Brief Definitive Report

Thymic Epithelial Cells Provide Unique Signals for Positive Selection of CD4+CD8+ Thymocytes In Vitro

By Graham Anderson, John J. T. Owen, Nel C. Moore, and Eric J. Jenkinson

From the Department of Anatomy, Medical School, University of Birmingham, Birmingham B15 2TT, UK

Summary

Using a novel system that supports positive selection in vitro, we have investigated the cellular requirements for this process by testing the ability of individual thymic and nonthymic stromal cell types to support the maturation of CD4+CD8+ thymocytes into CD4+ or CD8+ T cells. We show that thymic cortical epithelial cells are unique in their ability to mediate this maturation, and suggest that in addition to TCR ligation, these cells supply specific signals for positive selection. Moreover, by demonstrating positive selection on ECDI (1-ethyl-3-[3'dimethyl-aminopropyl]-carbodiimide)-fixed epithelial cells in this system, we provide direct evidence that the provision of these signals involves interactions with epithelial cell surface molecules rather than the release of soluble factors.

Signaling through the TCR complex on immature CD4+CD8+ thymocytes can lead to two distinct developmental fates: positive selection, which results in further maturation, or negative selection, which results in cell death by apoptosis (1–3). One explanation for this paradox is that additional signals regulate the outcome of TCR ligation, and that for positive selection, these are provided by interactions with thymic epithelial cells (4, 5). Recently, a specific requirement for interaction with thymic epithelial cells has been questioned by studies that demonstrate positive selection on various cell types, including fibroblasts and hemopoietic cells, that are introduced into the thymus in vivo (6–9). However, whereas these studies indicate that positive selection does not require recognition of peptide–MHC complexes unique to thymic epithelial cells, they do not exclude the possibility that endogenous epithelial cells are providing additional differentiation signals essential for positive selection and consequent maturation.

In this study, we have used a novel in vitro system for positive selection, based on reaggregate organ cultures (10, 11), to analyze the ability of individual stromal cell types to support the maturation of CD4+CD8+ thymocytes in the absence of any other thymic elements. We show that the ability to support the maturation of CD4+CD8+$\alpha\beta$TCR^{lo} thymocytes into CD4+8− and CD4−8+ cells bearing high levels of $\alpha\beta$TCR is a property unique to thymic cortical epithelium, and suggest that these cells provide specific differentiation signals for positive selection. Furthermore, we demonstrate that ECDI (1-ethyl-3-[3'dimethyl-aminopropyl]-carbodiimide-fixed, metabolically inert, thymic epithelial cells can support the maturation of CD4+CD8+ cells and thus provide direct evidence that positive selection by these cells involves interaction with surface molecules rather than the release of soluble factors.

Materials and Methods

Mice

BALB/c mice embryos at day 14 of gestation, produced by timed matings, were used as a source of fetal material.

Antibodies

For immunomagnetic selection, the following antibodies were bound to either anti-rat or anti-mouse coated magnetic beads (Dynal, Wirral, UK) as appropriate: anti-CD3 (clone KT-3; Seralab, Crawley, Down, UK), anti-CD8 (clone YTS 169.3; Serotec, Ltd., Oxford, Oxon., UK), anti-CD45 (clone M1/69; American Type Culture Collection, Rockville, MD), anti-Ia^d (clone MK-D6; Becton Dickinson & Co., Mountain View, CA), and anti-GQ ganglioside (clone A2B5; a gift of M. Raff, University College, London, UK). For flow cytometric analysis of thymocyte differentiation, the following reagents were used: anti-$\alpha\beta$TCR (clone H57.597; a gift from R. Kubo, National Jewish Centre for Immunology and Respiratory Medicine, Denver, CO), anti-CD4-PE (clone GK 1.5; Becton Dickinson & Co.), anti-CD8-FITC (clone 53-6.7; Becton Dickinson & Co.), anti–hamster F(ab)$_2$ biotin (Caltag Laboratories, San Francisco, CA), and streptavidin-APC (Becton Dickinson & Co.).

Preparation of Cell Types

Thymocytes. To obtain a population of CD4+CD8+ cells not yet exposed to TCR-mediated positive selection signals in vivo, CD4+CD8+TCR− cells were prepared from newborn thymocyte suspensions by immunomagnetic selection as described previously (10, 11).

Thymic and Nonthymic Stromal Cells. Stromal cell suspensions for incorporation into reaggregate cultures were prepared from thymic and other fetal rudiments as previously described in detail (10, 11). Briefly, dispersed suspensions of whole thymic stromal cells were prepared by trypsinization of 2-dGuo–treated fetal thymus lobes. Purified MHC class II+ thymic cortical epithelial cells were

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then selected from these suspensions by immunomagnetic depletion of residual CD45$^+$ hemopoietic elements and A2B5$^+$ medullary epithelium, followed by positive selection using anti-MHC class II coated beads with subsequent bead removal by a brief exposure to ice-cold pronase.

Fetal mesenchyme enriched suspensions were prepared from whole thymic stromal suspensions by immunomagnetic depletion of residual CD45$^+$ cells, and then by depletion of MHC class II$^+$ epithelial cells.

MHC class II$^+$ thymic dendritic cells were prepared from organ-cultured thymic lobes, using a modification of the technique described by Inaba et al. (12) for peripheral mouse blood. Briefly, cell suspensions obtained from 7-d-old organ cultures were cultured for 7–14 d in the presence of 40 U/ml GM-CSF (Genzyme Corp., Cambridge, MA). Colonies forming in these cultures were enriched in cells with dendritic morphology, strong MHC class I and II expression, B7 expression, and potent stimulatory function in proliferation assays (13).

Cell suspensions enriched for MHC class II$^+$ gut or salivary gland epithelial cells were prepared by disaggregating explants of fetal mid-gut or fetal salivary gland previously organ cultured for 2–3 d in 100 U/ml IFN-γ (Genzyme Corp.). The presence of MHC class II$^+$ epithelial cells in these preparations was verified by immunolabeling (data not shown).

ECDI-treated Thymic Stromal Cells. Freshly prepared CD45-depleted thymic stromal preparations were allowed to recover from trypsinization for 60 min at 4°C, pelleted by centrifugation, and fixed by resuspension in 440 μl of 75 mM ECDI (Calbiochem-Novabiochem (UK) Ltd., Nottingham, UK) in 0.8% normal saline (14). Incubation was for 60 min at 4°C, and cells were resuspended every 15 min to prevent agglutination. Treated cells were then washed three times in serum-free culture medium, and then incorporated into reaggregate cultures as described below.

Preparation of Reaggregate Organ Cultures

Reaggregate organ cultures were prepared by mixing together suspensions of the desired combination of lymphoid and stromal cells. For cultures involving freshly prepared thymic and nonthymic stromal cells, purified CD4$^+$CD8$^+$TCR$^-$ thymocytes and the appropriate stromal cell suspension were mixed at a ratio of 1:1. Mixed suspensions were pelleted by centrifugation and, after removal of the supernatant, the cell pellet was vortexed and placed as a standing drop on the surface of a nucleopore filter in organ culture, as described in detail elsewhere (10, 11). Intact rudiments reform from these standing drops of cell suspensions within a few hours, providing an optimal three-dimensional environment for lymphocyte–stromal cell interactions where stromal cells retain their in vivo phenotype (10).

For cultures involving ECDI-fixed thymic stromal cells, treated stromal preparations were mixed at a ratio of 2:2:1 with purified CD4$^+$CD8$^+$TCR$^-$ thymocytes and freshly trypsinized lung mesenchyme cells and reaggregated as described above. The addition of fetal mesenchyme preparations to cultures containing ECDI-fixed cells allowed incorporation of cells into a coherent reaggregate structure. For comparability, cultures with unfixed stroma also received fetal mesenchyme cells. It is important to note that reaggregates made with fetal lung mesenchyme and CD4$^+$CD8$^+$TCR$^-$ thymocytes alone yielded no viable thymocytes after 4 d culture (data not shown).

Flow Cytometry

Freshly purified CD4$^+$CD8$^+$TCR$^-$ thymocytes and thymocytes harvested from reaggregate organ cultures by mechanical disruption were pelleted by centrifugation and incubated sequentially in 100 μl of anti-$\alpha\beta$TCR (H57.597 tissue culture supernatant), anti-hamster F(ab')$_2$ biotin and a mixture of CD4-PE, CD8-FITC, and streptavidin-APC. Stained cells were fixed in 1% paraformaldehyde and analysis was performed on an Elite Dual Laser machine (Coulter Electronics Inc., Hialeah, FL) with forward and side scatter gates set to exclude nonviable cells. Gates defining TCR$^-$, TCR^{lo}, and TCR^{hi} populations were set by comparison with adult thymocyte suspensions stained in parallel.

Results and Discussion

Thymic Epithelial Cells Provide Unique Signals for Positive Selection of CD4$^+$CD8$^+$ Thymocytes In Vitro. Newly generated CD4$^+$CD8$^+$ thymocytes have an average life span of 3–4 d, after which they die unless rescued for further maturation into single positive CD4$^+$ or CD8$^+$ cells by positive selection (3, 15). Thus, to examine the ability of different stromal cells to mediate positive selection, each cell type was reaggregated individually with purified CD4$^+$CD8$^+$ thymocytes and the cultures were analyzed for cellular content and T cell differentiation after 4 d. As shown in Fig. 1 *a*, viable cells (~30% of the input number) were recovered from reaggregates with purified MHC class II$^+$ thymic epithelium. In contrast to freshly purified CD4$^+$CD8$^+$TCR$^-$ thymocytes used for the reaggregate input (Fig. 2, *a* and *b*), these cells were predominantly single positive CD4$^+$ or CD8$^+$ cell expressing high levels of $\alpha\beta$TCR (Fig. 1 *b*), indicating that positive selection had taken place. Moreover, cells recovered from these cultures were able to proliferate in response to stimulation by superantigen, indicating that they had acquired functional as well as phenotypic maturity (13, and data not shown).

In contrast to reaggregates with thymic epithelial cells, very few viable cells were recovered from cultures with other stromal cell types (Fig. 1 *a*), even though intact reaggregate structures, providing optimal conditions for interaction, formed in all cases. Thus, both fetal mesenchyme and thymus-derived dendritic cells failed to support maturation, even though the latter express both MHC class I and II molecules (13), providing the potential for selection of both CD8$^+$ and CD4$^+$ cells, respectively. Similarly, other types of fetal epithelium (gut and salivary gland), even when induced to express MHC class II molecules by prior culture in IFN-γ, also failed to support thymocyte survival and maturation, indicating that positive selection had not taken place.

It is important to note that the lack of positive selection by cells other than thymic epithelium was not due to the inability of CD4$^+$CD8$^+$TCR$^-$ cells to reach the CD4$^+$CD8$^+$TCR^{lo} stage (when selection can operate) in the absence of thymic support. Indeed, the majority of CD4$^+$CD8$^+$TCR$^-$ cells (>95%) survived for the first 18 h of culture and acquired low levels of $\alpha\beta$TCR expression, either when cultured in the complete absence of stromal cells (Fig. 2, *c* and *d*), or in the presence of IFN-γ–treated nonthymic stromal cells (Fig. 2, *e* and *f*). This latter observation also excludes the possibility that IFN-γ–treated stromal cells failed to sup-

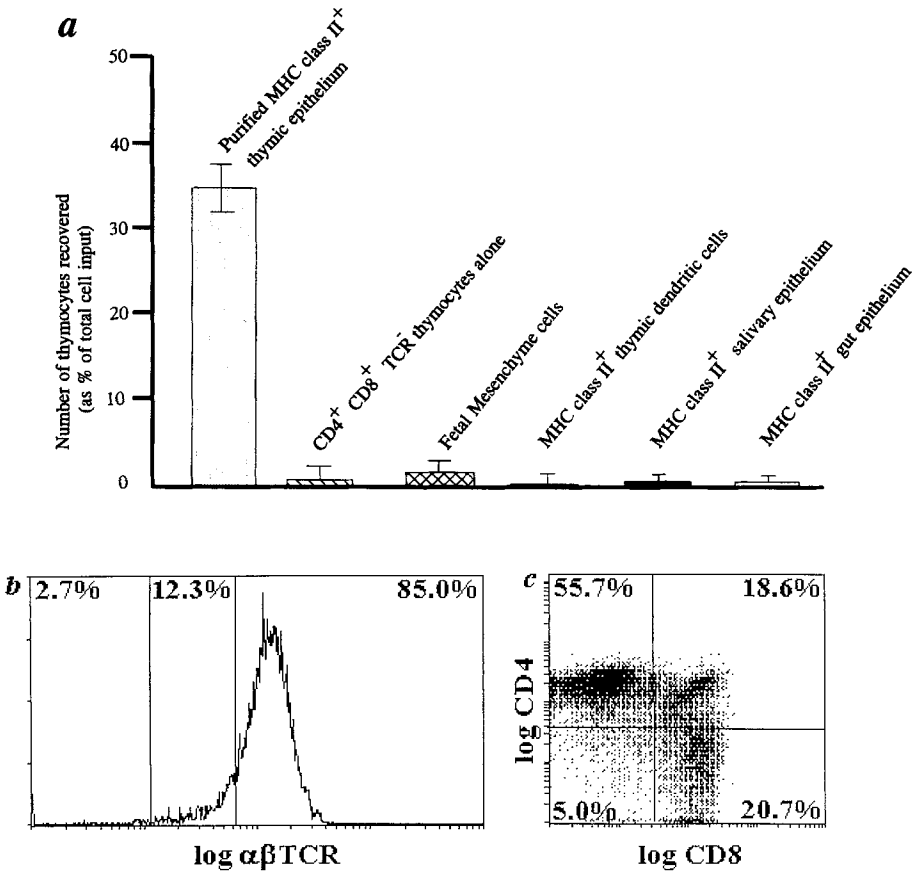

Figure 1. Comparison of the abilities of various thymic and nonthymic stromal cells to support positive selection of $CD4^+$ $CD8^+$ thymocytes in vitro. Reaggregate lobes, consisting of purified $CD4^+CD8^+$ TCR^- thymocytes and individual stromal cell types, were harvested after 4 d in culture and analyzed by microscopy for total thymocyte content (*a*). (*b* and *c*) Flow cytometric analysis of thymocytes harvested from reaggregate cultures formed from purified MHC class II^+ thymic epithelium. Note that TCR^{hi}, $CD4^+$, and $CD8^+$ cells have been generated from the $TCR^-CD4^+CD8^+$ input. After 4 d, insufficient viable thymocytes for analysis were recovered from reaggregates formed from other stromal cells.

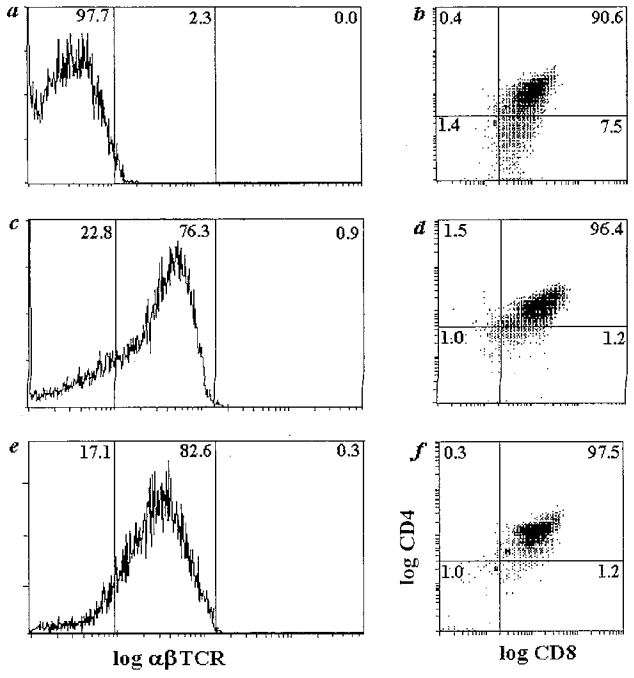

Figure 2. Purified $CD4^+CD8^+TCR^-$ thymocytes progress to a $\alpha\beta TCR^{lo}$ stage in short-term culture independent of stromal cell support. Immunomagnetically selected $CD4^+CD8^+TCR^-$ thymocytes were analyzed by flow cytometry for their expression of CD4, CD8, and αβTCR either immediately after isolation (*a* and *b*), or after culture for 18 h, either in the complete absence of stromal cell support (*c* and *d*), or in the presence of IFN-γ–treated salivary epithelium (*e* and *f*). Gates defining the levels of αβTCR expression were set by comparison with adult thymocytes stained in parallel. Although no viable cells could be found in these cultures after 4 d, consistent with the finite life span of $CD4^+CD8^+$ cells in the absence of positive selection, at 18 h the majority of cells (>95%) were viable, still $CD4^+CD8^+$ (*c* and *e*), and had progressed to a stage where most of the cells expressed low levels of αβTCR (*d* and *f*). Data shown are representative of three separate experiments.

port positive selection because they are toxic to $CD4^+CD8^+$ thymocytes. Moreover, in reaggregates formed from a mixture of stromal cells containing thymic epithelium and IFN-γ–treated salivary epithelium, positive selection was still observed (data not shown), providing further evidence that the latter are not inhibitory to thymocyte maturation.

In contrast to our findings, a recent study (16) suggests that stromal cells in fetal gut explants, when transplanted into thymectomised adult mice, are able to support the development of single positive $CD4^+$ and $CD8^+$ αβTCR-bearing cells. It is important to note, however, that it is not clear whether development in this model involves transition through an intermediate $CD4^+CD8^+$ stage, as examined in the present

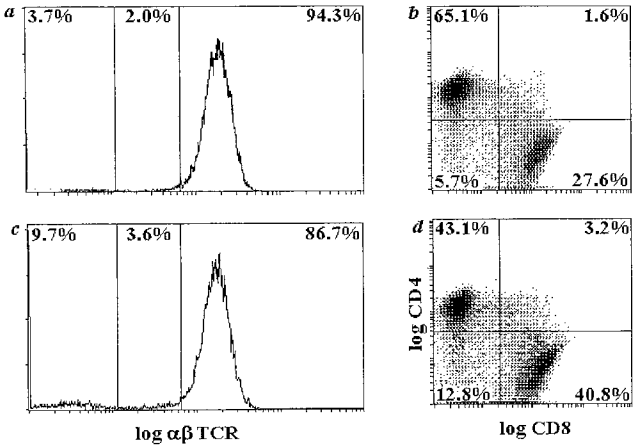

Figure 3. ECDI-treated thymic stromal cells can support positive selection in vitro. Thymocytes harvested from reaggregate cultures of either untreated (*a* and *b*) or ECDI-treated thymic stromal cells (*c* and *d*), together with mesenchyme cells to allow formation of a coherent structure, were harvested after 6 d and analyzed for evidence of thymocyte maturation. Recovery in cultures containing ECDI-treated cells was, on average 12–20% of the input as compared with 30% with untreated epithelium, suggesting that ECDI-treated cells are less efficient at mediating positive selection, possibly as a result of their smaller surface area for interaction. Note that in both cases, single positive $CD4^+$ and $CD8^+$ cells with upregulated levels of TCR have been generated from the input of $CD4^+CD8^+TCR^-$ thymocytes. Three separate experiments were performed with similar results.

study. Thus, our data do not rule out a role for gut epithelial cells in the maturation of T cells via other developmental pathways. In conclusion, the most likely explantation of our findings is that only thymic epithelial cells can supply all the signals required for positive selection and the consequent maturation of single positive cells from their $CD4^+CD8^+$ precursors.

Positive Selection of Thymocytes Involves Interactions with Surface Membrane Molecules on the Thymic Epithelium. In addition to TCR ligation, signals for positive selection provided by thymic epithelial cells might involve either interaction with cell surface ligands or the production of soluble factors. To investigate these possibilities, we tested the ability of thymic stromal cells to support positive selection after fixation using the cross-linking agent ECDI. Studies on ECDI-fixed APCs from spleen have shown that these cells are metabolically inactive but retain their surface expression of peptide–MHC complexes in a form able to interact with the TCR (14, 17). ECDI-treated thymic epithelial cells also showed no evidence of metabolic activity as determined by MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliam bromide) and FDA (fluorescein diacetate) assays (data not shown) and remained intact but unattached after 4 d in monolayer cultures, providing further evidence for their inactive state. When incorporated into reaggregate cultures, however, ECDI-treated epithelial cells were able to support the generation of single positive $CD4^+$ and $CD8^+$ cells from their $CD4^+CD8^+$ precursors, demonstrating their ability to support positive selection (Fig. 3, *c* and *d*). Thus, it is likely that the additional signals that thymic epithelial cells provide for positive selection of thymocytes involve ligands on the cell surface rather than soluble products.

Overall, our results show that thymic epithelial cells are unique in their ability to provide positive selection signals for the maturation of $CD4^+CD8^+$ thymocytes into $CD4^+$ or $CD8^+$ T cells. Since thymic epithelial cell–specific peptide–MHC complexes do not appear to be required for positive selection (8, 9), this suggests that as well as TCR ligation, thymic epithelial cells provide additional signals necessary for positive selection. Such a requirement for dual signaling in positive selection parallels that seen in the activation of mature T cells, where both TCR ligation and costimulatory signals are required (17). However, despite some similarities between positive selection and activation (18), the additional signals involved are unlikely to be the same, since MHC class II^+ thymic epithelial cells do not express the costimulatory ligand B7 and are unable to deliver functional costimulatory signals for the activation of mature T cells (13).

Finally, our results are in agreement with a recent study (19) where introduction of MHC class II–bearing, bone marrow–derived cells into the thymus of MHC class II–deficient mice failed to allow the positive selection of class II–restricted $CD4^+$ cells. However, they differ from other studies (8, 9) where MHC class I–bearing cells introduced into the thymus of class I–deficient mice by intrathymic injection or the construction of bone marrow chimeras did allow positive selection of class I–restricted $CD8^+$ cells. Whereas this may reflect differences in the selection signals required for $CD4^+$ and $CD8^+$ cells, we did not observe selection of either cell type by individual stromal elements other than thymic epithelium. This suggests that the formation of both T cell types requires interaction with thymic epithelial cells. Thus, positive selection by MHC–peptide complexes on cells other than the thymic epithelium may only be possible when thymic epithelial cells are also present to provide the additional accessory signals required for maturation. Our current studies are aimed at defining the surface molecules on thymic epithelial cells that provide these accessory signals.

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Address correspondence to Dr. G. Anderson, Department of Anatomy, The University of Birmingham Medical School, Vincent Drive, Edgbaston, Birmingham B15 2TT, UK.

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