

Laminin 5 in the Human Thymus: Control of T Cell Proliferation via $\alpha_6\beta_4$ Integrins

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Abstract. Laminin 5 ($\alpha_3\beta_3\gamma_2$) distribution in the human thymus was investigated by immunofluorescence on frozen sections with anti- α_3 , $-\beta_3$, and $-\gamma_2$ mAbs. In addition to a linear staining of subcapsular basal laminae, the three mAbs give a disperse staining in the parenchyma restricted to the medullary area on a subset of stellate epithelial cells and vessel structures. We also found that laminin 5 may influence mature human thymocyte expansion; while bulk laminin and laminin 2, when cross-linked, are comitogenic with a TCR signal, cross-linked laminin 5 has no effect. By contrast, soluble laminin 5 inhibits thymocyte proliferation induced by a TCR signal. This is accompanied by a particular pattern of inhibition of early tyrosine kinases, including

Zap 70 and p59^{lyn} inhibition, but not overall inhibition of p56^{lck}. Using a mAb specific for $\alpha_6\beta_4$ integrins, we observed that while $\alpha_3\beta_1$ are known to be uniformly present on all thymocytes, $\alpha_6\beta_4$ expression parallels thymocyte maturation; thus a correspondence exists between laminin 5 in the thymic medulla and $\alpha_6\beta_4$ on mature thymocytes. Moreover, the soluble Ab against $\alpha_6\beta_4$ inhibits thymocyte proliferation and reproduces the same pattern of tyrosine kinase phosphorylation suggesting that $\alpha_6\beta_4$ is involved in laminin 5-induced modulation of T cell activation.

Key words: laminin 5 • thymus • T cell • integrins • cellular activation

LAMININS are the major constituents of basal laminae; together with other extracellular matrix components (ECM)¹ they contribute, by binding cell surface receptors such as integrins, to maintain epithelial tissue integrity (3, 38, 76). In addition to their mechanical role, they are important signaling molecules with the ability to strongly influence cellular programs, promoting differentiation and migration, proliferation and activation (13, 39, 40). Thus, they are influential molecules in the development (1, 59, 62) and repair (29, 30, 53) of many tissues.

Laminins are heterotrimers where the α chain is critical for cell/matrix interactions (37). Among laminin isoforms

(4, 67), laminin 5 (epiligrin, kalinin, nicein) is somewhat unique and is preferentially found in basement membranes underlying squamous and transitional epithelia (4). From a structural viewpoint, the molecule displays a particular chain composition ($\alpha_3\beta_3\gamma_2$) and has been characterized as a 105-nm rod-like molecule from the conditioned medium of normal human keratinocytes (49). Studies performed with extracts of human amnion revealed that, in addition to monomeric molecules, much of the laminin 5 isolated is covalently associated with laminin 6 ($\alpha_3\beta_1\gamma_1$) and 7 ($\alpha_3\beta_2\gamma_1$) (8). Whether these complexes are located in other basement membranes remains to be shown. Laminin 5 has the ability to bind the NH₂-terminal domain of type VII collagen and is believed to strongly connect cells to anchoring fibrils in skin (51). Laminin 5 has been identified as a ligand for the integrins $\alpha_3\beta_1$ (7, 48) and $\alpha_6\beta_4$ (44, 60). From a functional viewpoint, laminin 5 plays a major mechanical role in maintaining the basal layer of epithelia (1, 3, 6, 7, 14, 15, 23, 49, 51, 52), and it is also increased in the early stages of wound repair (30, 53). Furthermore, accumulated evidence suggests that laminin 5 is a signaling molecule that is particularly involved in controlling cell migration and tumor cell expansion (16, 26, 27, 39, 46).

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1. *Abbreviations used in this paper:* APY, anti-phosphotyrosine; Ab, antibody; ECM, extracellular matrix; GAR, goat anti-rabbit immunoglobulins; IP, immunoprecipitation; pAb, polyclonal antibody; PE, phycoerythrin.

The thymus can be regarded as a mesh of epithelial cells surrounding maturing T cells; thus, ECM components and integrins displayed by thymocytes appear to strongly influence T cell differentiation/maturation (5, 10, 12, 20, 31, 43, 54–56, 69, 73, 74). Mice deficient in merosin, for example, suffer from abnormal T cell development (31). Since the presence of laminin 5 has been found within the human thymus (25, 42, 72), this work was undertaken to document expression and functional influence of laminin 5 and its cellular receptors during thymocyte maturation. We show here that laminin 5 displays a peculiar tissue distribution in the human thymus. In addition, we show that laminin 5, in soluble form, provides mature thymocytes with an inhibitory signal upon stimulation via the CD3–TCR complex which can be reproduced by an anti- α_6 mAb specific for $\alpha_6\beta_4$. We have already shown that ligands to β_1 integrins, when used in soluble form, inhibit T cell activation triggered via the CD3–TCR complex (19, 34, 65). We now report that $\alpha_6\beta_4$ integrins can also trigger a similar effect after ligation with soluble laminin 5.

Materials and Methods

Materials

Human laminin 5 was obtained by immunopurification of the conditioned medium of SCC25 cells (squamous cell carcinoma) using the 6F12 mAb coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals). Before this step, medium was passed sequentially over 25 ml of gelatin-Sepharose (Pharmacia Fine Chemicals) in order to remove fibronectin. Molecular mass of the eluted laminin 5 was analyzed by SDS-PAGE and revealed the presence of the trimer $\alpha_3\beta_3\gamma_2$ only. Purity of the molecule preparation was checked by immunoblotting using a panel of several monoclonal and polyclonal antibodies as previously described (48, 50). Laminin 2 was obtained from Chemicon. Human fibronectin, protein A-Sepharose CL 4B, PMSF, leupeptin, aprotinin, NP-40, and BSA were all purchased from Sigma Chemical Co.; human IL-2 was from R&D Systems Inc. Chemicals for PAGE were from Bio-Rad Laboratories. Anti-fading mounting medium Citifluor was obtained from Citifluor Chemical Laboratory. [3 H]thymidine and 125 I were obtained from Commissariat à l'Énergie Atomique. ECL reagents were purchased from Amersham.

Antibodies

The BM165 mAb (mouse IgG1), specific for α_3 chain of human laminin 5 was produced as previously described (49). The 6F12 mAb, specific for β_3 chain of human laminin 5, was produced as previously described (33). The GB3 mAb (70) specific for γ_2 chain of human laminin 5, was from Accurate and Specific Corporation. The human CD49f (α_6 integrin chain) mAb S3-41 (mouse, IgG1) was produced as previously described (24). The human CD49f mAb GOH3 (rat IgG1; 61) was obtained from Immunotech. The human CD49f mAbs 135 13C (rat IgG2a), J8H (mouse IgG1), BQ16 (mouse IgG1), and 450 30A1 (mouse IgG1) were obtained from the 5th International Workshop of Leukocyte Typing. The CD49c mAb (α_3 integrin chain) P1B5 was obtained from Becton Dickinson. Human CD104 (β_4 integrin chain) mAb 3E1F6 was a gift from Dr. Kennel (Oak Ridge National Laboratory, Oak Ridge, Tennessee). Anti-p67 high affinity non-integrin laminin receptor mAb MPLR2 was provided by Dr. Colnaghi (Istituto Nazionale Tumori, Milan, Italy) as ascites fluid. CD28 mAb 9.3 (mouse IgG2a) was kindly provided by Dr. Ledbetter (Pharmaceutical Research Institute, Bristol Myers Squibb, Seattle, WA). Human CD29 (β_1 integrin chain) mAb K20 (mouse IgG2a), CD3 mAb X3 (mouse IgG2a), and CD2 mAbs GT2 (mouse IgG1) and D66 (mouse IgM) were generated in our laboratory. The anti-Zap 70 rabbit antibody was directed against amino acids 485 to 499 of the human ZAP 70 sequence as previously described (68). Rabbit anti-human p56^{lck} and anti-p59^{lyn} Abs and peroxidase (HRP) conjugated anti-phosphotyrosine (4G10) were obtained from Upstate Biotechnology Inc. The goat anti-mouse, anti-rat, or anti-rabbit antibodies, alone or coupled with fluorochromes or peroxidase, as well as irrelevant mouse IgG1, were purchased from Dakopatts.

Cell Culture and Tissue

Normal thymi were obtained from children (<4 yr) undergoing cardiac surgery. Thymocytes were prepared by physical disruption of the tissue and washes in RPMI/10% FCS. Jurkat (T lymphoma), HT29 (human colon carcinoma), and K562 (myelogenous leukemia) cell lines were obtained from the American Type Culture Collection.

Thymocytes, human cell lines Jurkat and K562 were cultured in RPMI 1640 (GIBCO BRL) supplemented with 10% FCS. Human colon carcinoma cell line HT29 was cultured in DME (GIBCO BRL) 5% FCS. All media were supplemented with 50 U/ml penicillin, 50 μ g/ml streptomycin, 2 mM L-glutamine, and 1 mM pyruvate (Merck).

Immunohistochemistry

For immunohistochemistry, specimens of human thymi were fragmented, immediately frozen in liquid nitrogen, and stored at -70°C .

Frozen sections (4 μm thick) were processed for indirect immunofluorescence, using a single label fluorescein technique. Air-dried cryostat sections were incubated for 30 min with the primary antibody diluted in PBS, at room temperature. Antibody binding was then detected by incubating the sections with FITC-conjugated rat anti-mouse secondary antibody (F(ab) $'_2$) fragments (Dakopatts), diluted 1:50 in PBS, for 30 min at room temperature. Cell nuclei were labeled with 0.5% propidium iodide (Sigma Chemical Co.). After final washing in PBS, the sections were mounted in Citifluor (Plano) and examined with a laser scanning confocal microscope (Ultima Meridian; DGL Bioscience). Indirect double staining of thymic epithelial cells was performed with the anti-laminin 5 mAb BM165 (mouse IgG1) and the anti-keratin mAb CK19 (mouse IgM) using isotype-specific secondary antibodies, i.e., FITC-conjugated goat anti-mouse IgM, obtained from Chemicon, and rabbit anti-mouse IgG1 (Dakopatts) revealed with phycoerythrin (PE)-conjugated goat anti-rabbit Ig (Dakopatts). After final washing in PBS, sections were mounted in Citifluor (Plano) and examined with a laser scanning confocal microscope (Ultima Meridian; DGL Bioscience).

Proliferation Assays

Cell proliferation assays were performed in triplicate in 96-well culture plates (Nunc). Thymocytes at 10^5 cells/well were cultured in 0.2 ml of RPMI 1640 with 10% FCS. Coating of the CD3 mAb was carried out by overnight incubation at 4°C with CD3 mAb ($\times 3$, 10 $\mu\text{g}/\text{ml}$) diluted in PBS. Each well was then washed three times with PBS. To saturate the plastic before using soluble reagents, RPMI with 10% FCS was added for 2 h, then the wells were washed three more times with PBS. Cells were added in culture medium containing soluble laminins or Abs before the addition of rIL-2 (5 ng/ml).

For costimulation assays, CD3 mAb was coimmobilized with another mAb or with an ECM. A first overnight incubation at 4°C was carried out with an ECM component (laminin 5, laminin 2, or fibronectin) or mAbs diluted in PBS followed, after washing, by immobilization of CD3 mAb.

Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere for 4 d, and 1 μCi of [3 H]thymidine (2 Ci/mmol; CEA) was added for the final 18 h. Cells were harvested onto filter paper using a semiautomatic cell harvester (Skatron) and thymidine incorporation was measured in a liquid scintillation counter (Beckman Instruments Inc.).

Apoptosis Assays

Apoptosis was determined by incubating cells previously cultivated as described above with Hoechst 33342 (Interchim), 30 min at 37°C . Results were analyzed on a FACStar[®] cytometer (Becton Dickinson).

Immunofluorescence Analysis of Isolated Cells

Cells were washed three times with PBS and incubated at 4°C for 30 min in the dark in 100 μl PBS, 0.1% NaN_3 , and 0.1% BSA with saturating concentration of mAbs, washed three times and resuspended at a density of 3×10^6 cells/ml. Indirect labeling was performed by adding goat anti-mouse PE-conjugated F(ab) $'_2$ fragments (Dako). This reagent also binds to rat IgG. Double staining was performed using indirect labeling with a primary mAb revealed with anti-mouse (PE) conjugated F(ab) $'_2$ fragments (Dako) followed, after washes, by staining with (FITC) conjugated CD3. Controls included cells incubated with mouse IgG of the relevant subclasses. Analysis was performed on a FACScan[®] (Becton Dickinson).

Radioiodination of Cells, Immunoprecipitation, and Immunoblot Analysis

Surface labeling of cells with ^{125}I (1 mCi; Amersham) was performed by the lactoperoxidase method, as previously described (11). For immunoprecipitation, cells were washed three times, and lysed in ice-cold lysis buffer (stop buffer with 1% NP-40, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM PMSF, and 1,000 U/ml aprotinin) for 30 min on ice. Immunoprecipitation with indicated mAbs (10 μg) adsorbed on protein A-Sepharose 3 h at 4°C under agitation was then performed on precleared lysates. Sepharose-bound immune complexes were washed three times in TNN buffer (50 mM Tris [pH 7.8], 250 mM NaCl, 0.1% NP-40), then eluted into reduced Laemmli buffer. Immunoprecipitated proteins were analyzed by SDS-PAGE on a 7.5% gel and revelation was performed by autoradiography. Unlabeled immunoprecipitated proteins were analyzed by SDS-PAGE on a 7.5% gel and then electroblotted onto Immobilon P membrane (Millipore). The blotted membrane was saturated overnight at 4°C in a buffer containing 5% BSA, 100 mM Tris, 1.4 M NaCl, pH 7.4, then incubated overnight with the anti- $\alpha 6$ mAb S3-41. Immunolabeling was revealed by chemiluminescence (ECL) after reaction with goat anti-mouse immunoglobulins in conjunction with HRP.

Anti-Phosphotyrosine Immunoblot Analysis

Cells at $20 \times 10^6/\text{ml}$ in RPMI 5% FCS were incubated with Abs at 37°C for various time intervals. Activation was stopped by addition of 1 ml of ice-cold stop buffer (20 mM Hepes [pH 7.4], 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, and 2 mM Na_3VO_4). Cells were spun down and the pellets were resuspended in lysis buffer (stop buffer with 1% NP-40, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM PMSF, and 1,000 U/ml aprotinin) and incubated on ice for 30 min. After centrifugation (15,000 g for 15 min)

supernatants were incubated with Abs for 4 h at 4°C with shaking, followed by 1 h with RAM adsorbed on protein A-Sepharose. Pellets were washed four times with lysis buffer containing 1% NP-40, then once in stop buffer without detergent before mixing with Laemmli buffer containing 3% (final volume) SDS. For reduction, the loading buffer contained 0.75 M 2 ME. Proteins were analyzed by SDS-PAGE, then electroblotted onto Immobilon P membrane (Millipore). The blots were saturated overnight at 4°C in a buffer containing 5% BSA, 100 mM Tris, 1.4 M NaCl, pH 7.4, then incubated overnight with the appropriate antibodies (HRP conjugated anti-phosphotyrosine mAb [4G10], pAb anti-lck, pAb anti-fyn, or pAb anti-Zap 70). For anti-lck, anti-fyn, and anti-Zap 70 immunoblots, membranes were washed and incubated 1 h with a HRP-conjugated goat anti-rabbit antibody. After washing, the immunolabeling was revealed by ECL (enhanced chemiluminescence) analysis system. HRP-conjugated anti-phosphotyrosine Ab was also used followed by ECL. For reprobing, the membranes were submerged in stripping buffer (100 mM 2 NE, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) and incubated at 50°C for 30 min. After washing, the membrane was blocked and immunodetection was performed as described.

Results

Immunohistolocalization of Laminin 5 on Post-natal Human Thymus Sections

We investigated laminin 5 ($\alpha_3\beta_3\gamma_2$) expression in the human thymus by immunofluorescence, using mAbs specific for the $\alpha 3$ chain (BM165 [49]; Fig. 1, a and b), for the $\beta 3$

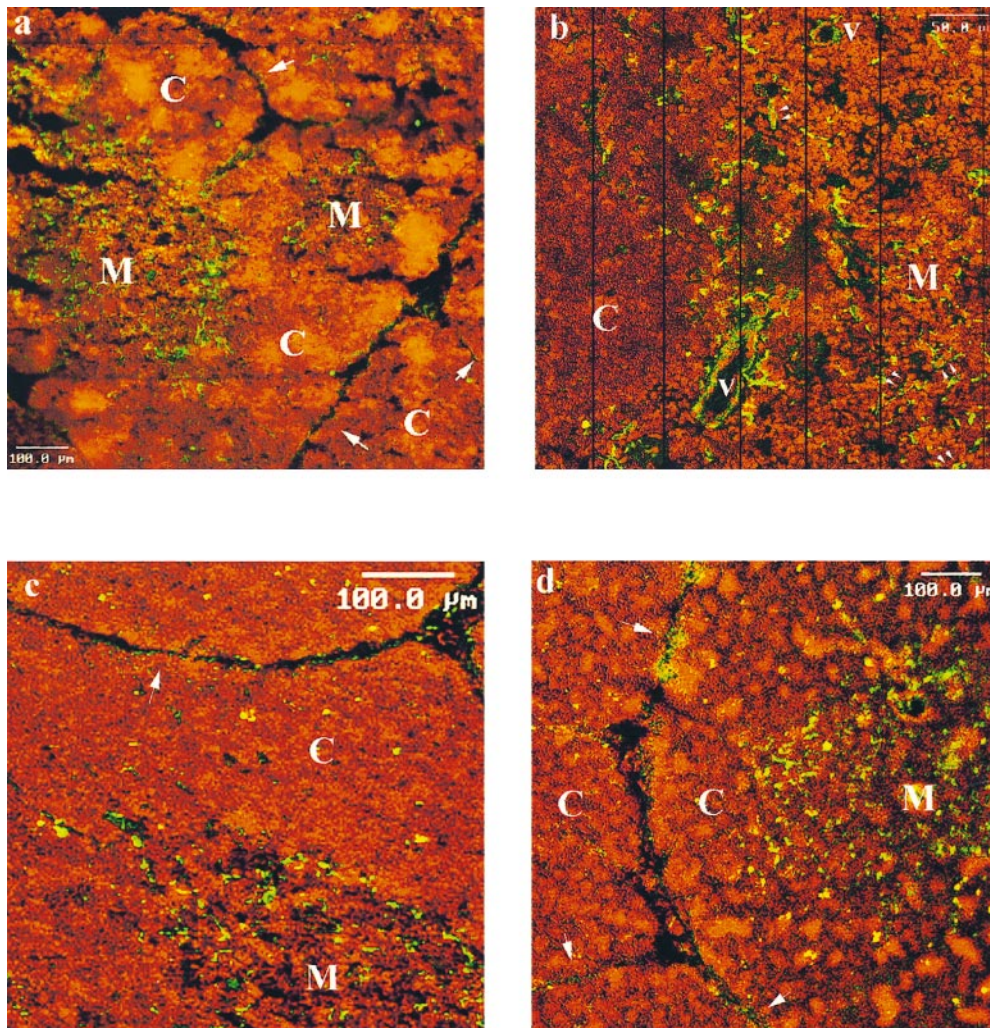


Figure 1. Immunofluorescence on frozen human thymus section was performed with anti-laminin 5 chain mAbs: the anti- $\alpha 3$ chain BM165 (a and b), the anti- $\beta 3$ chain 6F12 (c); the anti- $\gamma 2$ chain GB3 (d). mAb staining was revealed by FITC conjugated goat anti-mouse F(ab)'_2 fragments and analysis was performed by laser scanning confocal microscopy. Variations of cellular density between cortex (C) and medulla (M), were revealed by cell nuclear staining with propidium iodide. Single arrows designate the subcapsular basal laminae. Double arrows show examples of laminin 5 positive epithelial cells. b is a view constructed by the juxtaposition of successive areas scanned at a larger magnification.

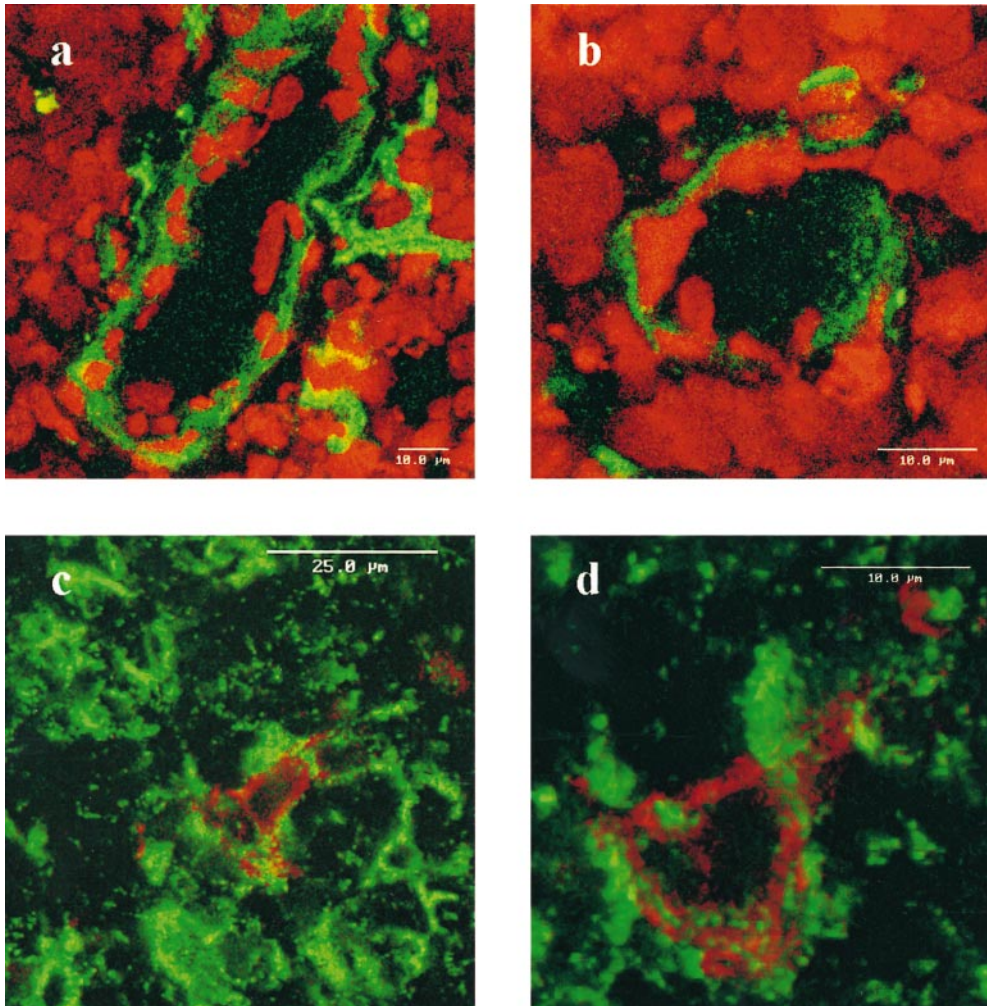


Figure 2. Frozen human thymus section stained with anti-laminin 5 α_3 chain mAb BM165. (a and b) BM165 staining on vessels; staining was revealed by FITC conjugated goat anti-mouse F(ab)'₂ fragments and analysis was performed by laser scanning confocal microscopy. Cell nuclei were stained with propidium iodide. (c and d) BM165 staining on stellate thymic epithelial cells; double staining obtained with mAb BM165 revealed by rabbit anti-mouse IgG1 F(ab)'₂ fragments followed by PE conjugated goat anti-rabbit F(ab)'₂ fragments, and the anti-keratin CK19 revealed by FITC conjugated goat anti-mouse IgM Ab. Controls made with secondary Abs gave no staining (not shown).

chain (6F12 [33]; Fig. 1 c), or for the γ_2 chain (GB3, [35]; Fig. 1 d). Staining of laminin 5 was performed together with a nuclear counter-staining with propidium iodide (Fig. 1, a–d; Fig. 2, a and b) in order to recognize the cortical (C) and the medullary (M) regions. All three mAbs stained strongly the medullary area of parenchyma and the basal laminae from subcapsular cortex, consistent with the presence of laminin 5 in these areas. More precisely, laminin 5 was localized in stellate keratin positive epithelial cells (Fig. 2, c and d) and in the basal laminae of vessels, including small vessels and capillary structures, together with endothelial cells of the larger vessels (Fig. 1 b, arrows; Fig. 2, a and b). Of note, a more intense staining at the corticomedullary junction was only visible with the anti- α_3 mAb BM165, suggesting that laminin 6 ($\alpha_3\beta_1\gamma_1$) and/or 7 ($\alpha_3\beta_2\gamma_1$) could be associated with laminin 5 in this area. As to the cortex, mAbs stained very scattered structures that corresponded for the most part to vascular structures.

Laminin 5 and Laminin 2 Have Distinct Effects on Thymocyte Proliferation: The Soluble Form of Laminin 5 Inhibits Proliferation of Human Thymocytes Stimulated via the CD3–TCR Complex

Since laminins were shown to influence the functional pro-

gram of numerous cell types, and in particular bulk preparations of laminins were shown to deliver coactivation signals to T cells (10, 58, 66), we investigated the effects of purified laminin 5 on thymocytes. When we coimmobilized laminin 5 on plastic, this induced a weak costimulation with a CD3–TCR signal, as compared with coated fibronectin or laminin 2 ($\alpha_2\beta_1\gamma_1$), another laminin isoform found in the thymus (10; Fig. 3). We then investigated the effect of laminin 5 and laminin 2 presented in soluble form; soluble laminin 5 clearly inhibited the proliferation of thymocytes stimulated via the CD3–TCR (Fig. 4 a). The inhibition was dose-dependent reaching a maximum of 50%, in terms of [³H]thymidine incorporation, at 5 μ g/ml. Over six independent experiments we saw a quite significant inhibition down to 1 μ g/ml (~30%). We checked the specificity of laminin 5–induced inhibition by adding the anti- α_3 chain mAb BM165, which recognizes an epitope involved in the interaction of laminin 5 with cells. BM165 abolished the inhibiting effect observed with soluble laminin 5 alone (Fig. 4 a). Conversely, no inhibition was seen when we added, instead of laminin 5, increasing amounts of soluble human laminin 2 (Fig. 4 a).

Inhibition was seen only when T cells were stimulated via the CD3–TCR; we observed no inhibition when thymocytes were induced to proliferate via a mitogenic pair

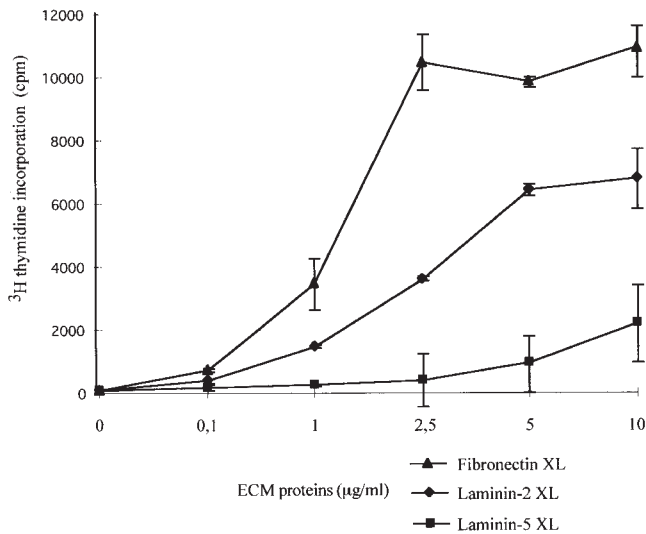


Figure 3. Comparison of costimulating effects of fibronectin, laminin 2 and laminin 5 when presented in cross-linked form to thymocytes. Indicated amounts of purified ECM components were first added to culture wells. After overnight incubation at 4°C, ECM solutions were discarded and wells were washed. CD3 mAb was next incubated in the culture wells for an additional 12 h at 4°C. After further washes, cells were added (10^5 /well) to the culture wells and incubated for 4 d with addition of [3 H]thymidine for the final 18 h.

of CD2 mAbs (Fig. 4 b). We verified that inhibition of proliferation was not related to apoptosis by incubating cells with Hoechst 342 30 min at 37°C. Results analyzed on a FACStar[®] cytometer (Becton Dickinson) did not reveal any sign of apoptosis (not shown).

Inhibition by Soluble Laminin 5 of Early Kinase Phosphorylations Activated via the CD3–TCR Complex

We tested soluble laminin 5 effects on early kinase phosphorylation events. Since Jurkat cells express a low density of $\alpha_6\beta_4$ we selected by cytofluorometry cells expressing $\alpha_6\beta_4$ in similar density as CD3^{high} thymocytes (not shown). These cells were activated with a CD3 mAb ($\times 3$, 10 μ g/ml) during 2.5 min at 37°C, with or without soluble laminin 5. Immunoprecipitation of Zap 70, p56^{lck}, or p59^{fyn} was carried out on activated cell lysates, after which precipitated proteins were transferred on Immobilon. Membranes were then incubated with HRP conjugated anti-phosphotyrosine antibody. Results were revealed by ECL system. We observed that the addition of 1 μ g/ml of soluble laminin 5 was sufficient to induce an inhibition of Zap 70 (Fig. 5 a) and p59^{fyn} tyrosine phosphorylation (Fig. 5 c). By contrast, the addition of soluble laminin 5, up to 5 μ g/ml, a concentration fully efficient in proliferation assays, did not induce a significant inhibition of p56^{lck} phosphorylation (Fig. 5 b). Membranes were stripped, blocked, and reprobed with the precipitating antibody, i.e., respectively anti-Zap 70 (Fig. 5 a), anti-lck (Fig. 5 b), and anti-fyn (Fig. 5 c), respectively, in order to verify amounts of precipitated material in each lane.

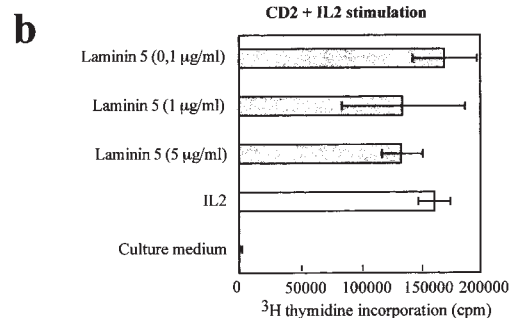
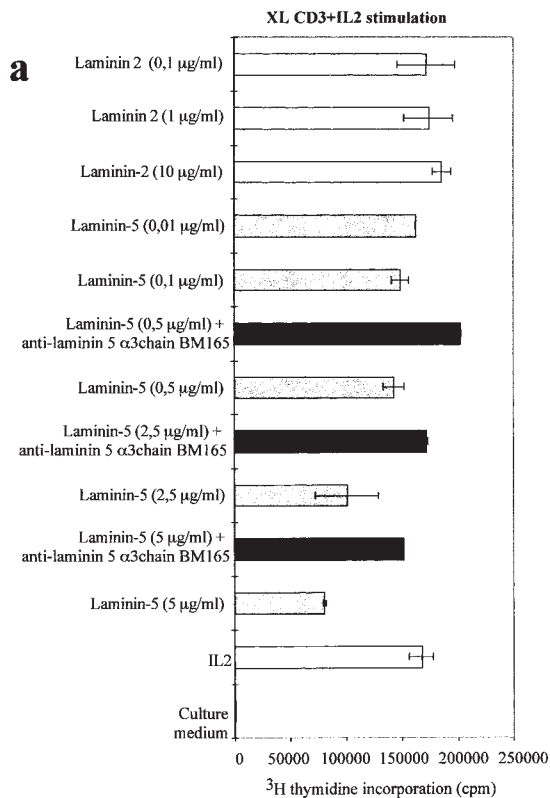
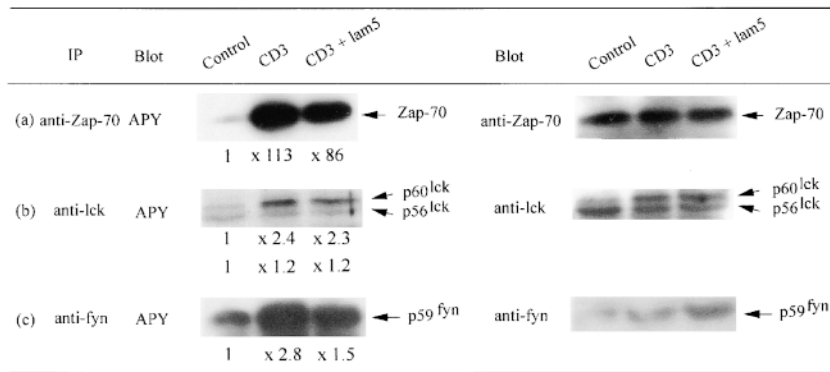


Figure 4. Laminin 5 but not laminin 2, when added in soluble form, inhibits human thymocyte proliferation induced by CD3 + IL-2. (a) CD3 antibodies were first incubated overnight in culture wells. Next, 10% FCS in RPMI was added and incubated in order to minimize further protein adsorption on plastic. After washing, cells (10^5 /well) were added in culture medium containing IL-2 (5 ng/ml) and laminin 5 or laminin 2. Cultures were also performed in the presence of 10 μ g/ml anti-laminin 5 mAb BM165. (b) Effect of soluble laminin 5 on thymocyte proliferation induced by a mitogenic CD2 mAb pair (GT2 + D66, 10 μ g/ml) plus IL-2.

Identification of an Anti- α_6 mAb Specific for $\alpha_6\beta_4$: Evidence for Distinct Forms of α_6 Chains Paired with β_1 and β_4 Chains

Next, we investigated whether the effect of laminin 5 would be mediated by $\alpha_6\beta_4$, $\alpha_3\beta_1$, or both. We tested a battery of anti- α_6 mAbs for their reactivity in flow cytometry with dispersed human thymocytes (135 13C [rat IgG2a]; J8H, BQ16, 450 30A1, S3-41 [mouse IgG1]; reference 24)



mentioned under each lane. For reprobing, the membranes were submerged in stripping buffer, blocked and immuno-detected with (a) pAb anti-Zap 70, (b) pAb anti-lck, and (c) pAb anti-fyn was performed. Reactions were revealed by incubating membranes with GAR HRP followed by ECL. Laminin 5 (lam5) was added in soluble form at 1 $\mu\text{g/ml}$ for Zap 70 and fyn analysis and 5 $\mu\text{g/ml}$ for lck analysis.

Figure 5. Inhibition by soluble laminin 5 (lam5) of early kinases phosphorylation events activated via the CD3-TCR complex. Jurkat cells at $20 \times 10^6/\text{ml}$ in RPMI 5% FCS were incubated with Abs at 37°C for 2.5 min, solubilized, and lysates were immunoprecipitated with (a) a pAb anti-Zap 70, (b) a pAb anti-lck, (c) a pAb anti-fyn. After SDS-PAGE separation and electrotransfer onto Immobilon P membrane, proteins were incubated with HRP conjugated anti-phosphotyrosine Ab (APY) followed by ECL. For densitometric analysis of APY immunoblotting, control values were reduced to 1 in order to compare signals after activation. Values are

and the widely used anti- $\alpha 6$ GOH3 [rat IgG1; reference 61]. We noticed that the mouse mAb S3-41 displayed a peculiar reactivity as compared with other anti- $\alpha 6$ mAbs, strikingly similar to the reactivity of the anti- $\beta 4$ mAb 3E1F6 (Fig. 6). S3-41, as 3E1F6, did not react with the most immature CD3^{low} thymocytes. Moreover, staining by S3-41 and 3E1F6 increased with the density of CD3, which indicates the level of maturation of thymocytes (Fig. 6). This pattern of reactivity was in marked contrast to the pattern given by the other anti- $\alpha 6$ mAbs tested including GOH3, which stained immature CD3^{low} thymocytes. Of note, peripheral blood T lymphocytes were equally stained with GOH3 and S3-41 (not shown).

To confirm that S3-41 would react only with the $\alpha 6$ chains linked to $\beta 4$, we performed sequential immunoprecipitations using the colon carcinoma cell line HT29, which expresses high densities of both $\alpha 6\beta 1$ and $\alpha 6\beta 4$. Cells were lysed with 1% NP-40 and lysates were depleted of $\beta 1$ or $\beta 4$ integrins by two successive immunoprecipitations either with the anti- $\beta 1$ mAb K20 or the anti- $\beta 4$ mAb 3E1F6. Depleted lysates were then immunoprecipitated with either the anti- $\alpha 6$ mAbs S3-41 or GOH3 (Fig. 7 a). It can be seen that GOH3 was still able to immunoprecipitate residual material when lysates were depleted of either $\beta 1$ or $\beta 4$ integrins (Fig. 7 a, middle, lane 3; right, lane 3). In contrast, S3-41 did not precipitate any residual material when lysates were depleted of $\beta 4$ integrins (Fig. 7 a, right, lane 2) whereas it remained fully reactive when lysates were depleted of $\beta 1$ integrins (Fig. 7 a, middle, lane 2). Thus, if GOH3 immunoprecipitates $\alpha 6$ chains associated with either $\beta 1$ or $\beta 4$ chains, S3-41 recognizes only $\alpha 6$ chains paired with $\beta 4$. These results were confirmed by immunoblotting experiments with S3-41. Cell lysates were first immunoprecipitated either with the anti- $\beta 1$ mAb K20 or the anti- $\beta 4$ 3E1F6. Fig. 7 b shows that S3-41 recognized only the $\alpha 6$ chains that coimmunoprecipitated with $\beta 4$. Note that these experiments were performed on two distinct cell lines that expressed different relative amounts of $\alpha 6\beta 1$ and $\alpha 6\beta 4$ (Fig. 7 b, left and right). Taken together, these experiments demonstrate that the anti- $\alpha 6$ mAb S3-41 reacts with an epitope present only when $\alpha 6$ chains are linked to $\beta 4$ chains.

When Used in Soluble Form the $\alpha 6\beta 4$ -specific mAb S3-41 Inhibits Proliferation of Thymocytes Stimulated via the CD3-TCR Complex

Anti- $\alpha 6$ antibodies were used in proliferation assays performed on human thymocytes. To determine which laminin 5 receptor is involved in the modulation of thymocyte proliferation, we used GOH3 mAb which recognizes all $\alpha 6$ chains and S3-41 mAb which is $\alpha 6\beta 4$ specific. As previously described (58, 66), the anti- $\alpha 6$ mAb GOH3 immobilized on culture wells delivered a strong costimulus for thymocyte proliferation in the presence of cross-linked CD3 mAb (Fig. 8 a). Under the same conditions, S3-41 delivered a weak proliferative costimulus to thymocytes when compared with GOH3. Moreover, there was no additive or synergistic effect when the two mAbs GOH3 and S3-41 were immobilized on plates with a CD3 mAb. When anti- $\alpha 6$ mAbs were used in soluble form, we observed that only S3-41 inhibited proliferation of thymocytes stimulated via the CD3-TCR complex (Fig. 8 b). Inhibition was very efficient with S3-41 (50%). We tested the same soluble mAbs on thymocytes stimulated with a mitogenic pair of CD2 mAbs (GT2 + D66) (Fig. 9 a), the CD28 mAb and immobilized CD3 mAb (Fig. 9 b), or the CD28 mAb and rIL-2 (Fig. 9 c). Again, an inhibitory effect was seen only when thymocytes were stimulated via the CD3-TCR pathway.

We also used the anti- $\alpha 3$ mAb P1B5, which recognizes the $\alpha 3\beta 1$ epitope involved in laminin 5 binding (7, 48). When added in soluble form in amounts ranging from 0.1 to 10 $\mu\text{g/ml}$, P1B5 did not inhibit proliferation of thymocytes stimulated via the CD3-TCR complex (not shown).

As it has been described that the non-integrin receptor p67 can associate with $\alpha 6\beta 4$ (2), it was of interest to test the effects of an anti-p67 mAb, namely MPLR2, on T cell proliferation. We did not observe a significant inhibition of soluble MPLR2 on thymocyte proliferation induced via the CD3-TCR complex (not shown).

Inhibition by Soluble S3-41 mAb of Early Kinase Phosphorylations Activated via the CD3-TCR Complex

We tested the effects of S3-41 and P1B5 mAbs, used in sol-

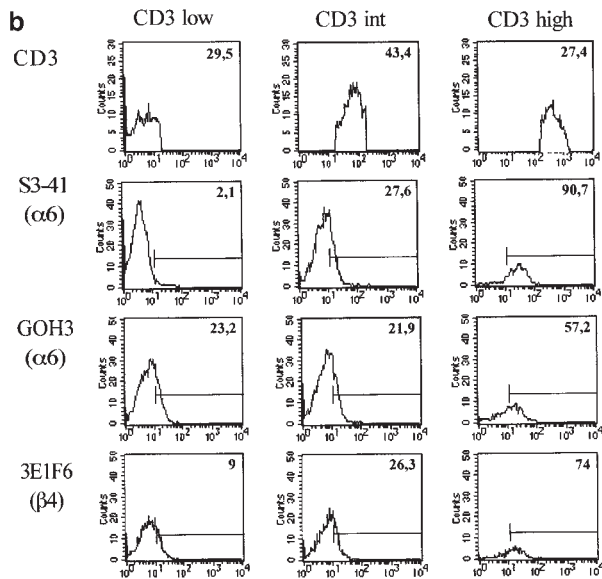
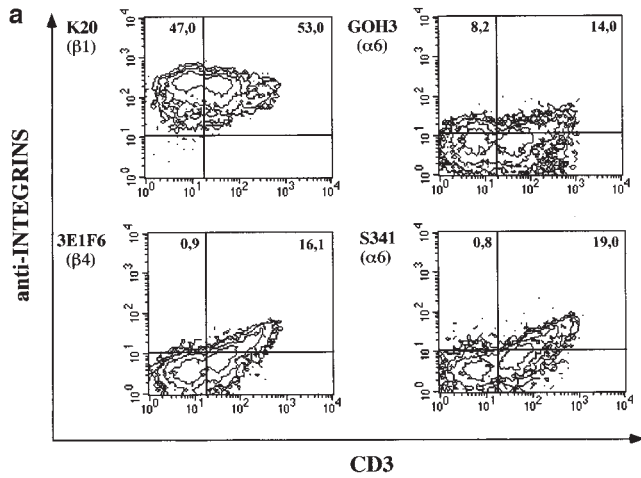


Figure 6. (a) The anti- α_6 integrin chain mAb S3-41 has a peculiar pattern of reactivity with human thymocytes, resembling the reactivity of the anti- β_4 chain mAb. Two color immunofluorescence was performed on human thymocytes with the CD3 mAb X3 directly conjugated with FITC and with the indicated amounts of anti-integrin mAb that was indirectly labeled with goat anti-mouse PE conjugated F(ab)₂ fragments. The percentage of positive cells for the integrin chain analyzed is noted in the upper corner of the corresponding area. α_6 expression was analyzed with the mAbs GOH3 and S3-41; β_1 expression was analyzed with the mAb K20 and β_4 expression was analyzed with the mAb 3E1F6. (b) Reactivities of the anti- α_6 mAb S3-41 and GOH3 and of the anti- β_4 mAb 3E1F6 with thymocyte subpopulations according to their surface density in CD3. The percentage in the upper right corner indicates the number of positively stained cells. All antibodies were used at saturating doses, i.e., 10 μ g/ml for S3-41, 3E1F6, K20 and 5 μ g/ml for GOH3 and X3.

uble form, on early kinase phosphorylation events (Fig. 10). Jurkat cells expressing $\alpha_6\beta_4$ in similar density as CD3^{high} thymocytes were activated with a CD3 mAb ($\times 3$, 10 μ g/ml) during 2.5 min at 37°C, with or without soluble mAbs. Immunoprecipitation of Zap 70, p56^{lck}, or p59^{fyn}

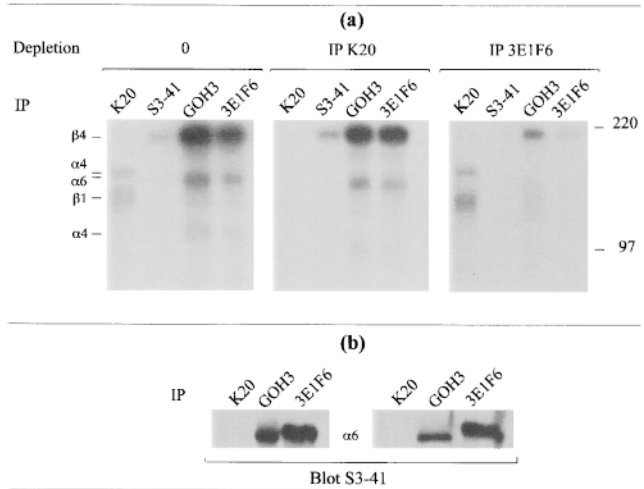


Figure 7. (a) Sequential immunoprecipitations of α_6 , β_1 , or β_4 chains from HT29 colon carcinoma cell line after anti- β_1 or anti- β_4 immunoprecipitations (IP). (Left) IP with anti- β_1 mAb K20, anti- α_6 mAb S3-41, anti- α_6 mAb GOH3, and anti- β_4 mAb 3E1F6 were performed on total cell lysates. (Middle) To deplete lysates of β_1 integrins, a first IP with the anti- β_1 mAb K20 was performed on cell lysates and a second IP was then carried out on the supernatants using the same mAb. Finally, a third immunoprecipitation was carried out on the supernatants with the indicated mAbs. (Right) To deplete lysates of $\alpha_6\beta_4$, a first IP with the anti- β_4 mAb 3E1F6 was performed on cell lysates and a second IP was then performed on the supernatants using the same mAb. Finally, a third immunoprecipitation was performed on the supernatants with the indicated mAbs. Controls showed that the material recovered was efficiently depleted in β_1 (middle panel, lane 1) or β_4 chains (right panel, lane 4), respectively. Immunoprecipitated proteins were separated on SDS-PAGE 7.5%, under reducing conditions. Results were revealed by autoradiography. (b) Immunoblots with the anti- α_6 mAb S3-41 were performed on immunoprecipitates obtained with mAbs K20 (anti- β_1), 3E1F6 (anti- β_4) and GOH3 (anti- α_6) on lysates from (left) HT29 cells that express $\alpha_6\beta_4$ at higher density than $\alpha_6\beta_1$ or (right) K562 cells that express $\alpha_6\beta_1$ at higher density than $\alpha_6\beta_4$. Immunoprecipitated proteins were separated on SDS-PAGE 7.5%, under reducing conditions before blotting. Immunolabeling was revealed by chemiluminescence (ECL) after reaction with goat anti-mouse immunoglobulins conjugated with HRP.

was carried out on activated cell lysates. Precipitated proteins were transferred onto Immobilon, then membranes were incubated with an anti-phosphotyrosine antibody coupled to peroxidase. Results were revealed by ECL. We observed that the addition of S3-41 (10 μ g/ml) induced the same pattern of inhibition as soluble laminin 5 with inhibition of Zap 70 and p59^{fyn} tyrosine phosphorylation (Fig. 10, a and c, respectively) but no inhibition of overall p56^{lck} (Fig. 10 b). In contrast, addition of soluble P1B5 (10 μ g/ml) did not induce this particular pattern of reactivity, supporting the hypothesis that $\alpha_6\beta_4$ is the laminin 5 receptor involved.

Discussion

Using mAbs specific for the three chains of laminin 5

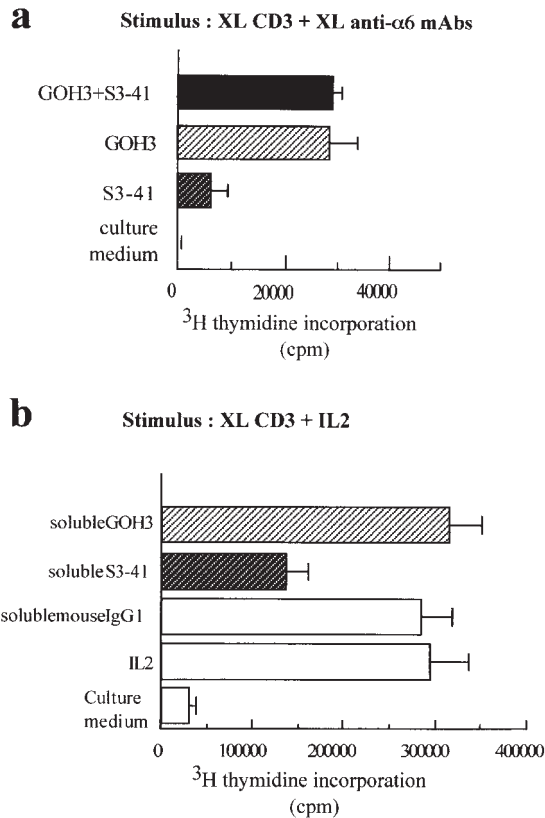


Figure 8. Effects of anti- $\alpha 6$ chain mAb S3-41, added, either in cross-linked or soluble form, on thymocyte proliferation. (a) S3-41 or GOH3 and CD3 were cross-linked on plastic from culture wells as indicated in Fig. 2 before adding the thymocyte suspension. (b) CD3 was first cross-linked on plastic from culture wells. FCS in culture medium was incubated for 2 h at 37°C to saturate the plastic, before adding IL-2, S3-41, or GOH3 in solution and the thymocyte suspension. The indicated mAbs were added at 10 $\mu\text{g}/\text{ml}$.

(anti- $\alpha 3$ mAb BM165, anti- $\beta 3$ mAb 6F12, anti- $\gamma 2$ mAb GB3), we have investigated the presence and cellular distribution of laminin 5 on frozen human thymus sections. It has been mentioned in earlier publications that laminin 5 is expressed in the human thymus (25, 42, 72). Our data focus on laminin 5 expression in thymic parenchyma and link up with functional effects of this molecule on thymocyte proliferation. Since we have found an identical reactivity of the three mAbs, it can be concluded that a complete form of laminin 5 is displayed by the thymus. Indeed, it is known that $\beta 3\gamma 2$ modules can be expressed without any detectable $\alpha 3$ chains, such as in choroid plexus (1). We have observed that laminin 5 has quite a particular expression in the thymus; it can be detected in the medulla where a subpopulation of epithelial cells displays laminin 5 as revealed by double staining with keratin. For double staining we used the anti-keratin Ab CK19 that recognizes all epithelial cells (71). Whether the laminin 5 epithelial cells form a specialized set of cells or whether any epithelial cells in these areas could become laminin 5 positive under certain circumstances of stimulus remains to be estab-

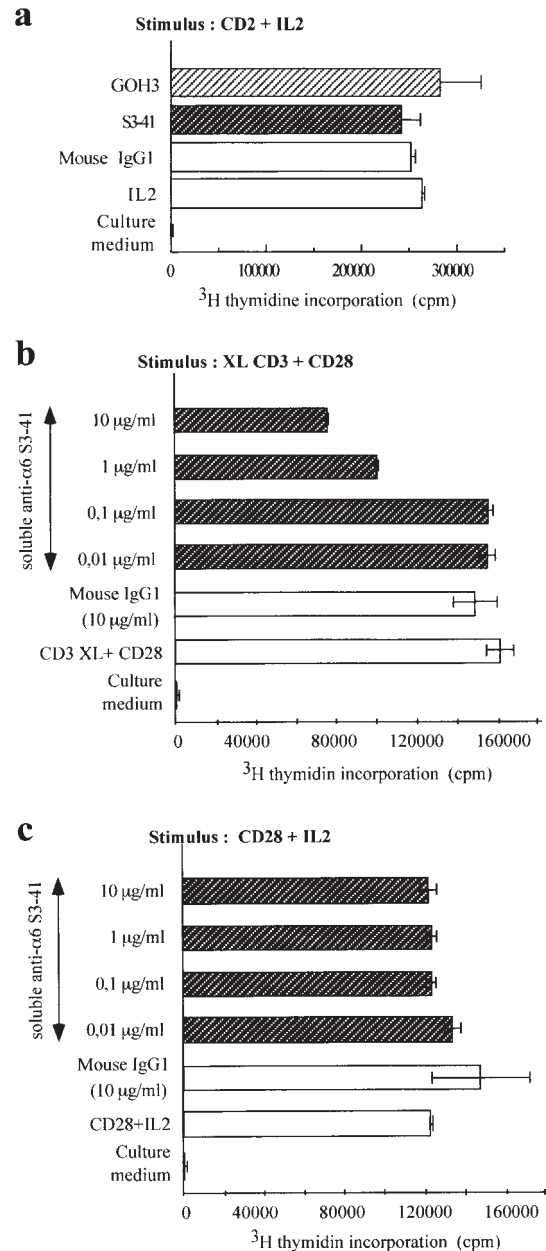


Figure 9. Effects of anti- $\alpha 6$ chain mAb S3-41, added in soluble form, on thymocyte proliferation induced by several pathways. (a) Cells were stimulated with a mitogenic pair of CD2 mAbs (GT2 and D66, 10 $\mu\text{g}/\text{ml}$) plus IL-2. The indicated mAbs were added in soluble form at saturating dose (10 $\mu\text{g}/\text{ml}$). (b) Cells were stimulated with plate-bound CD3 and soluble CD28 mAb (9.3, 1/500). (c) Cells were stimulated with soluble CD28 mAb 9.3 and rIL-2. Various amounts of S3-41 were added in soluble form before addition of cell suspension.

lished. However, this feature is additional to the similarities previously described between thymic epithelial cells and epidermal keratinocytes (45). Thymic vessels are also strongly stained with anti-laminin 5 antibody, particularly at the level of basal laminae, including capillary structures. It is more difficult to ascertain that endothelial cells are

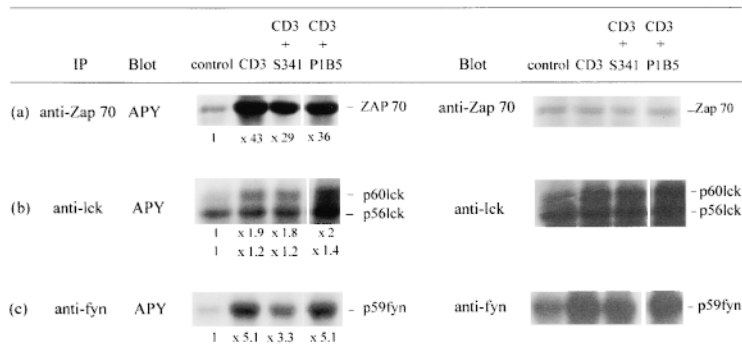


Figure 10. Inhibition by soluble anti- $\alpha 6$ mAb S3-41 of early kinases phosphorylation events activated via the CD3-TCR complex. Jurkat cells at $20 \times 10^6/\text{ml}$ in RPMI 5% FCS were incubated with Abs at 37°C for 2.5 min, solubilized, and lysates were immunoprecipitated with (a) a pAb anti-Zap 70, (b) a pAb anti-lck, (c) a pAb anti-fyn. After SDS-PAGE separation and electrotransfer onto Immobilon P membrane, proteins were incubated with HRP conjugated anti-phosphotyrosine Ab (APY) followed by ECL. For reprobing, the membranes were submerged in stripping buffer, blocked, and immunodetected with (a) pAb anti-Zap 70, (b) pAb anti-lck, (c) pAb anti-fyn was performed. Reactions were revealed by incubating membranes with GAR HRP followed by ECL. For densitometric

analysis of APY immunoblotting, control values were reduced to 1 in order to compare signals after activation. Values are mentioned under each lane. Anti-integrin antibodies were added in soluble form at $10 \mu\text{g}/\text{ml}$.

also stained; this staining is irregular and is restricted to larger vessels. Interestingly, preliminary results indicate that, in humans, small vessels and capillary structures from other organs appear devoid of laminin 5. In the thymus, vessels are abundant in the medulla, particularly at the corticomedullary junction and it is likely that emigration of thymocytes having reached maturation occurs in this area. Indeed, laminin 5 has been involved in various cell migration phenomena in wound repair (63) or tumor invasion (26, 27, 41) involving cleavage by metalloproteases (17). Therefore, the present data raise the possibility of a role for laminin 5 in T cell migration outside the thymus. The intensity of BM165 staining at the corticomedullary junction suggests that laminin 6 and/or 7 may be also present in these areas. Laminin 5 is also present in subcortical flat epithelial basement membrane that is also known to include hemidesmosome components like collagen type VII and $\alpha 6\beta 4$ integrins (50, 72). It is thus likely that laminin 5 in this region has a different function to the laminin 5 present in the thymus parenchyma, which would be similar to the structural role it exerts in the basement membrane of skin and other epithelia.

Whereas laminin 5 has been identified as a ligand for both $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins in vitro, (7, 44, 48, 60), we have observed that $\alpha 3\beta 1$ and $\alpha 6\beta 4$ have a quite distinct distribution on human thymocytes in vivo. Whereas it was found that $\alpha 3\beta 1$ is evenly distributed on all thymocyte subsets (43), we have shown here that $\alpha 6\beta 4$ density on the surface of thymocytes parallels their maturation. We have used the mAb S3-41 (24) because we found that it reacts only with the $\alpha 6$ chains linked to the $\beta 4$ chain, and not with the $\alpha 6$ chains linked to the $\beta 1$ chain. Although this mAb was classified as CD49f by the International Workshops on Leucocyte Antigens on the basis of its capacity to recognize human $\alpha 6$ transfected in RBL rat cells (22), a particular reactivity was noticed on skin sections, where $\alpha 6$ is associated with the $\beta 4$ chain, but not on kidney sections, where $\alpha 6$ is associated with the $\beta 1$ chain (28, 77). The sequential immunoprecipitations and immunoprecipitations followed by Western blots that were performed in the present study confirmed that S3-41 mAb reacts exclusively with the $\alpha 6$ chain bound to $\beta 4$. We have noticed that, on

total lysates, S3-41 immunoprecipitated much less material than anti- $\alpha 6$ mAb GOH3. This could be explained either by a difference of affinity between the two mAbs and/or by the staining of only a subpopulation of $\alpha 6$ chains with S3-41. Indeed, S3-41 mAb did not precipitate any material when lysates were depleted of $\beta 4$ integrins but it was fully reactive when lysates were depleted of $\beta 1$ integrins, and only $\alpha 6$ chains coimmunoprecipitated with $\beta 4$ carried the S3-41 epitope. Moreover, as S3-41 reacted with $\alpha 6$ chains separated from $\beta 4$ chains, this suggests that it recognizes an intrinsic isomorphism of the $\alpha 6$ chains and not a conformational epitope. Thus, strikingly, $\alpha 6\beta 4$ upregulation on mature thymocytes coincides with laminin 5 expression in the medullary area, the thymic region where mature thymocytes are located. In mice, it has been described that, on the contrary, downregulation of $\beta 4$ is simultaneous with the upregulation of CD4, CD8, and CD3 (73), but differences in integrin expression with T cell maturation between human and mouse have already been observed for $\alpha 4\beta 1$ (55).

Since it was described that bulk preparations of laminin coated on plastic are strongly mitogenic for T cells (58), it was of interest to investigate the effect of laminin 5 on human T cell proliferation. We show here that laminin 5 presented in a cross-linked form has only a slight coactivation effect on thymocytes. However, when presented in soluble form, a clear inhibition of proliferation triggered via the CD3-TCR complex was observed. It should be noted that this effect was reproduced by the anti- $\alpha 6$ mAb S3-41; however, it was not reproduced by the typical anti- $\alpha 3$ integrin mAb P1B5 in agreement with the view that the inhibitory influence of soluble laminin 5 on T cell proliferation is mediated via $\alpha 6\beta 4$. In addition, we did not observe an inhibition on TCR triggered proliferation with soluble mAb MPLR2, an antibody reacting with the high affinity non-integrin p67 laminin receptor, reported to be associated with $\alpha 6\beta 4$ (2). Soluble laminin can be detected in the serum of normal individuals, and its level was found to be strongly increased in inflammatory conditions and certain autoimmune disorders (57, 75). Laminin is also present in the extracellular spaces of secreting cells, and it was found that a significant proportion ($\sim 15\%$) of Engelbreth Holm

Swarm matrix proteins is present as a soluble form released into the supernatant (47). It is likely that an equilibrium takes place between the soluble laminins, either released by secreting cells or liberated from the ECM on the one hand, and the laminin deposited as an insoluble fraction in the ECM or on cell surface receptors on the other hand (47). The data presented here disclose a cross-talk event occurring between the laminin 5 receptor $\alpha_6\beta_4$ and the T cell receptor. Of note, inhibition induced by laminin 5 is restricted to signaling via the CD3-TCR complex, and we observed no inhibition when T cells were stimulated via other pathways such as the CD2 or the CD28 pathways. Inhibition by laminin 5 therefore shares similarities with the inhibition generated by β_1 integrin ligands we have described, either VCAM 1 or mAbs (19, 65, 34). It has been previously shown by other groups that integrins can negatively regulate T cell activation (21, 36, 64). Our data show that $\alpha_6\beta_4$ can also modulate T cell activation. Consistent with this view, it has been previously suggested that this integrin could, depending on the cell type, either increase or block normal cell proliferation (16). Besides, as an activation pathway was previously described when $\alpha_6\beta_4$ was ligated with coated antibodies or laminin 5 (32), our data obtained with soluble ligand and mAb suggest also that exposing cells to immobilized or soluble ligands can trigger different signaling pathways. Such influences, depending on the form of the ligand, fits well with the known feature of integrins signaling, whose pattern is critically dependent on sole ligand occupancy, sole integrin aggregation or both ligand occupancy and aggregation (40).

Moreover, we have observed here that laminin 5 inhibits the phosphorylation of Zap 70 and p59^{fyn} but has no effect on overall phosphorylation and activation of p56^{lck}, a pattern of kinase inhibition that was also observed in our laboratory by binding β_1 integrins with soluble ligand (34). Thus $\alpha_6\beta_4$, under the influence of laminin 5 appears to use, at least in part, the same intracellular signaling pathway as β_1 integrins under the influence of VCAM 1. Whether these signaling pathways are common or whether they diverge deserves further investigation.

There is increasing evidence that integrins and their ligands, including ECM components, play a critical role in T cell maturation (5, 10, 12, 20, 31, 43, 54–56, 69, 73, 74). The pattern of expression of the various integrins present in the thymus and the pattern of expression of their ligand are both subtle events that account for their influence at given stages of T cell differentiation/maturation (12, 54). Since, it is clear that thymocyte differentiation involves cycles of proliferation and arrest (18), the coordination of adhesion and proliferation events mediated by laminin sub-types is likely to be significant for T cell maturation. The results reported here suggest that laminin 5 might be an important signaling molecule in the control of expansion of mature thymocytes and possibly their migration outside the thymus, given its presence in vascular structures.

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