# Studies on T Cell Maturation on Defined Thymic Stromal Cell Populations In Vitro

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## Summary

We describe an in vitro system in which positive selection of developing T cells takes place on defined stromal cell preparations, which include major histocompatibility complex class $II^+$ epithelial cells but exclude cells of bone marrow origin. In this system, maturation of double-positive T cell receptor negative ($TCR^-$), $CD4^+8^+$ thymocytes into single-positive $TCR^+$, $CD4^+$ and $CD8^+$ cells takes place together with the development of functional competence. As in vivo, this maturation is associated with the upregulation of TCR levels as cells progress from double-positive to single-positive status. We also show that class $II^+$ epithelial cells in these cultures are less efficient than dendritic cells in mediating the deletion (negative selection) of $V\beta8^+$ cells by the superantigen staphylococcal enterotoxin B. For the first time, this approach provides a model in which the cellular interactions involved in both positive and negative selection can be studied under controlled in vitro conditions.

On entering the thymus from the blood stream, lymphoid stem cells are induced to undergo a program of proliferation, gene rearrangement, and differentiation resulting in the production of large numbers of immature double-positive $CD4^+8^+$ thymocytes expressing a diverse repertoire of TCR specificities. This repertoire is subject to stringent selection so that only those cells recognizing self-MHC molecules are signaled to mature into single-positive $CD4^+$ or $CD8^+$ T cells (positive selection), and autoreactive cells recognizing self-antigen/MHC complexes are induced to die (negative selection) (reviewed in references 1–4).

Interactions between thymocytes and stromal cells are known to be important in both positive and negative selection (1–5). However, the thymus is made up of a number of different stromal cell types, and this complexity has hindered attempts to analyze the precise role of individual stromal components in the selection of the TCR repertoire.

To overcome these difficulties, we have sought to reconstruct the thymic microenvironment so that interactions between defined stromal cell types and thymocytes can be investigated. We have devised an in vitro system in which positive selection of thymocytes can be obtained in the presence of purified thymic epithelial cells. This system, for the first time, opens the way to an analysis of positive selection under controlled conditions in vitro. In addition, we show that it is possible to use a similar approach to investigate the efficiency of various stromal cell types in inducing negative selection of the TCR repertoire.

## Materials and Methods

### *Embryonic Material*

Embryonic material was obtained from timed matings of BALB/c ($H\text{-}2^d$) mice. Fetal thymus lobes isolated at day 14 of gestation were organ cultured for 6 d in the presence of 1.35 mM dGuo to deplete lymphoid and dendritic cells as described previously (6). These lobes were used as a source of stromal cells for the preparation of reaggregate cultures.

### *Antibodies*

For immunomagnetic selection the following antibodies were bound onto either anti-rat Ig– or anti-mouse Ig–coated Dynal beads (Dynal, Wirrall, UK) as appropriate: anti-CD45 (clone M1-9; American Type Culture Collection, Rockville, MD), anti-$H\text{-}2K^d$ (clone MmH-2K-3PI; Pharmingen, AMS Ltd., Witney, UK), anti-CD4 (clone YTS 191.1.3; Sera-Lab., Crawley Down, UK), anti-CD8 (YTS 169.4; Sera-Lab.), and anti-CD3 (clone C363.29B; a kind gift from S. Carding, Yale University School of Medicine, New Haven, CT). Purified antibodies used for immunolabeling were: anti-$Ia^d$ (MK-D6; Becton Dickinson & Co., Oxford, UK), anti-IE (Clone AMS-16.2; Pharmingen, AMS Ltd.), anti-mouse dendritic cell (MIDC-8; Serotech Limited, Kidlington, UK), anti-CD4 PE (GK-1.5; Becton Dickinson & Co.), anti-CD8 FITC (clone 53-6.7; Becton Dickinson & Co.), and anti-mouse macrophage (clones 5C6 and F4/80 used as a mixture; a gift from S. Gordon, Sir William Dunn School of Pathology, Oxford University). Antibody TR-7, recognizing thymic fibroblasts (a gift from W. van Ewijk, Erasmus University, Rotterdam), anti-GQ ganglioside, a marker for medullary epithelial cells (7) (clone A2B5; a gift from


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M. Raff, University College, London), anti-V$\beta$8 (clone F.23.1), and pan anti-TCR $\alpha/\beta$ (H.57–597; a gift from R. Kubo, National Jewish Centre for Immunology and Respiratory Medicine, Denver, CO) were used as tissue culture supernatants. Second-step reagents were anti-mouse Ig biotin (Becton Dickinson & Co.) anti-hamster Ig biotin cross-absorbed against rat and mouse (Caltag, Bradshaw Biologicals, Market Harborough, UK), anti-rat Ig alkaline phosphatase (AP) (Sigma Chemical Co., Poole, UK), and anti-mouse IgM FITC (Vector, Peterborough, UK), and streptavidin-APC (Becton Dickinson & Co.).

### *Purification of Thymocyte Subpopulations*

Thymocyte suspensions were prepared from either 17-d fetal or newborn mice by teasing apart isolated lobes with fine knives. Purified subpopulations of thymocytes were prepared as follows.

*Purified CD4$^+$8$^+$ Thymocytes.* The initial strategy was based on evidence that most double-positive cells lack H-2K$^d$ expression, whereas a proportion of double-negative cells and single-positive cells express H-2K$^d$ (8). Hence, enrichment of CD4$^+$8$^+$ thymocytes was obtained by removing H-2K$^d$-positive cells with anti-H-2K$^d$-coated beads (Dynal). Further removal of any residual double-negative cells was obtained by rosetting the enriched CD4$^+$8$^+$ population on either anti-CD4- or anti-CD8-coated beads (Dynal) and recovering the rosetted cells from the beads using Detachabead (Dynal).

An initial population of 8 × 10$^6$ cells was subjected to three rounds of depletion with 100 $\mu$l per round of anti-H-2K$^d$-coated magnetic beads. Beads and cells were mixed in a 300-$\mu$l volume in round-bottomed freezing vials (Sterilin, Paisley, Scotland) and given a 10-min spin at 200 *g* in the first round, two 10-min spins in the second round, and three 10-min spins in the third round with resuspension of the pellet between each spin. Rosetted cells were removed on a magnet between each round. Cells remaining after depletion of H-2K$^d$-positive cells were spun together with anti-CD4- or anti-CD8-coated beads at a ratio of 3:1, and the rosetted cells were collected on a magnet and washed four times by magnetic separation to remove any unbound cells. Beads were removed from the rosetted cells using Detacha bead (Dynal). Cells were separated from the released beads using a magnet, washed, and counted.

*Purified TCR$^-$, CD4$^+$8$^+$ Thymocytes.* The strategy here was to first remove TCR$^+$ cells using three rounds of depletion by anti-CD3-coated Dynal beads. In this way, double-positive, single-positive, and double-negative TCR$^+$ thymocytes are removed. Rosetting on anti-CD8-coated beads was then used to produce a population of predominantly CD4$^+$8$^+$ cells (only a few TCR$^-$ cells express CD8 only). The protocols used were as above.

*Thymocytes Depleted of Ia$^+$ Dendritic Cells.* In some experiments, whole thymocyte populations were used but they were first depleted of dendritic cells using anti-Ia$^d$-coated beads (Dynal). This strategy would also remove any B cells that might be present in newborn thymocyte preparations.

### *Preparation of Dendritic Cells*

Dendritic cells were prepared from spleen cell suspensions as described previously (9). Briefly, spleen cell suspensions were treated with 0.84% $NH_4Cl$ to remove red cells and allowed to adhere to tissue culture quality petri dishes for 2 h. After vigorous washing to remove nonadherent cells, the plates were incubated overnight and detached cells collected in the supernatant. These were depleted of any contaminating T cells using anti-Thy-1-coated beads (Dynal), counted, and used as a source of enriched dendritic cells.

### *Preparation of Reaggregate Cultures*

This was carried out using the protocol illustrated in Fig. 1. To obtain thymic stromal cells depleted of lymphoid elements, dGuo-treated lobes were harvested, washed four times in $Ca^{2+}$ $Mg^{2+}$-free PBS, and incubated in 600 $\mu$l of 0.25% trypsin, 0.02% EDTA in $Ca^{2+}$ $Mg^{2+}$-free PBS for 30–40 min at 37°C. Trypsinisation was stopped by the addition of 400 $\mu$l of Hepes-buffered medium containing 10% FCS, and the suspension was vigorously pipetted. Debris was allowed to settle, and the dispersed stromal cells were removed in the supernatant, spun down, and resuspended in 200 $\mu$l. Depletion of any residual haemopoietic cells in this suspension was carried out by two rounds of interaction with 100 $\mu$l per round of anti-CD45-coated magnetic beads as described above.

To form reaggregates, selected thymocyte populations were mixed with dispersed stromal cells at a ratio of ~3:1 in 1.5-ml Eppendorf tubes and spun into a pellet. When required, dendritic cells at a final ratio of one cell per 30–50 thymocytes were also incorporated before pelleting. After removal of the supernatant, the cell pellet was dispersed into a slurry by careful mixing on a vibrating mixer and then drawn into a fine, mouth-controlled glass capillary pipette. The slurry was then expelled as a discrete standing drop on the surface of a nucleopore filter resting on gelatine foam sponge as used for conventional organ culture as described previously (6). Such standing drops reformed intact thymus lobes within 12 h. Where required, staphylococcal enterotoxin B (SEB)[1] (Sigma Chemical Co.) was added to cultures at a final concentration of 10 $\mu$g/ml at the time they were established. Reaggregated lobes were harvested after either 18 or 96 h, by gently teasing apart with fine knives to release the thymocytes into suspension for cell surface analysis or functional studies.

### *Flow Cytometry*

Three-color labeling of cells recovered from the reaggregate cultures was carried out by sequential incubation in anti-$\alpha/\beta$ TCR, anti-hamster Ig biotin, and a mixture of CD4-PE CD8-FITC and streptavidin-APC. Labeling of stromal cell suspensions was carried out using A2B5 followed by an anti-mouse IgM-FITC or anti-Ia$^d$ biotin followed by streptavidin APC. All analyses were performed on an Elite Dual Laser machine with light scatter gates set to exclude nonviable cells (Coulter Electronics Inc., Hialeah, FL).

### *Immunohistology*

Frozen sections, cut at 5–7 $\mu$m, were labeled with various stromal cell markers, followed by AP-conjugated second-step reagents, and developed using a Fast-Red TR salt substrate (Sigma Chemical Co.). Sections were viewed either in bright field or under fluorescence conditions to exploit the increased sensitivity afforded by the fluorescent properties of the Fast-Red reaction product (10).

### *Detection of SEB Binding to Stromal Cells*

SEB was biotinylated using long spacer arm water-soluble biotin (Vector Laboratories) according to the manufacturer's instructions. Suspensions of stromal cells were incubated in biotinylated SEB at a concentration of 0.5 mg/ml for 2 h at room temperature, washed, cytospun, and fixed for 10 min in 0.1% paraformaldehyde in PBS. Washed slides were incubated with streptavidin-AP and developed with Fast-Red substrate as above.

[1] *Abbreviation used in this paper:* SEB, staphylococcal enterotoxin B.

*Stimulation Assays*

The functional status of cells recovered from reaggregate cultures was examined by measuring their proliferative response to the superantigen SEB (11). Cells harvested from lobes were set up in triplicate wells in round-bottomed 96-well plates at $3 \times 10^3$ cells per well in the presence of $10^5$ mitomycin C (Sigma Chemical Co.)-treated spleen cells as a source of APC. SEB was added to some wells at a final concentration of 10 $\mu$g/ml. Control wells received no SEB. Mitomycin C-treated spleen cells without responders were also exposed to SEB to establish background levels of response in stimulator populations. Cultures were harvested for counting after 96 h. They were pulsed with [$^3$H]thymidine at a final concentration of 1 $\mu$Ci/ml (Amersham International, Amersham, UK) for the final 18 h of culture.

## Results

*Characterization of Thymic Stromal Cells Used in Reaggregate Cultures.* Alymphoid thymus lobes produced by treatment of embryonic lobes with dGuo were used as a source of stromal cells, as we have previously shown that they possess all the elements required to support T cell development in vitro (6, 12). These lobes contain both MHC class II$^+$ cortical epithelial cells and medullary epithelial cells (7, 13; and Fig. 2, *a* and *b*) but are poorly characterised in terms of their nonepithelial components. To obtain a more complete definition of the stromal cell input into our reaggregate cultures, we therefore examined dGuo lobes using a panel of markers identifying nonepithelial thymic stromal cell types.

As shown in Fig. 2, in addition to epithelial cells, dGuo-treated lobes contain numerous 5C6$^+$ and F4/80$^+$ macrophages (14) (Fig. 2 *c*), together with fibroblast-like cells (TR-7$^+$; reference 13) forming the capsule and septae (Fig. 2 *d*). However, dendritic cells were not found, even though they were readily detectable as MIDC-8$^+$ cells (15) in age-matched lobes cultured in the absence of dGuo (Fig. 2 *e*). Previously, the absence of dendritic cells in dGuo-treated lobes has only been inferred from the inability of these lobes to "prime" an allograft response when grafted into normal adult mice (16).

Thus, after immunomagnetic removal of CD45$^+$ macrophages, stromal cell suspensions prepared from dGuo-treated lobes contain MHC class II$^+$ cortical epithelial cells, medullary epithelial cells, and fibroblasts, but not dendritic cells. By flow cytometry, ~40% of cells in freshly prepared suspensions expressed MHC class II (Fig. 3), whereas ~5% of cells expressed the medullary epithelial marker A2B5 (data not shown). The remaining class II$^-$ cells may include some epithelial cells that have lost class II during trypsinisation in addition to the class II$^-$ fibroblasts derived from the capsule and septae. Such mixtures of epithelial cells and fibroblasts retain the ability to reform intact thymic lobes when reassociated with thymocytes, providing an optimal microenvironment for thymocyte/stromal cell interactions.

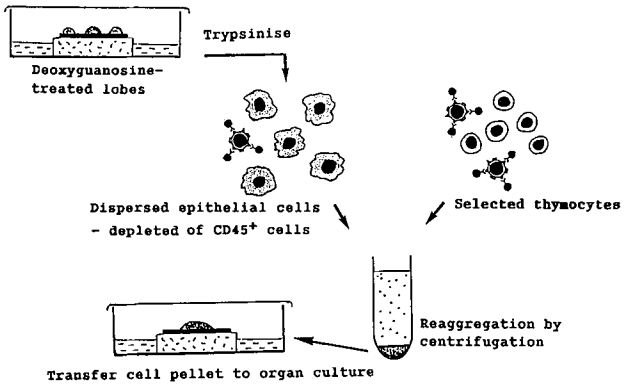


**Figure 1.** Protocol for the preparation of reaggregate thymus cultures from selected stromal and lymphoid components.

*Reaggregate Cultures Support the Maturation of Thymocytes Enriched for CD4$^+$8$^+$ Cells into Populations Enriched for Single-Positive CD4$^+$ or CD8$^+$ Cells and that Proliferate in Response to SEB.* These experiments were designed to investigate whether newborn thymocytes, enriched for CD4$^+$CD8$^+$ cells by the strategy outlined in Materials and Methods, would mature in reaggregate cultures of epithelial cells and fibroblasts. Newborn thymus contains ~88% CD4$^+$CD8$^+$ cells (Fig. 4, *left*). After removal of H-2K$^{d+}$ cells, the remaining population consists of 92.5% CD4$^+$8$^+$, 6.7% CD4$^+$, 0.3% CD8$^+$, and 0.5% CD4$^-$8$^-$ cells (average of two experiments). Thus, further enrichment by isolation on anti-CD8-coated beads (Dynal) results in a population consisting of 99.7% CD4$^+$8$^+$ and 0.3% CD8$^+$ cells, whereas enrichment on CD4 gives a population consisting of 93.3% CD4$^+$8$^+$ and 6.7% CD4$^+$ cells.

When such enriched populations were used as the input into reaggregate cultures, cells isolated on CD8 were found to give rise to output populations containing single-positive CD4$^+$ cells (54% in the experiment illustrated in Fig. 4), which is representative of three similar experiments. Similarly, an input isolated on CD4 was found to give rise to an output containing 18.6% single-positive CD8$^+$ cells (plus 10.7% CD4$^+$8$^+$, 58.5% CD4$^+$, and 12.2% CD4$^-$8$^-$ cells). Thus, either type of single-positive cell can develop from an input expressing the other marker (and consisting predominantly of double-positive cells), demonstrating phenotypic maturation in reaggregate cultures. Cell recoveries from these cultures after 4 d ranged from 30 to 60% of the input number.

Mature V$\beta$8$^+$ T cells are known to respond to SEB by proliferation (11), and a proportion of cells in newborn thymus respond in this way (Fig. 5 *C*). However, after enrichment for CD4$^+$8$^+$ cells, the input population does not respond (Fig. 5 *D*). In contrast, the output population derived from such cells gives a good proliferative response (Fig. 5, *A* and *B*), indicating that functional as well as phenotypic maturation takes place in reaggregate cultures.

*Reaggregate Cultures Support Positive Selection of TCR$^-$ Thymocytes into TCR$^+$ CD4$^+$ and CD8$^+$ Single-Positive Cells.* The experiments described above show that reaggregate cultures support the generation of single-positive cells and the maturation of functional competence, developments considered to be associated with positive selection (3). However,

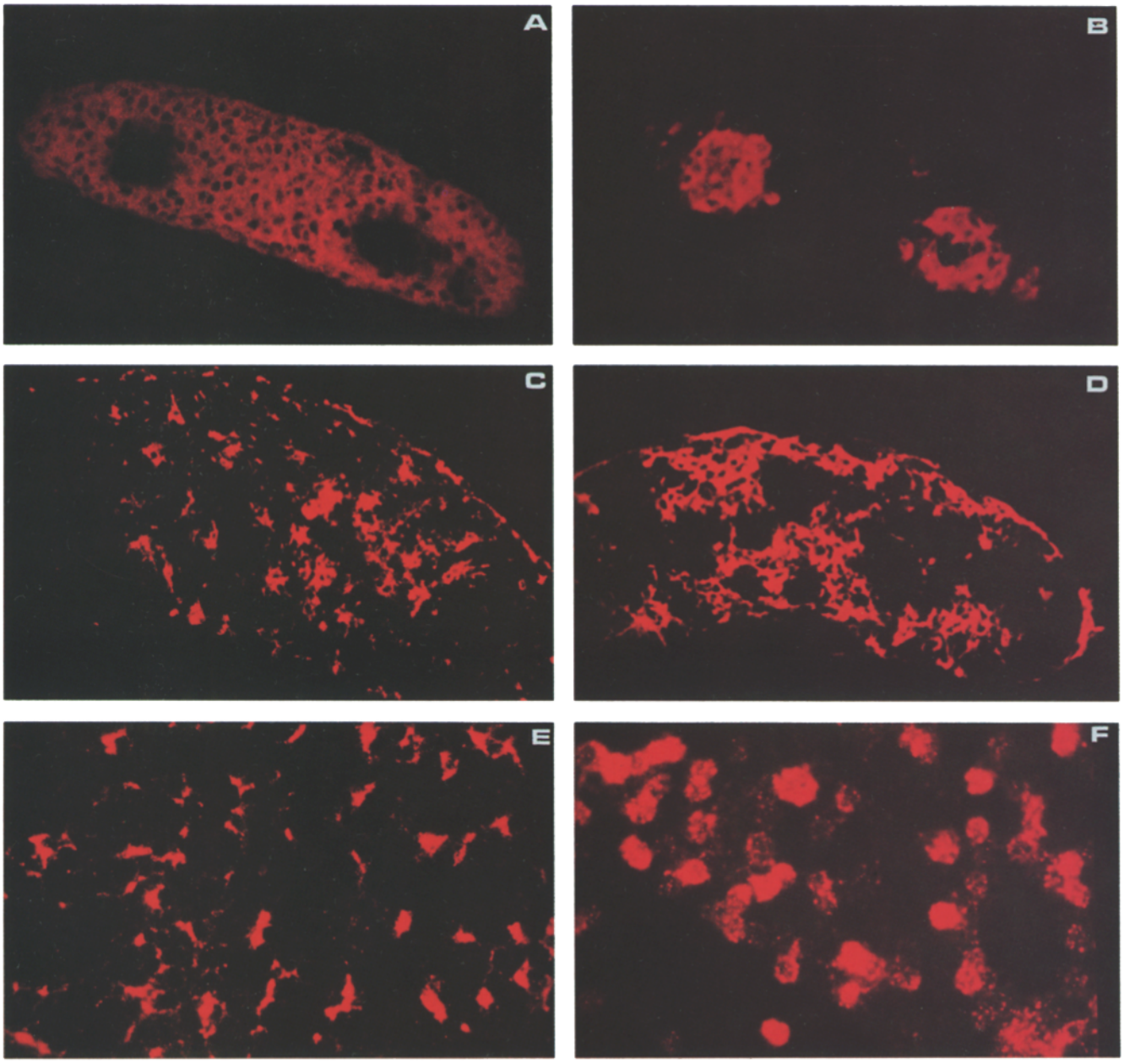


**Figure 2.** Identification of thymic stromal cells expressing various surface markers and able to bind SEB. (*a–d*) Frozen section of dGuo-treated lobes (*a*) labeled with anti-MHC class II. (*b*) Serial section labeled for the medullary marker A2B5. Note that the medullary and class II labeling are largely nonoverlapping. (*c*) Labeled with macrophage markers. (*d*) Labeled with antibody TR-7 recognizing fibroblasts in the capsule and septae. (*e*) Section of normal 6-d cultured thymus lobe labeled with the dendritic cell marker MiDC-8. Labeled cells are distributed throughout the lobe. Such cells are absent in dGuo-treated lobes. (*f*) Cytospin preparation of a macrophage-depleted stromal cell suspension prepared from dGua-treated thymus lobes and incubated with biotin-labeled SEB and streptavadin AP. Counts on this preparation showed that 46% of the cells had bound SEB. This was reduced to <5% when class $II^+$ cells were removed before addition of SEB.

$TCR^+$ cells in the newborn thymocyte input might have received signals for positive selection before removal from the thymus, and they are revealed on further maturation in reaggregate cultures. To exclude this possibility and to show that positive selection of thymocytes can be obtained in vitro, we designed experiments so that the input population was $TCR^-$ as well as predominantly $CD4^+8^+$. Removal of $TCR^+$ cells from newborn or 17-d fetal thymocytes was achieved using anti-CD3-coated beads (Dynal). Cells were then selected on anti-CD8-coated beads (Dynal) producing input populations that were >97% $CD4^+8^+$ with the remaining cells being single-positive $CD8^+$. Five separate experiments were carried out with similar results.

As shown in Fig. 6, $TCR^-$ input cells (third panel) were

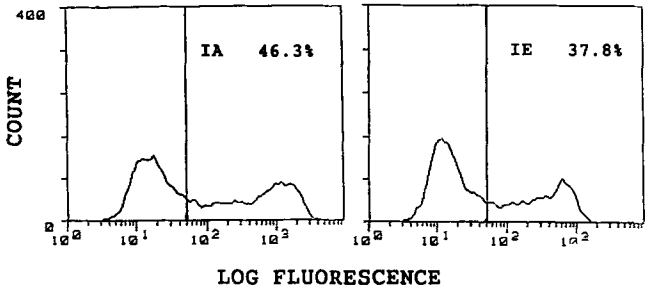


**Figure 3.** Expression of MHC class II antigens on stromal cell suspensions freshly prepared from dGuo-treated lobes and depleted of CD45$^+$ cells. Note that both IA and IE antigens are detectable after trypsinization.

able to generate TCR$^+$ cells in reaggregate cultures (fourth panel). Such cultures show an accumulation of cells with upregulated levels of TCR expression as compared with adult thymocytes where most TCR$^+$ cells show low levels of expression (second panel). Within the TCR$^+$ population, both double-positive and single-positive CD4$^+$ and CD8$^+$ cells are generated from the predominantly double-positive input originally isolated on CD8 (Fig. 7). In addition, three-color analysis shows that TCR upregulation is correlated with progression from double-positive to single-positive status (Fig. 8) in a manner comparable to that seen during thymocyte development in vivo (17). Thus, while the majority of double-positive cells express low TCR levels, some double-positive and all single-positive cells express high TCR levels comparable with those seen on some double-positive and all mature single-positive cells in the adult thymus (18).

*The Ability of Epithelial Cells in Reaggregate Cultures to Induce Negative Selection.* We have previously shown that the MHC class II–dependent superantigen SEB causes rapid depletion of immature V$\beta$8$^+$ cells by programmed cell death (apoptosis) when added to thymus organ cultures (19). These cultures contain two types of class II$^+$ cells: epithelial cells and dendritic cells (and possibly macrophages) derived from hemopoietic tissues. Either or both types of cell might be

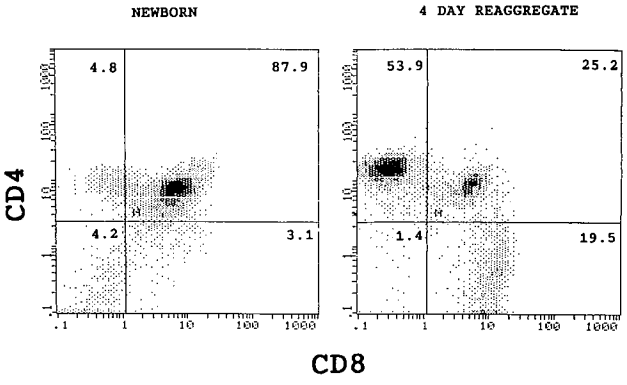


**Figure 4.** Comparison of CD4/CD8 phenotype of newborn thymocytes with that of cells recovered from a reaggregate culture where the thymocyte input was selected to be >99% CD4$^+$8$^+$. Note the accumulation of single-positive cells, especially CD4$^+$ cells, in the reaggregate culture.

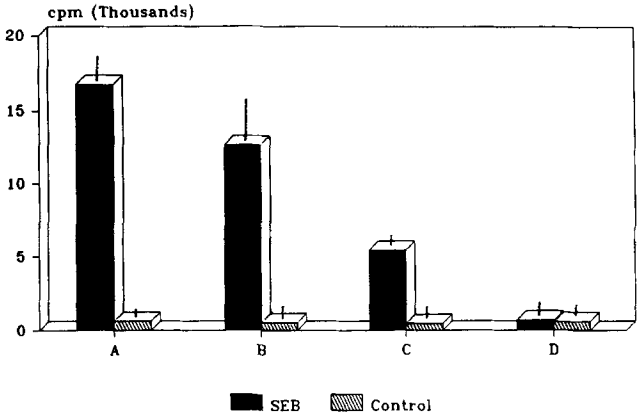


**Figure 5.** Response of various thymocyte populations to SEB in the presence of APC. (*a* and *b*) Cells recovered from 4-d reaggregate cultures. (*c*) Newborn thymocytes and (*d*) newborn thymocytes depleted of mature cells for use as an input into reaggregate.

involved in inducing negative selection. To test the efficiency of epithelial cells in this process, we added SEB to reaggregate cultures containing epithelial cells and newborn thymocytes (from which Ia$^+$ dendritic cells had been removed). Our results, summarized in Fig. 9, show that an 18-h exposure to SEB does not result in deletion of V$\beta$8$^+$ cells in these cultures, whereas substantial depletion does occur in cells from thymic organ cultures that have been trypsinized and reag-

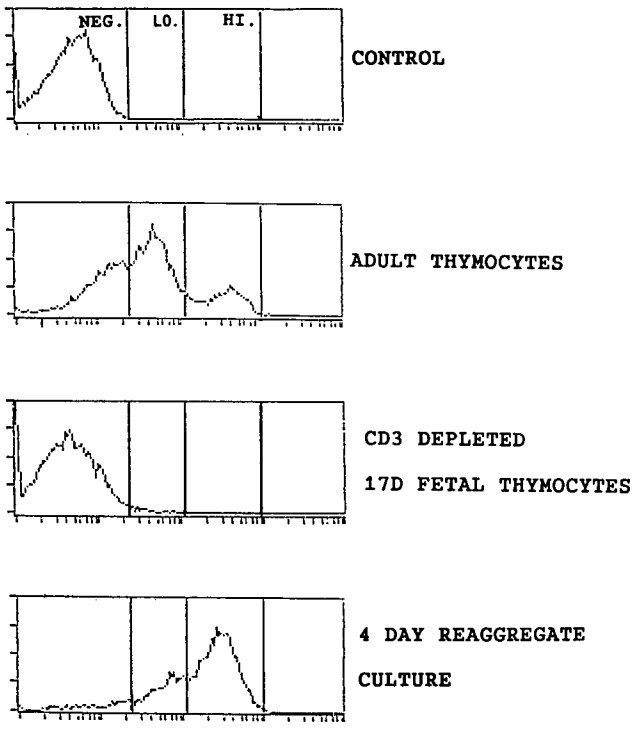


**Figure 6.** TCR$^-$ cells (*third panel*) give rise to TCR$^+$ cells (*fourth panel*) in 4-d reaggregate cultures. Note that reaggregate cultures (*fourth panel*) show an accumulation of cells with upregulated TCR levels as compared with adult thymocytes (*second panel*), where most TCR$^+$ cells have low levels of TCR expression.

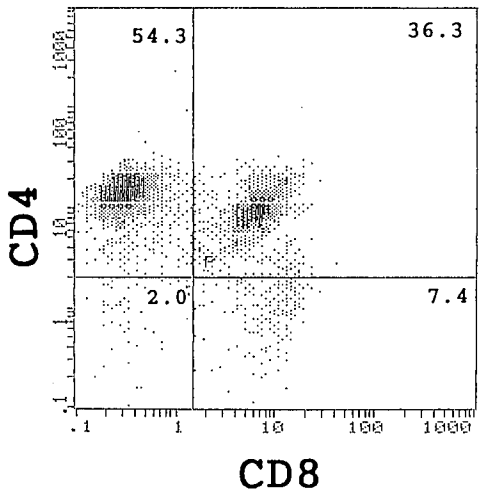


**Figure 7.** CD4/CD8 phenotype of TCR$^+$ cells recovered from a 4-d reaggregate culture. Both single-positive CD4$^+$ and CD8$^+$ TCR$^+$ cells have developed from an input consisting of >97% CD4$^+$8$^+$ TCR$^-$ cells originally isolated on CD8.

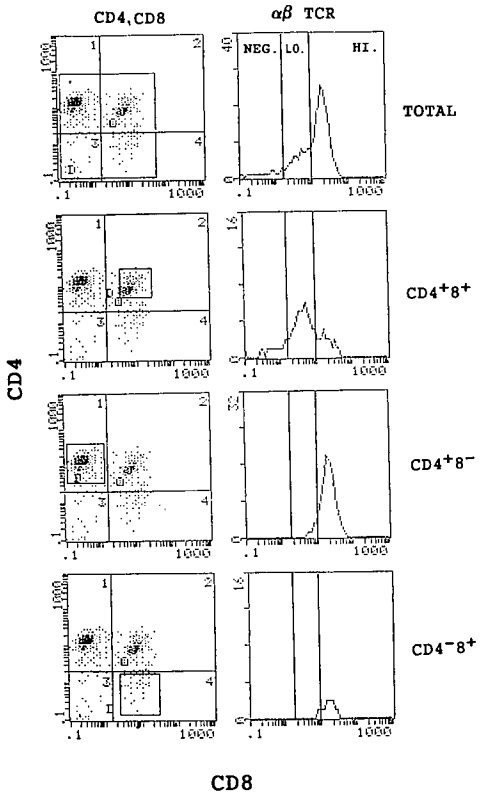


**Figure 8.** Correlation of TCR levels with CD4/CD8 phenotypes on cells generated from TCR$^-$ cells in reaggregate cultures. Three-color analysis was carried out followed by gating on individual double-positive and single-positive populations (*left*) to determine TCR levels (*right*). Note that higher levels of TCR are found on a cohort of double-positive cells and almost all single-positive cells.

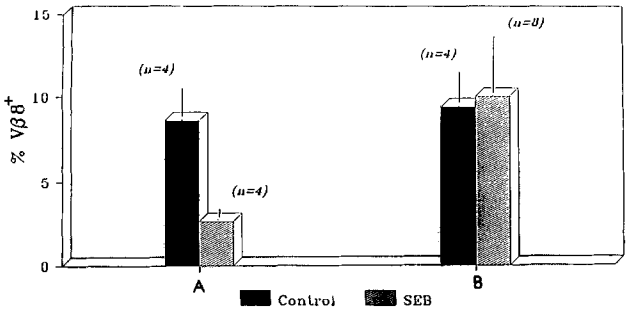


**Figure 9.** Effect of 18-h exposure to SEB on the number of V$\beta$8$^+$ cells in reaggregate cultures. (*A*) Whole thymus lobes disaggregated and allowed to reaggregate in the presence of SEB show a significant reduction ($p$ >0.001) in the number of V$\beta$8$^+$ cells as compared with controls. (*B*) Reaggregate cultures of epithelium and thymocytes without dendritic cells. Deletion of V$\beta$8$^+$ cells is not seen in the presence of SEB.

gregated in the same way. The latter cultures do contain dendritic cells (Fig. 2 *e*), suggesting that these cells play a crucial role in V$\beta$8$^+$ deletion. This suggestion receives additional support from the data in Fig. 10, which show that V$\beta$8$^+$ cells in newborn thymocyte populations are susceptible to depletion when small numbers of dendritic cells (10-fold fewer than epithelial cells) are incorporated into epithelial reaggregates. Thus, despite their greater abundance in reaggregates, epithelial cells appear to be less efficient than dendritic cells in mediating deletion of V$\beta$8$^+$ cells during an 18-h exposure to SEB. As shown in Fig. 2 *f*, this is not simply due to the inability of class II$^+$ epithelial to bind SEB, which can be directly visualized using biotinylated SEB preparations.

We have shown that continuous exposure of thymic organ cultures to SEB beginning before the development of TCR$^+$ cells results in a complete absence of V$\beta$8$^+$ cells at later stages (20). To determine the effect of continuous exposure to SEB on the generation of V$\beta$8$^+$ cells in epithelial reaggregate cultures, we exposed TCR$^-$ cells (obtained by depletion with anti-CD3-coated beads [Dynal] followed by selec-

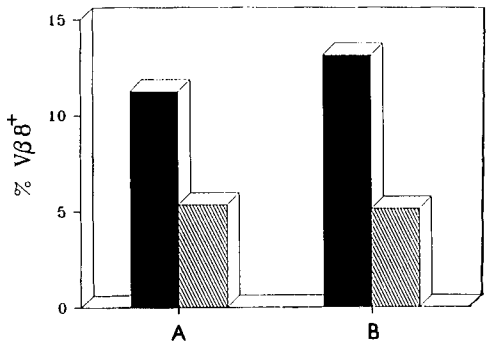


**Figure 10.** Introducing dendritic cells into reaggregate cultures of epithelium and thymocytes restores deletion of V$\beta$8$^+$ cells by SEB. Two replicate experiments are shown in which dendritic cells were added at the time of reaggregation at a ratio of 30–50 thymocytes per dendritic cells. Cultures with dendritic cells (*hatched columns*) show a reduction in the number of V$\beta$8 cells of ~50% as compared with epithelial reaggregates without dendritic cells (*filled columns*).

**Table 1.** *Effect of SEB on the Generation of TCR Vβ8⁺ Cells from TCR⁻ Cells in Epithelial Reaggregate Cultures*

| SEB treatment | | Phenotype | | | |
|---|---|---|---|---|---|
| | | $CD4^+8^+$ | $CD4^+8^-$ | $CD4^-8^+$ | $CD4^-8^-$ |
| – | Total cells | 43.3 | 42.9 | 9.8 | 4.0 |
| | $V\beta8^+$ cells (18%) | 52.5 | 37.0 | 7.8 | 2.6 |
| – | Total cells | 46.8 | 39.5 | 7.0 | 6.7 |
| | $V\beta8^+$ cells (19.4%) | 51.0 | 32.3 | 11.9 | 4.8 |
| + | Total cells | 34.4 | 52.2 | 7.1 | 6.2 |
| | $V\beta8^+$ cells (10.5%) | 27.6 | 38.6 | 14.9 | 18.9 |
| + | Total cells | 30.1 | 56.6 | 6.7 | 6.5 |
| | $\alpha/\beta+$ cells | 20.2 | 63.8 | 8.4 | 7.6 |
| | $V\beta8^+$ cells (5.8%) | 27.9 | 31.8 | 12.6 | 27.6 |

tion on CD8) to SEB over a 4-d culture period. As summarized in Table 1, $V\beta8^+$ cells are generated in the presence of SEB, including cells with $CD4^+8^+$ and single positive $CD4^+$ and $CD8^+$ phenotypes. However, as compared with control cultures without SEB, the number of $V\beta8^+$ cells accumulating over the 4-d culture period is reduced to 30–50%, and the pattern of phenotypes is perturbed, most noticeably in the accumulation of double-negative cells. Thus, although epithelial cells do not appear to be efficient in mediating rapid deletion, prolonged exposure to SEB on these cells does have an effect on the development of T cells expressing $V\beta8^+$.

## Discussion

Cell-cell interactions play a key role in T cell development in the thymus. Thus, cytokines have a limited ability to promote gene rearrangement and maturation in T cell precursors in vitro (reviewed in reference 21), suggesting that essential cues for differentiation derive from contact with thymic stromal cells. Furthermore, while $CD4^+8^+$ thymocytes have a 3–4-d lifespan in the intact thymus (22), when separated from the thymic epithelial microenvironment in vitro they die within 24 h (23). These observations suggest that thymic epithelial cells are important for the maintenance of thymocyte viability pending positive or negative selection of the TCR repertoire.

Attempts have been made to use thymic epithelial cell lines in order to obtain thymocyte differentiation and selection in vitro. However, the relationship of these lines to stromal cells in vivo is not clear, and they have failed to provide the signals necessary for normal thymocyte maturation either because they have lost important functional properties or because they derive from an epithelial type that does not possess these properties (24–26). Similarly, attempts to induce differentiation of isolated rat $CD4^+8^+$ cells using a combination of IL-2 and TCR crosslinking have resulted only in the generation of $CD8^+$ cells (27). In contrast, fetal thymus organ cultures support the full range of T cell maturation in an environment where the functional and phenotypic characteristics of thymic stromal cells are maintained (12). Although these lobes contain a complex network of stromal cells, we have previously shown that dGuo treatment eliminates lymphoid and possibly MHC class $II^+$ dendritic cells, while leaving the remaining stromal cells functionally intact (6, 12). In the present study we have confirmed the absence of dendritic cells directly by immunohistology and have shown that the remaining stroma includes macrophages and fibroblasts as well as cortical and medullary epithelial cells. Thus, trypsinization of these lobes followed by removal of macrophages provides a defined suspension of epithelial cells and fibroblasts that retain the ability to reform intact thymus lobes. This has enabled us to examine the functional properties of these cells in reaggregate cultures with selected thymocyte populations.

T cell development proceeds from a $TCR^-$ double-negative stage through a $CD4^+8^+$ stage where low levels of TCR are first expressed. TCR-mediated positive selection then allows selected cells to mature into single-positive $CD4^+$ or $CD8^+$ cells that display upregulated TCR levels and acquire functional competence (1–4, 18). A number of studies have now indicated that this positive selection step involves interaction with MHC antigens on thymic epithelial cells (3–5, 28). Thus, in a first series of experiments, we tested the ability of our stromal preparations to support the maturation of highly enriched $CD4^+8^+$ cells. These experiments show that double-positive cells (enriched to >99% in some experiments) give rise to single-positive $CD4^+$ and $CD8^+$ cells that contain functionally mature cells as evidenced by a proliferative response to SEB. To be certain that the interactions leading to positive selection occur in these cultures, we carried out a second series of experiments in which $TCR^-$ $CD4^+8^+$ cells were associated with thymic epithelial cells. The results clearly show the accumulation of single-positive cells with upregulated levels of TCR expression. Thus, positive selection, presumably mediated via MHC molecules

on the epithelial cells in the reaggregate cultures, can be obtained. These studies open the way, therefore, to experimental manipulation of positive selection in vitro.

Although dendritic cells have been implicated in negative selection (29–31), there is uncertainty over the ability of epithelial cells to provide signals for negative selection (reviewed in reference 32). Using SEB, which is known to produce deletion (by apoptosis) of $V\beta8^+$ cells in whole organ cultures (19), we studied the effect of SEB in reaggregate cultures where the only class $II^+$ cells are epithelial cells. In 18-h cultures, deletion of $V\beta8^+$ cells was not observed, in contrast to cultures where dendritic cells were also present. $V\beta8^+$ cells were also generated from $TCR^-$ $CD4^+8^+$ cells and underwent maturation into single-positive cells in the continuous presence of SEB in 4-d cultures. However, the accumulation of $V\beta8^+$ cells was reduced and the pattern of phenotypes perturbed as compared with untreated controls, indicating some effect of SEB. Thus, it is possible that epithelial cells can mediate negative selection but in a much less efficient way than dendritic cells. In this regard, it is of interest that thymic epithelial and dendritic cells also differ in their ability to deliver costimulatory signals to mature T cells (33). Alternatively, rather than inducing deletion, interaction with SEB on epithelium may result in anergy, as in the case of self-superantigens presented on thymic epithelial cells in vivo (30). There is evidence that newly formed $TCR^+$, $CD4^+$ cells in neonates are in cell cycle (34). Thus, inhibiting the proliferation of newly formed $V\beta8^+$ cells would result in the accumulation of fewer of these cells in SEB-treated cultures. This possibility is open to direct investigation by cell cycle analysis in our in vitro system.

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