E-cadherin-mediated interactions of thymic epithelial cells with CD103$^+$ thymocytes lead to enhanced thymocyte cell proliferation

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Summary

Cadherins are a family of cell adhesion molecules that mainly mediate homotypic homophilic interactions, but for E-cadherin, heterophilic interactions with the integrin α_E(CD103)β_7 have also been reported. In the human thymus, where thymocytes develop in close contact with thymic stromal cells, E-cadherin expression was detected on thymic epithelial cells. By immunofluorescence staining, the strongest expression of E-cadherin was observed on medullary thymic epithelial cells. These cells also express cytosolic catenins, which are necessary to form functional cadherin-catenin complexes. Regardless of their developmental stage, human thymocytes do not express E-cadherin, indicating that homophilic interactions cannot occur. Flow cytometric analysis revealed that the E-cadherin ligand CD103 is expressed on subpopulations of the early CD4$^-$ CD8$^-$ double-negative and of the more mature CD8$^+$ single-positive thymocytes. Using an in vitro cell adhesion assay, double-negative and CD8$^+$ single-positive thymocytes adhered strongly to isolated thymic epithelial cells. These adhesive interactions could be inhibited by antibodies against E-cadherin or CD103. CD8$^+$ thymocytes showed a proliferative response when incubated with thymic epithelial cells. This mitogenic effect was inhibited by antibodies against CD103, which strongly indicates a direct involvement of the adhesive ligand pair CD103–E-cadherin in human thymocyte cell proliferation.

Key words: Thymus, Cell-to-cell interactions, Cellular differentiation, Cell adhesion molecules, Cadherins

Introduction

The development of T-lymphocyte precursors into mature T-cells takes place in the thymus, where a specialised microenvironment for the control of thymocyte proliferation, differentiation and repertoire selection processes is provided (Chidgey and Boyd, 2001). Crucial elements of this microenvironment are a complex extracellular matrix and a heterogeneous class of thymic epithelial cells, which are found in the cortical as well as in the medullary regions of the thymic lobules (Owen et al., 1999; Anderson et al., 2000; Savino et al., 2000; Kutleša et al., 2002). Defined stages of thymocyte development within the thymic lobules can be monitored by the expression of accessory molecules, for example, CD4 and CD8 (Shortman and Wu, 1996; Res and Spits, 1999). The earliest double-negative (DN, CD4$^-$ CD8$^-$) thymocytes are mainly located at the subcapsular region of the thymic lobules. These DN thymocytes develop into double-positive (DP, CD4$^+$ CD8$^+$) cells, which are found throughout the cortical region and represent the vast majority of all thymocytes. In the medulla, the more mature CD4$^+$ or CD8$^+$ single-positive (SP) thymocytes can be detected. Although it is widely accepted that stromal cells of the thymic microenvironment including thymic epithelial cells play a pivotal role in thymocyte development, the molecular nature of these interactions is not fully understood.

The cell-cell adhesion molecule family of classical cadherins consists of more than 15 members (Kemler, 1992; Angst et al., 2001). Cadherins are not only critically involved in the maintenance of tissue architecture and embryonic development but also in cellular signalling processes (Knudsen et al., 1998). However, this gene family has not gained much attention in thymocyte development, although several cadherins, including E-cadherin, have been detected on murine thymocytes (Munro et al., 1996). The classical type I cadherins, to which E-, N- and P-cadherin belong, mediate homophilic adhesive interactions; however, a heterophilic interaction of E-cadherin with the integrin α_E(CD103)β_7 has been recently determined as an exception to the rule of homophilic cadherin interactions (Cepek et al., 1994; Higgins et al., 1998). The integrin $\alpha_E\beta_7$ is expressed on intraepithelial T lymphocytes of the skin and gut, but it is also found on a very small percentage of peripheral blood lymphocytes and developing T cells in the thymus (Andrew et al., 1996; Agace et al., 2000; Pauls et al., 2001). Analysis of CD103-deficient mice indicated an important role for $\alpha_E\beta_7$ in the localisation of mucosal T lymphocytes (Schön et al., 1999). Although the localisation of cutaneous lymphocytes is not affected in these knockout mice, the $\alpha_E\beta_7$ deficiency seems to be a risk factor for inflammatory skin diseases (Schön et al., 2000). Whether

the development of thymocytes in CD103-deficient mice is altered or impaired has not been reported so far.

A functional role for E-cadherin in the fetal thymus was determined in murine fetal thymic organ cultures (Müller et al., 1997). E-cadherin has been shown to be expressed by murine thymic epithelial cells as well as thymocytes at embryonic days 14 to 18, but not on thymocytes of adult animals (Lee et al., 1994). Antibodies against E-cadherin-mediated homophilic interactions blocked epithelial organisation and early thymocyte development in reaggregate fetal thymic organ cultures, whereas an antibody selectively inhibiting interactions of E-cadherin with the integrin $\alpha_E\beta_7$ did not interfere with these processes in the embryonic tissue (Müller et al., 1997). The functional significance of the adhesion pair E-cadherin–integrin $\alpha_E\beta_7$ in the postnatal murine thymus, however, has not been investigated.

In the present study we have analysed the expression of E-cadherin in human thymic tissue. Since E-cadherin was not detected on human postnatal thymocyte subpopulations, the expression pattern of CD103 on human thymocyte subpopulations was studied in detail. Functional analyses, including cell adhesion and proliferation studies, were performed to determine the role of E-cadherin and its ligand $\alpha_E\beta_7$ integrin during human thymocyte development.

Materials and Methods

Tissues and cells

Normal thymuses were obtained from children (<8 years) undergoing cardiac surgery after informed consent of their parents. Thymocytes were isolated from the thymuses by disrupting the thymic tissue and flushing the thymocytes out of the tissue with a syringe filled with RPMI 1640 cell culture medium.

Antibodies

Two different monoclonal antibodies against human E-cadherin were used in this study. The antibody HECD-1 (R&D Systems, Wiesbaden, Germany) was used for immunoprecipitation, flow cytometric analysis and adhesion blockade, whereas the antibody 67A4, which was recently characterised as another human E-cadherin-specific antibody (Bühring et al., 1996), was used for immunoblotting and immunohistochemistry. The anti-P-cadherin antibody NCC-CAD 299 (Shimoyama et al., 1989) was a kind gift of Dr Hirohashi (National Cancer Center Research Institute, Tokyo, Japan). Two polyclonal rabbit antisera against human α- und β-catenin were purchased from Sigma (Deisenhofen, Germany). Monoclonal antibodies generated against α-, β- or γ-catenin were purchased from Transduction Laboratories (α and β; BD Biosciences, Heidelberg, Germany) or Sigma (β and γ). The mouse monoclonal anti-CD103 antibody (clone 2G5.1) was obtained from Serotec (Biozol, Eching, Germany). Medullary thymic epithelial cells were specifically labelled with the monoclonal antibody TE4 (IgM) obtained from the American Type Culture Collection (ATCC; Manassas, VA).

Isolation of thymocyte subpopulations by magnetic cell sorting

Using the MACS CD4 Multisort kit (Miltenyi Biotec, Bergisch Gladbach, Germany), $CD4^-$ $CD8^-$ double-negative (DN) thymocytes, $CD4^+$ $CD8^+$ double-positive (DP) thymocytes, and the $CD4^+$ and $CD8^+$ single-positive (SP) thymocyte cell populations could be sorted according to the manufacturers' instructions. Briefly, thymocytes were collected by density-gradient centrifugation on a Ficoll® cushion and incubated with CD4 Multisort CD4 microbeads for 30 minutes. After washing with 5 mM EDTA and 0.5% BSA in PBS, the labelled cells were separated on magnetic columns. Positively selected thymocytes, which were retained on the magnetic columns, contained the $CD4^+$ SP and the $CD4^+$ $CD8^+$ DP cell populations, whereas the CD4-depleted cell population, which ran through the columns, contained the $CD8^+$ SP and the $CD4^-$ $CD8^-$ DN thymocytes. To remove the CD4 microbeads from the CD4-positively selected cell populations, the cells were incubated with MACS Multisort release reagent. After 20 minutes, the digestion was stopped, and the cells were labelled for 30 minutes with CD8 microbeads. $CD4^+$ $CD8^+$ DP thymocytes were obtained by positive selection, whereas $CD4^+$ SP cells were found in the depleted cell population.

The CD4-depleted cell population was incubated for 30 minutes with CD8 microbeads. After applying the labelled cells on magnetic columns, $CD8^+$ SP cells could be separated from the double-negative thymocytes. The purities of the four different thymocyte subpopulations were controlled by flow cytometric analysis.

For the isolation of $CD103^-$ $CD8^+$ SP thymocytes, the CD4-depleted thymocyte cell population (which contains $CD8^+$ SP and $CD4^-$ $CD8^-$ DN thymocytes) was incubated for 1 hour with the anti-CD103 antibody 2G5.1, which is of Ig2a isotype. Rat-anti-murine Ig2a+b microbeads (Miltenyi) were applied as secondary antibodies. After magnetic separation $CD103^-$ $CD8^+$ SP and $CD103^-$ $CD4^-$ $CD8^-$ DN thymocytes were found in the run-through of the columns. Following incubation with CD8 microbeads $CD103^-$ $CD8^+$ thymocytes were obtained by positive selection.

Isolation of primary thymic epithelial cells

Thymic tissues were cut with a scalpel into small pieces of about 1 cm^3 and digested at 37°C with 1 mg/ml collagenase A and 50 μg/ml DNase I in RPMI 1640 medium for 45 minutes (Roche, Mannheim, Germany). After this digestion step, the tissue fragments were collected by gravity sedimentation. The supernatants contained mainly thymocytes, which were discarded. The tissue fragments were digested a second time with collagenase A and DNase I for 30 minutes. The cell suspension obtained after the second digestion was washed with RPMI 1640 medium. Then, by repeated 5 minutes gravity sedimentation, the thymic epithelial cells could be isolated from thymocytes, which were found in the supernatants. The combined thymic epithelial cells were washed with PBS and cultured in serum-free OptiMem™ medium (GIBCO-BRL, Eggenstein, Germany) on Petri dishes coated with collagen type I (Roche). The serum-free medium was changed every third day. After one week of culture, the first clusters of adherent thymic epithelial cells could be observed, which could be expanded to almost confluent monolayers by extended culture periods of up to eight weeks.

Immunohistochemistry

Specimens of human thymuses were frozen in Tissue Tek embedding medium (Vogel, Gießen, Germany) and stored at –70°C until use. 5 μm thymic cryostat sections, as well as primary thymic epithelial cells grown on eight-well chamber CultureSlides coated with collagen type I (Falcon, Heidelberg, Germany), were fixed with methanol or acetone at –20°C for 5 minutes and washed with PBS. For indirect immunofluorescence staining, the tissue sections and the adherent thymic epithelial cells were incubated for 1 hour with the primary antibodies diluted 1:100 in PBS containing 0.1% BSA. After washing with PBS, bound antibodies could be detected by Cy3™-conjugated goat anti-mouse or Cy3™-conjugated goat anti-rabbit antibodies (Dianova) diluted 1:500 or 1:1000, respectively. Cell nuclei could be identified by counterstaining with 4′,6-diamino-2-phenylindol-dihydrochloride (DAPI; 1 μg/ml). Control staining was performed by omitting the first antibody. Photographs were taken on a Zeiss axiophot microscope.

For double-immunofluorescence staining of E-cadherin with

epithelial cell markers, the labelled sections were also visualised by epifluorescence light microscopy. Digital pictures from every fluorescence channel were taken and superimposed for the specific antibody stains as well as for each negative control labelling using the software DOKU® from Soft Imaging Systems (Leinfelden-Echterdingen, Germany).

For enzymatic horseradish peroxidase staining, the EnVision™ system of Dako Diagnostics (Hamburg, Germany) was applied. After blocking of endogenous peroxidase activity the cryostat sections were first incubated with normal rabbit antiserum for 30 minutes and then with the first antibodies for another 30 minutes. For control staining, the sections were labelled with the antibody W6/32.HL, which recognises the heavy chain of MHC class I antigens (positive control) or the antibody W6/32.HK, an inactive variant of W6/32.HL (negative control). After extensive washing, the sections were incubated with EnVision™-horseradish peroxidase-coupled goat anti-mouse antibodies for 30 minutes. Incubation with the chromogenic substrate DAB (3,3′-diaminobenzidine tetrahydrochloride) revealed specific signals. The sections were counterstained with Mayer's hemalaun.

Flow cytometric analysis

Unfractionated thymocytes were labelled with the individual cadherin antibodies as described recently (Armeanu et al., 1995). Expression of the integrin receptor α_E (CD103) on the different thymocyte subpopulations isolated by magnetic cell sorting was studied by single-fluorescence analysis. Briefly, for each staining 1-5×10^5 cells were incubated with 20 μl polyglobin® (Bayer, Leverkusen, Germany) for 30 minutes to block unspecific binding of antibodies, washed with PBS containing 0.1% BSA and 0.05% NaN_3 and incubated for 45 minutes with the primary anti-CD103 antibody 2G5.1. After intensive washing, a FITC-conjugated anti-mouse-IgG2a (Caltag Laboratories, Hamburg, Germany) was applied for 30 minutes as secondary antibody.

For dual, triple and four colour flow cytometric analysis, DN and $CD8^+$ SP thymocyte subpopulations were analysed using FITC- or PE-conjugated antibodies against α/β TCR and γ/δ TCR, a PE-conjugated antibody against CD24, APC-conjugated antibodies against CD25 and CD69, and a Cy-Chrome-conjugated antibody against CD62L. All these fluorochrome-conjugated antibodies were purchased from BD Biosciences (Heidelberg, Germany). In the multiple immunofluorescence analyses, the CD103 antibody was detected with secondary FITC- or PE-conjugated anti-IgG2a antibodies (Caltag). The purity of the DN and $CD8^+$ SP thymocyte subpopulations were assessed with the directly labelled CD4-PE (clone B-F5) and CD8-FITC (clone MCD8) antibodies, which were obtained from DPC-Biermann (Bad Nauheim, Germany). Isotype control antibodies, obtained from DPC-Biermann, were used as negative controls. After labelling and extensive washing, 10,000 thymocytes were analysed for cell-surface antigen expression in the dual colour analysis using a FACSort flow cytometer (Becton Dickinson, Heidelberg, Germany) and the WinMDI 2.8 software. For the triple and four colour flow cytometric analysis, at least 20,000 events were analysed on an FACScalibur (Becton Bickinson).

Immunoblotting and immunoprecipitation

Thymic protein extracts were obtained by homogenisation and sonication of the tissue in Tris-buffered saline (TBS) containing 1% NP-40, 1% Triton X-100, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF and 1 mM aprotinin and subsequent incubation on ice for 30 minutes. After centrifugation at 12,500 ***g***, the resulting protein extracts were separated on 10% polyacrylamide gels and transferred to nitrocellulose filters. Non-specific protein-binding sites were blocked with a TBS solution containing 0.1% Tween-20 (TTBS) and 5% skimmed milk powder. The filters were probed for 1 hour with the primary monoclonal or polyclonal antibodies diluted in blocking solution. After washing with TTBS, bound antibodies were detected either by alkaline-phosphatase-conjugated rabbit α-mouse or goat α-rabbit immunoglobulins (Dako Diagnostics) followed by colorimetric reaction with the Fast BCIP/NBT system (Sigma).

For non-radioactive immunoprecipitations, the thymic cell lysates were incubated with protein-A-sepharose (Amersham Pharmacia Biotech, Freiburg, Germany) for 1 hour for preclearing the lysates. After centrifugation, specific immune complexes were formed by the addition of anti-cadherin or anti-catenin antibodies to the supernatants followed by the addition of protein-G-sepharose. After rotation for 1 hour at 4°C, the precipitated antigens were washed several times and dissolved by boiling in SDS-PAGE sample buffer. After separation in 10% polyacrylamide gels, the precipitated antigens were detected by immunoblotting using antibodies against β-catenin or E-cadherin.

Cell adhesion assay

Isolated thymic epithelial cells were cultured in serum-free medium in 24-well plates coated with collagen type I (Roche). The non-thymic human epithelial carcinoma cell line A431, which expresses high levels of E-cadherin, was used in parallel experiments. The adherent epithelial cells were washed twice with pre-warmed PBS and incubated with 2.5% BSA/PBS for 1 hour at 37°C to block unspecific cell binding sites of the wells. After blocking, the wells were washed again with medium. Isolated thymocyte subpopulations were labelled with 2 μl BCECF-AM [2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxylmethyl ester; Sigma], dissolved in DMSO at 37°C for 15 minutes. After washing, 3×10^5 thymocytes were added to each well and allowed to adhere to the confluent monolayers of thymic epithelial cells for 1 hour at 37°C. To inhibit cell-cell interactions, either the thymocytes were preincubated under constant rotation for 30 minutes with anti-integrin antibodies or the thymic epithelial cells were preincubated with anti-E-cadherin antibodies. The murine antibody W6/32. HL, which recognises HLA-A, -B and -C molecules on the surface of the cells was used as an irrelevant control antibody. Then the cell adhesion assays were performed in the presence of the respective antibodies. After 1 hour of incubation, the non-adherent thymocytes were removed by gently washing with pre-warmed PBS. Finally, 500 μl PBS were added to each well, and the BCECF-AM-fluorescence was quantified with a fluorometer (Fluoroskan Ascent; Thermo Biosciences, Egelsbach, Germany) at excitation wavelength 485 nm and emission wavelength 538 nm. The ratio (%) of the adhering thymocytes was calculated as follows:

$$\frac{(\text{fluorescence from experimental sample} - \text{fluorescence from negative control sample})}{\text{total fluorescence added to well}}.$$

The assays were carried out in triplicate. After quantification, attached thymocytes were fixed with 2% formaldehyde and stained with 0.5% crystal violet to visualise cell attachment also under the light microscope.

Cell proliferation assay

$CD8^+$ SP or $CD103^-$ $CD8^+$ thymocytes isolated by magnetic cell sorting were incubated with confluent monolayers of primary thymic epithelial cells cultured in 96-well plates. 1×10^5 thymocytes in MEM D-valine medium containing 10% human AB serum were added to each well. The combined cell populations were cultured for 48 hours without addition of any cytokine. During the last 16 hours, 1 μCi [^{3}H]thymidine was added to each well. To analyse the influence of various antibodies on cell proliferation, the antibodies were present during the whole culture period. After incubation, the thymocytes together with the thymic epithelial cells were harvested on filter papers. [^{3}H]thymidine incorporation was measured in a liquid scintillation counter. All experiments were carried out in triplicate.

Results

Expression of E-cadherin on human thymic epithelial cells

The expression pattern of E-cadherin in the human thymus was determined by immunofluorescence staining of thymic cryostat sections. The strongest staining signals were found on medullary thymic epithelial cells, but immunoreactivity was also detectable on cortical epithelial cells and in the subcapsular epithelium (Fig. 1A). Antibodies against P-cadherin, another classical type I cadherin, showed the opposite staining pattern: strong labelling of cortical epithelial cells, weaker signals in the medulla (Fig. 1B). A double immunofluorescence staining of E-cadherin with the antibody TE4 specific for medullary thymic epithelial cells revealed a colocalisation of the TE4 antigen with E-cadherin on epithelial cells of the medulla (Fig. 2). However, this double labelling also showed that there were other cells than $TE4^+$ cells in the medulla that express E-cadherin. These medullary $TE4^-$ E-$cadherin^+$ cells most probably represented dendritic cells of the medulla.

The expression of E- and P-cadherin on stromal cells of the human thymus was also analysed on isolated thymic epithelial cells grown in culture for several weeks. These primary cells express both E- and P-cadherin mainly on their cell borders (Fig. 3A,B).

For functional interactions, cadherins have to be associated with cytoplasmic catenins. Therefore the distribution of α-, β- and γ-catenin in human thymus was determined by immunofluorescence staining. β-catenin was found to be equally distributed on cortical and medullary thymic epithelial cells, whereas a prevalence of medullary epithelial cells for γ-catenin could be observed (Fig. 1C,D). α-catenin was evenly expressed on cortical and medullary epithelial cells (data not shown). On isolated thymic epithelial cells, β-catenin and γ-catenin were found to be strongly enriched at the sites of cell-cell contact (Fig. 3C,D).

The expression of both cadherins and catenins in thymic tissue as well as in cultured primary thymic epithelial cells was confirmed by western blotting. The specific antibodies detected the 120 kDa bands of E- and P-cadherin and also the 102, 95 and 86 kDa bands of the three different catenins (Fig. 4A,C). Several additional immunoreactive bands were detected with the antibodies against β- and γ-catenin, which most probably represent proteolytically degraded products. The existence of intact E-cadherin–catenin complexes in the thymus was investigated by co-immunoprecipitation. In these experiments, the cadherin-catenin complexes could be precipitated by antibodies against E-cadherin, α-, β- or γ-catenin, respectively

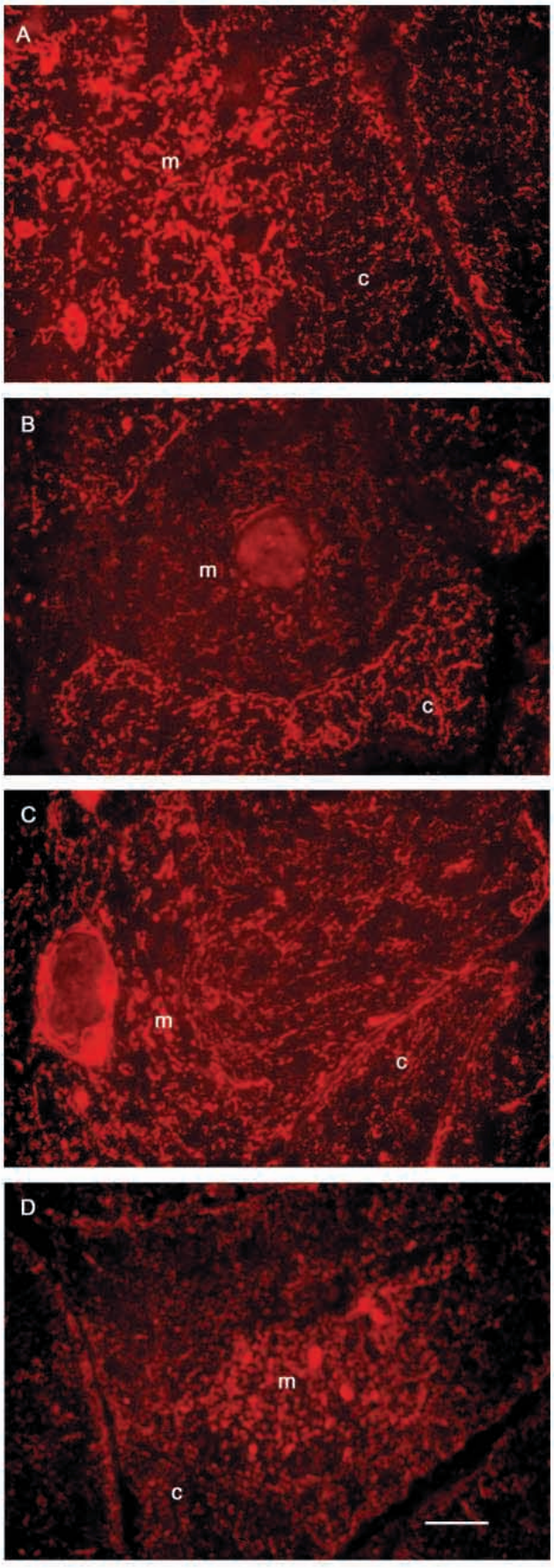

Fig. 1. Expression of cadherins and catenins in the human thymus. The micrographs show immunofluorescence staining of human thymus cryostat sections stained with monoclonal antibodies against E- and P-cadherin and β- and γ-catenin. (A) Labelling with anti-E-cadherin antibodies revealed strong staining signals in the medulla (m) and weaker signals in the cortex (c) of the thymic lobules. (B) P-cadherin expression seemed to be restricted to epithelial cells of the cortical area, since medullary epithelial cells did not show any significant staining signals. (C) β-catenin expression was evenly distributed over the medullary and cortical areas, whereas γ-catenin expression was found predominantly in the medulla (D). Bar, 100 μm.

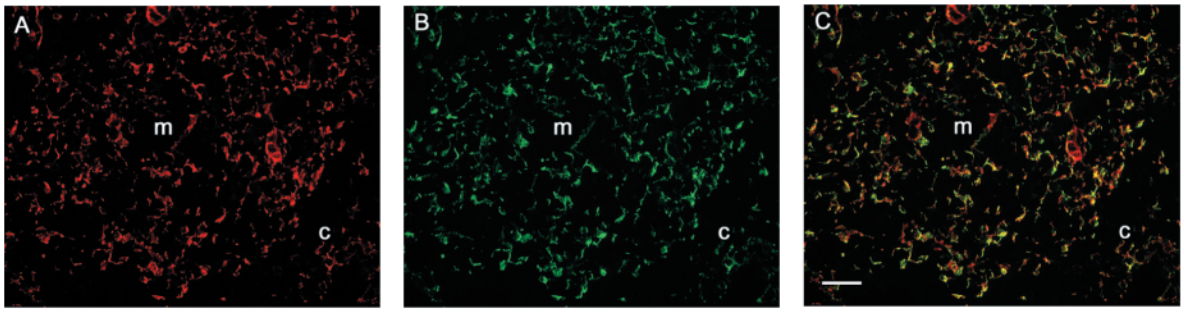

Fig. 2. Expression of E-cadherin on medullary epithelial cells. Double-immunofluorescence staining of human thymus cryostat sections with the anti-E-cadherin antibody HECD-1 (A) and the antibody TE4 (B) specific for medullary thymic epithelial cells showed colocalization (C) of both antigens on medullary epithelial cells. Note that not all E-cadherin-expressing cells in the medulla express the TE4 antigen. Bar, 100 μm.

(Fig. 4B), which suggested direct functional interactions in thymic epithelial cells.

By immunofluorescence staining of thymic tissue, no staining of thymocytes for E- or P-cadherin could be detected. However, this type of analysis is not sensitive enough to exclude the possibility that no thymocyte subpopulation could express cadherins on their cell surface. Therefore, flow cytometric analyses of isolated thymocytes were performed (Fig. 5). The results confirmed that no thymocyte subpopulation in the postnatal human thymus expressed E- or P-cadherin, indicating that no cadherin-mediated homophilic interaction of thymocytes with thymic epithelial cells is likely to occur.

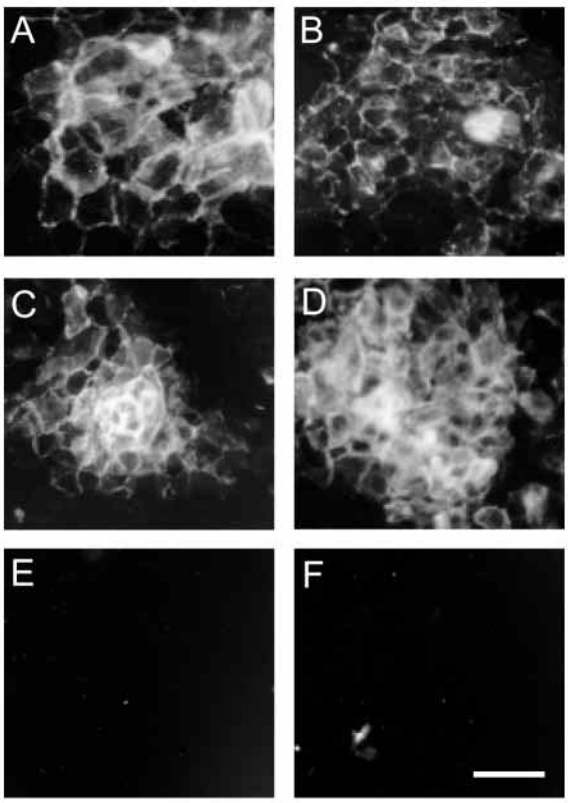

Fig. 3. Expression of cadherins and catenins on isolated thymic epithelial cells. Isolated primary thymic epithelial cells cultured on chamber slides were fixed and stained with antibodies against E-cadherin (A), P-cadherin (B), β-catenin (C) and γ-catenin (D) followed by Cy3^{TM}-labelled second antibodies. Control cultures were incubated only with the Cy3^{TM}-labelled anti-mouse (E) or anti-rabbit (F) antibodies. The cultured thymic epithelial cells showed expression of all four analysed antigens. Expression was mainly found on the cell surfaces at sites of cell-cell contacts. Bar, 100 μm.

Expression of CD103 on subpopulations of human thymocytes

The integrin $\alpha_E(CD103)\beta_7$ is the only heterophilic ligand for E-cadherin known so far. Since thymocytes did not express E-cadherin, the expression pattern of CD103 on human thymocyte subpopulations was determined by flow cytometric analysis (Fig. 6A). About 12% (normal range: 10-17%) of the early $CD4^-$ $CD8^-$ DN thymocyte subpopulation expressed CD103, whereas the more mature $CD4^+$ $CD8^+$ DP thymocytes did not express CD103 at all. On $CD4^+$ SP thymocytes, CD103 was also hardly detectable (<2%). However, a strong expression was observed on $CD8^+$ SP thymocytes where more than one third of the cells (normal range: 30-50%) expressed CD103. An immunohistochemical staining of human thymus with the anti-CD103 antibody revealed strong surface labelling of thymocytes in the medullary region where $CD8^+$ SP thymocytes are expected to be located (Fig. 7).

To determine whether the $CD103^+$ $CD8^+$ SP cells represent a specific subpopulation of $CD8^+$ SP thymocytes, $CD8^+$ SP cells were isolated by MACS technology and stained with antibodies against the T-cell receptors (TCR) αβ and γδ (Fig. 6C). A more than 90% enrichment of the $CD8^+$ SP cells was achieved using MACS separation. Only 4.3% of these cells expressed the γδ TCR, whereas 89% expressed the αβ TCR. Double labelling of the $CD8^+$ SP cells with the anti-CD103 antibody and the anti-αβ and γδ TCR antibodies clearly showed that the overwhelming majority of $CD103^+$ $CD8^+$ SP thymocytes expressed the αβ TCR. A further characterisation of the $CD103^+$ $CD8^+$ SP thymocyte subpopulation was performed with antibodies against CD24, CD62L and CD69 in a four colour flow cytometric analysis (Fig. 6D). Whereas CD24 was hardly detected on the $CD103^+$ $CD8^+$ SP thymocyte subpopulation, a strong expression of CD62L and CD69 was detected on these cells. These results show that a prominent subpopulation of TCR$\alpha\beta^+$ $CD62L^+$ $CD69^+$ $CD8^+$ SP thymocytes is characterised by CD103 expression.

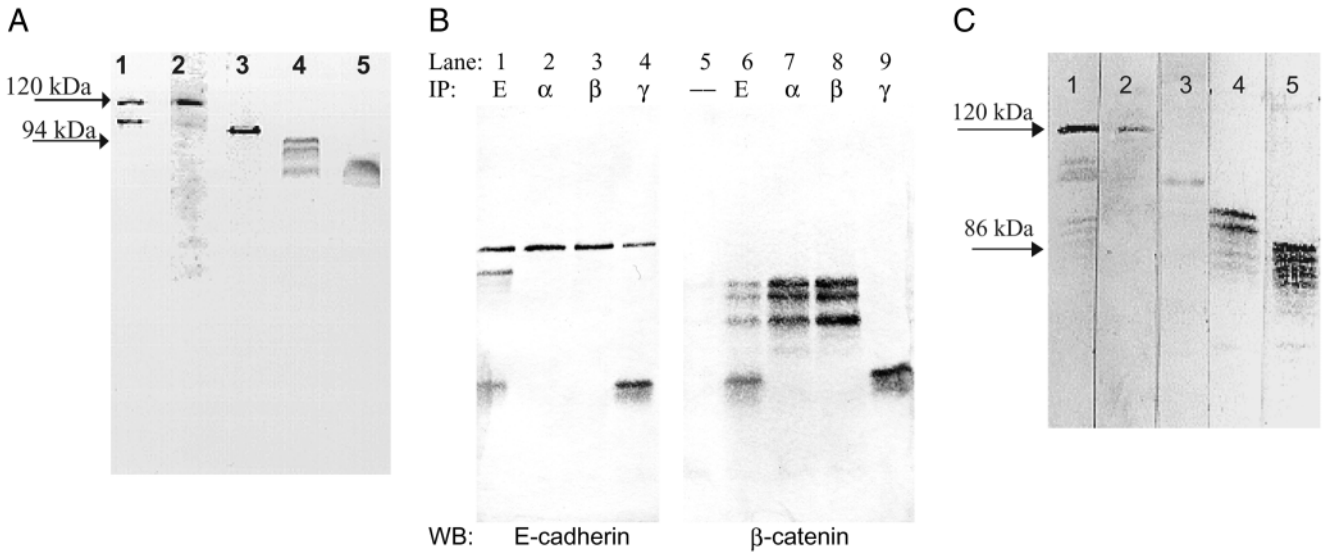

Fig. 4. (A) Immunoblot analysis of cadherin-catenin expression in the human thymus. Protein extracts of human thymic tissue were separated on 10% polyacrylamide gels and transferred to nitrocellulose filters. After blocking, the filters were divided into five lanes and incubated with antibodies against E-cadherin, P-cadherin, α-catenin, β-catenin and γ-catenin. After colorimetric development, the 120 kDa bands of E-and P-cadherin (lane 1 and 2), the 102 kDa band of α-catenin (lane 3), the 94 kDa band of β-catenin (lane 4) and the 86 kDa band of γ-catenin (lane 5) were detected in the thymic extracts. Partial degradation products were observed for E-cadherin and β-catenin. The positions of 120 and 94 kDa are indicated on the left side. (B) Co-immunoprecipitation of cadherins and catenins isolated from human thymus. Cell lysates of freshly isolated thymic tissue were immunoprecipitated (IP) with monoclonal antibodies against E-cadherin (lanes 1,6) and γ-catenin (lanes 4,9), with polyclonal antisera against α-catenin (lanes 2,7) and β-catenin (lanes 3,8) or without antibody (lane 5). The precipitated immune complexes were immunoblotted with antibodies against E-cadherin (lanes1-4) and β-catenin (lanes 5-9). Co-precipitation of E-cadherin with the different catenins and of β-catenin with α-catenin and E-cadherin, but not with γ-catenin, revealed functional cadherin-catenin complexes. (C) Immunoblot analysis of cadherin-catenin expression in thymic epithelial cells. Protein extracts of isolated thymic epithelial cells were separated on 10% polyacrylamide gels. After transfer to nitrocellulose, the filters were incubated with antibodies against E-cadherin (lane 1), P-cadherin (2), α-catenin (3), β-catenin (4) and γ-catenin (5). After colorimetric development, specific bands for all analysed antigens were detected. Partial degradation products were observed for β-catenin and γ-catenin (lane 4 and 5). The positions of 120 and 86 kDa are indicated on the left side.

The CD103$^+$ DN thymocyte subpopulation was also characterised in more detail (Fig. 6B). Flow cytometric analysis revealed a more than 97% enrichment of these cells by MACS separation. Triple staining with antibodies against CD103, CD25 and αβ TCR or γδ TCR showed that the CD103$^+$ DN thymocyte subpopulation do not express CD25 or αβ TCR significantly. However, about 20% of the CD103$^+$ DN thymocytes showed an expression of γδ TCR, whereas the remaining 80% of these cells do not. These results strongly indicate that the majority of the CD103$^+$ DN thymocytes are immature precursors.

Adhesion of CD8$^+$ CD103$^+$ thymocytes to thymic epithelial cells

CD103$^+$ thymocytes were evaluated for adhesive interactions with E-cadherin$^+$ thymic epithelial cells. After incubation of CD8$^+$ SP thymocytes for 1 hour with a confluent layer of primary thymic epithelial cells, strong adhesive interactions between these two cell types could be observed (Fig. 8A). The adhesive interactions were strongly inhibited by pre-incubation of the CD8$^+$ SP thymocytes with anti-CD103 antibody (Fig. 8B), indicating a direct involvement of the E-cadherin–integrin $\alpha_E\beta7$ ligand pair in the observed attachment of the cells. To quantify the adhesive interactions, the isolated CD8$^+$ SP thymocytes were labelled with a fluorescent dye that does not interfere with the cell-binding process. About 40% of the labelled CD8$^+$ SP thymocytes attached to the thymic epithelial cell layer (Fig. 8C), corresponding to the amount of CD103$^+$ thymocytes in the CD8$^+$ SP cell population. Addition of the control antibody W6/32. HL, which binds to the surface of the thymocytes, did not influence the adhesive interactions (Fig. 8C). However, addition of either anti-CD103 antibody or anti-E-cadherin antibody to the interacting cells drastically diminished cellular interactions (Fig. 8D). No synergistic effect was observed when both antibodies were simultaneously added. Although cell binding of CD103$^+$ CD8$^+$ SP cells to

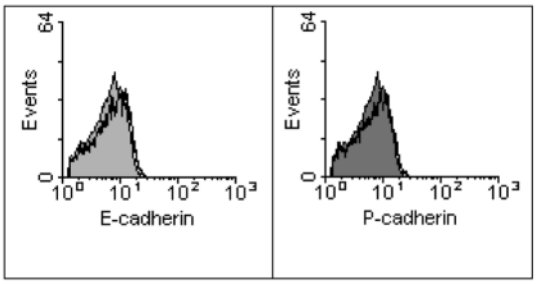

Fig. 5. Flow cytometry analysis of cadherin expression on human thymocytes. Isolated thymocyte suspensions were stained with antibodies to E- and P-cadherin. The staining pattern showed no difference from the isotype control staining (thin line), indicating no expression of both cadherins on human thymocytes.

isolated thymic epithelial cells could be drastically inhibited by anti-CD103 and anti-E-cadherin antibody treatment, a 100% inhibition could never be observed, indicating that other adhesive mechanisms might still play a role in the binding of thymocytes to thymic epithelial cells.

Expression of E-cadherin on the cell surface was sufficient for binding of $CD103^+$ $CD8^+$ SP thymocytes. This was shown using the non-thymic epithelial cell line A431 instead of primary thymic epithelial cells. The isolated thymocytes attached well to A431 cells, and again the attachment could be blocked by antibodies against E-cadherin or CD103 (data not shown).

The DN thymocytes also contain a subpopulation (>10%) that express CD103. Therefore, DN thymocytes were also evaluated for cellular interactions with primary thymic epithelial cells and the non-thymic epithelial carcinoma cell line A431. DN thymocytes attached to both E-cadherin$^+$

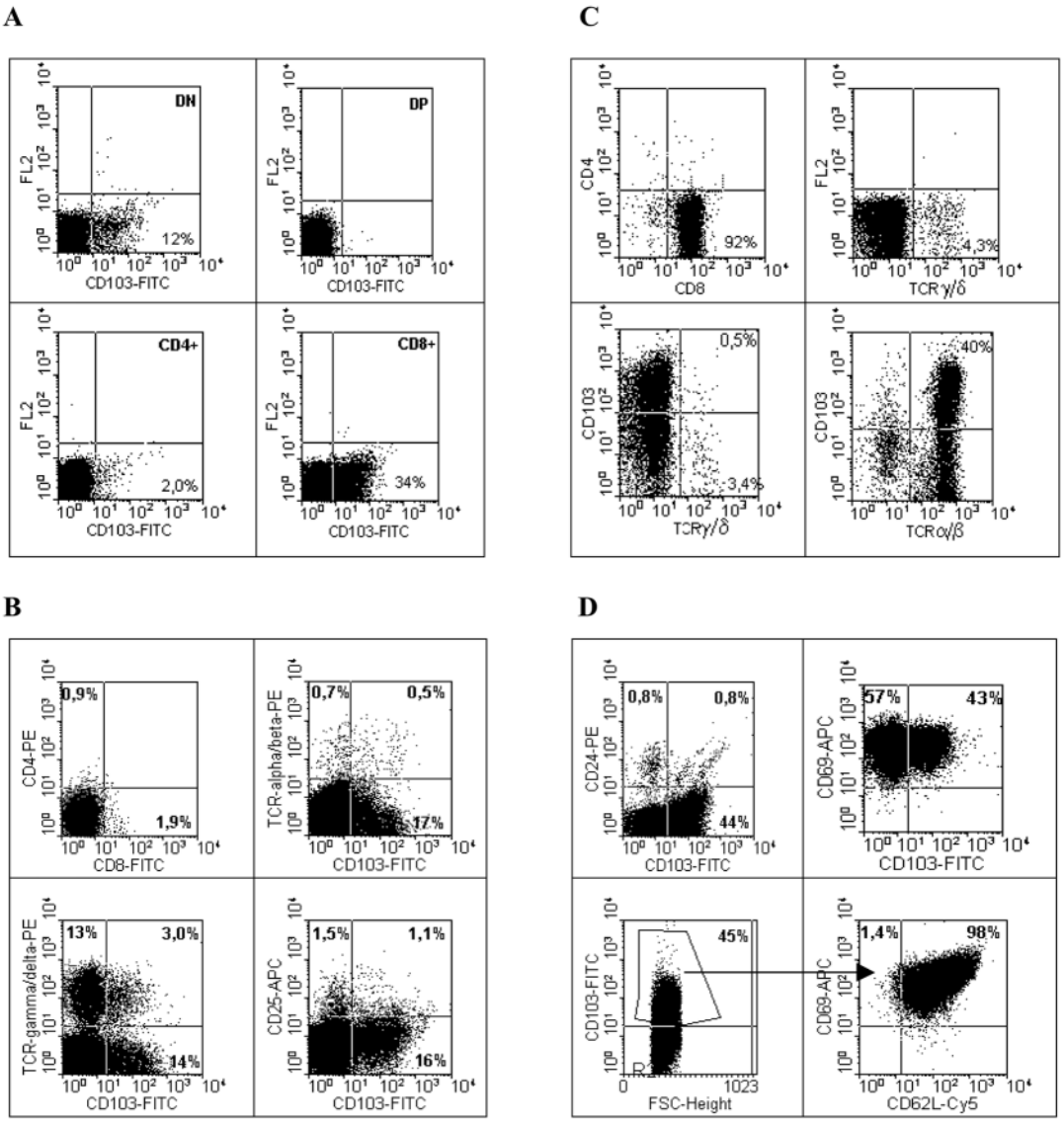

Fig. 6. Detection of integrin α_E receptor (CD103) on human thymocyte subpopulations. (A) DN, DP and SP thymocyte subpopulations were isolated by magnetic cell sorting and subsequently labelled with the antibody against CD103. No expression of CD103 was observed in the DP thymocyte cell population, and only a marginal expression was seen in the $CD4^+$ SP population. 12% of the DN subpopulation expressed CD103. The highest expression, however, was observed in the $CD8^+$ SP population, in which more than one third of this subpopulation expressed CD103. (B) MACS-isolated $CD4^-$ $CD8^-$ DN thymocytes were triple-stained with antibodies against CD103 (FITC), CD25 (APC), and TCR α/β (PE) or TCR γ/δ (PE), respectively. The $CD103^+$ DN thymocytes did not show any significant expression of TCR α/β or CD25. A subpopulation of $CD103^+$ DN thymocytes expressed the TCR γ/δ, the majority of the $CD103^+$ DN cells, however, did not express TCR γ/δ. (C) Isolated $CD8^+$ SP thymocytes were analysed for TCR expression. By one round of magnetic cell sorting, a purity of >92% of $CD8^+$ cells was achieved. 4.3% of the $CD8^+$ cells expressed the γ/δ TCR, whereas 89% of these cells expressed the α/β TCR. By double staining of $CD8^+$ cells it was shown that $CD103^+$ $CD8^+$ cells mostly expressed the α/β TCR, but not the γ/δ TCR . (D) Four-colour flow cytometric analysis was performed with MACS-sorted $CD8^+$ SP thymocytes, which were labelled with antibodies against CD103 (FITC), CD24 (PE), CD62L (Cy5) and CD69 (APC). All $CD103^+$ $CD8^+$ SP thymocytes expressed CD69, but not CD24. When the $CD103^+$ $CD8^+$ cells were gated it could be demonstrated that all of these cells express L-selectin (CD62L) and CD69 simultaneously.

epithelial cell types, and antibodies against E-cadherin or CD103 showed a strong inhibitory effect (data not shown).

Co-culturing CD8$^+$ CD103$^+$ thymocytes with thymic epithelial cells induces cell proliferation

The functional significance of the observed adhesive interactions between CD103$^+$ thymocytes and an adherent thymic epithelial cell layer was determined in cell proliferation assays. Incubation of confluent epithelial cell layers with CD8$^+$ SP thymocytes consistently increased cell proliferation, which could not be blocked by incubation with the control antibody W6/32. HL (Fig. 9A). Addition of antibodies against E-cadherin resulted in a modest blockade of cell proliferation. However, antibodies against CD103 drastically diminished cell proliferation in the co-culture assay (*n*=5; Fig. 9A). Incubation of CD8$^+$ SP thymocytes with the CD103 antibody without epithelial cells did not show an influence on cell proliferation (data not shown).

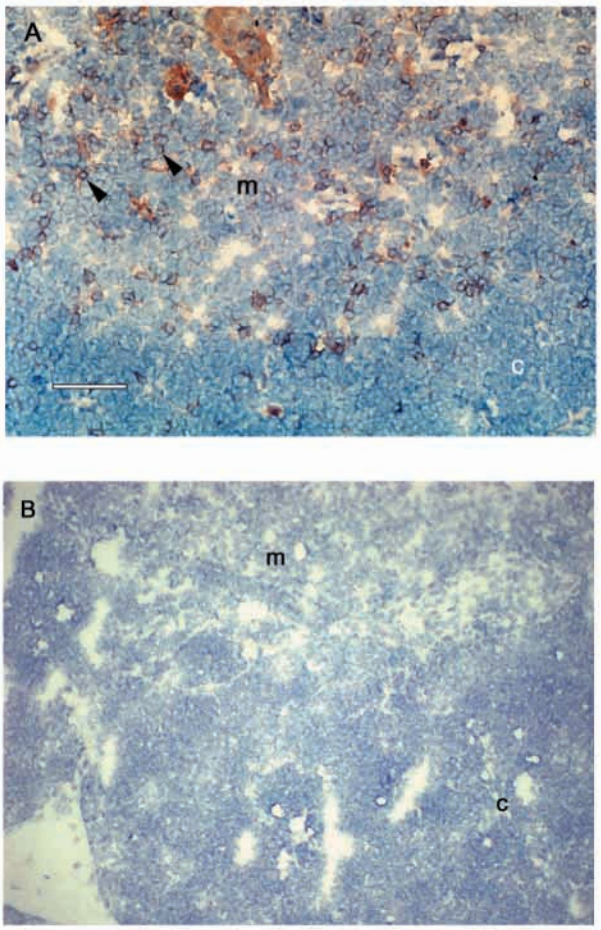

Fig. 7. Localisation of integrin α_E (CD103) in the human thymus. (A) Staining of thymus cryostat sections with the antibody against CD103 revealed that the cell surfaces of individual thymocytes (arrowheads) within the medulla (m) are strongly labelled. Note that only a few thymocytes within the cortical region (c) were stained with the CD103 antibody. (B) Control staining was performed with the antibody W6/32.HK, an inactive variant of the anti-MHC class I antibody W6/32.HL. No background staining was observed with this antibody. Bar, 100 μm.

Since CD8$^+$ SP thymocytes contain CD103$^+$ and CD103$^-$ subpopulations, the CD103$^-$ CD8$^+$ SP cells were also analysed for cell proliferation with primary thymic epithelial cells. CD103$^+$ CD8$^+$ SP cells could not be directly analysed since the anti-CD103 antibody used for cell separation was shown to be strongly inhibitory in cell proliferation studies. CD103$^-$ CD8$^+$ SP thymocytes did not show a proliferative response when co-cultivated with thymic epithelial cells (*n*=3), whereas unseparated CD8$^+$ SP thymocytes did show a proliferative response (Fig. 9B). Again this mitogenic effect could be

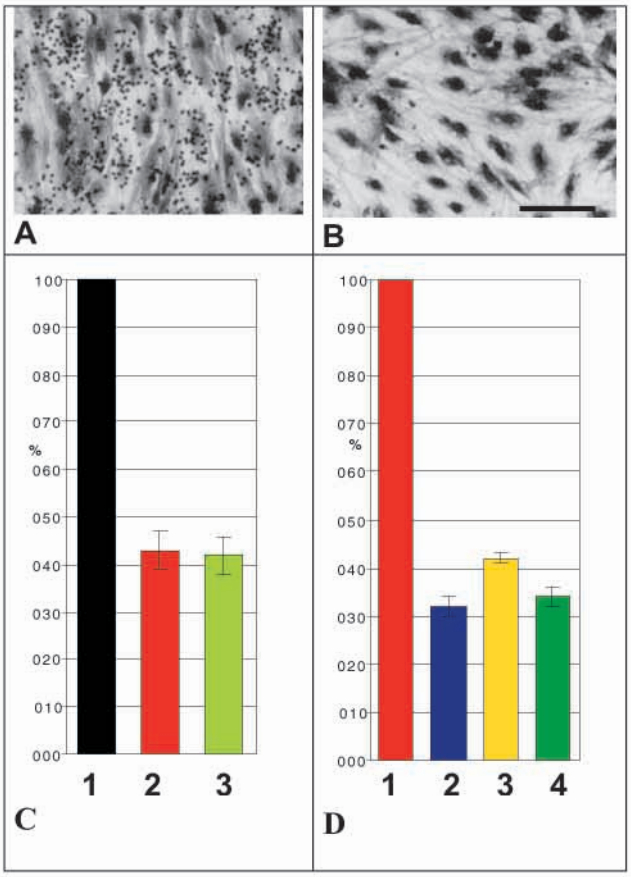

Fig. 8. Inhibition of thymocyte adhesion to thymic epithelial cells by function-blocking antibodies to integrin α_E and E-cadherin. (A,B) The micrographs show adhesive interactions of MACS-isolated CD8$^+$ thymocytes with primary thymic epithelial cells in the absence (A) or presence (B) of anti-CD103 antibodies. Without antibody treatment, the small, round and darkly stained thymocytes adhered strongly to the adherent epithelial cells. By contrast, anti-CD103 treatment strongly diminished thymocyte adhesion. Bar, 100 μm. (C) Thymocyte cell adhesion to thymic epithelial cells was quantified after BCECF-labelling of CD8$^+$ thymocytes. Fluorescence intensity of the labelled cells used for the cell adhesion assay was set to 100% (lane 1). Around 40% of the CD8$^+$ thymocytes attached to the adherent thymic epithelial cell layer (lane 2). The adhesion process was not influenced by addition of the W6/32. HL control antibody (lane 3). (D) Adhesive interactions of CD8$^+$ thymocytes with thymic epithelial cells were inhibited by antibodies against E-cadherin and CD103. Fluorescence intensity of cell binding in the absence of the antibodies was set to 100% (lane 1). Both anti-E-cadherin (lane 2) and anti-CD103 (lane 3) antibodies diminished cell binding to 30-40%. Combined addition of both antibodies did not show an enhanced inhibitory effect (lane 4).

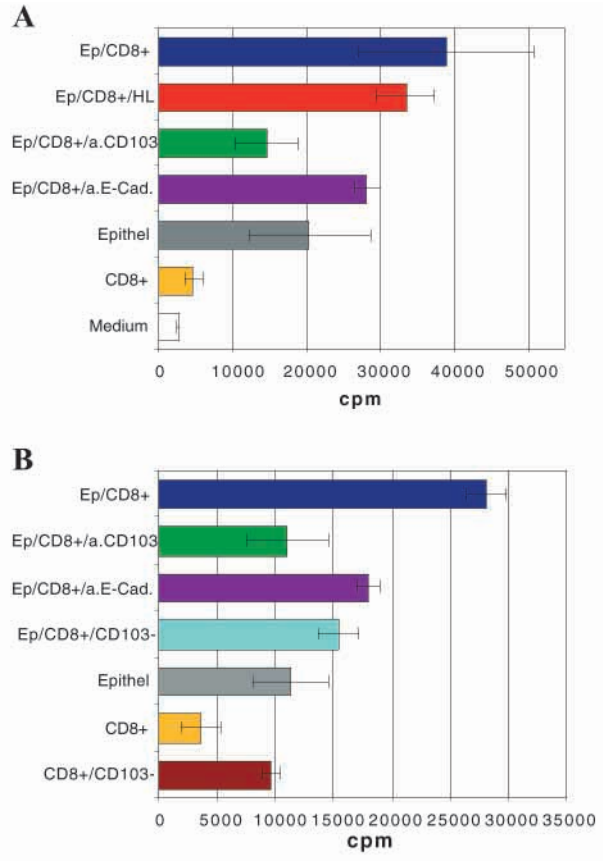

Fig. 9. Inhibition of cell proliferation by anti-CD103 antibodies. (A) Isolated $CD8^+$ SP thymocytes were co-cultured with primary thymic epithelial cells (Ep/CD8+), which induces a net increase in cell proliferation after 48 hours of culture. Antibodies against HLA-A,B,C (Ep/CD8+/HL) used as control antibodies did not significantly interfere with the proliferation process, whereas antibodies against E-cadherin (Ep/CD8+/a.E-cad) slightly blocked cell proliferation. Anti-CD103 antibodies (Ep/CD8+/a.CD103), however, drastically reduced cell proliferation. Results represent the mean of triplicate experiments. (B) $CD103^-$ $CD8^+$ SP thymocytes, when co-cultured with primary thymic epithelial cells for 48 hours, did not show a net increase in cell proliferation (Ep/CD8+/CD103–) whereas $CD8^+$-SP-containing $CD103^+$ $CD8^+$ SP thymocytes did (Ep/CD8+). Antibodies against CD103 inhibited cell proliferation drastically (Ep/CD8+/a.CD103).

efficiently blocked by antibodies against CD103. $CD103^+$ $CD8^+$ SP thymocytes attached well to E-cadherin$^+$ A431 cells. However, this adhesive interaction did not lead to an induction of cell proliferation (n=2; data not shown).

These results showed that the E-cadherin–integrin $\alpha_E\beta_7$ ligand pair is necessary but not sufficient to induce cell proliferation, suggesting that primary thymic epithelial cells can provide additional signals for cell proliferation not found on non-thymic epithelial cells.

Discussion

The thymus and its specialised microenvironments play a pivotal role in multiple developmental processes leading to fully mature T-lymphocytes. In the present study we have shown that E-cadherin, predominantly expressed by medullary thymic epithelial cells, is a functional counter-receptor for the integrin $\alpha_E\beta_7$, which is present on a subset of medullary $CD8^+$ SP thymocytes and also on a subpopulation of cortical $CD4^-$ $CD8^-$ DN thymocytes. Adhesive interactions between $CD8^+$ SP thymocytes and thymic epithelial cells mediated by the E-cadherin–$\alpha_E\beta_7$ integrin ligand pair led to an increased cell proliferation. However, although the E-cadherin–$\alpha_E\beta_7$ integrin interaction is necessary, it is not sufficient to induce thymocyte cell proliferation. Only a co-cultivation of thymocytes with primary thymic epithelial cells, but not with E-cadherin$^+$ epithelial carcinoma cells, led to an increased cell proliferation. This result strongly suggested that primary thymic epithelial cells can provide at least one additional signal necessary for the induction of cell proliferation that is not present on epithelial carcinoma cells.

The cadherin family shows a very restricted expression pattern during hematopoietic cell development. In the human bone marrow, N-cadherin is present on very early $CD34^+$ $CD19^+$ hematopoietic progenitor cells but is downregulated on more mature progenitor cells (Puch et al., 2001). N-cadherin can also be detected on T cell leukaemia and lymphoma cells, but not on normal leukocytes (Tsutsui et al., 1996; Kawamura-Kodama et al., 1999). E-cadherin expression is found on bone-marrow-derived dendritic Langerhans cells of the epidermis (Tang et al., 1993). On myeloid blood cells E-cadherin expression is restricted to distinct developmental stages of erythropoiesis, whereas other myeloid cells do not seem to express E-cadherin (Armeanu et al., 1995; Armeanu et al., 2000; Bühring et al., 1996; Lammers et al., 2002). On developing T-lymphocytes, Munro and colleagues detected E-cadherin as well as the classical type II cadherin-6, -8 and -11 by RT-PCR. These results were obtained with murine adult $CD4^+$ $CD8^+$ thymocytes, but they were not corroborated by any immunological technique (Munro et al., 1996). On the other hand, by flow cytometry analyses, Lee and co-workers only detected E-cadherin on murine fetal thymocytes and on a very small percentage of postnatal thymocytes, but not on adult thymocytes (Lee et al., 1994). The expression of E-cadherin on the cell surface of murine fetal thymocytes was confirmed in subsequent studies with fetal thymic organ cultures. Antibodies that block homophilic E-cadherin interactions inhibited epithelial organization and thymocyte development in reaggregate fetal thymic organ cultures (Müller et al., 1997). However, antibodies that were able to block heterophilic interactions between $\alpha_E\beta_7$ integrin and E-cadherin did not interfere with early thymocyte development, indicating an important role for homophilic E-cadherin interactions during murine fetal thymocyte development. Homophilic E-cadherin-mediated interactions between thymocytes and thymic epithelial cells did not seem to play a role in the postnatal human thymus, since no human postnatal thymocyte subpopulation seemed to express E-cadherin, as shown in the present study. Whether human fetal thymocytes, like their murine counterparts, also express E-cadherin has not been analysed so far.

Although no E-cadherin expression was found on postnatal human thymocytes, cortical and medullary thymic epithelial cells still express E-cadherin with the highest expression level found on medullary thymic epithelial cells. Using suitable culture conditions, primary thymic epithelial cells strongly

expressing functional E-cadherin-catenin complexes could be grown in vitro. However, the expression of E-cadherin on the primary thymic epithelial cells was critically dependent on the culture conditions used, as slight changes in these conditions led to a downregulation of E-cadherin as well as an upregulation of N-cadherin (S.K. and G.K., unpublished). Therefore, all primary thymic epithelial cells used in the present study for functional analyses had to be tested for E-cadherin expression.

The integrin α_E(CD103)β_7 is the heterophilic counter-receptor for E-cadherin and is mainly expressed on intraepithelial lymphocytes (Cepek et al., 1993; Higgins et al., 1998). In the skin, $\alpha_E\beta_7$ seems to contribute to tissue-specific epidermal localization of $CD8^+$ lymphocytes (Pauls et al., 2001). $\alpha_E\beta_7$ is also expressed on developing T-cells as shown for murine and human thymocytes. Whereas Andrew and co-workers found that $\alpha_E\beta_7$ was expressed on comparable subsets of both $CD4^+$ SP and $CD8^+$ SP murine thymocytes, the study of Lefrancois and colleagues showed that the majority of murine $CD103^+$ thymocytes were medullary $CD8^+$ SP thymocytes (Andrew et al., 1996; Lefrancois et al., 1994). In the human thymus, an average of 26% of $CD8^+$ SP thymocytes were quantified as CD103-expressing thymocytes (McFarland et al., 2000). These cells were predominantly $\alpha\beta$ TCR^+ $\gamma\delta$ TCR^-, which is in agreement with our present study. An analysis of T-cell-receptor rearrangement excision circles (TREC) showed that the $CD103^+$ $CD8^+$ cells within the human thymus represent an actual stage of thymopoiesis and not a subset of mature peripheral memory T cells recirculating in the thymus (McFarland et al., 2000).

Our present functional studies showed that the expression of CD103 on human thymocytes led to adhesive interactions with thymic epithelial cells. This was shown for $CD4^-$ $CD8^-$ DN and $CD8^+$ SP thymocytes. Quantification of the adhering thymocytes revealed that only the fraction of thymocytes expressing CD103 attached to thymic epithelial cells. However, adhesion of $CD103^+$ thymocytes was not restricted to thymic epithelial cells, since the $CD103^+$ thymocytes could also adhere to epithelial carcinoma cells expressing E-cadherin. The specificity of these interactions was verified by inhibition studies using antibodies against either CD103 and/or against E-cadherin. Recently, it has been shown that the integrin $\alpha_E\beta_7$ can also mediate cell adhesion in an E-cadherin-independent way (Strauch et al., 2001). However, the antibodies against CD103 and E-cadherin caused similar inhibitory effects on thymocyte adhesion to epithelial cells, suggesting that an E-cadherin-independent binding mechanism of $CD103^+$ thymocytes does not seem to play a role.

Cell proliferation of thymocytes can be directly influenced by adhesive interactions with cellular and extracellular matrix components. Fibronectin and laminins as prominent representatives of extracellular matrix molecules have been shown to either enhance or downregulate cell proliferation of developing T cells (Halvorson et al., 1998; Vivinus-Nebot et al., 1999). Cellular adhesion of human $CD103^+$ $CD8^+$ SP thymocytes to epithelial carcinoma cells did not lead to enhanced thymocyte proliferation, whereas adhesive interactions of these thymocytes with cultivated thymic epithelial cells did. The proliferative response seemed to be directly linked to CD103 expression since (1) antibodies against CD103 inhibited cell proliferation in the co-culture system and (2) $CD103^-$ $CD8^+$ thymocytes did not proliferate on thymic epithelial cells. Although the expression of CD103 on $CD8^+$ SP thymocytes seemed to be necessary, it was not sufficient to induce $CD8^+$ thymocyte cell proliferation, since only the interaction with E-cadherin$^+$ thymic epithelial cells, but not with E-cadherin$^+$ epithelial carcinoma cells, led to an enhanced proliferative response. The nature of the additional signal(s) present on thymic epithelial cells is, however, not known.

There is increasing evidence that integrins play a pivotal role in regulating T-cell maturation (Mojcik et al., 1995; Andrew et al., 1996; Salomon et al., 1997; Halvarson et al., 1998; Vivinus-Nebot et al., 1999; Savino et al., 2000; Schmeissner et al., 2001). Integrins mainly interact with extracellular matrix molecules but also with cellular adhesion molecules like VCAM-1 and E-cadherin. The expression pattern of the integrin ligands in the thymus as well as the integrin expression on thymocytes seems to be finely tuned, being responsible for their influence at defined stages of T cell maturation (Crisa et al., 1996; Kutleša et al., 2002). Developing T cells have to undergo various cycles of proliferation, migration, adhesion and arrest during their differentiation process. Therefore, a coordination of adhesive interactions and induction of proliferation as shown here for the integrin $\alpha_E\beta_7$ and its ligand E-cadherin is most likely to be of high significance for human T cell development.

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