Immunity, Vol. 6, 257–264, March, 1997, Copyright ©1997 by Cell Press

Involvement of E-Cadherin in Thymus Organogenesis and Thymocyte Maturation

Kai M. Müller,* Carrie J. Luedecker,* Mark C. Udey,† and Andrew G. Farr*
*Departments of Biological Structure and Immunology
School of Medicine
University of Washington
Seattle, Washington 98195
†Dermatology Branch
National Cancer Institute
Bethesda, Maryland 20892

Summary

We examined the role of E-cadherin expressed by thymic epithelial cells and immature thymocytes in thymus organogenesis and thymocyte development. ECCD-1 MAb, which blocks homotypic E-cadherin interactions, inhibited epithelial organization and thymocyte development in reaggregate fetal thymic organ cultures. It also interfered with the differentiation of fetal liver cells or fetal thymocytes within deoxyguanosine-treated thymic lobes, but did not affect thymocyte development in intact cultured fetal thymic lobes. In contrast, antibodies that selectively inhibit interactions between E-cadherin and $\alpha_E\beta_7$ integrins did not perturb thymic epithelial organization or thymocyte development, suggesting that homotypic E-cadherin interactions play an important role in thymus development and function.

Introduction

The cadherin superfamily encompasses a number of structurally related cell adhesion receptors that mediate highly specific Ca^{2+}-dependent homotypic adhesion (Grunwald, 1993; Takeichi, 1990, 1991, 1993) and play a fundamental role in cell sorting in vitro and in vivo (Nose et al., 1988). Specificity of cadherin adhesive interactions is dictated by residues within the amino-terminal domains of these molecules, while their cytoplasmic domains interact with a group of three proteins, the catenins, that mediate essential associations with the cytoskeleton (Takeichi, 1990, 1991).

E-cadherin/uvomorulin is expressed by all early embryonic cells and mainly by epithelial cells later in development. Its importance in morphogenesis is illustrated by the observation that homozygous E-cadherin-deficient mice have a lethal defect in early embryonic development, whereas their heterozygous parents appear normal (Larue et al., 1994; Riethmacher et al., 1995). Although adhesion through cadherins usually involves lineage-related cells, E-cadherin has been implicated in a number of interactions between bone marrow–derived cells and epithelia. Murine epidermal Langerhans cells and dendritic epidermal $\gamma\delta$ T cells have both been shown to bind to keratinocytes via E-cadherin (Lee et al., 1993, Clin. Res., abstract; Tang et al., 1993).

E-cadherin is also a counterreceptor for the integrin $\alpha_E\beta_7$ ($\alpha_{IEL}\beta_7$, $\alpha_{M290}\beta_7$) expressed by lymphocytes (Cepek et al., 1994; Karecla et al., 1995). While $\alpha_E\beta_7$–E-cadherin interactions contribute to mucosal lymphocyte localization (Shaw and Brenner, 1995), expression of this integrin by thymocytes (Andrew et al., 1996; Lefrançois et al., 1994) raises the possibility that this receptor–counterreceptor pair may participate in thymocyte–thymic stromal cell interactions as well.

We have previously shown that E-cadherin is synthesized and expressed by immature thymocytes as well as by thymic epithelial cells and that levels of E-cadherin expression by thymocytes is particularly elevated during fetal life (Lee et al., 1994). In the present study, we investigated the possible contribution of E-cadherin to the establishment of a functional thymic environment and its involvement in thymocyte–stromal cell interactions required for thymocyte maturation. To this end, we took advantage of the differential activities of two monoclonal antibodies (MAbs) to E-cadherin: ECCD-1, which blocks homotypic adhesion between E-cadherin molecules, but does not inhibit $\alpha_E\beta_7$–E-cadherin interactions, and ECCD-2, which inhibits the binding of E-cadherin to the integrin $\alpha_E\beta_7$, but does not block homotypic E-cadherin-mediated adhesion (Karecla et al., 1995; Shirayoshi et al., 1986; Yoshida-Noro et al., 1984). We report here that ECCD-1 MAb strongly inhibited both the reassociation of thymic epithelial cells and subsequent thymocyte development in a fetal thymus reaggregate culture system. Furthermore, although this MAb had no demonstrable effect on thymocyte development occurring within intact cultured fetal thymus lobes, ECCD-1 impaired the differentiation of fetal liver cells or fetal thymocytes within 2-deoxyguanosine (DOG)-treated fetal thymus lobes in vitro. The inhibitory activity of MAb ECCD-1 in two different fetal thymic organ culture systems and the failure of MAb ECCD-2 to affect thymocyte maturation in reaggregated fetal thymic lobes collectively indicate that homotypic E-cadherin interactions play a critical role in the establishment of a functional thymic environment and in the cellular interactions that facilitate proximal stages of thymocyte maturation.

Results

ECCD-1 Blocks the Spontaneous Reaggregation of Enzymatically Dissociated Fetal Thymi

Various cell adhesion molecules contribute to the organization of the thymic microenvironment and to its interaction with thymocytes. These include extracellular matrix components that constitute ligands for integrins (Chang et al., 1993; Lannes-Vieira et al., 1991), in addition to thymocyte cell surface molecules that can interact with ligands expressed by thymic stromal cells or their products (Denning et al., 1987; Fine and Kruisbeek, 1991; Hueber et al., 1992; Liu et al., 1992; Nelson et al., 1996; Sawada et al., 1992; Utsumi et al., 1991; Wadsworth et al., 1992; Wee et al., 1994). Since these potential adhesion mechanisms could obscure the functional

consequences of disrupting E-cadherin function, we assessed the impact of anti-E-cadherin MAb on the reaggregation and subsequent functional activity of enzymatically dissociated thymus tissue where many of these other interactions have been disrupted. Cell suspensions from enzymatically dispersed day 16 fetal thymic lobes were established as standing drops on membrane filters in the presence or absence of anti-E-cadherin or control MAb. After 7–10 days of culture, dissociated thymus cells established in medium or in medium containing control MAb 1B1 formed plump "solid" reaggregates that could be lifted from the filter with forceps and that yielded substantial numbers of viable thymocytes upon mechanical dispersion (25%–89% of the average number of input cells with viabilities at 59.7%–91.2%). In sharp contrast, addition of ECCD-1 MAb to standing drop cultures resulted in flattened fragile discs of cells with little mechanical stability. Recoveries of viable cells from ECCD-1-treated cultures were consistently very low (<1% of the input cells; viability 5.2%–14.6%; three independent experiments).

While thymocytes recovered from medium or 1B1 control reaggregate cultures predominantly displayed a mature phenotype (Figure 1a), the few viable cells recovered from ECCD-1-treated reaggregate cultures expressed low levels of CD3 and were essentially double negative (DN) (Figure 1). The ECCD-1 concentration used in most of these experiments (100 μg/ml) was identical to that used in previous functional studies (Hirai et al., 1989a, 1989b), although almost equivalent inhibitory activity was seen at 30 μg/ml (data not shown). DECMA-1, another anti-E-cadherin MAb that blocks E-cadherin-mediated homotypic interactions (Vestweber and Kemler, 1985), also inhibited thymus reaggregation and subsequent thymocyte development, although not as effectively as ECCD-1 (data not shown). In contrast, the anti-E-cadherin MAb, ECCD-2, which blocks heterotypic E-cadherin–$\alpha_E\beta_7$ integrin interactions (Karecla et al., 1995) but not homotypic E-cadherin interactions (Shirayoshi et al., 1986), did not affect thymocyte development in reaggregate cultures after 7 days (Figure 1b) or 10 days of culture (data not shown).

The reaggregates that formed in medium alone or in the presence of 1B1 or ECCD-2 MAbs were well organized. As shown in Figure 2a, these cultures were stratified, with a layer of vacuolated cuboidal cells oriented along the filter, a thin layer of stratified stromal cells and lymphocytes above the basal layer of cells, and a more extensive upper layer, which contained the majority of thymocytes. Immunohistochemical analysis of these reaggregate cultures revealed scattered foci of cells bearing a medullary phenotype, as defined by reactivity with MAb 10.1.1 (Farr et al., 1993; Figure 2c). The 10.1.1$^+$ cells were oriented toward the free surface of the reaggregates and were surrounded by stromal elements with a cortical phenotype, as defined by reactivity with MAb NLDC-145 (Kraal et al., 1986; Figure 2b). The distribution of thymocytes expressing Vβ8 TCR (Figure 2d) was similar to that of CD3$^+$ cells (data not shown) in that they were preferentially located in the upper two layers of the reaggregates and were largely excluded from the basal layer. Furthermore, Vβ8$^+$ (Figure 2d) or Vβ6$^+$ thymocytes (data not shown) were not uniformly distributed throughout the reaggregates, but were predominantly

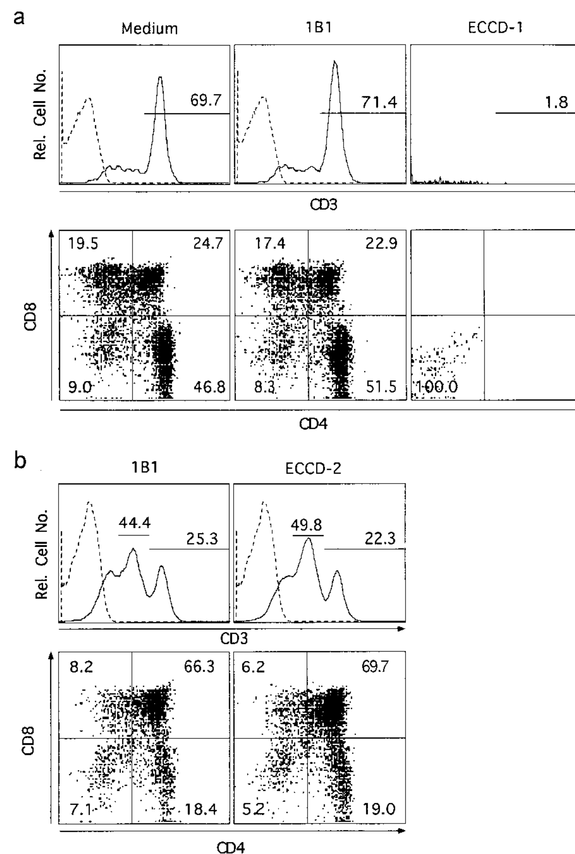

Figure 1. Effects of Anti-E-Cadherin MAbs on Fetal Thymic Reaggregate Cultures

(a) MAb ECCD-1 perturbs thymocyte maturation in reaggregate cultures. Day 16 fetal thymic reaggregates were allowed to form in the presence of 100 μg/ml MAbs as indicated and cultured for 12 days. Reaggregates were mechanically dispersed, and the resulting single cells were analyzed by flow cytometry. Upper panels depict cells stained with anti-CD3ε-FITC (solid line) or isotype control MAb (broken line). Numbers in lower panels indicate percentages of cells in the relevant region or quadrant. Representative of six experiments.
(b) MAb ECCD-2 has no effect on thymocyte maturation in reaggregate cultures. Reaggregates that were allowed to form in the presence of 100 μg/ml of the indicated MAb were mechanically dispersed after 9 days of culture, and the cells were analyzed by flow cytometry. Upper panels depict cells stained with anti-CD3ε-FITC (solid line) or isotype control MAb (broken line). CD3 is expressed at high levels by mature thymocytes bearing αβ TCR and at lower levels by immature thymocytes. Numbers in lower panels indicate percentages of cells in the relevant region or quadrant. Representative of three experiments.

found as small clusters of 2–8 cells. The clustered distribution of TCR$^+$ thymocytes within the reaggregate cultures was much more apparent than in fetal or neonatal thymus tissue in situ (Farr and Anderson, 1988). This may reflect the cessation of progenitor cell influx in vitro, leading to a paucity of immature thymocytes expressing cytoplasmic β chain and the accumulation of thymocytes bearing a mature single positive (SP) phenotype during the culture period. Clusters of thymocytes utilizing the same Vβ element may correspond to the population of cycling mature thymocytes previously described in fetal thymus reaggregate cultures (Ernst et al., 1995).

This pattern of stromal cell organization was abrogated in thymic reaggregate cultures established in the

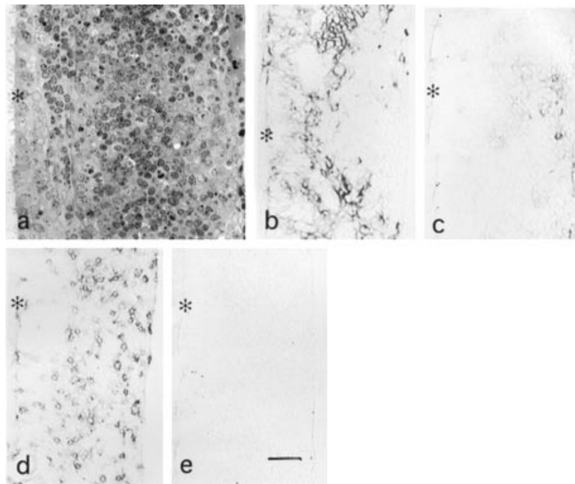

Figure 2. Morphologic and Phenotypic Characterization of Reaggregate Cultures

Reaggregates established and cultured for 12 days in 1B1 were embedded in epoxy, and sections were stained with Richardson's stain (a) or frozen and stained with MAb NLDC-145 to cortical epithelium (b), MAb 10.1.1 to medullary epithelium (c), MAb F23.1 to TCR Vβ8 (d), or no primary antibody (control; e). Asterisks indicate the original location of the supporting filter. Bar is 60 μm. Representative of two experiments.

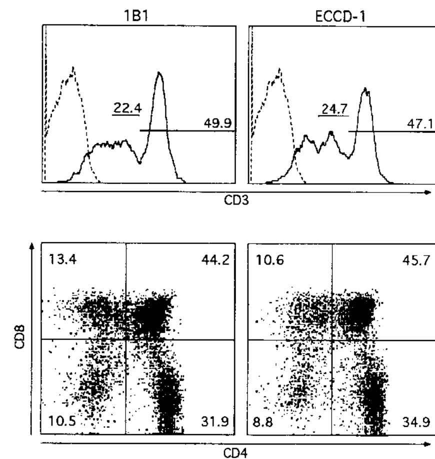

Figure 4. ECCD-1 MAb Does Not Affect Thymocyte Differentiation within Intact Cultured Thymic Lobes

Day 16 fetal thymic lobes cultured in FTOC for 10 days with MAb as indicated were mechanically dispersed, and the resulting cells were analyzed by flow cytometry. Upper panels show cells stained with anti-CD3ε-FITC (solid line) or isotype control MAb (broken line). Lower panels demonstrate cells stained with anti-CD4-FITC and anti-CD8-PE. Numbers in lower panels indicate percentages of cells in the relevant region or quadrant. Representative of three experiments.

presence of ECCD-1. In these cultures, the basal layer of cells was present, but appeared to be slightly disorganized. More dramatically, the superficial layers of the ECCD-1-treated reaggregate cultures did not develop properly and the cultures were hypocellular (Figure 3a). NLDC-145$^+$ cortical cells (Figure 3b) or 10.1.1$^+$ medullary epithelial cells (Figure 3c) were not detected in ECCD-1-treated reaggregate cultures, and thymocytes expressing cell surface or cytoplasmic TCR Vβ8 were absent (Figure 3d).

MAb ECCD-1 Perturbs the Differentiation of T Cell Progenitors in DOG-Treated Thymic Lobes

The inhibitory effects of ECCD-1 MAb on the organization and function of enzymatically dissociated thymic epithelium indicated an important role for E-cadherin in the establishment of a productive thymic microenvironment. However, this outcome could reflect disruption of E-cadherin-mediated interactions between T cell progenitors and thymic epithelial cells or result from the disruptive effects of ECCD-1 MAb on thymic epithelium. To assess more directly the role of E-cadherin in interactions between progenitor cells and thymic epithelial cells, we examined the effects of ECCD-1 MAb on the ability of T cell progenitors from fetal liver or fetal thymus to repopulate and differentiate within intact fetal thymic lobes that had been previously depleted of hematogenous elements by culture in medium containing DOG. This strategy was appropriate because ECCD-1 MAb had no effect on stromal cell organization of intact day 16 fetal thymus lobes or the ability of intact fetal thymic lobes to support thymocyte development (Figure 4; data not shown).

Day 15 fetal liver cells, which contained T cell progenitors still dependent on the most proximal environmental cues provided by the thymic environment, were CD3$^-$ CD4$^-$CD8$^-$ and expressed high levels of E-cadherin (Figure 5a; data not shown). Both ECCD-1 and ECCD-2 MAbs detected the expected 120 kDa E-cadherin protein (and only E-cadherin) in lysates from fetal liver cells used in the reconstitution studies or from a thymic epithelial cell line (Figure 5b), ruling out the possibility that the inhibitory effects of ECCD-1 were due to cross-reactivity with recently described cadherins that are expressed by T cells and have a M_r approaching 130 kDa (Cepek et al., 1996).

In the absence of antibody or the presence of irrelevant control MAb, DOG fetal thymic lobes repopulated with fetal liver cells yielded about 10^5 viable cells per lobe after 12 days of culture. As shown in Figure 6a, most of the recovered cells expressed low or intermediate levels of CD3 and were either DN (39.3%) or double

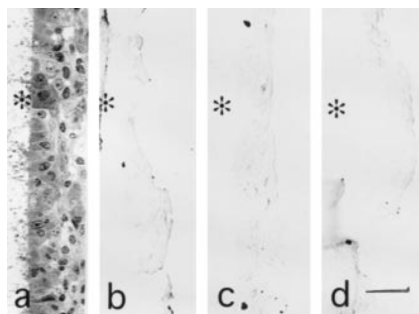

Figure 3. Disrupted Organization of Reaggregates Treated with MAb ECCD-1

Reaggregates established in ECCD-1 were embedded in epoxy, and sections were stained with Richardson's stain (a) or frozen and stained with MAb NLDC-145 (b), 10.1.1 (c), or F23.1 (d). Asterisks indicate the original location of the supporting filter. Bar is 60 μm. Representative of three experiments.

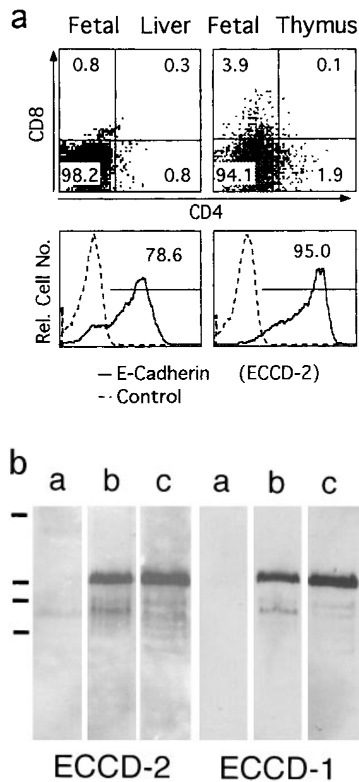

Figure 5. Characterization of Input Cells for Repopulation Assays

(a) Upper panels depict labeling of indicated cell populations with anti-CD4-FITC and anti-CD8-PE. Lower panels depict labeling of indicated cell populations with ECCD-2 anti-E-cadherin MAb. Representative of two experiments.

(b) Immunoblot analysis of fetal liver reactivity with anti-E-cadherin MAbs. Whole-cell lysates from different sources were probed with anti-ECCD-1 and anti-ECCD-2 MAbs. Lane a, lysate from E-cadherin^{-} thymic stromal cell line (ANV 41-2); lane b, lysate from E-cadherin^{+} thymic stromal cell line (TE-71); lane c, lysate from mechanically dispersed fetal liver cells used in repopulation assay. Marks indicate M_r of molecular weight standards (from top, 205, 116, 97.5, and 66). Representative of two experiments.

positive (DP) (44.1%), although small subpopulations reached more distal stages of development (8.2% CD8 SP and 8.4% CD4 SP). The ECCD-1 MAb dramatically reduced the recovery of viable thymocytes ($\sim 10^3$ per lobe), and most of the thymocytes recovered from the ECCD-1-treated lobes were CD4^{-}CD8^{-} ($\sim$85%). Representation of CD4 and CD8 SP thymocytes in the small number of cells recovered from these reaggregates was similar to that observed in the control cultures.

To define better the stage of thymocyte development affected by the ECCD-1 MAb, we also assessed the effects of this MAb on the repopulation/differentiation potential of day 15 fetal thymocytes in the DOG-treated thymic lobes. Day 15 fetal thymocytes, while predominantly expressing a E-cadherin^{+} phenotype (Figure 5a; data not shown), contain a small population of CD4^{-} CD8^{low} and CD4^{low}CD8^{-} cells that have interacted with the thymic environment and thus may exhibit different sensitivities to the inhibitory effects of the ECCD-1 MAb. Repopulation of DOG-treated day 16 fetal thymic lobes with 2.5×10^4 day 15 fetal thymocytes in the presence of 100 μg/ml MAb 1B1 yielded 5.4×10^4 viable cells per lobe. Representation of thymocytes defined by CD4/CD8 expression was skewed toward CD4 and CD8 SP cells, 53% and 17%, respectively, although there was also substantial representation of CD4^{-}CD8^{-} and CD4^{+} CD8^{+} thymocytes as well (12% and 17%, respectively) (Figure 6b). As with fetal liver cell reconstitution, cell recoveries from ECCD-1-treated cultures were reduced in comparison (6.0×10^3 viable cells per lobe). Although ECCD-1 treatment reduced overall cell recoveries, it did not prevent the development of mature SP thymocytes from the input fetal thymocytes. As shown in Figure 6b, thymocytes recovered from ECCD-1-treated reaggregates were mostly CD3^{bright} (92.5%) and displayed increased representation of CD4 SP and CD4^{-}CD8^{-} cells. The marked reduction of DP cells in response to treatment with ECCD-1 MAb (2.2% versus 17.6%), suggests that progression from the DN to DP stage was perturbed or that the size of the progenitor cell population capable of differentiation was reduced. The preferential representation of mature SP thymocytes (albeit dramatically reduced in absolute numbers) within the ECCD-1-treated lobes may reflect the E-cadherin-independent differentiation of the more differentiated progenitors initially present within the thymocyte suspension used for repopulation.

Although ECCD-1 interfered with the capacity of fetal liver cells and fetal thymocytes to differentiate productively in DOG-treated thymic lobes, it had no discernible effect on the organization of the cortical and medullary epithelial components. As shown in Figure 7, lobes cultured in ECCD-1 MAb displayed well-defined cortical and medullary compartments and were comparable with lobes cultured in irrelevant MAb, although stromal elements in the ECCD-1-treated lobes appeared more prominent owing to the relative paucity of lymphoid cells.

Discussion

The data described in this report demonstrate that E-cadherin plays an important role in the organization or development (or both) of thymic epithelium and in the interactions mediating early stages of thymocyte development. In reaggregate culture systems, where potentially redundant mechanisms that maintain epithelial integrity have been compromised, anti-E-cadherin MAbs that inhibit homophilic cadherin-mediated adhesion inhibited the ability of thymic epithelial cells to form a productive lymphopoietic environment in vitro. Stromal elements that reassociated in the presence of the ECCD-1 MAb did not exhibit differentiation antigens that define cortical and medullary epithelial components, suggesting that E-cadherin interactions were involved, directly or indirectly, in the maintenance of distinct thymic epithelial cell phenotypes. Thymic stromal elements that reassociated in the presence of ECCD-1 also did not support the proliferation, survival, or differentiation of thymocyte progenitors.

In addition to preventing reaggregation of enzymatically dissociated thymic epithelial cells and the accompanying thymocyte differentiation, ECCD-1 also impaired the in vitro repopulation of DOG-treated fetal

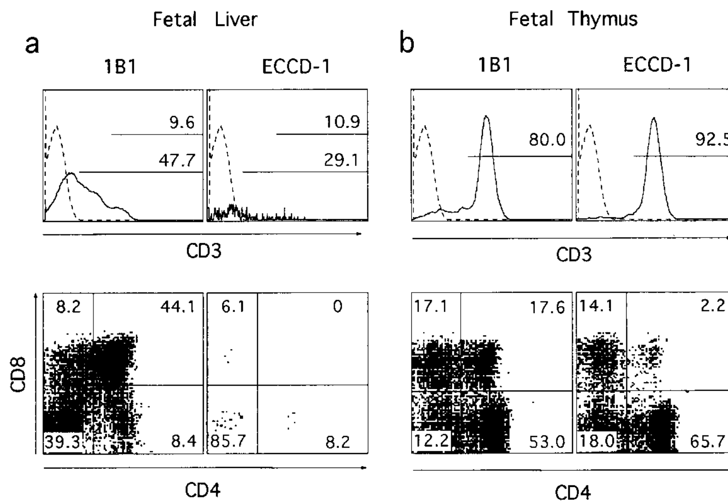

Figure 6. MAb ECCD-1 Perturbs Repopulation of Thymic Lobes with Fetal Liver or Thymus-Derived Progenitors

DOG-treated lobes that were recolonized with 2.5×10^4 of the indicated lymphoid progenitor cells and cultured in FTOC for an additional 12 days were mechanically dispersed and analyzed by flow cytometry. Shown are lobes recolonized with fetal liver cells (a) and lobes recolonized with fetal thymocytes (b). Upper panels depict cells stained with anti-CD3ε-FITC (solid line) or control (broken line). Numbers in lower panels indicate percentages of cells in the relevant region or quadrant. Representative of three experiments.

thymic lobes by E-cadherin$^+$ fetal liver cells or thymocytes. In the case of fetal liver cell progenitors, the dramatic reduction in the frequency and absolute number of CD4$^+$CD8$^+$ cells and the corresponding increase in the representation of CD4$^-$CD8$^-$ cells suggest that the progression of CD4$^-$CD8$^-$ cells to the CD4$^+$CD8$^+$ stage does not proceed normally in the presence of ECCD-1 MAb, although the reproducible recovery of a small but signficant population of SP thymocytes from these lobes indicates that some progenitor cells have undergone productive differentiation. Repopulation of DOG-treated thymic lobes with fetal thymocytes generated substantial percentages of phenotypically mature thymocytes, albeit reduced in absolute number. In this latter case,

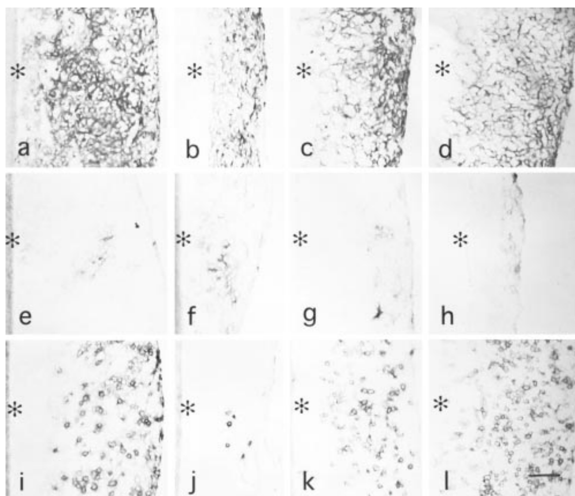

Figure 7. MAb ECCD-1 Does Not Affect Epithelial Organization within Intact Cultured Thymus Lobes

DOG-treated lobes repopulated with 2.5×10^4 fetal liver cells (a, b, e, f, i, and j) or 2.5×10^4 fetal thymocytes (c, d, g, h, k, and l) and cultured in FTOC for an additional 12 days were frozen, and sections were stained with MAb NLDC-145 (a–d), MAb 10.1.1 (e–h), or MAb F23.1 (i–l). (a), (c), (e), (g), (i), and (k), 1B1 cultures; (b), (d), (f), (h), (j), and (l), ECCD-1 cultures. Asterisks indicate the original location of the supporting filter. Bar is 60 μm. Representative of two experiments.

the significant representation of CD8 SP and CD4 SP thymocytes, and the lack of DP cells, suggests that ECCD-1 prevents progression of the CD4$^-$CD8$^-$ population to the CD4$^+$CD8$^+$ stage and that it has little effect on the transition from the DP to the SP stage, or other distal stages of thymocyte development. The mature thymocytes that accumulate following recolonization with fetal thymocytes in the presence of ECCD-1 may represent a wave of progeny derived from more mature CD3^{dull}CD4$^-$CD8^{low} and CD4^{low}CD8$^-$ thymocyte progenitors that are committed to becoming CD4$^+$CD8$^+$ thymocytes and that spontaneously do so in suspension culture (Nikolic-Zugic and Bevan, 1988; Nikolic-Zugic et al., 1989; Takahama et al., 1994). Alternatively, the differential sensitivity of fetal liver and fetal thymus progenitors to ECCD-1-mediated inhibition could also reflect a role for E-cadherin in progenitor cell homing to the thymus, where fetal thymocytes, having successfully homed to the thymus, would be expected to be less affected by a loss of E-cadherin function.

Based on the documented inhibitory activities of ECCD-1 and DECMA-1 anti-E-cadherin MAbs, blockade of homotypic E-cadherin interactions is a likely explanation for the inhibitory activity of these MAbs in thymus organogenesis and thymocyte development in vitro, particularly since no inhibitory activity was noted with ECCD-2 MAb, which selectively blocks E-cadherin interaction with $\alpha_E\beta_7$ integrin and does not inhibit homophilic E-cadherin-mediated interaction. Importantly, the molecules expressed by fetal liver cells and recognized by ECCD-1 MAbs displayed electrophoretic mobility identical to that of E-cadherin, excluding reactivity with recently described novel members of the cadherin superfamily associated with T lineage cells (Cepek et al., 1996; Munro et al., 1996) or their progenitors. The finding that ECCD-1 MAb did not affect the organization of functional activity of intact cultured fetal thymus lobes is consistent with the results of previous studies of lung and skin morphogenesis in which ECCD-1 caused only subtle alterations in organ culture but had significant effects on the ability of epithelial cells from these tissues to reaggregate in vitro (Fujita et al., 1992; Hirai et al., 1989a, 1989b). The resistance of intact tissues to disruptive or inhibitory activity of ECCD-1 MAb may reflect the

multiple adhesive interactions between epithelial cells and between epithelial cells and extracellular matrix components. Alternatively, this low affinity anti-E-cadherin MAb may be unable to disrupt preformed cadherin multimers.

In light of the inability of ECCD-1 to perturb thymocyte development within intact cultured fetal thymus lobes or epithelial organization of intact DOG-cultured thymic lobes, the inhibitory activity of this anti-E-cadherin MAb in thymic lobe repopulation experiments suggests that E-cadherin participates in interactions involving T cell progenitors and epithelial cells, as well as those beween thymic epithelial cells themselves. The high level expression of E-cadherin by fetal and adult thymic epithelial cells and early fetal thymocytes and the selective expression of E-cadherin by $CD4^{-}CD8^{-}$ thymocytes in the adult thymus (Lee et al., 1994) are consistent with this possibility. Molecular approaches to direct selective expression of different forms of E-cadherin within the thymic environment are presently being developed to explore this question.

The results presented in this study reemphasize the importance of cadherins in adhesive interactions involving hematopoietic cells. Studies of human bone marrow indicate that E-cadherin is expressed by immature cells of the erythroid lineage and that antibodies directed against E-cadherin interfere with the erythropoietin-stimulated erythropoiesis in vitro (Armeanu et al., 1995). These findings, together with earlier studies demonstrating that the adhesion of Langerhans cells to keratinocytes involves E-cadherin (Tang et al., 1993) clearly indicate that E-cadherin expression is not restricted to epithelial cells and that E-cadherin-mediated cell interactions participate in a wide range of biological processes beyond homotypic epithelial adhesion.

Experimental Procedures

Reagents

The tissue culture media was RPMI 1640 supplemented with 1% nonessential amino acids, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-ME (all from Sigma Chemical Co., St. Louis, MO), and 10% heat-inactivated FCS (Summit Biotechnology, Fort Collins, CO). MAbs reactive with E-cadherin (ECCD-1 [Yoshida-Noro et al., 1984] and ECCD-2 [Shirayoshi et al., 1986]) were provided by Dr. M. Takeichi (Kyoto University, Kyoto, Japan), and DECMA-1 (Vestweber and Kemler, 1985) was purchased from Sigma. The rat MAb 1B1 (IgG2A, κ) was generated in our laboratory against the murine thymic epithelial cell line TEC (Glimcher et al., 1983). Although the specificity of this MAb is unknown, it has no effect in a number of biological assays (including those used in the present study) and reacts weakly with a small subset of medullary thymic cells by immunohistochemistry (Nelson et al., 1996; A. G. F. and A. J. Nelson, unpublished data). These MAb were purified from serum-free hybridoma supernatants by chromatography on protein G–Sepharose (Pharmacia, Uppsala, Sweden) and dialyzed extensively against serum-free RPMI 1640 before use. Endotoxin was undetectable by Limulus lysate assay (<1 pg/ml).

Animals and Cell Preparation

Timed pregnant female BALB/c mice (Universal Banting & Kingman, Kent, WA) were sacrificed on day 16 of gestation (counting the day of the appearance of the vaginal plug as day 0). Fetal thymi and livers were dissected aseptically and kept in media on ice until further processing (usually <2 hr). All procedures involving animals followed institutional guidelines established by the Department of Comparative Medicine of the University of Washington.

Fetal Thymic Organ Culture

Fetal thymic organ culture (FTOC) was performed as described previously (Ramsdell, 1992). In brief, day 16 fetal thymic lobes were cultured on polyvinylidene filters (Millipore, Bedford, MA) supported on gelatin sponges (Upjohn, Kalamazoo, MI) in 2.5 ml of media containing the antibody to be tested. The cultures were maintained in 35 mm Petri dishes (Corning, New York, NY) in a humidified atmosphere containing 5% CO_2/95% air at 37°C.

Thymic Reaggregation Assay

Fetal thymic lobes were digested for 15 min at 37°C with a cocktail consisting of 0.8 U/ml dispase I, 0.1 U/ml collagenase, and 150 U/ml DNase (all from Boehringer Mannheim, Indianapolis, IN) in HBSS. Cells were dispersed by vigorous pipetting, washed in media containing 10% FCS, and suspended in media containing the antibody to be tested. After centrifugation and aspiration of the supernatant, the cell pellet was dispersed into a thick slurry, drawn into a fine glass pipette, and placed as a standing drop on the surface of a cellulose acetate filter (Millipore; Jenkinson et al., 1992). Individual drops had a volume of 1.5–2 μl and contained cells from 3–5 lobes. Culture of the thymus reaggregates was performed as described for FTOC.

Thymic Recolonization Assay

Fetal thymic lobes and livers were mechanically dispersed, and cells were washed with media and resuspended (7.5×10^5 cells per milliliter) in medium containing 100 μg/ml of the antibody to be tested. We placed 30 μl of these cell suspensions (2.5×10^3 cells) into each well of a Terasaki plate (Falcon, Lincoln Park, NJ). Fetal thymic lobes that had been rendered alymphoid by culture for 4–5 days in FTOC with 1.35 mM DOG (Sigma; Jenkinson et al., 1982) were then added (one to each well), and the plate was inverted to allow the formation of hanging drops (Kingston et al., 1985). After 2 days of culture, the thymic lobes were washed and placed into FTOC in the presence of MAb for 12 days to allow the development of colonizing cells. The efficacy of the DOG treatment was confirmed by the absence of thymocytes recovered from DOG-treated lobes that had not been repopulated with fetal thymocytes or fetal liver cells.

Flow Cytometry

Fetal thymic lobes or reaggregate cultures were mechanically dispersed in cold medium, passed through nylon mesh, and washed with additional medium. To minimize Fc receptor–mediated labeling, cells were incubated with anti-FcγRII MAb 2.4G2 (Unkeless, 1979; 50% hybridoma supernatant in HBSS supplemented with 0.1% NaN_3), 1% FCS, 10% rat serum, and 10% goat serum prior to labeling with fluorochrome-conjugated MAbs. Cells were then stained with either anti-CD3ε–fluorescein isothiocyanate (FITC) (clone 500A2; Havran et al., 1987), anti-CD4-FITC (clone RM4-5; Pharmingen, San Diego, CA), and/or anti-CD8-phycoerythrin (PE) (clone 3B5; Caltag, Burlingame, CA) and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). All data shown are with gates set on living cells, as assessed by their exclusion of the fluorescent dye 7-amino-actinomycin D (Molecular Probes, Inc., Eugene, OR; Schmid et al., 1992). In some experiments, cells were stained with anti-E-cadherin (ECCD-2) MAb followed by PE-conjugated goat anti–rat immunoglobulin (Pierce, Rockford, IL); in these experiments, MAb 2.4G2 and rat serum were omitted.

Histology

Filters bearing thymus cultures were immersed in cold 100 mM cacodylate buffer (pH 7.2) containing 2% glutaraldehyde, 2% paraformaldehyde, and 1 mM $CaCl_2$. After 30 min at 4°C, the samples were washed with additional buffer, exposed to 1% aqueous OsO_4 for 30 min on ice, and then processed for conventional epoxy embedding.

Immunohistochemistry

Phenotypic analyses of thymic reaggregates and recolonized thymi utilized indirect enzyme immunohistochemical procedures (Farr and Nakane, 1981). In brief, frozen sections of tissue were serially incubated with optimal dilutions of antigen-specific or control primary

antibodies that had been modified with N-hydroxysuccinimidyl-digoxigenin (Boehringer Mannheim) according to the instructions of the manufacturer. Horseradish peroxidase–conjugated Fab fragments of sheep anti-digoxigenin antibodies (Boehringer Mannheim) were used to detect digoxigenin-modified primary antibodies. Peroxidase activity was revealed with 3,3′-diaminobenzidine (Sigma) in the presence of hydrogen peroxide. Primary MAb used included NLDC-145 (Kraal et al., 1986), 10.1.1 (Farr et al., 1993), and F23.1 (Staerz et al., 1985).

Immunoblotting

The methodology for immunoblotting with ECCD-1 and ECCD-2 MAbs was a modification of the work of Yoshida-Noro et al. (1984). Aliquots of dissociated fetal liver cells or thymic stromal cell lines were suspended in 20 mM Tris–HCl buffer containing 0.5% Triton X-100, 50 mM NaCl, 0.2 mM Na vanadate, 1 mM each of $CaCl_2$ and $MgCl_2$, 1 mM phenyl-methylsulfonyl fluoride, and 1 mM p-tosyl-L-arginine methyl ester. The cell lysates were kept on ice for 90 min with occasional vortexing. After low speed centrifugation to remove nuclei and other insoluble cellular debris, the supernatants were processed for SDS–PAGE and electrophoretic transfer to nitrocellulose membranes. Nitrocellulose membranes were reversibly stained with Ponceau red to identify molecular weight markers. Blots were rinsed in 10 mM Tris-buffered saline containing 0.05% Tween 20 (TBS-20) and then incubated in TBS-20 containing 5% nonfat milk powder for 1 hr. Incubation in primary MAbs (5 μg/ml) was overnight. After washing five times in TBS-20, the blots were briefly immersed in TBS-20 containing 0.2% glutaraldehyde. All solutions through the TBS-20–glutaraldehyde step contained 1 mM $CaCl_2$. After fixation, the blots were washed repeatedly with TBS-T20 and sequentially incubated for 1 hr at 4°C with digoxigenin-modified goat anti–rat immunoglobulin antibodies (250 ng/ml) and peroxidase-conjugated sheep anti–digoxigenin antibody conjugates (Boehringer Mannheim; 100 ng/ml) with intervening washing with TBS-20. Peroxidase activity was detected with chemiluminescence (New England Nuclear).

Acknowledgments

Correspondence should be addressed to A. G. F. We thank Dr. M. Takeichi for the generous gift of hybridomas producing ECCD-1 and ECCD-2 and Dr. T. Jakob for helpful discussions. This work was supported in part by National Institutes of Health grants AI 24137 and AG 04360. K. M. M. was supported by a postdoctoral fellowship from the Swiss National Foundation for Scientific Research.

Received December 13, 1996; revised January 20, 1997.

References

Andrew, D.P., Rott, L.S., Kilshaw, P.J., and Butcher, E.C. (1996). Distribution of $\alpha_4\beta_7$ and $\alpha_E\beta_7$ integrins on thymocytes, intestinal epithelial lymphocytes and peripheral lymphocytes. Eur. J. Immunol. *26*, 897–905.

Armeanu, S., Bühring, H.-J., Reuss-Borst, M., Müller, C.A., and Klein, G. (1995). E-cadherin is functionally involved in the maturation of the erythroid lineage. J. Cell Biol. *131*, 243–249.

Cepek, K.L., Shaw, S.K., Parker, C.M., Russell, G.J., Morrow, J.S., Rimm, D.L., and Brenner, M.B. (1994). Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the $\alpha_E\beta_7$ integrin. Nature *372*, 190–193.

Cepek, K.L., Rimm, D.L., and Brenner, M.B. (1996). Expression of a candidate cadherin in T lymphocytes. Proc. Natl. Acad. Sci. USA *93*, 6567–6571.

Chang, A.C., Wadsworth, S., and Coligan, J.E. (1993). Expression of merosin in the thymus and its interaction with thymocytes. J. Immunol. *151*, 1789–1801.

Denning, S.M., Tuck, D.T., Vollger, L.W., Springer, T.A., Singer, K.H., and Haynes, B.F. (1987). Monoclonal antibodies to CD2 and lymphocyte function-associated antigen 3 inhibit human thymic epithelial cell-dependent mature thymocyte activation. J. Immunol. *139*, 2573–2578.

Ernst, B., Surh, C.D., and Sprent, J. (1995). Thymic selection and cell division. J. Exp. Med. *182*, 961–972.

Farr, A.G., and Anderson, S.K. (1988). *In situ* localization of T cell receptor β chain in the murine thymus: changes in the intra thymic distribution of thymocytes expressing β chain during fetal development. Cell. Immunol. *116*, 135–148.

Farr, A.G., and Nakane, P.K. (1981). Immunohistochemistry with enzyme labeled antibodies: a brief review. J. Immunol. Meth. *47*, 129–144.

Farr, A., Nelson, A., Hosier, S., and Kim, A. (1993). A novel cytokine-responsive cell surface glycoprotein defines a subset of medullary thymic epithelium *in situ*. J. Immunol. *150*, 1160–1171.

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

Fujita, M., Furukawa, F., Fujii, K., Horiguchi, Y., Takeichi, M., and Imamura, S. (1992). Expression of cadherin cell adhesion molecules during human skin development: morphogenesis of epidermis, hair follicles and eccrine sweat ducts. Arch. Dermatol. Res. *284*, 159–166.

Glimcher, L.H., Kruisbeek, A.M., Paul, W.E., and Green, I. (1983). Functional activity of a transformed thymic epithelial cell line. Scand. J. Immunol. *17*, 1–11.

Grunwald, G.B. (1993). The structural and functional analysis of cadherin calcium-dependent cell adhesion molecules. Curr. Opin. Cell Biol. *5*, 797–805.

Havran, W.L., Poenie, M., Kimura, J., Tsien, R., Weiss, A., and Allison, J.P. (1987). Expression and function of the CD3-antigen receptor on murine $CD4^+8^+$ thymocytes. Nature *330*, 170–173.

Hirai, Y., Nose, A., Kobayashi, S., and Takeichi, M. (1989a). Expression and role of E- and P-cadherin adhesion molecules in embryonic histogenesis. I. Lung epithelial morphogenesis. Development *105*, 263–270.

Hirai, Y., Nose, A., Kobayashi, S., and Takeichi, M. (1989b). Expression and role of E- and P-cadherin adhesion molecules in embryonic histogenesis. I. Skin morphogenesis. Development *105*, 271–277.

Hueber, A.-O., Pierres, M., and He, H.-T. (1992). Sulfated glycans directly interact with mouse Thy-1 and negatively regulate Thy-1-mediated adhesion of thymocytes to thymic epithelial cells. J. Immunol. *148*, 3692–3699.

Jenkinson, E.J., Franchi, L.L., Kingston, R., and Owen, J.J.T. (1982). Effect of deoxyguanosine on lymphopoiesis in the developing thymus rudiment *in vitro*: application in the production of chimeric thymus rudiments. Eur. J. Immunol. *12*, 583–587.

Jenkinson, E.J., Anderson, G., and Owen, J.J.T. (1992). Studies on T cell maturation on defined thymic stromal cell populations *in vitro*. J. Exp. Med. *176*, 845–853.

Karecla, P.I., Bowden, S.J., Green, S.J., and Kilshaw, P.J. (1995). Recognition of E-cadherin on epithelial cells by the mucosal T cell integrin αM290β7 (αEβ7). Eur. J. Immunol. *25*, 852–856.

Kingston, R., Jenkinson, E.J., and Owen, J.J.T. (1985). A single stem cell can recolonize an embryonic thymus, producing phenotypically distinct T-cell populations. Nature *317*, 811–813.

Kraal, G., Breel, M., Janse, M., and Bruin, G. (1986). Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. J. Exp. Med. *163*, 981–997.

Lannes-Vieira, J., Dardenne, M., and Savino, W. (1991). Extracellular matrix components of the mouse thymus microenvironment: ontogenetic studies and modulation by glucocorticoid hormones. J. Histochem. Cytochem. *39*, 1539–1546.

Larue, L., Ohsugi, M., Hirchenhain, J., and Kemler, R. (1994). E-cadherin null mutant embryos fail to form a trophectoderm epithelium. Proc. Natl. Acad. Sci. USA *91*, 8263–8267.

Lee, M.G., Sharrow, S.O., Farr, A.G., Singer, A., and Udey, M.C. (1994). Expression of the homotypic adhesion molecule E-cadherin by immature murine thymocytes and thymic epithelial cells. J. Immunol. *152*, 5653–5659.

Lefrançois, L., Barrett, T.A., Havran, W.L., and Puddington, L. (1994).

Developmental expression of the $\alpha_{IEL}\beta_7$ integrin on T cell receptor $\gamma\delta$ and T cell receptor $\alpha\beta$ T cells. Eur. J. Immunol. *24*, 635–640.

Liu, Y., Jones, B., Aruffo, A., Sullivan, K.M., Linsley, P.S., and Janeway, C.A., Jr. (1992). Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. J. Exp. Med. *175*, 437–446.

Munro, S.B., Duclos, A.J., Jackson, A.R., Baines, M.G., and Blaschuk, O.W. (1996). Characterization of cadherins expressed by murine thymocytes. Cell. Immunol. *169*, 309–312.

Nelson, A.J., Dunn, R.J., Peach, R., Aruffo, A., and Farr, A.G. (1996). The murine homolog of human Ep-CAM, a homotypic adhesion molecule, is expressed by thymocytes and thymic epithelial cells. Eur. J. Immunol. *26*, 401–408.

Nikolic-Zugic, J., Moore, M.W., and Bevan, M.J. (1989). Characterization of the subset of immature thymocytes which can undergo rapid *in vitro* differentiation. Eur. J. Immunol. *19*, 649–653.

Nikolic-Zugic, N., and Bevan, M.J. (1988). Thymocytes expressing CD8 differentiate into CD4+ cells following intrathymic injection. Proc. Natl. Acad. Sci. USA *85*, 8633–8637.

Nose, A., Nagafuchi, A., and Takeichi, M. (1988). Expressed recombinant cadherins mediate cell sorting in model systems. Cell *54*, 993–1001.

Ramsdell, F. (1992). Fetal thymus organ culture for T cell development studies. In Current Protocols in Immunology, J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds. (New York: John Wiley & Sons), pp. 3.18.1–3.18.10.

Riethmacher, D., Brinkmann, V., and Birchmeier, C. (1995). A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. Proc. Natl. Acad. Sci. USA *92*, 855–859.

Sawada, M., Nagamine, J., Takeda, K., Utsumi, K., Kosugi, A., Tatsumi, Y., Hamaoka, T., Miyake, K., Nakajima, K., Watanabe, T., Sakakibara, S., and Fujiwara, H. (1992). Expression of VLA-4 on thymocytes: maturation stage-associated transition and its correlation with their capacity to adhere to thymic stromal cells. J. Immunol. *149*, 3517–3524.

Schmid, I., Krall, W.J., Uittenbogaart, C.H., Braun, J., and Giorgi, J.V. (1992). Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. Cytometry *13*, 204–208.

Shaw, S.K., and Brenner, M.B. (1995). The β7 integrins in mucosal homing and retention. Semin. Immunol. *7*, 335–342.

Shirayoshi, Y., Nose, A., Iwasaki, K., and Takeichi, M. (1986). N-linked oligosaccharides are not involved in the function of a cell-cell binding glycoprotein E-cadherin. Cell Struct. Funct. *11*, 245–252.

Staerz, U.D., Rammensee, H.-G., Benedetto, J.D., and Bevan, M.J. (1985). Characterization of a murine monoclonal antibody specific for an allotypic determinant on T cell antigen receptor. J. Immunol. *134*, 3994–4000.

Takahama, Y., Letterio, J.J., Suzuki, H., Farr, A.G., and Singer, A. (1994). Early progression of thymocytes along the CD4/CD8 developmental pathway is regulated by a subset of thymic epithelial cells expressing transforming growth factor β. J. Exp. Med. *179*, 1495–1506.

Takeichi, M. (1990). Cadherins: a molecular family important in selective cell-cell adhesion. Annu. Rev. Biochem. *59*, 237–252.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. Science *251*, 1451–1455.

Takeichi, M. (1993). Cadherins in cancer: implications for invasion and metastasis. Curr. Opin. Cell Biol. *5*, 806–811.

Tang, A., Amagai, M., Granger, L.G., Stanley, J.R., and Udey, M.C. (1993). Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. Nature *361*, 82–85.

Unkeless, J.C. (1979). Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. J. Exp. Med. *150*, 580–596.

Utsumi, K., Sawada, M., Narumiya, S., Nagamine, J., Sakata, T., Iwagami, S., Kita, Y., Teraoka, H., Hirano, H., Ogata, M., Hamaoka, T., and Fujiwara, H. (1991). Adhesion of immature thymocytes to thymic stromal cells through fibronectin molecules and its significance for the induction of thymocyte differentiation. Proc. Natl. Acad. Sci. USA *88*, 5685–5689.

Vestweber, D., and Kemler, R. (1985). Identification of a putative cell adhesion domain of uvomorulin. EMBO J. *4*, 3393–3398.

Wadsworth, S., Halvorson, M.J., and Coligan, J.E. (1992). Developmentally regulated expression of the β4 integrin on immature mouse thymocytes. J. Immunol. *149*, 421–428.

Wee, S.-F., Wang, W.-C., Farr, A.G., Nelson, A.J., Patel, D.D., Haynes, B.F., Linsley, P.S., and Aruffo, A. (1994). Characterization of a CD6 ligand(s) expressed on human- and murine-derived cell lines and murine lymphoid tissues. Cell. Immunol. *158*, 353–364.

Yoshida-Noro, C., Suzuki, N., and Takeichi, M. (1984). Molecular nature of the calcium-dependent cell-cell adhesion system in mouse teratocarcinoma and embryonic cells studied with a monoclonal antibody. Dev. Biol. *101*, 19–27.