twice with 0.2 $\times$  SSC at 50°C for 20 min.

Detection of in situ hybridization was conducted using the Genius Detection system (Boehringer Mannheim). Specifically, transcripts were detected with an anti-digoxigenin mAb conjugated to alkaline phosphatase in solution 1 (0.1 M Maleic acid and 0.15 M NaCl). The slides were washed several times with solution 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl<sub>2</sub>) and then immersed in the color-development solution (0.3 mg/ml nitroblue tetrazolium and 0.15 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M NaHCO<sub>3</sub>) (Boehringer Mannheim). Color development was stopped by immersing the slides in solution 4 (10 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA).

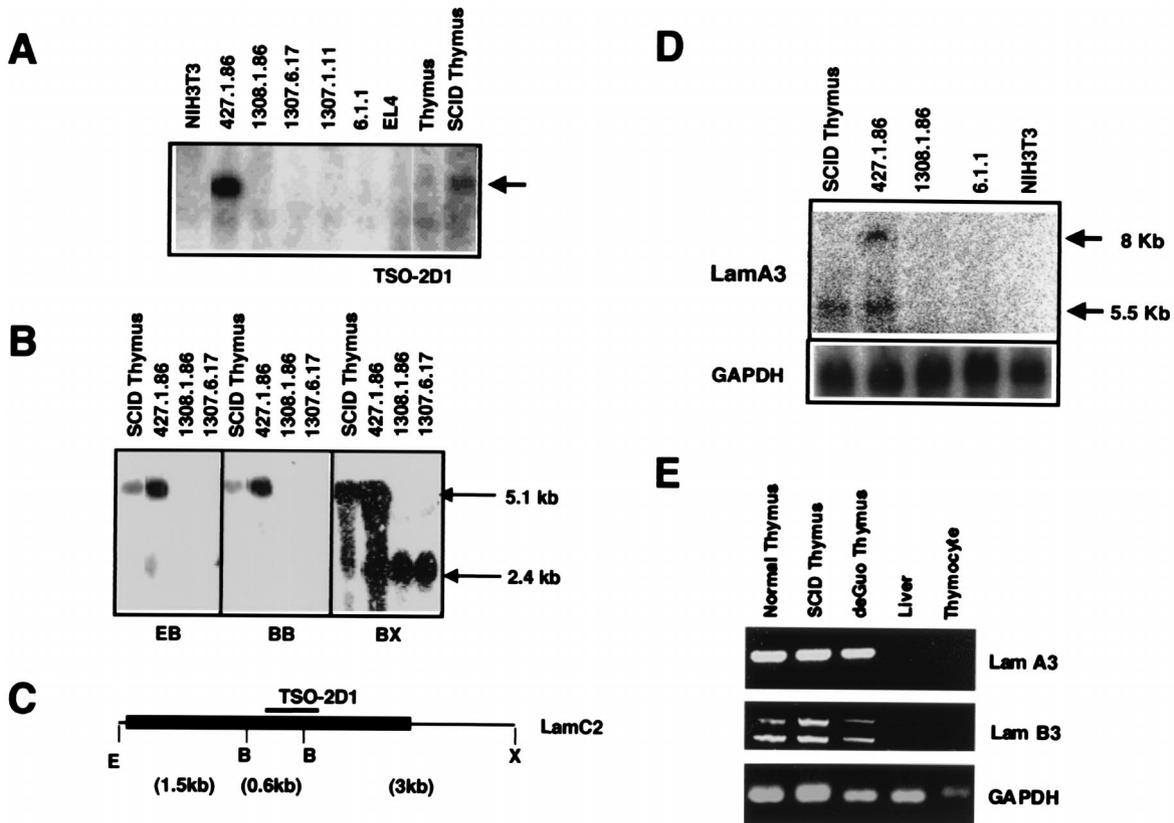
### Immunohistochemical and immunocytochemical staining

Tissue sections of thymii from newborn mice and 4-wk-old mice (C57BL/6) were deparaffinized and hydrated. Endogenous peroxidases were then inactivated using 3% hydrogen peroxide for 10 min (34). The sections were blocked using normal goat serum, and three-layer immunolabeling was performed. The sections were incubated for 1 h with the primary Ab: two anti-human laminin 5 mAb, the P3H9-2 blocking mAb, or the P3E4 nonblocking mAb, followed by incubation with a biotinylated secondary anti-mouse IgG Ab and then streptavidin-peroxidase labeling. The immunolocalization was visualized by diaminobenzidine (HistoMark; Kirkegaard & Perry Laboratories, Gaithersburg, MD). For the negative control, the sections were not incubated with the primary Ab.

The 427.1.86 cell line was grown in a four-well chamber slide for 1 day (Nalge Nunc International, Naperville, IL), washed with PBS, and fixed with 3.7% paraformaldehyde in PBS for 2 h. The wells were then rinsed four times with PBS and incubated in a blocking solution (10% goat serum in PBS) for 20 min. Primary Ab diluted with the blocking solution was then added overnight at 4°C. After washing the cells three times, FITC-conjugated anti-mouse rat IgG1 (R2-40; PharMingen, San Diego, CA), diluted in the blocking solution, was added for 20 min at room temperature. The glass slides were then washed with PBS and mounted in 70% glycerol containing 1% *p*-phenylene diamine (Katayama Chemical, Osaka, Japan) in PBS. The slides were analyzed using a fluorescence microscope (Axioplan 2; Zeiss, Jena, Germany) using filter set 10. Microscopic images were taken using the ZVS-47DE CCD camera (Optronics Engineering, Goleta, CA) and processed with Adobe Photoshop 4.0.1 software (Adobe Systems, Mountain View, CA).

### Northern blotting

Total RNAs from different cell lines and tissues of mice were prepared by using the Triazol reagent (Molecular Research Center, Cincinnati, OH)



**FIGURE 1.** Message expression of laminin 5 chains in thymic epithelial cell lines and in the thymus. Ten micrograms of total RNA were loaded in each lane, and the gel was blotted with the TSO-2D1 probe (A) or the subfragment probes from the *LamC2* full-length cDNA clone (B). The subfragments were generated by cleaving the plasmid containing the 5.1-kb *LamC2* cDNA with *Bam*HI and *Xho*I. Probes are indicated in the map (C). In the map, B is *Bam*HI, E is *Eco*RI, and X is *Xho*I. LamA3 and B3 expression is shown in RNA from various cell lines (D) and tissues (E). A probe for the LamA3 (D) and primers for RT-PCR (E) are described in *Materials and Methods*. GAPDH signals for RNA loading controls are shown at the bottom of each lane.

after homogenizing the samples with a hand-held polytron (Omni International, Waterbury, CT). Poly(A)<sup>+</sup> RNA was prepared using oligo(dT) columns (Quik polyA; Pharmacia). Ten micrograms of total or 2  $\mu$ g of poly(A)<sup>+</sup> RNA from each sample was separated on a 1% agarose gel containing 0.7% formaldehyde and transferred onto a nylon membrane (Nytran maximum strength; Schleicher and Schuell, Keene, NH). The membrane was hybridized with a <sup>32</sup>P-labeled probe in a hybridization solution (QuikHyb; Stratagene) for 2 h. The membrane was washed twice with 2 $\times$  SSC-0.1% SDS and once with 0.1 $\times$  SSC-0.1% SDS at 65°C for about 20 min each and then analyzed on a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

#### RT-PCR

Ten micrograms of total RNA prepared from tissues or 500 CD45<sup>+</sup> thymocytes (99% purity) (31) were reverse transcribed using Moloney murine leukemia virus (MMTV) reverse transcriptase (Stratagene) in a total volume of 50  $\mu$ l. One microliter of each generated cDNA was then amplified for 40 cycles with ExTag (Takara Shuzo, Shiga, Japan). The gene-specific primers for MML5A3B (X84014) are nucleotides 7008–7030, TCAGC CCCAGAGAACAGGTTTAC, and nucleotides 7438–7417, CGAGTGC CATTGTCCATCACAC; the primers for LamB3 (MMU43298) are nucleotides 2965–2988, AGGCTCCCTAATGTGGACTCAGTG, and nucleotides 3469–3449, TGATGTCAGGATTCGATTGC. The sequences of primers used for the GAPDH controls are GGTGAAGGTCGGTGTGAACGGA for the 5' primer and TGTTAGTGGGGTCTCGCTCTCTG for the 3' primer. The PCR product of the LamA3 reaction was used as a probe for the Northern blotting.

#### Western blotting

SCID thymii and C57BL/6 kidneys were snap-frozen, ground under liquid nitrogen, and suspended in the lysis buffer (1% Triton X-100, 25 mM Tris-HCl, pH7.5, 250 mM NaCl) at 4°C for 1 h. Then the samples were centrifuged at 13,000 rpm at 4°C for 20 min, washed with the lysis buffer for 10 min, and centrifuged again. The pellet was resuspended in 10 mM

EDTA, 50 mM Tris-HCl, pH 7.5, and the ECM proteins extracted by rotating overnight at 4°C. After centrifugation at 10,000 rpm for 20 min, the supernatant was saved. Protein concentrations were estimated by the bicinchoninic acid method (Pierce, Rockford, IL). Then, 50  $\mu$ g of each protein sample were electrophoresed on a 6% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dried milk (v/v) in TBS-0.1% Tween 20 (v/v) (TBS-T) at room temperature for 2 h. The P3H9-2 and P3E4 mAbs were then added in 1% nonfat dried milk in TBS-T for 3 h. The membrane was washed with TBS-T three times for 10 min each. HRP-conjugated anti-mouse Ab (Amersham, Arlington Heights, IL) was then added at a 1/3,000 dilution for 2 h. After washing three times with TBS-T, the blot was developed using the enhanced chemiluminescent method (Amersham).

#### FTOC

Thymic lobes were collected at fetal day 14.5 and placed in transwell plates (0.4- $\mu$ m pore size; Costar, Cambridge, MA) in IMDM containing 10% FBS with or without purified anti-laminin 5 Ab preparations that did not contain any sodium azide. Any lobes of obviously smaller size were assumed to be behind in their development and excluded. The medium was changed every other day with fresh medium containing the Ab, or every day with half of the medium and the Ab, for a total of 5–8 days. The thymocytes from the culture were recovered by scraping the tissue through nylon mesh. The recovered cells were stained with either anti-CD24 (ML/69) FITC, anti-CD44 (IM7) PE, anti-CD69 (H1.2F3) FITC, anti-Sca-I (D7) biotin, and/or anti-CD25 (7D4) biotin plus a streptavidin-allophycocyanin conjugate (PharMingen), as well as anti-CD4 (H129.19) Red 613 and anti-CD8 (53-6.7) Red 613 conjugates (Life Technology, Gaithersburg, MD). Alternatively the cells were stained with anti-CD4 (H129.19) Red 613, anti-CD8 (53-6.7) PE, anti-TCR $\gamma$  $\delta$  (GL3) FITC, and anti-TCR $\alpha$  $\beta$  (H57.597) biotin/streptavidin-allophycocyanin (PharMingen). The propidium iodide (PI)-negative gated cells were analyzed on a FACScalibur using two lasers and the CellQuest program (Becton Dickinson, San Jose,

CA). For three-color staining with annexin V, the thymocytes were recovered from FTOC after 2 days of culture and stained with three different combinations of mAb. The first used PE-conjugated anti-CD25 mAb with biotinylated anti-CD44 mAb plus streptavidine-conjugated Quantum Red (Sigma). The second reversed this and used PE-conjugated anti-CD44 and biotinylated anti-CD25. The third involved PE-conjugated anti-CD4 mAb and biotinylated anti-CD8 mAb. For all three combinations, 1  $\mu$ l of FITC-conjugated annexin V (PharMingen) was added in 200  $\mu$ l of buffer and incubated for 5–10 min at room temperature. Finally, PI (20  $\mu$ g/ml final concentration) was added just before flow cytometry analysis on a FACStar using a single laser (Becton Dickinson). All analyses were done on PI-negative cell populations except as indicated in Fig. 7.

#### Statistical analysis

Paired or unpaired Student's *t* tests were performed on the numbers or a log transformation of the numbers in the various sets of data. The results are presented as the arithmetic mean  $\pm$  SEM. Statistical calculations and graphical analyses were conducted using JMP software, version 3.2 (SAS, Cary, NC) or PRISM, version for Macintosh computers (GraphPad Software, San Diego, CA).

## Results

### *cDNA clone TSO-2D1 from a thymic stromal cell library encodes a component of laminin 5*

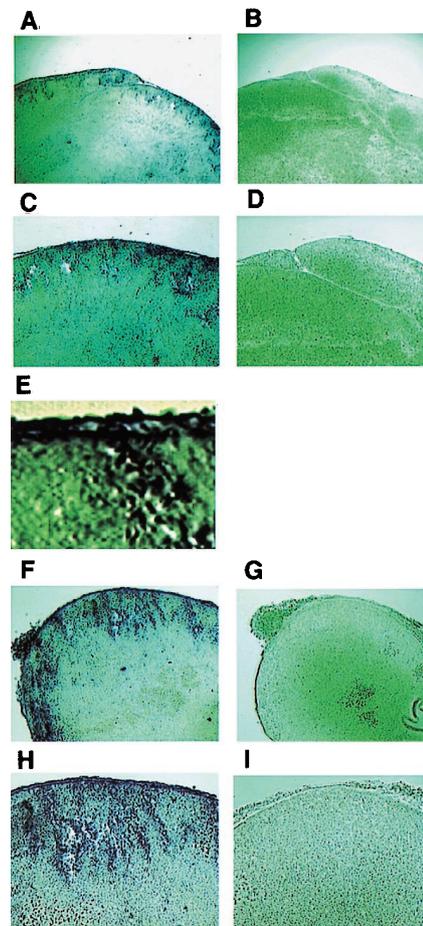
In the initial screening of a PCR-based, subtractive thymic stromal cell cDNA library by Northern blot analysis (18), expression of mRNA corresponding to clone TSO-2D1 was detected only in a thymic epithelial cell line of the nurse cell type and not in other cell lines (line 427.1.86 in Fig. 1A). This cell line has been shown to function in positive and negative selection of T cells when injected intrathymically (35–37). Weak expression of message was also detected in normal thymus, and a strong signal was observed with RNA from SCID thymus. Expression of the message was also detected in the kidney, but not in muscle or liver (data not shown).

A full-length clone was then isolated from a SCID thymus cDNA library (31). The nucleotide sequence of this 5.1-kb clone was identical with GenBank accession number S75987 with a few minor exceptions. This gene (*LamC2*) encodes the mouse  $\gamma_2$  chain of laminin 5, which is known as an epithelial cell-specific ECM protein (19, 20). Northern blotting done with three separate non-overlapping fragments of the 5.1-kb clone revealed two different forms of the message. Using the 5' and central probes, EB and BB, only the thymic nurse cell line and the SCID thymus showed a 5.1-kb mRNA (Fig. 1, B and C). In contrast, the 3' probe, BX, revealed an additional smaller 2.4-kb message, and this mRNA was also present in the other thymic epithelial cell lines. Genomic sequencing is in progress to determine the derivation and significance of these two alternative messages.

We also tested the expression of the other components of laminin 5 in the thymic epithelial cell lines and thymic tissues. As shown in Fig. 1D, the thymic nurse cell line 427.1.86 showed clear expression of both the 5.5-kb LamA3A and 8-kb LamA3B messages (38), while other thymic epithelial and fibroblast cell lines did not. SCID thymus appears to express only the 5.5-kb message. LamA3 and LamB3 expression was also tested by RT-PCR (Fig. 1E). As expected, all the thymic tissue preparations, including thymocyte-depleted thymic rudiment (deGuo Thymus in Fig. 1E), express LamA3 and LamB3 genes, while FACS-separated thymocytes (99% purity) and liver did not show any significant level of expression. The reason for more than a single band in the LamB3 RT-PCR needs to be investigated further.

### *Laminin 5 expression is confined to the subcapsular region of the thymus*

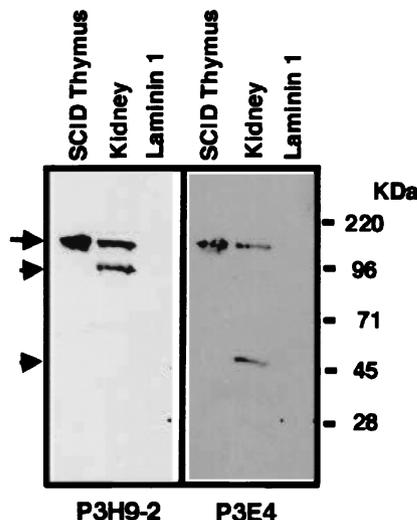
To localize the expression of the *LamC2* gene in the thymus, *in situ* hybridization was performed on paraformaldehyde-fixed sections of thymus from 4- to 6-wk-old mice. A unique digoxigenin-tagged



**FIGURE 2.** *In situ* hybridization with a unique *LamC2* probe. Antisense (A, C, E, F, and H) or sense (B, D, G, and I) RNA probes were used to label 4-wk-old (A–E) or newborn (F–I) thymic sections. Positive signals appear as blackish blue spots against the green background. The magnification in A, B, F, and G is  $\times 200$ ; in C, D, H, and I is  $\times 400$ ; and in E is  $\times 1000$ .

antisense probe from the middle of the cDNA was designed such that it would not cross-hybridize with *LamC1* mRNA and would only detect the long form of the message. This probe hybridized predominantly to the subcapsular region of the thymus sections (Fig. 2, A and C). The control sense probe showed little or no signal in this region (Fig. 2, B and D). Newborn thymus showed staining of both the subcapsular region and the outer cortex (Fig. 2, F and H). In higher magnification (Fig. 2E), it is clear that the message is expressed not only in the area of the basement membrane, but also extends into the subcapsular region. Overall, these results demonstrate that the long form of the mRNA encoding the full  $\gamma_2$  chain of laminin 5 is selectively expressed in the region of the thymus where DN thymocytes develop.

To verify the presence of laminin 5 at the protein level and to confirm its selective location, two available anti-human laminin 5 mAb specific for the  $\alpha_3$  chain (21, 30) were tested to determine whether they would react with the mouse protein. Initially, these Ab, P3E4 and P3H9-2, were used in a flow cytometric analysis to stain the thymic nurse cell line 427.1.86. Nonenzymatically detached 427.1.86 cells were uniformly stained at similar levels of intensity with both of these mAb (data not shown). Next, Western blotting was employed to examine their specificity for mouse laminin 5 protein (Fig. 3). ECM proteins prepared from SCID thymus and kidney revealed a band of  $\sim 135$ – $145$  kDa with both P3E4 and P3H9-2 mAb. This is around the molecular size of the processed



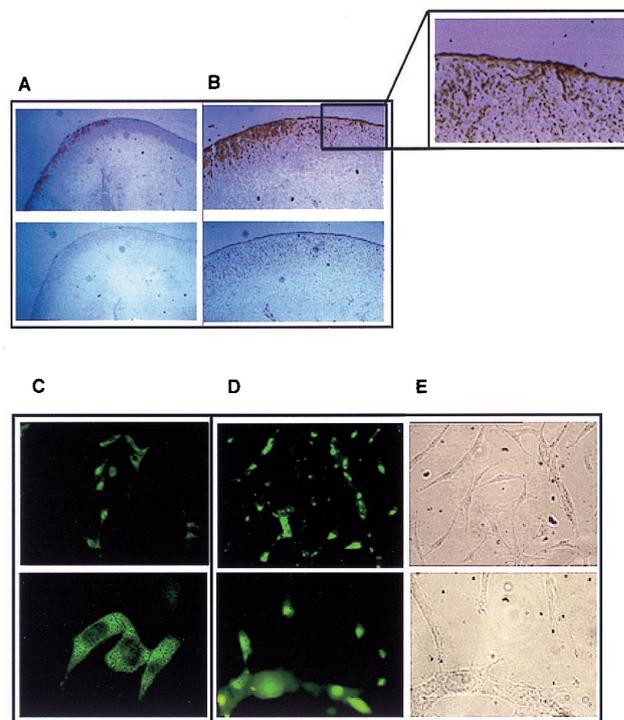
**FIGURE 3.** Western blots demonstrating the cross-reaction of two human mAb on mouse laminin 5  $\alpha_3$  protein. ECM proteins from SCID thymus and normal mouse kidney were prepared as described in *Materials and Methods*. The results with the P3H9-2 mAb are shown on the left and those with the P3E4 mAb are shown on the right. Neither of the mAb cross-react with purified laminin 1 protein.

form of the  $\alpha_3$  chain of human laminin 5 (39). Interestingly, in the kidney the two mAb detected different sized smaller fragments of the  $\alpha_3$  protein, indicating that they recognize different epitopes on the same molecule. Neither of these mAb reacted with purified mouse laminin 1 protein (Fig. 3, last lane), suggesting that both are specific for the laminin 5 protein.

Finally, the P3H9-2 mAb was used in an immunohistochemical stain of thymic sections. Four-week-old thymuses were stained almost exclusively in the subcapsular region, while newborn thymus sections showed a slightly deeper penetration into the cortex (Fig. 4, *A* and *B*). In the absence of the primary mAb, there was no detectable staining. The two mAb were also used to stain the thymic nurse cell line after the cells had been in culture for 1 day on chamber slides and fixed in situ with 3.7% paraformaldehyde (Fig. 4, *C* and *D*). Both mAb stained the fixed cells; however, the staining patterns were different. The P3E4 mAb staining was generally punctate and diffuse, filling all of the cytoplasmic area. In contrast, the staining with the P3H9-2 mAb was often clustered in large clumps (usually two to three per cell). Similar results were seen when 6% paraformaldehyde was used in the fixation process (data not shown), suggesting that the clusters existed before the addition of the Ab. Based on all these results, we concluded that these two mAb are reactive against the mouse  $\alpha_3$  chain of laminin 5 and therefore they could be used in our functional inhibition studies. The different staining patterns observed might be relevant to their functional differences in blocking cell adhesion (21) as well as their different effects in thymic organ culture described below.

#### *The P3H9-2 anti-laminin 5 mAb decreases the yield of thymocytes in FTOC*

To determine whether anti-laminin 5 Ab had any effect on thymic development, different concentrations of the two purified mAb were added into the culture medium of FTOC starting at fetal day 14.5, and the remaining thymocytes were analyzed 5 days later. As shown in Fig. 5, the nonblocking, diffusely staining mAb P3E4 did not produce any effect on the total cell recovery up to a concentration of 10  $\mu\text{g/ml}$ . In contrast, the blocking, cluster-staining mAb P3H9-2 gave a dose-dependent reduction in the cell recovery. The

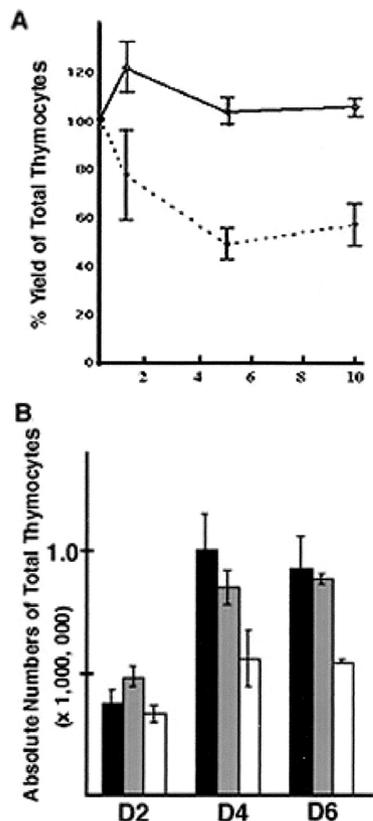


**FIGURE 4.** Immunohistochemical and immunocytochemical staining with an anti-laminin 5  $\alpha_3$  Ab. The staining patterns (brown color against a purplish blue background) of 4-wk-old (*A*) or newborn (*B*) thymic sections are shown in the upper block of pictures. The negative controls without the primary Ab are shown in the panels underneath. The boxed area in *B* is a blown-up in the picture on the right. In the lower group of pictures, immunofluorescent staining with the nonblocking anti-laminin 5 mAb P3E4 (*C*) or the blocking mAb P3H9-2 (*D*) of adherent thymic nurse cells (line 427.1.86) is shown at two magnifications:  $\times 100$  in the upper panel and  $\times 630$  in the lower panel. *E* is a phase contrast image of *D*.

maximum decrease was 50%, and this was achieved at a concentration of 5  $\mu\text{g/ml}$ . In 12 experiments in which the yields were compared after 5–8 days between cultures treated with 10  $\mu\text{g/ml}$  of P3H9-2 and either 10  $\mu\text{g/ml}$  of P3E4 or no Ab, the average yield in the presence of the blocking Ab was  $60 \pm 4\%$  of the control. The total thymocyte recovery for the control groups was  $8.5 \pm 0.36 (\times 10^5)$  cells and for the P3H9-2-treated group was  $5.0 \pm 0.55 (\times 10^5)$  cells. These two means were significantly different ( $p < 0.0001$ ) in an unpaired Student's *t* test.

The kinetics of the blocking effect is shown in Fig. 5*B*. After 2 days of culture, there was little difference in the thymocyte recoveries among untreated, P3E4-treated, or P3H9-2-treated (10  $\mu\text{g/ml}$ ) cultures. By day 4, however, P3H9-2-treated cultures yielded fewer thymocyte numbers than the P3E4-treated or no mAb control cultures. This pattern was also observed at day 6. An unpaired Student's *t* test on the pooled data from days 4 and 6 showed that the yields from the cultures with the P3H9-2 mAb were significantly less than those from the cultures with either the P3E4 mAb or no Ab and that the yields from the two control cultures were not significantly different.

Cells bearing the TCR- $\alpha\beta$  constitute the vast majority of TCR<sup>+</sup> cells in the thymus and so perhaps not surprisingly their yield was also decreased. For five experiments, the average yield was  $54 \pm 7\%$  in the presence of the blocking mAb. We also examined the yield of  $\gamma\delta$  TCR<sup>+</sup> cells, which are a much smaller fraction (5%) of the total thymocytes. They too showed a consistent pattern of inhibition in the presence of the blocking mAb. In five experiments,

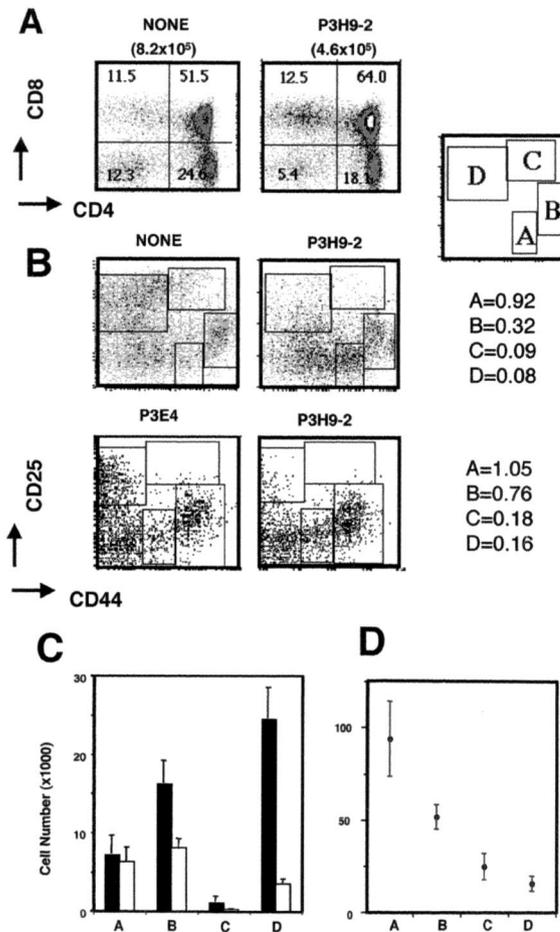


**FIGURE 5.** Dose- and time-dependent reduction of thymocyte yield in the presence of blocking anti-laminin 5 mAb. *A*, Recovery of total thymocytes from FTOC treated with two anti-laminin 5 mAb, P3E4 and P3H9-2, after 5 days of culture. In each experiment, the averages of the day 5 recovery of live cells per lobe from 6 to 20 lobes were calculated and the yield expressed as a percentage of the recovery from untreated cultures. The average total thymocyte yield for untreated FTOC was  $9.6 \pm 0.34 \times 10^5$ /lobe in this set of experiments. The numbers in the graph for each concentration of mAb represent the mean percentage values from three to five different experiments. *B*, Total thymocyte yields from 2-, 4-, and 6-day-old cultures that were either untreated (■), treated with P3E4 mAb (▨), or treated with P3H9-2 mAb (□). The lines indicate the SEs of the means.

the average yield was  $66 \pm 6\%$ . Thus, the development of both major T cell lineages is affected.

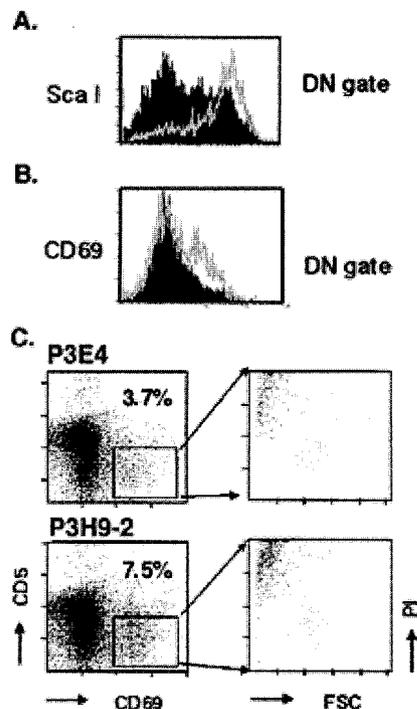
#### *The P3H9-2 anti-laminin 5 mAb blocks $\alpha\beta$ T cell lineage development predominantly at the $CD4^-CD8^-$ DN stage*

We next examined which subsets of CD4- and CD8-bearing cells were most affected by the treatment. As shown in Fig. 6*A*, the greatest reduction in yield in cultures treated with the P3H9-2 blocking mAb occurred in the  $CD4^-CD8^-$  DN thymocytes. In this experiment, the yield was 25% of that from the untreated control cultures:  $[(460,000 \text{ total thymocytes} \times 5.4\% \text{ DN cells for the P3H9-2 Ab}) \div (820,000 \text{ total} \times 12.3\% \text{ DN for no Ab})] \times 100$ . For four experiments, the average yield was  $34 \pm 7.5\%$ . The values for the DP, CD4 SP, and CD8 SP cells were  $61 \pm 11\%$ ,  $40 \pm 3\%$ , and  $50 \pm 5\%$ , respectively. To determine which subpopulations among the DN thymocytes were being affected, we further analyzed this subset (DN gated) with Ab to CD44 and CD25 (Fig. 6, *B–D*). The most profoundly affected subpopulations were the  $CD25^+CD44^+$  (subpopulation C) and  $CD25^+CD44^-$  (subpopulation D) thymocytes (see the subset labeling scheme and ratios on the right side of Fig. 6). Compared with either untreated cultures or cultures treated with the nonblocking P3E4 mAb, the yields of these two subpopu-



**FIGURE 6.** Subset analysis of recovered thymocytes from anti-laminin 5  $\alpha_3$ -treated FTOC. *A*, The upper panels show the CD4 and CD8 staining pattern of recovered live ( $PI^-$ ) cells after culturing 8 days. The numbers at the top of the figure are the total cell recoveries for each group. The numbers in the quadrants represent the percentage of cells in each subset. *B*, The panels show a further fractionation of the DN subpopulation into  $CD25^+$  and  $CD44^+$  subsets from two separate experiments, one with the P3E4 mAb used as a control and the other in which the control group received no treatment (none). The latter is an analysis of the DN subpopulation shown in *A*. A quantitative analysis was performed on the boxed subsets shown in the dot plots and in the scheme on the right (subpopulations A–D, which follows the known developmental pathway for DN thymocytes). The data shown underneath the scheme are the ratios of the absolute number of cells recovered for each subset in the P3H9-2-treated cultures divided by the number recovered in the control culture. The average and SEs of the ratios for each subpopulation measured in six independent experiments are shown in *C*. Open bars represent the means for P3H9-2-treated cultures, and filled bars represent the means for the controls. *D*, The data in *C* are displayed as percentages of control  $\pm$  SEM.

lations were only 8–18%. A quantitative analysis of six experiments is shown in Fig. 6, *C* and *D*. The average yield of subpopulation C was  $25 \pm 7\%$ , while the yield of subpopulation D was  $16 \pm 4\%$  (Fig. 6*D*). These decreases are statistically significant ( $p < 0.001$ ) using a paired *t* test done on a log transformation of the data. In contrast, the earliest subpopulation,  $CD25^-CD44^+$  (subpopulation A), was unaffected ( $95 \pm 19\%$ ). Interestingly, the transitional  $CD25^0CD44^+$  subpopulation B was significantly ( $p < 0.002$ ) decreased to  $52 \pm 7\%$ , suggesting that the loss begins at this stage. The remaining DN subpopulation, the  $CD44^-CD25^-$  cells, was also only partially affected; the average yield was  $46 \pm 6\%$ . Finally, the same



**FIGURE 7.** Flow cytometric analysis of the DN thymocytes from anti-laminin 5-treated FTOC for Sca-I, CD69, CD5, and PI. *A*, Sca-I expression of DN thymocytes recovered on day 8. *B*, CD69 expression of DN thymocytes recovered at day 8. *C*, On day 2 of culture, CD69<sup>+</sup>, CD5<sup>low</sup> cells were further analyzed for PI staining and forward scatter (FSC) to show that most of them are dying in both P3E4 and P3H9-2 mAb-treated cultures. Note that there are twice as many CD69<sup>+</sup> dead cells in the P3H9-2 mAb-treated cultures.

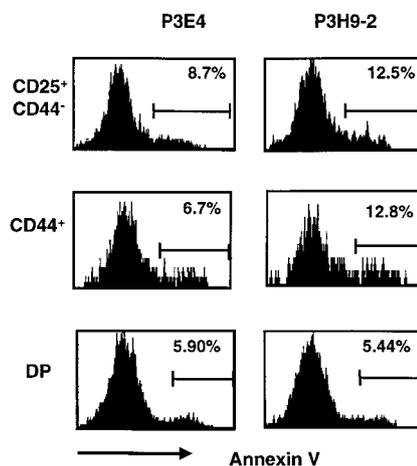
depletion was observed in the presence of the 2.4.G2 mAb at concentrations that blocked low-affinity Fc receptor binding to our DN fetal thymocyte preparations (data not shown).

Overall, these results suggest that early thymocyte differentiation starts normally and then aborts from a failure of the DN cells to interact properly with laminin 5. However, the cells that survive seem to be able to expand after signaling through their pre-T $\alpha$ /TCR $\beta$  receptor ( $\beta$  selection) and then move into the DP subpopulation.

*The phenotype of the DN thymocytes affected by the anti-laminin 5 Ab suggests that the cells are dying as they start to differentiate*

In the blocking mAb-treated cultures, the percentage of DN thymocytes expressing Sca-I was greatly increased after 8 days in culture (Fig. 7A). Normally, about 23% of the DN cells are Sca-I<sup>high</sup>. In addition, 23% express intermediate levels of Sca-I, and a significant fraction (54%) are Sca-I-low or -negative cells. In contrast, the DN population recovered from the mAb-treated cultures was mostly Sca-I<sup>high</sup> (71%), with 15% intermediates and 12% negative cells. The Sca-I marker is present on the bone marrow precursors that enter the thymus, and it is subsequently lost as the cells differentiate. Thus, the shift in the Sca-I profile observed with the mAb treatment is consistent with the idea that many of the DN cells recovered are still in an early stage of development. There is also the possibility that the cells arrest in development at this stage and thus fail to down-regulate the Sca-I molecule normally.

The percentage of DN thymocytes expressing the CD69 marker was also increased in Ab-treated cultures (Fig. 7B), and the majority of these cells were c-Kit positive (data not shown). CD69 is



**FIGURE 8.** The profile of annexin V staining of selected thymic subpopulations after 2 days in culture with nonblocking or blocking mAb treatment. Profiles are from PI-gated populations. Gates for each figure are on CD25<sup>+</sup>CD44<sup>-</sup> cells (*top*), CD44<sup>+</sup> cells (*middle*), and CD4<sup>+</sup>CD8<sup>+</sup> DP cells (*bottom*). P3E4 mAb-treated cultures are shown on the left, and P3H9-2 mAb-treated cultures are shown on the right. The percentage of annexin V-positive cells are shown with each histogram.

an early activation marker that is often associated with proliferation and/or differentiation of T cells; however, it can also up-regulate before the onset of cell death (40). To determine which fate the CD69<sup>+</sup> cells were undergoing in our cultures, we examined their staining with PI at an earlier time point, after only 2 days in culture with the blocking or nonblocking anti-laminin 5 mAb. As shown in Fig. 7C, most of the CD69<sup>+</sup> cells at this time were PI<sup>+</sup>, and there were twice as many in the presence of the P3H9-2 mAb as found in the presence of the nonblocking P3E4 mAb. In addition, the CD69<sup>+</sup> cells were mostly CD5<sup>low</sup>, suggesting that the cells were dying at an early stage in their differentiation.

To determine whether the dying cells were undergoing apoptosis, we looked at the profile of annexin V staining of different subpopulations in the two mAb-treated groups after culturing for 2 days. As shown in one experiment in Fig. 8, cells in the CD25<sup>+</sup>CD44<sup>-</sup> and CD44<sup>+</sup> compartments from the P3H9-2-treated cultures showed slightly higher levels of annexin V-positive staining in the gated PI-negative channel. In contrast, the CD4<sup>+</sup>CD8<sup>+</sup> DP cells appearing at day 2 showed comparable levels of annexin V staining. For 10 independent determinations, the CD44<sup>+</sup> compartment from the P3H9-2-treated cultures showed 62% more annexin V-positive cells than either P3E4 mAb or no Ab controls. For the CD25<sup>+</sup> subpopulation, the increase was 22%. Both increases were statistically significant using a paired Student's *t* test, while a comparison of annexin V-positive DP cells done for seven of these determinations were not significantly different. These observations support the notion that blocking thymocyte interactions with laminin 5 leads to more apoptotic cell death in DN thymocytes.

## Discussion

Laminin 5 is an important ECM component of the basement membrane underlying the basal epithelial cells in the skin (7, 39). In the human thymus, it has been located with anti- $\alpha_3$  and - $\beta_3$  Ab to the basement membrane of the capsule (9) and recently in the medulla with Ab against all three chains (10). Expression of mRNA for the *LamC2* gene has also been identified in the human thymus by Northern blotting, but never localized to a particular anatomic sub-region (6, 7, 19). In our studies, we localized expression of the

full-length mouse *LamC2* message primarily to the subcapsular region of the adult thymus by in situ hybridization (Fig. 2). In addition, an anti-human  $\alpha_3$  mAb, which we demonstrated cross-reacts with the mouse  $\alpha_3$  protein, was shown to stain mainly the subcapsular region of a 4-wk-old thymus. Both the epithelial cells in the basement membrane and the neighboring subcapsular epithelial cells were positive for expression. Staining in newborn thymus extended deeper into the cortex, but we think this is because the subcapsular region occupies a larger fraction of the thymus at that stage of development. Thus, two components of laminin 5 in the mouse thymus are confined in their expression to the region where DN thymocytes interact with nurse cells to undergo the earliest steps in thymocyte differentiation.

Laminin 5 is normally produced by the basal epithelial cells in the skin. In the thymus, there are two types of epithelial cells that have been described in the subcapsular region (41, 42). One (E1) abuts against the basement membrane and is a likely source for the laminin 5 in that structure. The other (E2) is the thymic nurse cell that is located slightly further inside the thymus in the subcapsular region. The relationship between these two cell types, if any, is unclear. Our studies show that an SV40 T Ag-transformed epithelial cell line with subcapsular nurse cell characteristics makes laminin 5; however, we are not sure which normal epithelial cell it represents. The cell line expresses two forms of the *LamC2* gene, one encoding the full-length message of 5.1 kb and the other a shortened form of 2.4 kb (Fig. 1). The full-sized message is only expressed in the nurse cell line. In contrast, the short form is present in other types of thymic epithelial cell lines including both medullary and cortical epithelial cells (Fig. 1). The short form may encode a truncated version of the protein with an alternative function. A shorter form of the human *LamC2* cDNA has been described; however, it is truncated at the 3' end of the message by an alternative splicing mechanism, and the long form is more widely expressed than the short form (19, 43). Whether the mouse also has a 3' truncated version of the mRNA expressed in tissues other than thymus remains to be determined.

A laminin 5 receptor,  $\alpha_6\beta_4$  integrin, has been identified in the subcapsular region of the human thymus (13) and shown to be expressed on the surface of mouse DN thymocytes (12). During fetal thymic development, the  $\beta_4$  chain was detected on virtually all CD25<sup>+</sup> cells, and 25–40% of  $\beta_4$ <sup>+</sup> cells expressed CD44. Another laminin 5 receptor,  $\alpha_3\beta_1$  integrin, was also found in the cortical epithelial area of human thymus (13), and Abs to both the  $\alpha_3$  and  $\alpha_6$  integrin chains were shown to block mouse immature thymocyte adhesion to laminin (15). Furthermore,  $\alpha_6\beta_4$  integrin expression is maximal at mouse fetal day 14–15 when only DN thymocytes and stromal cells are present in the organ (12). These observations suggest that early developing thymocytes are equipped to interact with laminin 5 for either anchoring and/or signaling purposes.

To test whether laminin 5 might be important for thymocyte development, we added anti-laminin 5 ( $\alpha_3$ ) mAb to day 14.5 FTOC and determined their impact after 5–8 days. The two mAb we chose had known differences in blocking function in a cell adhesion assay to laminin 5, and we could visualize a difference in their binding to the nurse cell line after it had adhered in culture, presumably to the laminin 5 and other ECM proteins it made and secreted. The nonblocking mAb showed a diffuse punctate staining pattern, while the blocking mAb tended to stain in big clumps. These differences presumably relate to recognition of different determinants on the  $\alpha_3$  chain by the two mAb as shown in our Western blot of kidney ECM (Fig. 3). The clumps are not an artifact produced by Ab cross-linking, as they were seen even after fixation with 6% paraformaldehyde. Instead, they could be equivalent to

the “desmosomal” structures observed by others in electron micrograph sections of cultured 427.1.86 cells (26). Hemidesmosomes in the skin play an important anchoring function and are known to contain  $\alpha_6\beta_4$  integrin receptors interacting with laminin 5 (7). Hemidesmosomes have also been observed in the thymus, but their function there is unknown (7). It is thus possible that this nurse cell line in culture sets up stable anchoring contacts (44, 45) in culture involving laminin 5 and  $\alpha_6\beta_4$  integrin receptors. Preliminary data from our laboratory show that an Ab to the  $\alpha_6$  integrin chain does indeed colocalize in the clumps with the blocking anti-laminin 5 ( $\alpha_3$ ) mAb. Why only the P3H9-2 mAb to  $\alpha_3$  laminin shows this unusual reactivity is not clear, but it could be the key to its functional blocking effects. Finally, it should be noted that because the blocking experiments were done with an anti- $\alpha_3$  mAb and not an anti- $\gamma_2$  mAb, it is formally possible that the target of the inhibition is laminin 6 or 7 instead of laminin 5, because those molecules also possess the  $\alpha_3$  chain.

The P3H9-2 mAb had an inhibitory effect on thymocyte development in FTOC, whereas the nonblocking P3E4 mAb did not. Both  $\alpha\beta$  and  $\gamma\delta$  T cell production were reduced by about 35–45%. One reason this inhibition might only appear to be partial is because at 14.5 days of fetal development a significant fraction of thymocytes are already beyond the point at which laminin 5 interactions are required for T cell development. In our hands, thymocytes from fetal day 14 contain over 60% CD25<sup>+</sup> cells. Consistent with this idea is the fact that the major effect of the Ab was on the DN subpopulation within which the CD25<sup>+</sup>CD44<sup>+</sup> and CD25<sup>+</sup>CD44<sup>-</sup> subsets were the most profoundly decreased (75–85%). Interestingly, the earliest DN cells persisted in normal numbers, and, as a consequence of the loss of the other subsets, Sca-I staining was proportionally increased. However, the observed increase is greater than the expected enrichment, suggesting that there also might be some sustained Sca-I expression on surviving later subsets because of disruptive effects on differentiation. Finally, DN cells expressing the activation marker CD69 were also increased, and early on in culture this was clearly associated with dying cells (Figs. 7 and 8). The possibility that blocking laminin 5 leads to an early thymocyte death mechanism is consistent with the observation that there is no accumulation of the CD44<sup>+</sup>CD25<sup>-</sup> precursors and that there are higher numbers of annexin V-positive cells in the CD44<sup>+</sup> subsets.

The simplest model to explain our observations is one in which the T cell precursors differentiate into CD44<sup>+</sup>CD25<sup>-</sup> thymocytes and begin to express  $\alpha_6\beta_4$  integrin receptors, which then interact with laminin 5 secreted by the thymic nurse cells. If the response to further differentiation signals requires trophic signaling through this integrin receptor, then blocking such signals with the P3H9-2 mAb could result in cell death. Less likely is the possibility that the Ab signals the cells to die by cross-linking laminin 5 bound to their surface integrin receptors. In either case, the CD44<sup>+</sup>CD25<sup>-</sup> cells would not accumulate because their differentiation is not inhibited, only their survival as they attempt to differentiate. In other model systems, epithelial and endothelial cells have been shown to undergo apoptosis when they are detached from an ECM (46–48). This process has been termed anoikis (47, 48). Protection from death is only mediated by certain integrins and is currently thought to involve signaling through focal adhesion kinase with subsequent activation of the phosphatidylinositol-3 kinase pathway (47). An alternative model consistent with our data is that the bone marrow thymocyte precursors require interaction with laminin 5 to successfully complete their homing to the subcapsular region of the thymus. If they don't receive this signal, they die. We favor the first model, however, because previous studies have shown that  $\alpha_6\beta_4$  integrin receptors display their highest level of expression

(and presumably function) on CD25<sup>+</sup> cells (12), a stage well into thymocyte differentiation and one at which we see the greatest effect on DN cell survival.

If somehow the cells pass through the critical laminin 5-dependent DN stage of development without undergoing apoptosis, they might then proceed normally with the rest of thymocyte differentiation, perhaps including extra proliferation after  $\beta$  selection. This would explain why we observed only a moderate reduction in DP and SP cells even though there was a severe reduction in DN cells. Such a phenotype is also observed in the absence of IL-7 (49). In our data, the CD44<sup>-</sup>CD25<sup>-</sup> DN subset appears to be less affected and the DP subset is the least affected, suggesting that some of the loss can be made up at the post- $\beta$  selection expansion phase. However, there is some suggestion that the yield of SP thymocytes is lower than what we would have expected based on the yield of the DP cells (40–50% for SP vs 60% for DP). Interestingly, a recent publication (10) has described the presence of laminin 5 in the medulla of the human thymus and shown that soluble laminin 5 as well as an anti- $\alpha_6\beta_4$  Ab could partially inhibit anti-CD3-induced thymocyte proliferation. Thus, laminin 5 might also play a role in the generation or survival of SP thymocytes. Alternatively, because we did not detect any laminin 5 in the medulla of the mouse thymus, the effect of the blocking P3H9-2 mAb on SP cells could be a secondary consequence of the earlier deprivation of laminin 5 signaling that manifests itself during positive or negative selection, e.g., an effect leading to lower bcl-2 levels in the surviving DP thymocytes that would make them more vulnerable to failing selection.

In conclusion, our experiments suggest that laminin 5 is an important ECM protein secreted by subcapsular thymic epithelial cells. We propose that it functions during the DN stage of thymocyte development to provide a temporary anchorage site for these cells. This presumably is required for maintaining the cells in the right environment for the completion of this stage in their differentiation. We interpret the fact that this interaction appears to be required for DN thymocyte survival as a mechanism for maintaining fidelity in this process.

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