Early Progression of Thymocytes along the CD4/CD8 Developmental Pathway Is Regulated by a Subset of Thymic Epithelial Cells Expressing Transforming Growth Factor β

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Summary

Precursor cells differentiate into mature $CD4^+$ and $CD8^+$ T cells in the inductive environment of the thymus by undergoing a series of distinct developmental steps marked by expression of the coreceptor molecules CD4 and CD8. Among the earliest cells to enter the CD4/CD8 developmental pathway are $CD4^-CD8^{lo}$ precursors cells that differentiate into $CD4^+CD8^+$ thymocytes. Here we show that differentiation of precursor cells into $CD4^+CD8^+$ thymocytes requires at least one cell division and that their progression through a cell cycle is specifically retarded in the thymus by interaction with thymic epithelial cells that express transforming growth factor β (TGF-β) proteins. We also demonstrate that TGF-β proteins, either in solution or bound to cell membranes, can regulate cell cycle progression and differentiation of $CD4^-CD8^{lo}$ precursor cells into $CD4^+CD8^+$ thymocytes. The regulatory effect of TGF-β is specific for $CD4^-CD8^{lo}$ precursor cells as TGF-β proteins do not regulate the earlier generation of $CD4^-CD8^{lo}$ precursor cells from $CD4^-CD8^-$ thymocytes. Finally, we demonstrate that TGF-β proteins are expressed in vivo in the intact thymus on subcapsular and cortical thymic epithelium where they can contact developing $CD4^-CD8^{lo}$ precursor cells. Thus, thymic epithelial cells expressing TGF-β proteins can actively regulate the rate at which $CD4^+CD8^+$ thymocytes are generated from $CD4^-CD8^{lo}$ precursor cells.

Mature T cells expressing TCR-α/β arise from TCR^- precursor cells in the inductive environment of the thymus. Differentiation of precursor cells into mature T cells proceeds via a series of distinct steps that are defined by levels of expression of the CD4 and CD8 coreceptor molecules (1–3), referred to as the CD4/CD8 developmental pathway. Immature precursor cells that express neither CD4 nor CD8 ($CD4^-CD8^-$) enter the CD4/CD8 developmental pathway by first expressing low levels of CD8 (4–9). These $CD4^-CD8^{lo}$ cells are the immediate precursors of $CD4^+CD8^+$ thymocytes, the cell type in which most repertoire selection events are thought to occur (10–13). Upon being positively selected, $CD4^+CD8^+$ thymocytes extinguish expression of one coreceptor molecule and differentiate into mature $CD4^+$ or $CD8^+$ T cells (14–17). Whereas little is known about the signals responsible for committing $CD4^+CD8^+$ thymocytes to become either $CD4^+$ or $CD8^+$ T cells (18–23), even less is known about the mechanisms regulating CD4 and CD8 expression in early precursor thymocytes differentiating along the CD4/CD8 development pathway.

The first cells in the thymus that can be identified as having entered the CD4/CD8 developmental pathway are $CD4^-CD8^{lo}$ precursor cells that are transcriptionally committed to becoming $CD4^+CD8^+$ thymocytes, and spontaneously do so when placed in single cell suspension culture. Despite their commitment to becoming $CD4^+CD8^+$, differentiation of $CD4^-CD8^{lo}$ precursor cells into $CD4^+CD8^+$ thymocytes can be actively inhibited by TCR engagement which signals the posttranscriptional elimination of mRNA species encoding two families of developmentally important molecules: (*a*) CD4 and CD8 coreceptor molecules, and (*b*) RAG-1 and RAG-2 recombination activating molecules (24). Consequently, this TCR-mediated regulatory mechanism functions in $CD4^-CD8^{lo}$ precursor cells to abort further development along the CD4/CD8 developmental pathway of early thymocytes expressing autoreactive TCR specific for self-antigens (25).

In this study, we have identified a novel regulatory mechanism that acts on all developing $CD4^-CD8^{lo}$ precursor cells independently of their TCR specificity and that is mediated

by a subset of thymic epithelial (TE)[1] cells. We found that progression through at least one cell cycle is necessary for differentiation of $CD4^-CD8^{lo}$ precursor cells into $CD4^+$ $CD8^+$ thymocytes, and that their progression through the cell cycle is specifically regulated by interaction with cortical TE cell lines. Furthermore, we found that inhibitory TE cells express TGF-β1 and TGF-β2 proteins which regulate the rate at which $CD4^-CD8^{lo}$ precursor cells can progress through the cell cycle. In addition, we found that the regulatory effects of TGF-β proteins on early thymocyte development are stage specific in that they do not affect the generation of $CD4^-$ $CD8^{lo}$ cells from $CD4^-CD8^-$ precursors. Finally, we found that TGF-β1 and TGF-β2 are primarily expressed by subcapsular and cortical thymic epithelium in vivo precisely in those areas of the thymus in which $CD4^-CD8^{lo}$ precursor thymocytes are located. Thus, this study demonstrates that interaction of lymphoid precursors with cortical TE cells expressing TGF-β proteins can regulate the rate at which early thymocyte development proceeds.

Materials and Methods

Mice. C57BL/6 (B6) mice were obtained from the National Cancer Institute. Fetal mice were obtained from time pregnancies at gestational times as indicated.

mAbs and Reagents. FITC-anti-CD4 (Rm4-4 and Rm4-5) and anti-TCR-β (H57-597) mAb were obtained from Pharmingen (San Diego, CA). FITC-conjugated and biotinylated anti-CD8 (53-6-72) mAb, as well as anti-human Leu4 negative control mAbs were obtained from Becton Dickinson Immunocytometry Systems (BDIS, San Jose, CA). All reagents were pretitrated and used in saturating amounts. Nocodazole and PMA were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human TGF-β1, recombinant mouse TNF-α, recombinant human IL-8, and anti-human TGF-β1 Ab were obtained from R&D Systems, Inc. (Minneapolis, MN). Recombinant human TGF-β2 and TGF-β3 were provided by Dr. M. Sporn at the National Institutes of Health.

Cell Lines. Cortical TE cell lines utilized were TEP1 (26), TEC (27), and 1308.1 (28, 29). Medullary TE cell lines utilized were TE71 (30), Z172, Z199R, and Z210 derived from a normal BALB/c thymus (Farr, A., unpublished observations). Also utilized were the keratinocyte line PAM212 (31) and the L cell fibroblast line DAP.3 (32).

Cell Preparations and Culture. Preparation of precursor thymocytes was described previously (24). For isolation of $CD4^-$ thymocytes, single cell suspensions of fetal thymocytes were treated with IgM anti-CD4 mAb RL172 plus C. For isolation of purified populations of $CD4^-CD8^+$ fetal thymocytes, $CD4^-$ thymocytes were stained with anti-CD8 mAb, and $CD8^+$ cells were sorted by using FACStar Plus® (BDIS). Cells were cultured in 24-well plates at 37°C in RPMI 1640-based medium as described (24). Where indicated, anti-TCR-β mAb was immobilized on plastic at 50 μg/ml in PBS at 4°C overnight.

Immunofluorescence and Flow Cytometry. Cultured cells (10^6) were washed in HBSS containing 0.2% BSA and 0.1% NaN_3. For staining, cells were first incubated with anti-FcγR mAb 2.4G2 (33) to block FcR-mediated binding, and then sequentially incubated with FITC-labeled anti-CD4 (mAb Rm4-5) and biotinylated anti-CD8 (mAb 53-6-72), followed by Texas red-streptavidin. Flow cytometry analyses were performed as described (34). The two anti-CD4 mAbs used for staining and for cytolytic depletion bind to different epitopes on CD4 and do not interfere with each other's binding.

CD4 fluorescence intensity on $CD8^+$ cells was quantitated in linear fluorescence units (25). Fluorescence units (FU) are the median intensity of CD4 staining minus median intensity of control Leu4 staining. Median intensity was calculated by converting median log channel number to linear units using an empirically derived calibration curve for each logarithmic amplifier used.

Cell Cycle Analysis. Cells (10^6) were stained with FITC-anti-CD8 mAb in the presence of anti-FcγR mAb 2.4G2, as described above. Cells were then incubated with hypotonic 3.4-mM citrate buffer containing 25 μg/ml propidium iodide (PI), 0.1% Triton X-100, and 0.1 mg/ml RNase A (DNase free) at 4°C overnight (35). Two-color flow cytometry analysis was performed by using a FACScan® (BDIS) for CD8 cell surface expression and DNA content detected by PI. Cell cycle status on electronically gated $CD8^+$ cells was analyzed by using Cellfit software (BDIS).

Cell Binding Analysis of TGF-β. Cross-linking analysis of TGF-β binding to cellular proteins using radiolabeled TGF-β was described previously (36). Confluent monolayers of TE cell lines were incubated with ^{125}I-labeled TGF-β1 (200 pM) in the absence or presence of excess amount of unlabeled TGF-β1 (10 nM) at 37°C for 2 h. Proteins on washed cells were cross-linked by using disuccinimidyl suberate (Pierce, Rockford, IL), and cell lysates were resolved by 7% SDS-PAGE and analyzed by subsequent autoradiography.

Immunohistochemical Analysis of TGF-β Expression. Anti-TGF-β antibodies used for immunohistochemical studies were developed by K. C. Flanders et al. (37–39) and raised in rabbits against: (*a*) synthetic peptides of amino acids 1–30 of mature TGF-β1; (*b*) synthetic peptides of amino acids 267–278 of the pro region of precursor TGF-β1; and (*c*) synthetic peptides of amino acids 50–75 of mature TGF-β2. All antibodies exhibit intracellular localization, and react specifically with the isoforms to which they were raised.

For cytospin preparations, cell cultures were harvested using a solution of 0.5 mM EDTA in PBS without calcium and magnesium. Cells were washed twice in PBS with 1 mg/ml of BSA, and then resuspended in PBS with BSA at a concentration of 10^6/ml. TGF-β isoforms were localized in cytospin preparations fixed in 4% paraformaldehyde. 5-μm sections of thymic lobes were dissected from day 17 fetal, neonatal, or adult mice. Tissues were fixed in 10% buffered formalin and embedded in paraffin for sectioning, and sections were deparaffinized before staining. Both tissue sections and cytospins were incubated in 2% hydrogen peroxide in methanol to block endogenous peroxidase, and then permeabilized with a hyaluronidase solution (1 mg/ml, Calbiochem-Novabiochem Corp., La Jolla, CA). Slides were blocked with 5% normal goat serum, and 1% BSA fraction V (Miles, Kankakee, IL) for 1 h at room temperature, and then incubated overnight with 3–5 μg/ml of anti-TGF-β IgG or normal rabbit IgG in Tris-buffered saline. Slides were washed with Tris-buffered saline/0.1% BSA and with a biotinylated goat anti-rabbit IgG followed by avidin-peroxidase. Slides were developed in an 0.5% solution of 3,3-diaminobenzidine (Sigma Chemical Co.), counterstained in Mayer's hematoxylin, and photographed at magnifications of either 200 or 400 using a Zeiss Axiophot microscope.

The binding of rabbit anti-TGF-β antibodies to frozen thymus sections was performed as previously described (40). Affinity-purified rabbit antibodies were diluted to final concentrations of 0.5–1 μg/ml in PBS containing 10 mg/ml BSA. Polyclonal mouse anti-rabbit

[1] *Abbreviations used in this paper:* FU, fluorescence units; PI, propidium iodide; TE, thymic epithelial.

IgG antibodies were derivized with *N*-Hydroxysuccinimidodigoxigenin and detected with sheep anti-digoxigenin Fab antibody fragments conjugated with horseradish peroxidase. Hydrogen peroxide and 3,3-diaminobenzidine were used to demonstrate peroxidase activity.

Results

Generation of CD4$^+$CD8$^+$ Thymocytes from CD4$^-$CD8^{lo} Precursor Cells Requires One Cell Division. We began by assessing the ability of CD4$^-$CD8^{lo} precursor cells to differentiate into CD4$^+$CD8$^+$ thymocytes either in intact thymic lobes or in single cell suspension culture in which they were not in contact with TE cells. Intact day 14 fetal thymus lobes that had been cultured for 5 d were either continued for an additional 16 h or were dispersed into single cell suspension cultures for an additional 16 h. Whereas total numbers of thymocytes recovered after 16 h were comparable in both situations, the frequency of CD4$^+$CD8$^+$ thymocytes generated in suspension culture (61%) was significantly higher than that generated in intact organ cultures (33%) (Fig. 1). Thus, dissociation of CD4$^-$CD8^{lo} precursor cells from intrathymic stromal components appeared to augment their differentiation into CD4$^+$CD8$^+$ thymocytes.

To determine if precursor cells proliferated in suspension culture during differentiation into CD4$^+$CD8$^+$ thymocytes, we purified CD4$^-$CD8^{lo} precursor cells from day 19 fetal thymuses and determined the number of starting cells and the subsequent number of CD4$^+$CD8$^+$ progeny. As shown in Fig. 2 *A*, purified CD4$^-$CD8^{lo} fetal thymocytes proliferated during suspension culture as their cell number increased 1.5-fold after 16 h. Cell cycle analysis of precursor thymocytes at the initiation of culture revealed that 53% had a DNA content greater than 1× and so were in S or G2+M phase and were actively progressing through the cell cycle (Fig. 2 *B*). After 16 h in suspension culture, all the cultured thymocytes were CD4$^+$CD8$^+$ and only 3% had DNA content greater than 1× indicating that they were no longer cycling (Fig. 2 *B*). However, it was not possible to discern if the 50% of precursor cells which were not in cycle became CD4$^+$CD8$^+$ without cell cycle progression at all, or if all precursor cells progressed through one cell division during their differentiation into CD4$^+$CD8$^+$ thymocytes. To distinguish between these possibilities, we added the mitosis inhibitor nocodazole (41) to the suspension cultures so that all cells that had entered the cell cycle would be captured in M phase. In the presence of nocodazole, virtually all cultured cells were found to be arrested with DNA content of 2× (Fig. 2 *B*), indicating that virtually all precursor thymocytes

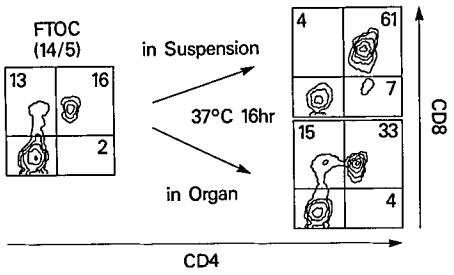

Figure 1. Generation of CD4+CD8+ cells in fetal thymic organ culture (*FTOC*) versus suspension culture. After 5 d of culture, day 14 fetal thymus lobes (*FTOC, 14/5*) were divided into two groups: one group was dispersed into single cell suspension culture, and the other group was washed but kept intact in organ culture. Cells were then cultured for an additional 16 h, and analyzed for CD4 and CD8 expression by two-color flow cytometry. Expression of CD4 and CD8 is displayed as dual parameter contour plots with increasing FITC fluorescence on the x-axis (3 decade log scale) versus increasing Texas red fluorescence on the y-axis (4 decade log scale). Numbers within each box of contour diagrams indicate the frequency of cells within that box. Compared with starting cell numbers before the 16-h culture, 105% were recovered from suspension cultures and 88% were recovered from thymus organ cultures.

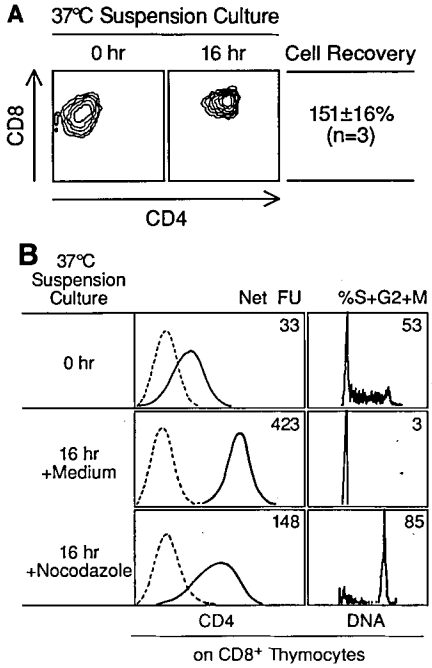

Figure 2. Differentiation of CD4$^-$CD8^{lo} precursor cells into CD4$^+$CD8$^+$ thymocytes requires one cell division. (*A*) CD4$^-$CD8^{lo} precursor cells were purified by cell sorting of CD4$^-$ day 19 fetal B6 thymocytes, and cultured for 16 h in suspension. CD4 and CD8 expression before and after suspension culture are displayed as dual parameter contour plots with increasing FITC fluorescence on the x-axis (3 decade log scale) versus increasing Texas red fluorescence on the y-axis (4 decade log scale). Relative viable cell recoveries from three experiments are indicated. (*B*) CD4$^-$ thymocytes from day 19 fetal B6 mice were cultured for 16 h in the absence or presence of 2 μM nocodazole. After culture, cells were assessed for CD4 and CD8 expression by two-color flow cytometry. CD4 expression on software gated CD8$^+$ cells is displayed as single-color histograms with increasing FITC fluorescence on the x-axis (3 decade log scale). Net CD4 fluorescence intensity on CD8$^+$ cells was quantitated in linear fluorescence units (*FU*) such that FU = median intensity of CD4 staining − median intensity of Leu4 staining. Cells were also stained with FITC-anti-CD8 mAb and with PI in the presence of 0.1% Triton X-100. PI staining profiles of DNA in electronically gated CD8$^+$ cells are displayed in linear scale, and the percentage of cells having a DNA content greater than 1x (*%S+G2+M*) is listed. Relative viable cell recoveries after culture were 85 ± 15% in medium alone and 48 ± 12% in the presence of nocodazole in three experiments.

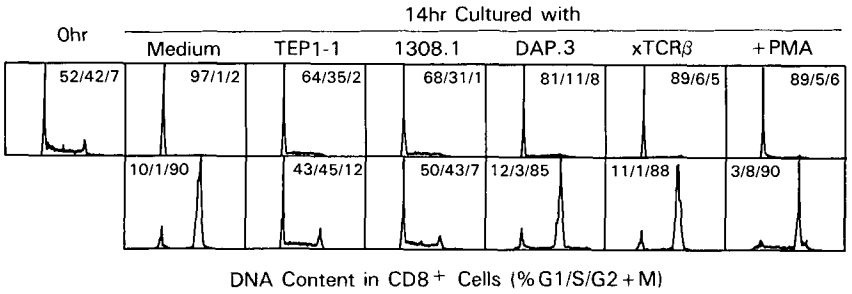

Figure 3. Cell cycle analysis of CD4$^-$CD8^{lo} precursor cells cultured with thymic epithelial cells or with TCR signals. CD4$^-$ thymocytes from day 19 fetal B6 mice were cultured for 14 h in the absence or presence of the indicated cell lines, platebound anti-TCR-β antibody (H57-597), or 100 ng/ml PMA. Nocodazole (10 μM) was added where indicated. After culture, cells were stained with FITC-anti-CD8 mAb and with PI in the presence of 0.1% Triton X-100. Cell cycle distribution based on PI staining profiles of DNA in electronically gated CD8$^+$ cells are displayed in linear scale, and percentage of cells in each cell cycle phase is listed (*%G1/S/G2 + M*).

were cycling cells that had progressed to M phase. It is interesting to note that arrest in M phase by nocodazole also resulted in significantly reduced quantities of surface CD4 levels by cultured precursor cells (Fig. 2 *B*). Thus, at least one cell division is required for complete expression of CD4 to generate CD4^{hi}CD8^{hi} thymocytes.

TE Cells Inhibit Cell Cycle Progression and Differentiation of CD4$^-$CD8^{lo} Precursor Cells. Because CD4$^-$CD8^{lo} precursor cells differentiated into CD4$^+$CD8$^+$ cells more efficiently in single cell suspension culture than within intact thymus lobes, we considered the possibility that interaction of precursor cells with TE cells might be inhibitory. Consequently, we examined the effect of TE cell lines on cell cycle progression of precursor cells during their differentiation in suspension culture into CD4$^+$CD8$^+$ thymocytes. At the initiation of culture, 52% of precursor cells were in G1 whereas the remaining half were cycling with DNA content greater than 1× (Fig. 3, column *1*). At the conclusion of cultures in medium alone, 97% of thymocytes were in G1 phase with DNA content of 1×, having completed one cell cycle as verified by nocodazole treatment in which 90% of cells were found frozen with DNA content of 2× (Fig. 3, column *2*). Similar results were observed at the conclusion of cultures that contained the L cell fibroblast DAP.3, platebound anti-TCR-β mAb, or the phorbol ester PMA (Fig. 3, columns *5–7*), indicating that none of these treatments affected cell cycle progression of precursor cells. In contrast, results were significantly different in cultures containing the TE cell lines TEP1-1 or 1308.1 (Fig. 3, columns *3* and *4*). At the conclusion of cocultures with either TEP1-1 or 1308.1, >30% of precursor cells had DNA content greater than 1×, and 12% or fewer were frozen in M phase by nocodazole, indicating that the majority of cultured precursor cells had not completed one cell cycle. However, the most profound effect of TEP1-1 and 1308.1 cell lines was that they inhibited precursor cells that were in G1 at the initiation of culture from progressing beyond G1, as ~50% of precursor cells were found in G1 even in the presence of nocodazole (Fig. 3, column *3* and *4*).

To assess the effect of TE cell lines on differentiation of CD4$^-$CD8^{lo} precursor cells into CD4$^+$CD8$^+$ thymocytes, we examined their effect on induction of CD4 expression by cultured precursor cells. The TE cell lines that inhibited cell cycle progression of precursor cells (TEP1-1 and 1308.1) also inhibited their expression of CD4 (Fig. 4). In contrast, cell lines that did not inhibit their progression through the cell cycle, such as the L cell line DAP.3 and the keratinocyte cell line PAM212, interfered with neither their expression of CD4 nor their differentiation into CD4$^+$CD8$^+$ thymocytes (Fig. 4).

Together these results indicate that the TE cell lines TEP1-1 and 1308.1 arrested most CD4$^-$CD8^{lo} precursor cells at the G1 stage of the cell cycle, delayed progression of precursor cells already in cycle, and, as a consequence, delayed the differentiation of precursor cells into CD4$^+$CD8$^+$ thymocytes.

Characterization of Inhibitory TE Cells. The inhibitory TEP1-1 and 1308.1 TE cell lines are both considered to be of thymic cortical rather than medullary origin (26, 29). Consequently, we wished to determine if the ability to regulate the differentiation of CD4$^-$CD8^{lo} precursor cells into CD4$^+$CD8$^+$ thymocytes was also a function of other cortical TE cell lines. We found that a third independently derived cortical TE cell line, TEC (27), similarly inhibited CD4 expression by cultured CD4$^-$CD8^{lo} precursor cells (Fig. 4). In contrast, four medullary TE cell lines Z172, Z199R, TE-71, and Z210 (30), had no inhibitory activity at all (Fig. 4 and data not shown).

To begin to characterize the inhibitory molecule(s) expressed by cortical TE cells, we wished to determine if the inhibitory molecule(s) was membrane bound or secreted. We found that treatment of the inhibitory TE cell line 1308.1 with trypsin removed its inhibitory ability, suggesting that the inhibitory molecule(s) was a membrane-bound protein (Table 1). Consistent with this conclusion, we also found that su-

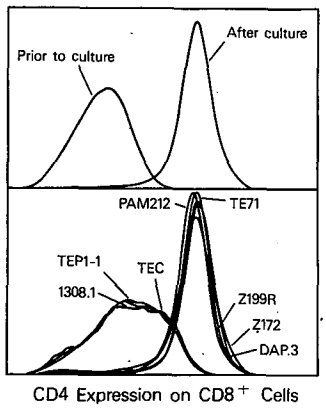

Figure 4. Effect of various adherent cell lines on the differentiation of CD4$^-$ CD8^{lo} precursor cells into CD4$^+$CD8$^+$ thymocytes. CD4$^-$ thymocytes from day 19 fetal B6 mice were cultured for 16 h in the absence or presence of confluent monolayers of the indicated cell lines. Cells were assessed for CD4 and CD8 expression before and after culture as indicated, with CD4 expression on software gated CD8$^+$ cells displayed as single-color histograms with increasing FITC fluorescence on the x-axis (3 decade log scale). The presence of monolayer cells did not affect viable cell recovery.

pernatants from inhibitory cell lines, such as TEC, did not contain any inhibitory activity (Table 1).

Cell Cycle Progression and Differentiation of CD4⁻CD8^{lo} Precursor Cells Can Be Inhibited by TGF-β. Because our results indicated that cortical TE cells inhibited the differentiation of CD4⁻CD8^{lo} precursor cells into CD4⁺CD8⁺ thymocytes by primarily affecting their ability to progress through the cell cycle, we focused our attention on molecules that can regulate cell cycle progression and that can be expressed on cell membranes. TGF-β is known to arrest cell cycle progression at G1 in many cells (42–44). And, whereas TGF-β is a soluble cytokine, it can also be bound to cell membranes by a membrane-associated β-glycan (36, 45, 46).

Consequently, we examined the effect of TGF-β1 on cell cycle progression of cultured CD4⁻CD8^{lo} precursor cells (Table 2). At the initiation of culture, 52% of precursor cells were in G1. Nocodazole treatment revealed that only 9% remained in G1 at the conclusion of cultures with medium alone, indicating that the vast majority of precursor cells had entered the cell cycle during the culture. In contrast, nocodazole treatment of TGF-β1 treated cultures revealed that 42% of precursor cells remained in G1 at the conclusion of culture, indicating that TGF-β1 inhibited most precursor cells from progressing beyond G1 (Table 2). These results demonstrate that, like cortical TE cells, TGF-β1 can arrest cell cycle progression of CD4⁻CD8^{lo} precursor cells at G1.

Next, we examined the effect of TGF-β on the differentiation of precursor cells into CD4⁺CD8⁺ thymocytes (Fig. 5). TGF-β1, TGF-β2, and TGF-β3 each inhibited the differentiation of CD4⁻CD8^{lo} precursor cells. In contrast, no other cytokine tested showed any inhibitory effect, including IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IFN-γ, and

Table 2. *Cell Cycle Analysis of CD4⁻CD8^{lo} Precursor Cells Cultured with TGF-β*

Suspension culture		Cell cycle distribution of CD8⁺ cells					
		No nocodazole			Nocodazole		
Time	Added reagent	G1	S	G2+M	G1	S	G2+M
h							
0	–	52*	34	14			
14	Medium	97	2	1	9	1	91
14	TGF-β1	85	13	2	42	21	37

CD4⁻ thymocytes from day 19 fetal B6 mice were cultured for 16 h in the absence or presence of 1 ng/ml TGF-β1. Nocodazole (10 μM) was added where indicated. After culture, cells were stained with FITC-anti-CD8 mAb and with PI in the presence of 0.1% Triton X-100. The cell cycle distribution based on PI staining profiles of DNA in electronically gated CD8⁺ cells were analyzed by flow cytometry.
* Percentage of total CD8⁺ cells.

Table 1. *The Inhibitory Ligand Expressed on Cortical TE cells is a Membrane-bound Protein*

Expt.	Suspension culture of CD4⁻ fetal thymocytes*			CD4 Expression on CD8⁺ cells (FU)§	Inhibition‖
	Time in culture	Added cell lines	Treatment of added cell lines‡		
	h				%
1	0	–		16	
	14	None		145	
	14	1308.1	Medium	34	86
	14	1308.1	Trypsin	147	0
2	0	–		17	
	14	None		234	
	14	TEC		142	42
	14	TEC supernatant		234	0

* CD4⁻ thymocytes (0.5 × 10⁶/culture) from day 19 fetal B6 mice were cultured for 14 h in the absence or presence of the indicated cell lines (10⁶). Where indicated, cultures included supernatant (final concentration at 50% vol/vol) from confluent TEC cell line.
‡ Cell lines were pretreated with trypsin (0.5 mg/ml in 0.2 mg/ml EDTA; GIBCO BRL, Gaithersburg, MD) for 10 min at 37°C, and washed thoroughly.
§ Cells were assessed for CD4 and CD8 expression at the conclusion of culture. CD4 expression on CD8⁺ cells is displayed as net CD4 fluorescence intensity expressed in linear fluorescence units (FU), where FU = median intensity of CD4 staining – median intensity of control staining. Median intensity was calculated by converting median log channel number to linear units using an empirically derived calibration curve for each logarithmic amplifier used.
‖ Percent inhibition of CD4 expression was calculated as follows: 100 × [(FU of cultured cells with medium alone – FU of experimental groups)/(FU of cultured cells with medium alone – FU of uncultured precursor cells)].

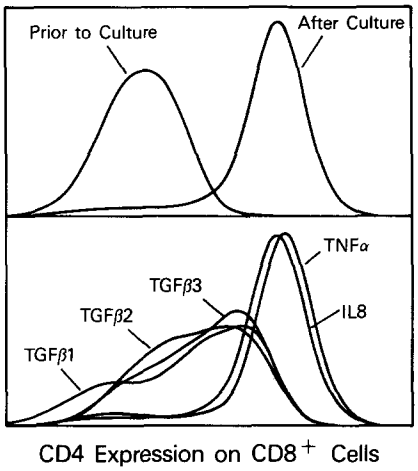

Figure 5. Effect of recombinant soluble cytokines on the in vitro differentiation of CD4$^-$CD8^{lo} precursor cells into CD4$^+$CD8$^+$ thymocytes. CD4$^-$ thymocytes from day 19 fetal B6 mice were cultured for 16 h in the absence or presence of the indicated recombinant cytokines. At the conclusion of culture, cells were assessed for CD4 and CD8 expression with CD4 expression on software gated CD8$^+$ cells displayed as single-color histograms with increasing FITC fluorescence on the x-axis (3 decade log scale). Concentration of cytokines used was 10 ng/ml for human TGF-β1, TGF-β2, TGF-β3, and mouse TNF-α; and 20 ng/ml for human IL-8.

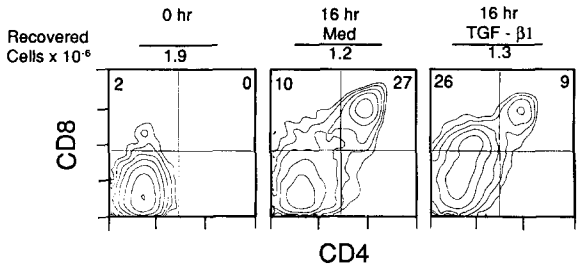

Figure 6. TGF-β does not effect the differentiation of CD4$^-$CD8$^-$ thymocytes into CD4$^-$CD8^{lo} cells. Unfractionated day 15 fetal thymocytes were placed in single cell suspension cultures at 37°C in either medium alone or in the presence of recombinant TGF-β1 (1 ng/ml). After 16 h of culture, cells were assessed for CD4 and CD8 expression by two-color flow cytometry. CD4 and CD8 expression are displayed as dual parameter contour plots with increasing FITC fluorescence on the x-axis (3 decade log scale) versus increasing Texas red fluorescence on the y-axis (4 decade log scale). The number of recovered cells are indicated and were not affected by the presence or absence of TGF-β1.

TNF-α. The effects of TNF-α and IL-8 are displayed as representative of cytokines without inhibitory effect (Fig. 5). Thus, soluble TGF-β also mimicked the inhibitory effects of cortical TE cell lines on the differentiation of CD4$^-$CD8^{lo} precursor cells.

Inhibitory Effect of TGF-β is Specific for CD4$^-$CD8^{lo} Precursor Cells. To determine if soluble TGF-β also interfered with the preceding developmental step, namely the generation of CD4$^-$CD8^{lo} precursor cells from CD4$^-$CD8$^-$ thymocytes, we dispersed untreated and unfractionated day 15 fetal thymocytes into single cell suspension cultures for 16 h in the presence or absence of soluble TGF-β1 (Fig. 6). Because of the early (i.e., day 15) gestational age of fetal thymocytes used in this experiment, 98% of cells were initially CD4$^-$CD8$^-$ with many differentiating into CD8$^+$ cells during 16-h suspension culture so that they were either CD4$^-$CD8^{lo} or CD4$^+$CD8$^+$ (Fig. 6, *left* and *center*). It is interesting that soluble TGF-β1 did not interfere with the differentiation of CD4$^-$CD8$^-$ cells into CD8$^+$ thymocytes as the percentage of CD8$^+$ cells was the same in medium (37%) or TGF-β1 (35%) (Fig. 6, *center* and *right*). However, at the end of culture in TGF-β1, more cells were CD4$^-$CD8^{lo} and fewer were CD4$^+$CD8$^+$. In medium alone three quarters of the CD4$^-$CD8$^-$ cells that differentiated into CD8$^+$ cells during the 16-h culture differentiated all the way to the CD4$^+$CD8$^+$ stage. In contrast, in the presence of TGF-β1, three quarters of the cells that became CD8$^+$ during culture remained at the CD4$^-$CD8^{lo} stage of development, with only one quarter becoming CD4$^+$CD8$^+$ (Fig. 6). Thus, soluble TGF-β is not inhibitory of all steps in early thymocyte differentiation as it does not inhibit the differentiation of CD4$^-$CD8$^-$ thymocytes into CD4$^-$CD8^{lo} precursor cells, although it does interfere with the subsequent differentiation of CD4$^-$CD8^{lo} precursor cells into CD4$^+$CD8$^+$ thymocytes.

TGF-β is Expressed by Inhibitory Cortical TE Cells. Because the inhibitory effect of TGF-β was specific for CD4$^-$CD8^{lo} precursor cells and so closely resembled that of cortical TE cells, we wondered if TGF-β could bind to the surface of TE cells and function as a membrane-bound inhibitory molecule. Indeed, we found that radiolabeled TGF-β1 could specifically bind to the surface of both inhibitory (TEP1-1 and TEC) and noninhibitory (Z199R) TE cell lines to form a high molecular weight complex with a membrane protein, presumably β-glycan (Fig. 7). It is interesting to note that when pulsed with TGF-β1, medullary Z199R cells inhibited the differentiation of CD4$^-$CD8^{lo} precursor cells to the same extent as inhibitory cortical TEP1-1 cells (Table 3, groups 1–5). The ability to confer an inhibitory potential on Z199R cells was dependent upon the expression of a membrane protein capable of binding TGF-β, as trypsinization of Z199R cells before TGF-β1 pulsing prevented the cells from becoming inhibitory (Table 3, group 7). Similarly, trypsinization of Z199R cells that had previously been pulsed with TGF-β1 reversed its inhibitory activity (Table 3, group 8). Thus, TGF-β can bind to the membrane of TE cells and, when present on the surface of TE cells, does function to inhibit the differentiation of precursor cells into CD4$^+$CD8$^+$ thymocytes.

We then wished to use neutralizing anti-TGF-β1 antibodies to determine if membrane-bound TGF-β1 molecules were the inhibitory molecule(s) expressed on the surface of inhibitory cortical TE cells. Unfortunately, we found that anti-TGF-β1 antibodies were unable to neutralize the inhibitory activity of membrane-bound TGF-β1 even though they could neutralize soluble TGF-β1 molecules (Fig. 8), presumably because antibody epitopes were obscured by TGF-β1 binding to membrane proteins. Specifically, we found that anti-TGF-β1 antibodies reversed inhibition of CD4 expression induced by soluble TGF-β1, but did not affect inhibition of CD4 expression induced by TGF-β1–pulsed medullary Z199R cells

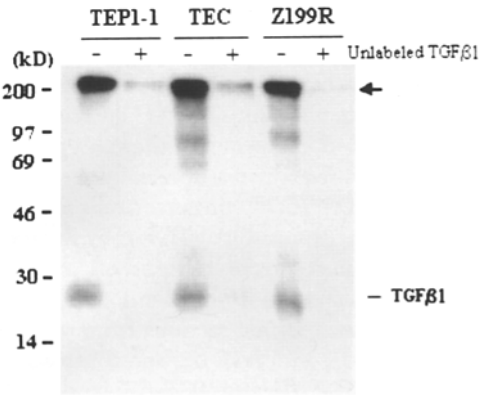

Figure 7. Binding of TGF-β to thymic epithelial cell lines. Monolayers of indicated TE cell lines were incubated with ^{125}I-labeled TGF-β1 in the absence (−) or presence (+) of unlabeled competitor TGF-β1. Cell surface proteins were cross-linked by using disuccinimidyl suberate, and cell lysates were resolved by SDS-PAGE and analyzed by subsequent autoradiography. (*Arrow*) The major TGF-β1–associated complex, with the molecular weight of β-glycan (36, 46). These bands were not observed in the presence of unlabeled competitor TGF-β1, indicating that the complexes were specific for TGF-β. Bands detected at molecular weight of approximately 25,000 represent cross-linked dimers of TGF-β1 molecules.

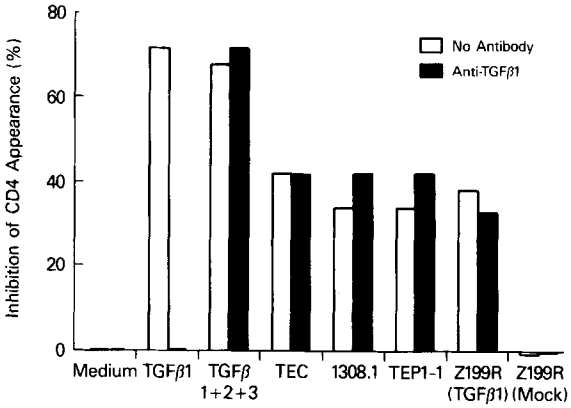

Figure 8. Effect of anti-TGF-β1 antibodies on the ability of TE cells to regulate in vitro thymocyte differentiation. CD4$^-$ thymocytes from day 19 fetal B6 mice were cultured for 14 h in the absence (*unfilled bars*) or presence (*filled bars*) or anti-TGF-β1 antibodies. Cultures also included either soluble TGF-β or TE cell lines as indicated. Z199R cells were pretreated with either TGF-β1 or medium alone (*Mock*). After culture, cells were assessed for CD4 and CD8 expression with CD4 expression on software gated CD8$^+$ cells determined in linear FU. Results are displayed as percent inhibition of CD4 appearance (see Table 1). Soluble recombinant TGF-β1, TGF-β2, and TGF-β3 were added at 10 ng/ml each.

(Fig. 8, groups *2* and *7*). In addition, anti-TGF-β1 antibodies were specific for soluble TGF-β1 and could not reverse inhibition induced by soluble TGF-β2 and TGF-β3 when these TGF-β forms were also present (Fig. 8, group *3*).

Consequently, we directly examined whether inhibitory TE cell lines expressed TGF-β by immunohistochemical analysis. In these experiments, we stained all fixed cells with rabbit antibodies specific for: (*a*) the latent form of TGF-β1; (*b*) the mature form of TGF-β1; (*c*) the mature form of TGF-β2; and (*d*) the mature form of TGF-β3. All three inhibitory cortical TE cell lines were stained positively with antibodies specific for TGF-β1 and TGF-β2, but were not stained with antibodies specific for TGF-β3. Staining with anti-TGF-β2 antibodies is displayed in Fig. 9 (*C–E*). In contrast, staining of noninhibitory medullary TE cell lines, such as Z199R, and the L cell line DAP.3 was clearly negative with all anti-TGF-β

Table 3. *Effects of Pulsing Noninhibitory Medullary TE Cell Lines with TGF-β*

	Suspension culture of CD4$^-$ fetal thymocytes*					
		Added cell lines‡				
Group	Time in culture	Name	1st treatment	2nd treatment	CD4 expression on CD8$^+$ cells*	Inhibition*
	h					%
1	0	–	–	–	17	
2	14	–	–	–	234	
3	14	TEP1-1	–	–	160	34
4	14	Z199R	Medium	Medium	236	0
5	14	Z199R	TGF-β1	–	152	38
6	14	Z199R	Trypsin	–	233	0
7	14	Z199R	Trypsin	TGF-β1	234	0
8	14	Z199R	TGF-β1	Trypsin	234	0

* Suspension culture of CD4$^-$ fetal precursor thymocytes, measurement of CD4 expression on CD8$^+$ cells in linear FU, and calculation of percent inhibition were carried out as described in Table 1.

‡ Cell lines were pretreated with trypsin for 10 min at 37°C, or pulsed with 10 ng/ml TGF-β1 for 30 min at 37°C, followed by three washings. Where indicated, cells were trypsinized and pulsed with TGF-β1 in the indicated sequence.

antibodies (Fig. 9, *A* and *B*), as was staining of thymocytes. Thus, the inhibitory ability of TE cell lines correlated with their expression of TGF-β1 and TGF-β2 proteins.

In situ Expression of TGF-β by Cortical TE Cells. Finally, to relate our in vitro studies of TE cells to the in vivo situation within the intact thymic environment, we performed immunohistochemical studies on both fixed and frozen thymic sections from day 16–17 fetal, newborn, and young adult mice to determine if TGF-β proteins are expressed by intact thymic epithelium in the thymus. Thymic sections were stained with rabbit anti-TGF-β1 (latent form), rabbit anti-TGF-β1 (mature form), rabbit anti-TGF-β2 (mature form), and rabbit anti-TGF-β3 (mature form). Staining of both fixed and frozen thymic sections with anti-TGF-β antibodies was similar regardless of the age of the mouse from which the thymic sections were obtained. Positive staining was observed with anti-TGF-β1 and anti-TGF-β2 antibodies, but not with anti-TGF-β3 antibodies. Fig. 10 displays staining of fixed thymic sections from newborn mice with anti-TGF-β2 and control antibodies, and reveals TGF-β expression in the subcapsular and cortical areas of the thymus which are precisely the areas of the thymus in which $CD4^-CD8^{lo}$ precursor cells are located (4). To better visualize expression of TGF-β by thymic stromal cells, we also stained frozen sections of unfixed thymuses with anti-TGF-β1 and anti-TGF-β2 antibodies. Fig. 11 displays staining of frozen thymic sections from young adult mice with anti-TGF-β1, anti-TGF-β2, and control antibodies. As can be seen, TGF-β1 and TGF-β2 expression was observed primarily by subcapsular and cortical epithelial cells, which are precisely the TE cells that contact immature

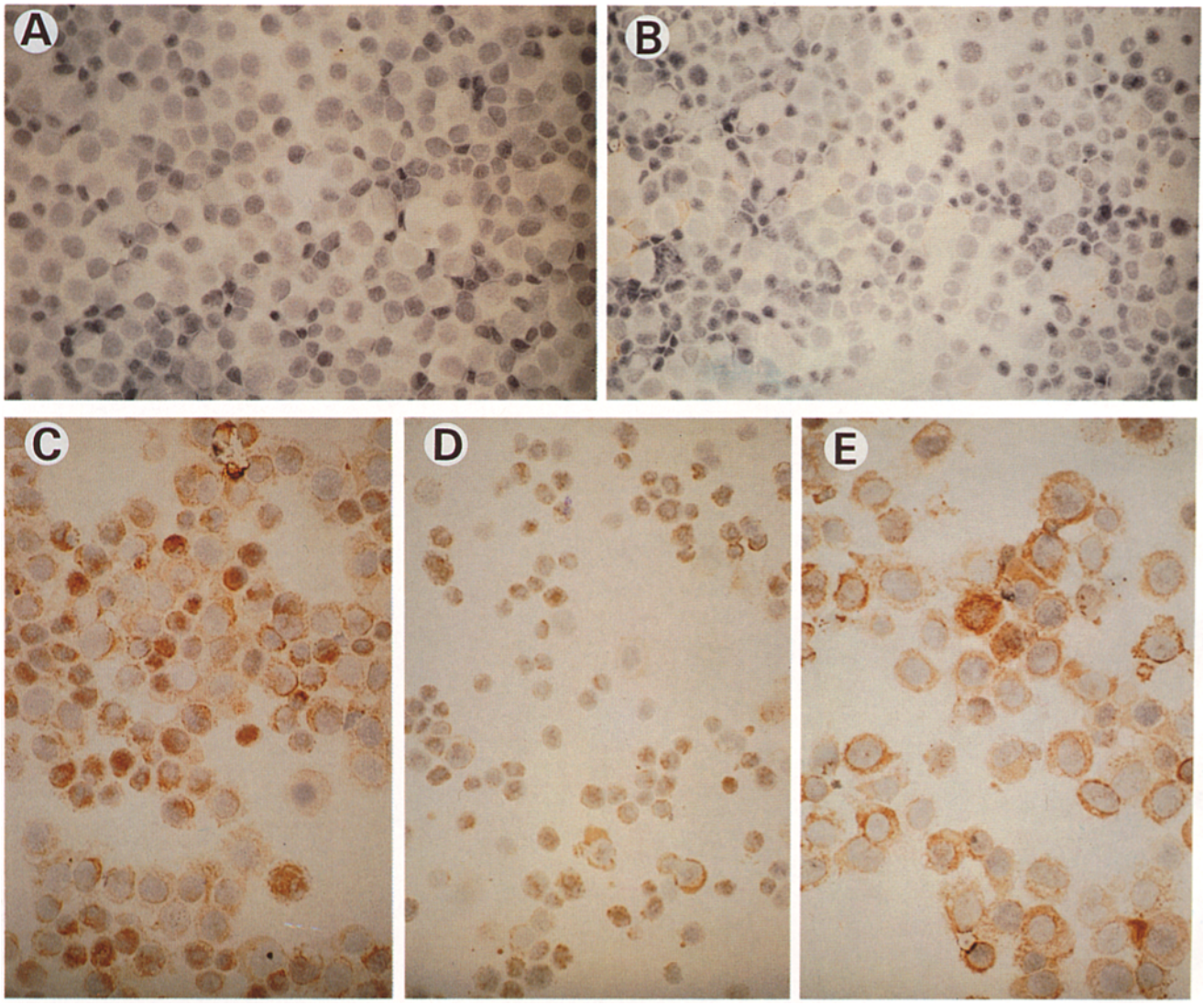

Figure 9. Expression of TGF-β by TE cell lines as determined by immunohistochemical analysis. DAP.3 (*A*), Z199R (*B*), TEP1-1 (*C*), 1308.1 (*D*), and TEC (*E*) were assessed for TGF-β expression. The indicated cell lines were all stained with hematoxylin and with rabbit antibodies specific for TGF-β1 (latent form), TGF-β1 (mature form), TGF-β2 (mature form), or with control rabbit antibodies. All three rabbit anti-TGF-β antibodies gave identical results. The results of staining with rabbit anti-TGF-β2 (mature form) are displayed. Antibody binding was visualized by indirect peroxidase staining. None of the cell lines stained with control rabbit antibodies.

$CD4^-CD8^{lo}$ precursor cells in the thymus (4). Thus, TGF-β1 and TGF-β2 are expressed in the intact thymus by thymic epithelium where they can contact developing $CD4^-CD8^{lo}$ precursor cells and regulate their progression through the cell cycle and, consequently, their further differentiation into $CD4^+CD8^+$ thymocytes.

Discussion

This study demonstrates that the rate at which $CD4^-CD8^{lo}$ precursor cells progress through the cell cycle and differentiate into $CD4^+CD8^+$ thymocytes is regulated by interaction with cortical TE cells expressing TGF-β proteins. Moreover, TGF-β proteins themselves, either in solution or bound to cell membranes, were capable of regulating cell cycle progression and differentiation of $CD4^-CD8^{lo}$ precursor cells into $CD4^+CD8^+$ thymocytes. Thus, thymic epithelium actively regulates the rate at which immature $CD4^-CD8^{lo}$ thymocytes become $CD4^+CD8^+$.

The majority of cells in the thymus are $CD4^+CD8^+$ thymocytes, whose immediate precursors are $CD4^-CD8^{lo}$ cells. The differentiation of $CD4^-CD8^{lo}$ precursor cells into $CD4^+CD8^+$ thymocytes was found to require at least one cell division, but the interaction of $CD4^-CD8^{lo}$ precursor cells with cortical TE cells interfered with their progression beyond the G1 phase of the cell cycle. The inhibitory activity of cortical TE cells was found to reside in a membrane-bound protein, which we tried unsuccessfully to identify by blocking with antibodies specific for a wide variety of known molecules expressed on thymocytes or TE cells, including adhesion and major histocompatibility molecules. We then considered the possibility that TGF-β might be the inhibitory molecule expressed by cortical TE cells since TGF-β was known to induce cell cycle arrest at G1, it could bind to cell membranes, and it was expressed in developing tissues. We found that soluble TGF-β did resemble cortical TE cells in its ability to inhibit both proliferation and differentiation of $CD4^-CD8^{lo}$ precursor cells. We also found that soluble TGF-β could bind to TE cell membranes, and, when present, would function as a membrane-bound inhibitory molecule. That TGF-β was actually expressed by inhibitory cortical TE cells was demonstrated by immunohistochemical staining with anti-TGF-β antibodies. Finally, we found that TGF-β was primarily expressed in the intact thymus by subcapsular and cortical epithelial cells that contact $CD4^-CD8^{lo}$ precursor cells in the thymus. Thus, expression of TGF-β proteins by cortical TE cells was consistent with their ability to regulate cell cycle progression and differentiation of immature precursor cells into $CD4^+CD8^+$ thymocytes.

The differentiation of $CD4^-CD8^{lo}$ precursor cells into $CD4^+CD8^+$ thymocytes has now been shown to be regulated by two distinct molecular mechanisms, one that specifically regulates differentiation of $CD4^-CD8^{lo}$ precursor cells expressing self-reactive TCR, and one that regulates differentiation of $CD4^-CD8^{lo}$ precursor cells regardless of their TCR specificity. The first mechanism is antigen specific, mediated by TCR signals, and aborts the further differentiation along the CD4/CD8 developmental pathway of precursor

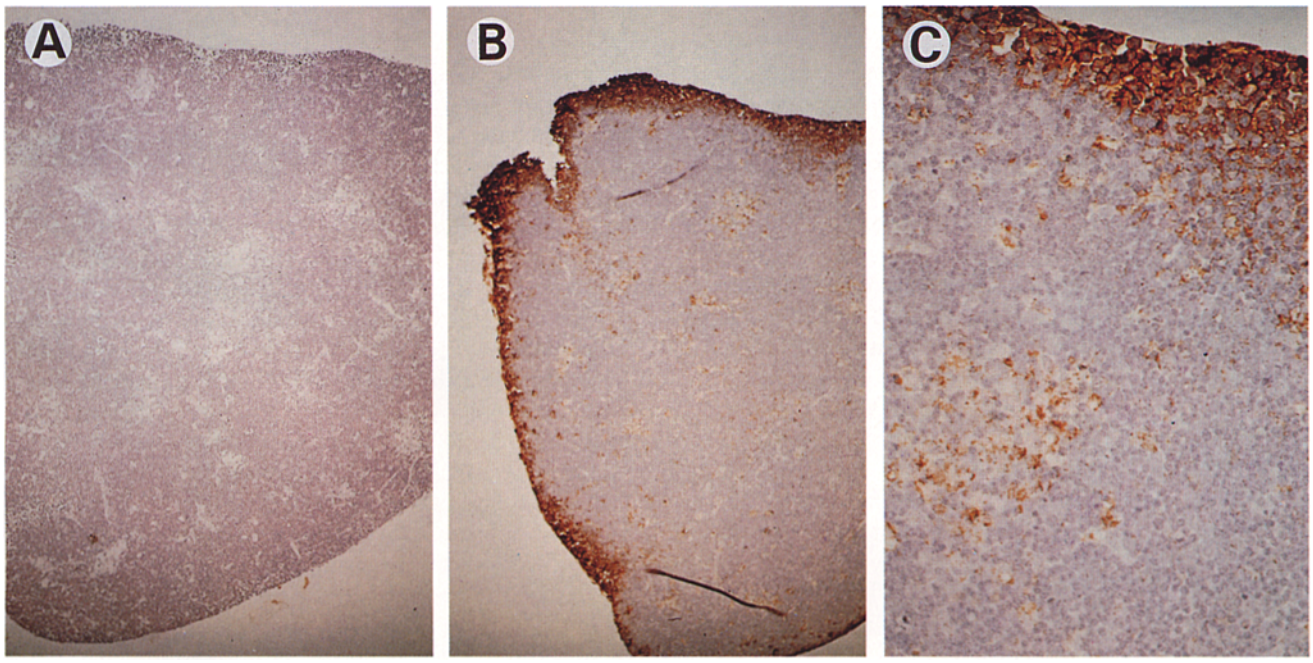

Figure 10. In situ expression of TGF-β in the thymus. Fixed thymus sections from day 17 fetal, newborn, and young adult B6 mice were stained with hematoxylin and with rabbit antibodies specific for TGF-β1 (latent form), TGF-β1 (mature form), TGF-β2 (mature form), or with control rabbit antibodies. All three rabbit anti-TGF-β antibodies gave identical results on all three thymus preparations. The results of staining newborn thymus with rabbit anti-TGF-β2 (mature form) (*B*, ×200 magnification; *C*, ×400) or control rabbit antibodies (*A*) are displayed. Antibody binding was visualized by indirect peroxidase staining.

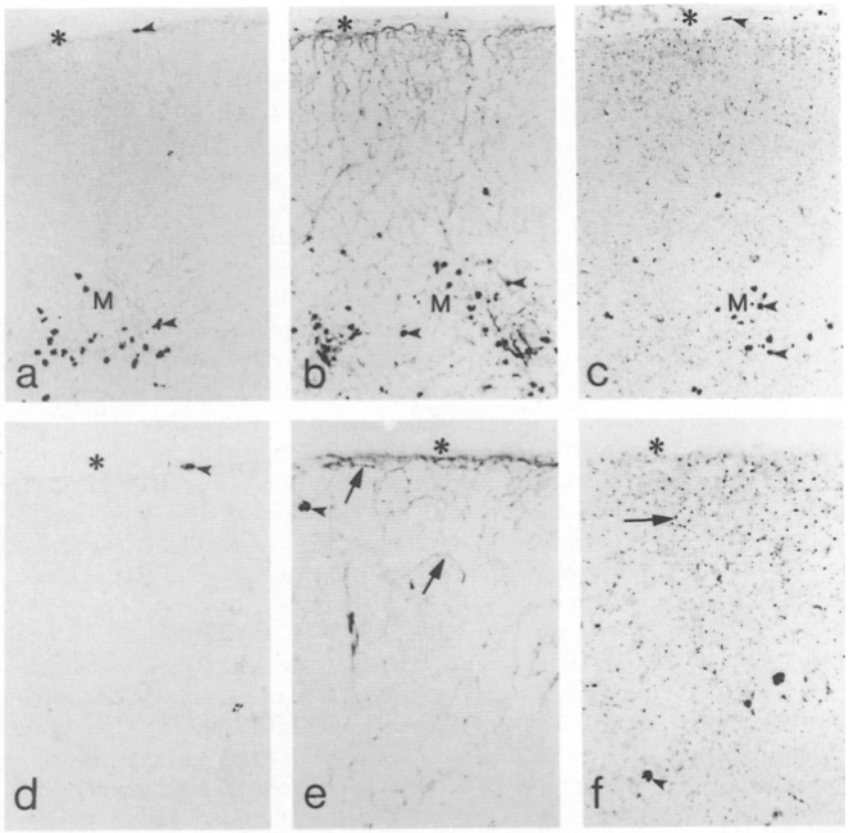

Figure 11. In situ expression of TGF-β in the thymus. Thymus frozen sections from young adult B6 mice were stained with rabbit antibodies specific for TGF-β1 (latent form), TGF-β2 (mature form), or control rabbit antibodies, and antibody binding was visualized with indirect peroxidase staining. Similar results were obtained with thymus frozen sections from day 16 fetal and newborn mice. (*a* and *d*) Display labeling with normal rabbit Ig; (*b* and *e*) depict labeling with anti-TGF-β1 antibodies; (*c* and *f*) depict labeling with anti-TGF-β2 antibodies. (*a–c*) Were obtained with a magnification of 500; (*d–f*) were obtained with a magnification of 1,000. Within the cortex, TGF-β1 is perferentially expressed by subcasular epithelial cells, although some cortical stromal cell processes are also labeled (*e*). With anti-TGF-β2 antibodies, the distribution of reaction product was punctate and also exhibited a preferential labeling of the subcapsular and outer cortical areas of the thymus. (*) Capsule; (*M*) medulla; (*arrows*) reaction product generated by enzyme-labeled antibodies; (*arrowheads*) endogenous peroxidase activity.

cells expressing autoreactive TCR against intrathymic self-antigens. TCR engagement induces signals in $CD4^-CD8^{lo}$ precursor cells that lead to the rapid elimination of mRNAs encoding two families of molecules: the coreceptor molecules CD4 and CD8, and the recombination activating molecules RAG-1 and RAG-2 (24). The second mechanism is described in this report and is mediated by TGF-β expressing TE cells that regulate the ability of $CD4^-CD8^{lo}$ precursor cells to progress through the cell cycle and to become $CD4^+CD8^+$. These two regulatory mechanisms are quite distinct as TCR engagement did not affect cell cycle progression, and cell cycle arrest did not rapidly eliminate CD4 mRNA expression (Takahama, Y., and A. Singer, unpublished results). The existence of two distinct mechanisms regulating the differentiation of $CD4^-CD8^{lo}$ precursor cells into $CD4^+CD8^+$ thymocytes identifies this developmental step as being tightly controlled during early thymocyte development.

TGF-β proteins are members of a superfamily of secreted growth factors that contain a cysteine knot motif and that function as dimeric molecules (47). At least five different TGF-β molecules have been identified in different species, but only TGF-β1, -β2, and -β3 have been found in mammalian cells (for reviews, see references 48 and 49). TGF-β is expressed in early fetal tissues and has been suggested as a potentially important regulator of early development (39, 50–52), but a role for TGF-β in regulating mammalian development has not previously been defined. It is interesting that we found that TGF-β expressing TE cells determine the rate at which $CD4^+CD8^+$ thymocytes are generated, for it has been suggested that TGF-β (in the presence of IL-7) induces $CD4^-CD8^-$ thymocytes to express CD8 (9, 53), and it is generally thought that TE cells provide an inductive environment for T cell development (15, 17, 26, 28, 54). However, control of the rate at which $CD4^+CD8^+$ thymocytes are generated might be important for subsequent developmental events in the thymus, such as selection of the T cell repertoire. Indeed, without TGF-β, $CD4^+CD8^+$ thymocytes might be generated too rapidly for autoreactive cells to be appropriately screened out in the thymus. It is interesting to note that whereas the basis for the pathology is not yet known, TGF-$\beta1^-$ mice whose TGF-β1 gene loci were disrupted by homologous recombination do suffer from an apparent autoimmune disorder with massive lymphocyte infiltration of multiple organs and early death (55). Unfortunately, our attempts to use TGF-$\beta1^-$ mice to assess the generation of $CD4^+CD8^+$ thymocytes in the absence of TGF-β1 proteins have so far been unsuccessful because of the presence of maternal TGF-β proteins during early life which is then followed by the rapid onset of disease and stress-induced thymic atrophy.

Regulation of cell cycle progression by TGF-β has been extensively analyzed but is still not completely understood. It is thought that TGF-β inhibits cyclin E assembly with cyclin-dependent kinase 2, and that this assembled complex functions to phosphorylate retinoblastoma protein that is required for progression beyond G1 phase of the cell cycle

(42–44). The present study demonstrates that TGF-β is biologically active in regulating cell cycle progression of precursor thymocytes even when bound to the surface of TE cells. Its binding to TE cells was found to be specific as it was competed with cold TGF-β, and it formed a high molecular weight protein complex on the cell surface that resembled β-glycan, a ubiquitous TGF-β binding protein. Thus, the binding of TGF-β to β-glycan effectively converts it into a membrane-bound cell interaction molecule on cortical TE cells. A precedent for conversion of soluble molecules into functional cell interaction molecules has recently been suggested for proteoglycan-immobilized macrophage inflammatory protein 1β which induces T cell adhesion to vascular cell adhesion molecule 1 and so can potentially attract T cells to inflammatory tissues (56).

In conclusion, this study demonstrates that TGF-β proteins are expressed by thymic epithelial cells that contact $CD4^-CD8^{lo}$ precursor cells developing in the thymus and regulate the rate at which they can differentiate into $CD4^+$ $CD8^+$ thymocytes.

We thank Drs. Takashi Suda and Anita Roberts for helpful discussion; Dr. Kathleen Flanders for development and characterization of anti-TGF-β antibodies; and Drs. K. Katz, M. Kuehn, J. Punt, S. Sharrow, D. Singer, and M. Zauderer for critically reading the manuscript.

This work was supported in part by grants from the National Institutes of Health (AI-24137 and AG-04360) and the Department of Energy (DE F006-ER60409).

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Received for publication 18 October 1993 and in revised form 31 January 1994.

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