Regulation of Cytokine Production in the Human Thymus: Epidermal Growth Factor and Transforming Growth Factor α Regulate mRNA Levels of Interleukin 1α (IL-1α), IL-1β, and IL-6 in Human Thymic Epithelial Cells at a Post-transcriptional Level

By Phong T. Le, Suzanne Lazorick, Leona P. Whichard, Barton F. Haynes, and Kay H. Singer

From the Department of Medicine, Division of Rheumatology and Immunology, and Department of Microbiology and Immunology, Division of Immunology, Duke University Medical Center, Durham, North Carolina 27710

Summary

Human thymic epithelial (TE) cells produce interleukin 1α (IL-1α), IL-1β, and IL-6, cytokines that are important for thymocyte proliferation. The mRNAs for these cytokines are short-lived and are inducible by multiple stimuli. Thus, the steady-state levels for IL-1 and IL-6 mRNAs are critical in establishing the final cytokine protein levels. In this study we have evaluated the effect of epidermal growth factor (EGF), a growth factor for TE cells, and its homologue transforming growth factor α (TGF-α), on primary cultures of normal human TE cells for the levels of IL-1α, IL-1β, IL-6, and TGF-α mRNA. We showed that TE cells expressed EGF receptors (EGF-R) in vitro and in vivo, and that treatment of TE cells with EGF or TGF-α increased IL-1 and IL-6 biological activity and mRNA levels for IL-1α, IL-1β, and IL-6. Neither EGF nor TGF-α increased transcription rates of IL-1α, IL-1β, and IL-6 genes, but rather both EGF and TGF-α increased cytokine mRNA stability. By indirect immunofluorescence assay, TGF-α was localized in medullary TE cells and thymic Hassall's bodies while EGF-R was localized to TE cells throughout the thymus. Thus, TGF-α and EGF are critical regulatory molecules for production of TE cell–derived cytokines within the thymus and may function as key modulators of human T cell development in vivo.

Epidermal growth factor (EGF)[1] is a potent growth factor for a variety of cells including epidermal keratinocytes and other epithelial cell types (1). EGF binds to EGF-R, a protein tyrosine kinase (PTK), and initiates a series of events that include activation of PTK activity of EGF-R, phosphorylation of the EGF-R and phospholipase C-γ, an increase in levels of intracellular Ca^{2+}, and stimulation of phosphoinositide turnover (2). EGF has also been shown to increase the rate of transcription of certain proto-oncogenes (3, 4). Recently, it has been shown that EGF can also increase stability of short-lived mRNA for EGF-R in a human epithelial cell line (5).

TGF-α is structurally and functionally related to EGF (6, 7). TGF-α binds to EGF-R with an affinity comparable to that of EGF and activates EGF-R PTK activity (8, 9). TGF-α was originally detected in culture supernatants of transformed rodent fibroblasts (10–12), and is also produced by a variety of normal cell types (13–16). TGF-α is mitogenic for cultured fibroblasts (10), endothelial cells (17), and epidermal keratinocytes (18). Since certain cell types that produce TGF-α can also respond to TGF-α, TGF-α has been implicated in normal and neoplastic growth regulation through autocrine and paracrine pathways (19). Whether TGF-α and EGF can affect autocrine and/or paracrine pathways in cells producing immunoregulatory cytokines has not been reported.

Thymic epithelial (TE) cells, a major cellular component of thymic stroma, play a critical role in T cell development (20, 21). Human TE cells produce multiple immunoregulatory cytokines including IL-1α, IL-1β (22, 23), M-CSF (24, 25), G-CSF (25), GM-CSF, leukemia inhibitory factor (LIF), and IL-6 (26). The mRNAs for most of these cytokines are short-lived and their synthesis is inducible by a myriad of stimuli (27). Thus, to establish an appropriate level of cytokine biological activity, it is important to maintain a certain level of mRNA for these cytokines, either by regulating mRNA transcription or by regulating mRNA stability. With regard

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[1] *Abbreviations used in this paper:* EGF, epidermal growth factor; G, granulocyte; LIF, leukemia inhibitory factor; M, monocyte; PTK, protein tyrosine kinase; TE, thymic epithelial.

to T cell development in the thymus, the regulation of production of TE cell-derived cytokines that affect T cell proliferation and differentiation is a critical aspect of thymic function, yet virtually nothing is known about regulatory mechanisms of human TE cell function (28, 29). TE cell binding to thymocytes has been shown to increase IL1 release, and triggering of TE cell surface lymphocyte function-associated antigen-3 (LFA-3), a ligand for CD2 on T cells, increases both IL-1α and IL-1β mRNA levels in TE cells (23).

In this study, we have investigated regulatory signals involved in cytokine production by human TE cells and defined a novel role for EGF and TGF-α in control of human TE cell-derived cytokine production at a posttranscriptional level. These studies suggest an important role for EGF and/or TGF-α in stabilizing short-lived mRNA for TE cell-derived cytokines. Since TGF-α is available within human thymic microenvironment, TGF-α may provide a novel mechanism for regulating the availability of T cell trophic cytokines that affect human T cell development.

Materials and Methods

Initiation and Culture of Human Thymic Epithelial (TE) Cells. Thymic tissues were obtained as discarded tissue through the Department of Pathology, Duke University Medical Center, from children undergoing select corrective cardiovascular surgical procedures. TE cell cultures were initiated by an explant technique and propagated in enriched medium as described previously (30). Contaminating fibroblasts were removed by treatment with 0.02% EDTA (30) and by complement-mediated lysis with a monoclonal antibody 1B10 which binds to a cell surface antigen on human fibroblasts (31). TE cell preparations were 95% to 98% positive for the keratin marker AE-3 (32) and negative for the macrophage marker Leu-M3 (33) and the thymocyte marker CD7 (34). For EGF or TGF-α treatment, confluent TE cell cultures were removed from enriched medium, washed twice with PBS and cultured for 24 h in DMEM medium containing 5% heat inactivated FCS, 10 μg/ml gentamicin sulfate, and 10 mM Hepes. Mouse EGF (Collaborative Research, Bedford, MA) and recombinant human TGF-α (Peninsula Labs, Belmont, CA; or Amgen, Thousand Oaks, CA) were used at concentrations described in figure legends.

Isolation of Total RNA and RNA Blot Analysis. TE cells were washed three times with ice cold PBS and lysed directly on the plate with 4.0 M guanidine isothiocyanate. Total RNA was pelleted by centrifugation through a 5.7 M cesium chloride cushion as described previously (35). Total RNA of 8.0 μg per lane was electrophoresed in 1.2% agarose gels containing 2.2 M formaldehyde in 40 mM 3-(N-morpholino) propanesulphonic acid (MOPS) buffer at pH 7.0, transferred to nitrocellulose membranes and probed with human cytokine cDNA as described elsewhere (23, 26).

Preparation of Nuclei. Nuclei were isolated from untreated TE cells or TE cells treated with EGF (5.0 ng/ml) or TGF-α (100 ng/ml) according to a modified method described by Bitter et al. (36). Briefly, TE cells were detached from culture dishes with 0.05% trypsin in PBS, pH 7.4, containing 0.02% EDTA and washed three times in ice cold PBS. TE cells were washed once in ice cold hypotonic buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3.0 mM MgCl$_2$), resuspended in the same buffer at 5.0 $\times$ 10^6 to 10^7 cells/ml and allowed to swell for 5.0 min on ice. NP-40 was added to the cell suspension to achieve a final concentration of 0.125% and the cells were incubated on ice for 5 min. This resulted in >95% lysis as

determined by phase contrast microscopy and trypan blue dye exclusion. The nuclei were collected by centrifugation at 500 g for 5 min at 4°C, washed and resuspended in 200 μl of 50 mM Tris, pH 8.0 buffer containing 40% glycerol, 5.0 mM MgCl$_2$ and 0.1 mM EDTA. Nuclei were either stored in liquid nitrogen or used immediately in nuclear transcription assays.

In Vitro Nuclear Transcription Assay. In vitro elongation of nascent RNA in isolated nuclei was performed according to a modified method of Groudine et al. (37). Nuclear transcription was performed in a total volume of 400 μl reaction buffer containing 5.0 $\times$ 10^6–10^7 nuclei, 30 mM Tris pH 8.0, 5 mM MgCl$_2$, 0.15 M KCl, 0.05 mM EDTA, 2.5 mM DTT, 20% glycerol, 250 U/ml RNAse inhibitor, 0.5 mM of α-ATP, α-GTP, α-CTP (Pharmacia-LKB, Piscataway, NJ) and 100 μCi of ^{32}P-α-UTP (3,000 mCi/mmole) (Amersham, Arlington Heights, IL). The transcription reaction was allowed to take place at 30°C for 30 min and terminated by adding 20 μg DNAse I in high salt buffer (10 mM Tris pH 7.4, 50 mM MgCl$_2$, 2.0 mM CaCl$_2$), and incubated at 30°C for 15 min. Proteinase treatment was carried out with 200 μg/ml of proteinase K in 0.8% SDS and 20 mM EDTA at 42°C for 30 min. The transcription reaction was extracted with phenol/chloroform/isoamylalcohol (20:20:1; v/v) in the presence of 4.0 μg/ml yeast tRNA. The aqueous layer was adjusted to 2.4 M ammonium acetate, precipitated with equal volume of isopropylalcohol in a dry ice-methanol bath for 1 h and centrifuged. The pellets were dissolved in 100 μl of 1$\times$ Tris-EDTA, pH 7.4 buffer. Unincorporated ^{32}P-α-UTP was separated from labeled nuclear RNA by centrifugation through a G-25 sephadex column. The procedure allowed the incorporation of 2.0 $\times$ 10^6 to 3.5 $\times$ 10^6 CPM into 3.0 $\times$ 10^6–5.0 $\times$ 10^6 isolated nuclei. The labeled RNA was incubated in 0.2 N NaOH on ice for 15 min, neutralized with equal molar concentration of 0.1 N HCl and mixed with 0.5 ml of hybridization buffer containing 6$\times$ SSPE (1$\times$ SSPE is 0.15 M NaCl, 0.01 M NaH$_2$PO$_4$, 1 mM EDTA, pH 7.4), 50% deionized formamide, 5$\times$ Denhardt's solution, 0.1% SDS, and 200 μg/ml denatured salmon sperm DNA. The purified labeled RNA was hybridized to an excess amount of plasmids (5.0 μg/slot) containing human cDNA specific sequences that were immobilized on nitrocellulose filters according to the method of Greenberg and Ziff (38) using a slot-blot apparatus (Schleicher & Schuell, Keene, NH). Hybridization was carried out at 42°C for 5 d and filters were washed in 2$\times$ SSC, 0.1% SDS, 2 mM EDTA at room temperature for 30 min (three times), followed by 0.1$\times$ SSC, 0.1% SDS, 2.0 mM EDTA at 50°C for 30 min (three times). Autoradiography was carried out for 7 d at −70°C using Kodak Xomat AR-5 film (Eastman Kodak Co., Rochester, NY) with intensifying screens.

cDNA Probes. The following human specific cDNA probes were used in this study: IL-1α (1.7 kb Xho fragment in pXM plasmid), IL-1β (1.4 kb Pst fragment in pSP64), IL-6 (0.7 kb Xho fragment in pXM), TGF-α (1.8 kb EcoRI fragment in SP65C17N3, a subclone of pTGF C-1), and β-actin (0.8 kb EcoRI-BamHI fragment of pHFβA3'UT). After digestion with the appropriate restriction endonuclease, cDNA inserts were separated from the plasmid in which they were propagated by gel electrophoresis and extracted from gel slices with Geneclean kit (Bio 101, La Jolla, CA). The cDNA inserts were labeled with ^{32}P-α-dCTP (3,000 mCi/mmole) (Amersham) by a multiprime DNA labeling technique (Pharmacia-LKB) to a specificity of 2–3 $\times$ 10^9 dpm/μg DNA.

Indirect Immunofluorescence Assay. 4 μm sections were prepared from frozen thymus tissue and fixed in acetone at −20°C for 5 min. Tissues were stained as described previously (39) with purified mAb against human EGF-R (20 μg/ml) (Oncogene Science Inc., Mineola, NY), isotype control antibody (P3x63/Ag8), affinity

purified polyclonal chicken anti–human TGF-α (IgG, 3.0 μg/ml) (gift from Dr. K. Hashimoto, Osaka University, Japan), or IgG fraction (3.0 μg/ml) from chicken serum (Organon Teknika Corp., Westchester, PA). Flow cytometry using trypsinized TE cells and anti–EGF-R receptor was performed as previously described using FACS Star flow cytometer (Becton-Dickinson and Co., Mountain View, CA).

IL-1 and IL-6 Assay. IL-1 biological activity was determined using the D10S assay as described elsewhere (40). IL-6 biological activity was determined with a mouse plasmacytoma cell line T1165 (41). TE cell culture supernatants were tested at 1/8 final dilution in both the IL-1 and IL-6 assays.

Results

Human Thymic Epithelial Cells Express Epidermal Growth Factor R (EGF-R) In Vitro and In Vivo. TE cells were cultured in medium without exogenous EGF and analyzed for EGF-R expression using indirect immunofluorescence assay and flow cytometry with anti–human EGF-R mAb. All TE cells expressed EGF-R as detected by flow cytometry (Fig. 1 A). However, TE cells cultured continuously with 20 ng/ml of EGF to saturate all receptors did not bind monoclonal anti–EGF-R antibody (Fig. 1 B). These results indicated that TE cells expressed EGF-R and that EGF specifically bound to EGF-R on TE cells. Using indirect immunofluorescence assays on frozen sections of human thymus, we detected EGF-R on TE cells in both the thymic cortex and medulla (Fig. 2 A–C). Bright staining of EGF-R was observed with cells of the subcapsular cortex (Fig. 2 A) and with cells surrounding the Hassall's bodies (Fig. 2 C); however, we did not detect EGF-R on epithelial cells within Hassall's bodies (Fig. 2 C). Previous studies have shown the presence of EGF-R in normal human thymus, and in thymoma (42, 43). Thus, TE cells expressed EGF-R both in vitro and in vivo.
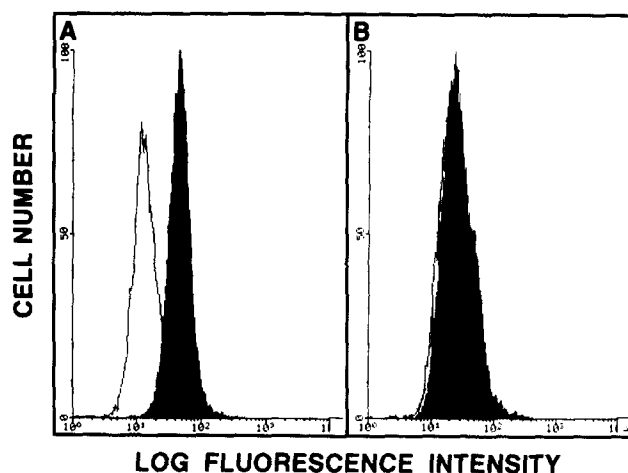

LOG FLUORESCENCE INTENSITY

Figure 1. Expression of EGF-R by human TE cells in vitro. Flow cytometry analysis of EGF-R expression on human TE cells that were removed from EGF containing medium and cultured in D-MEM + 5% FCS for 24 h before assay (A) and TE cells that were cultured continuously with 20 ng/ml of EGF (B). The filled peak represents reactivity with anti–EGF-R mAb, and the open peak represents reactivity with control antibody P3x63/Ag8. Purified anti–EGF-R (IgG1) was used at 20 μg/ml. P3x63/Ag8 ascites was used at a 1:500 dilution.

Epidermal Growth Factor (EGF) Regulates Thymic Epithelial (TE) Cell IL-1α, IL-1β, and IL-6 mRNA Levels. TE cells were cultured with various concentrations of EGF (1.0–200 ng/ml) for 16 h and cytokine mRNA levels were determined using RNA blot analysis. Laser scanning densitometry of RNA blot analysis of total RNA showed that EGF at 1.0 ng/ml increased the level of IL-1α mRNA 1.4-fold, and IL-1β mRNA 3 fold (Fig. 3 A–C). The increase in IL-1α and IL-1β mRNA

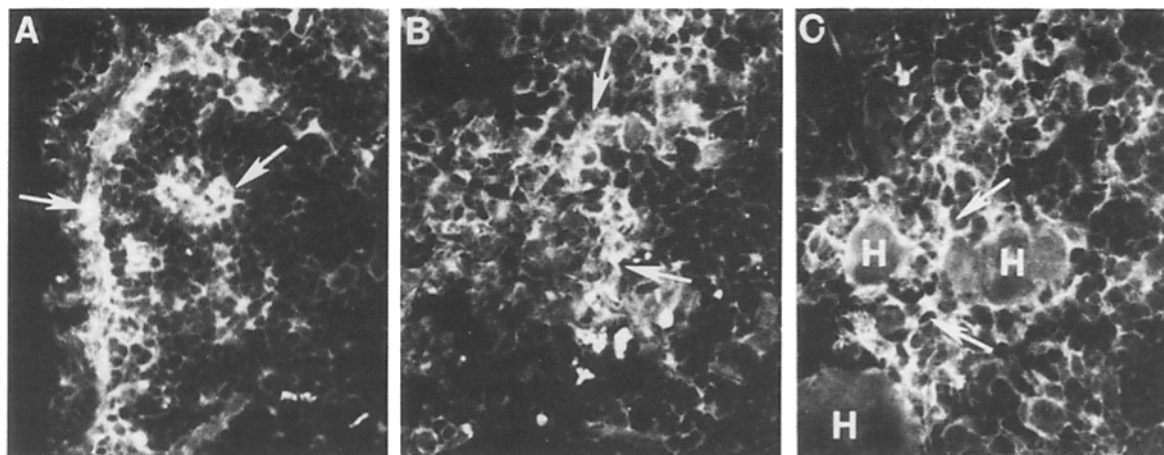## EXPRESSION OF EGF RECEPTORS IN HUMAN THYMUS

Figure 2. Expression of EGF-R in human thymus. Frozen sections of human thymus were fixed in acetone and stained with purified anti–EGF-R antibody at 20 μg/ml. (A) Thymic cortex. (B and C) Thymic medulla. *Arrows* indicate EGF-R positive cells. H indicates Hassall's bodies. No positive staining was detected with control antibody P3x63/Ag8 (data not shown).
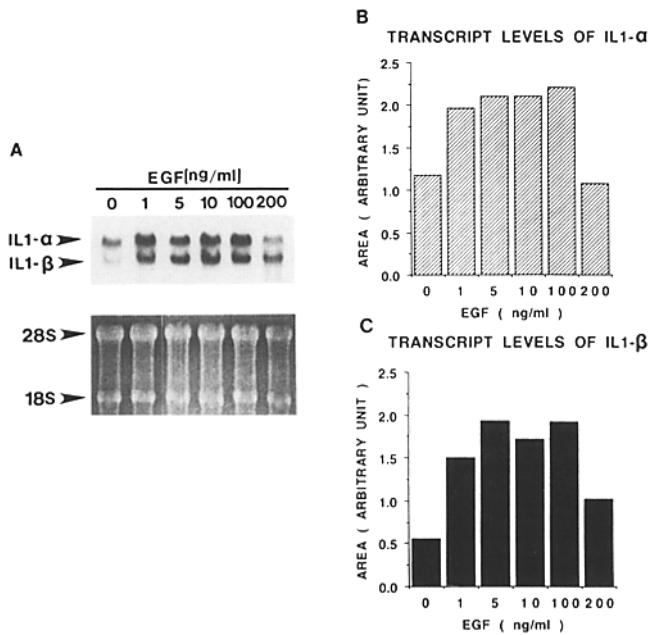
B

TRANSCRIPT LEVELS OF IL1-α

C

TRANSCRIPT LEVELS OF IL1-β

Figure 3. EGF increased mRNA levels for IL-1α and IL-1β in a concentration dependent fashion. TE cells were treated for 16 h with various concentrations of EGF. TE cells were then lysed and total RNA extracted and analyzed for IL-1α and IL-1β mRNA levels. *A* shows the actual mRNA levels of IL-1α and IL-1β (*upper panel*) and the levels of the 28S and 18S ribosomal RNA of identical lanes, stained with acridine orange (*lower panel*). *B* and *C* represent scanning densitometer tracings of the densities of the IL-1α and IL-1β bands, respectively.

levels was maintained over EGF concentrations ranging from 1.0 ng/ml to 100 ng/ml (Fig. 3 *A–C*). However, at EGF concentration of 200 ng/ml the IL-1α mRNA levels were not increased, and IL-1β mRNA levels increased to a lesser degree than at lower EGF concentrations (Fig. 3 *A–C*). EGF at 1.0 ng/ml increased the IL-6 mRNA level four-fold (Fig. 4 *A* and *B*). In contrast to IL-1α and IL-1β, an increase in the level of IL-6 mRNA above the control level was sustained even at a concentration of 200 ng/ml of EGF (Fig. 4 *A* and *B*). As shown in Fig. 5, EGF induced a transient increase in mRNA levels for all three cytokines. Increases in mRNA levels of IL-1α, IL-1β, and IL-6 were observed as early as 1 h after the addition of EGF (Fig. 5). An optimal increase in IL-1α and IL-6 mRNA was observed 1 h after the addition of EGF, while an optimal increase in IL-1β levels was observed 5 h after EGF addition. By 24 h, the mRNA levels for the three cytokines returned to baseline levels (Fig. 5). In the same kinetic studies, a parallel increase in biological activity of IL-1 and IL-6 in TE cell culture supernatants was also observed (Fig. 6 *A* and *B*). IL-1 biological activity, as determined by the D10S proliferation assay, increased at 1 h and reached an optimal level 10 h after EGF addition (Fig. 6 *A*). IL-1 activity returned to baseline levels after 24 h. Similar to IL-1 biological activity, IL-6 biological activity, determined by the T1165 proliferation assay, increased at 1 h following EGF stimulation of TE cells. However, optimal IL-6 activity was achieved at 5 h, and remained elevated 24 h after the addition of EGF (Fig. 6 *B*). We also demonstrated that the EGF concentrations (1.0 and 5.0 ng/ml) which increased
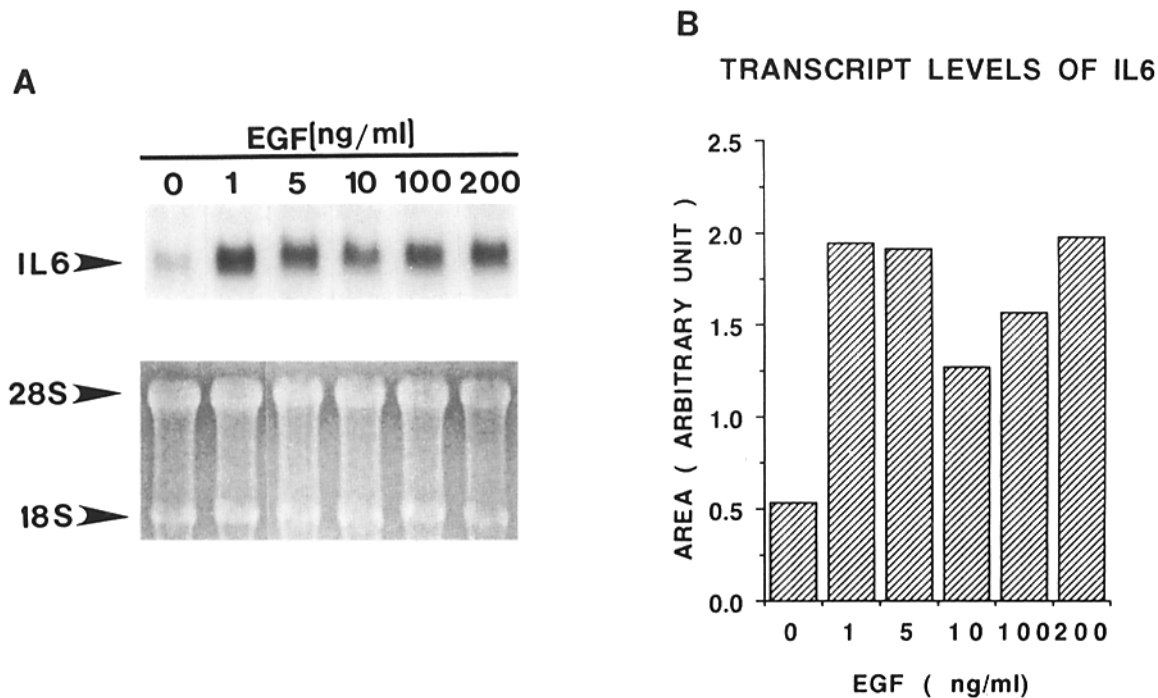
B

TRANSCRIPT LEVELS OF IL6

A

Figure 4. EGF increased mRNA levels for IL-6. RNA blot analysis of total RNA for IL-6 mRNA levels from the same experiment described in Fig. 5.
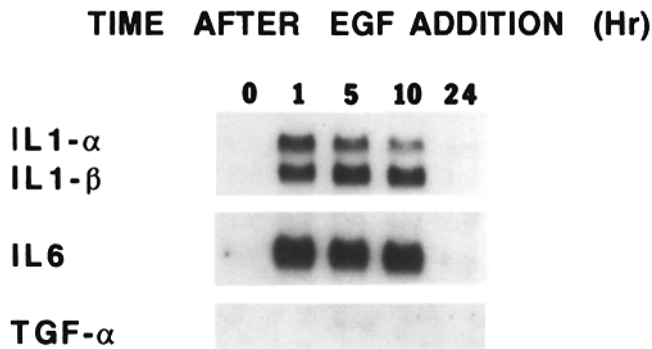
TIME AFTER EGF ADDITION (Hr)

Figure 5. EGF increased the levels of mRNA for IL-1α, IL-1β, and IL-6 in a transient fashion. TE cells were treated with 5.0 ng/ml of EGF for various time periods, the cells were then lysed and total RNA extracted for RNA blot analysis.
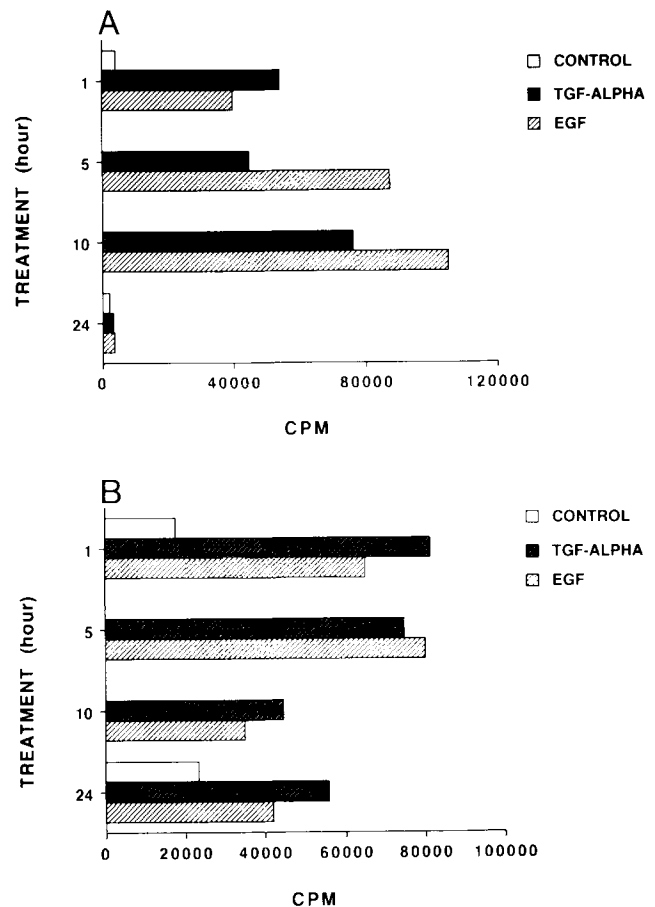

Figure 6. EGF and TGF-α induced a time dependent increase in IL-1 (A) and IL-6 (B) activity in TE cell supernatants. TE cell culture supernatants were collected at various time points after the addition of EGF and assayed for IL-1 activity in the D10S proliferation assay, and for IL-6 activity in the T1165 proliferation assay. Proliferation was measured by the incorporation of ³H-thymidine after a 4-h pulse. All samples were measured in triplicate.

cytokine mRNA levels and biological activities were not mitogenic for TE cells. A mitogenic effect of EGF on TE cells was observed at 10–200 ng/ml of EGF (data not shown).

Transforming Growth Factor α (TGF-α) Is Produced by Thymic Epithelial (TE) Cells, Is Regulated by Epidermal Growth Factor (EGF) and Can Increase TE Cell mRNA Levels of IL-1α, IL-1β, and IL-6. We also demonstrated that human TE cells in vitro produce TGF-α mRNA (Fig. 5 and 7). Optimal increase in TGF-α mRNA level was observed at 5 h after EGF treatment (Fig. 5). The level of TGF-α mRNA in TE cells increased in a concentration dependent fashion when TE cells were treated with EGF. A three- to four-fold increase in the level of TGF-α mRNA in TE cells was obtained between 5.0–100 ng/ml of EGF (Fig. 7 A and B). Since TGF-α also binds to EGF-R, we determined the effect of TGF-α on the levels of IL-1α, IL-1β, IL-6 mRNA, and on its own mRNA level in cultured TE cells. TGF-α induced a transient increase in IL-1α, IL-1β, and IL-6 mRNA levels, and as well IL-1 and IL-6 biological activity with kinetics similar to those observed with EGF (Fig. 8, Fig. 6 A and B). Increases in IL-1α, IL-1β, and IL-6 mRNA levels were observed as early as 1 h after the addition of TGF-α. Optimal increases in mRNA levels were seen at 1 h for IL-1α, and at 5 h for IL-1β and IL-6 (Fig. 8). TGF-α however did not increase its own mRNA level in TE cells (Fig. 8). Indirect immunofluorescence assay of acetone-fixed frozen sections of human thymus showed that TGF-α was present in medullary TE cells and in Hassall's bodies of the thymus medulla (Fig. 9).

Epidermal Growth Factor (EGF) and Transforming Growth Factor α (TGF-α) Increase mRNA Levels for IL-1α, IL-1β, and IL-6 At A Posttranslational Level. We next determined the effect of EGF and TGF-α on the rates of transcription of IL-1α, IL-1β, and IL-6 genes. Using an in vitro nuclear transcription assay, we demonstrated that the rates of transcription of IL-1α, IL-1β, and IL-6 genes in nuclei isolated from EGF and TGF-α treated TE cells were identical to those from untreated TE cells (Fig. 10). The transcription rate of the TGF-α gene was also not altered by either EGF or TGF-α treatment (Fig. 10). These data indicated that EGF and TGF-α did not initiate new transcription, but rather suggested that EGF and TGF-α increased the steady-state levels of cytokine mRNA levels by a posttranscriptional mechanism involving mRNA stability. Therefore, we determined the effect of EGF and TGF-α on cytokine mRNA stability following treatment of TE cells with either EGF plus actinomycin-D or TGF-α plus actinomycin-D. If EGF and TGF-α regulated stability of IL-1α, IL-1β, and IL-6 mRNA, we expected that the stability of mRNA at two different time points in the course of EGF or TGF-α treatment would be different since EGF and TGF-α mediated increases in mRNA levels were transient. We showed that the stability of IL-1α and IL-1β mRNA was much higher at 1 h after EGF (Fig. 11 A, B, and G) or TGF-α (Fig. 11 D, E, and H) treatment than at 5 h after EGF or TGF-α treatment, while optimal stability of IL-6 was obtained at 5 h after EGF (Fig. 11 C and G) or TGF-α treatment (Fig. 11 F and H). An additional increase in the IL-1α and IL-1β mRNA levels was also observed when TE cells were treated
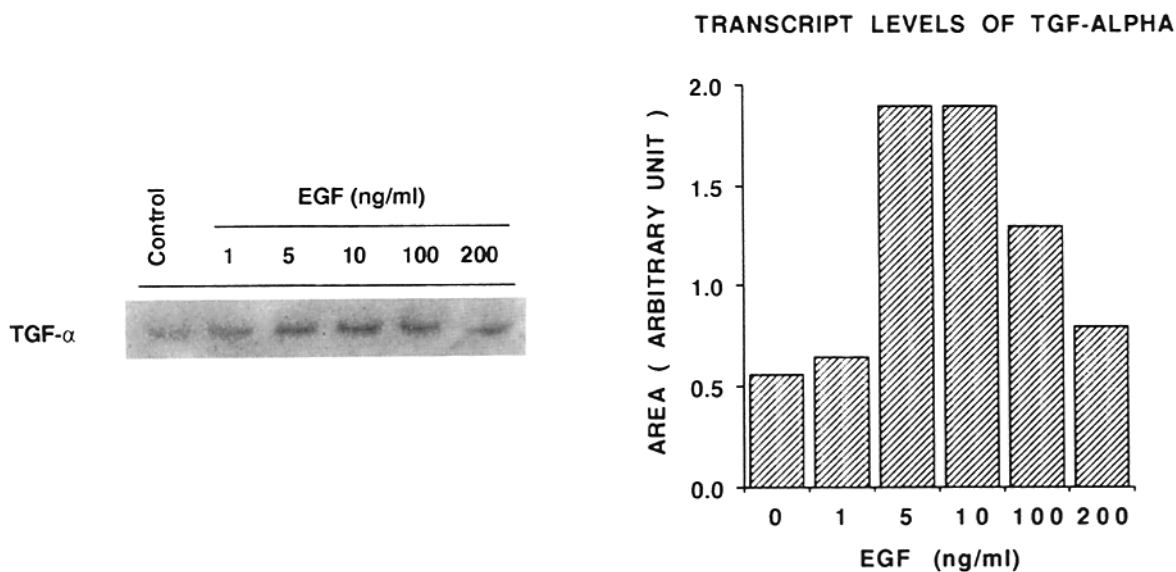
TRANSCRIPT LEVELS OF TGF-ALPHA

Figure 7. Effect of EGF on the level of TGF-α. TE cells were treated with EGF at various concentrations for 5 h, the cells were lysed, total RNA isolated and analyzed for TGF-α mRNA levels by RNA blot. (*A*) Actual levels of TGF-α mRNA. (*B*) Laser scanning densitometer tracings of the TGF-α bands shown in *A*.

with either EGF or TGF-α plus actinomycin-D (Fig. 11 *A*, *B*, *D*, *E*, *G* and *H*). This phenomenon, termed "superinduction," has been described previously for interferon and for IL-1 when the cytokine producing cells were treated with the combination of cycloheximide and actinomycin-D (44, 45). Superinduction of IL-6 mRNA was not observed when TE cells were treated with either EGF or TGF-α plus actinomycin-D (Fig. 11 *C*, *F*, *G*, and *H*). These results confirmed that in human TE cells, EGF and TGF-α regulated the mRNA levels for IL-1α, IL-1β and IL-6 at a posttranscriptional level by increasing stability of mRNA.

Discussion

Although transcription is an important event in regulation of gene expression, control of cytoplasmic mRNA turn-over at a posttranscriptional level is also critically important in establishing the final level of proteins. The turnover rate of a given mRNA determines the steady-state level of mRNA in cytoplasm and is affected by mRNA stability. Stable mRNA species that have a long half-life often encode proteins required for maintaining basic cellular functions, while unstable mRNA species with a short half-life are inducible by internal stimuli or external growth conditions (27, 38, 44, 45). Stability of mRNA is known to play an important role in controlling expression of cytokine, lymphokine and proto-oncogene genes that are responsive to changes in growth conditions (27, 38, 46-48).

In this paper, we document for the first time that the potent growth factors EGF and TGF-α affect the stability of mRNAs for three human TE cell-derived cytokines, IL-1α, IL-1β, and IL-6. The effect of EGF and TGF-α on cytokine mRNAs is immediate and transient with optimal response
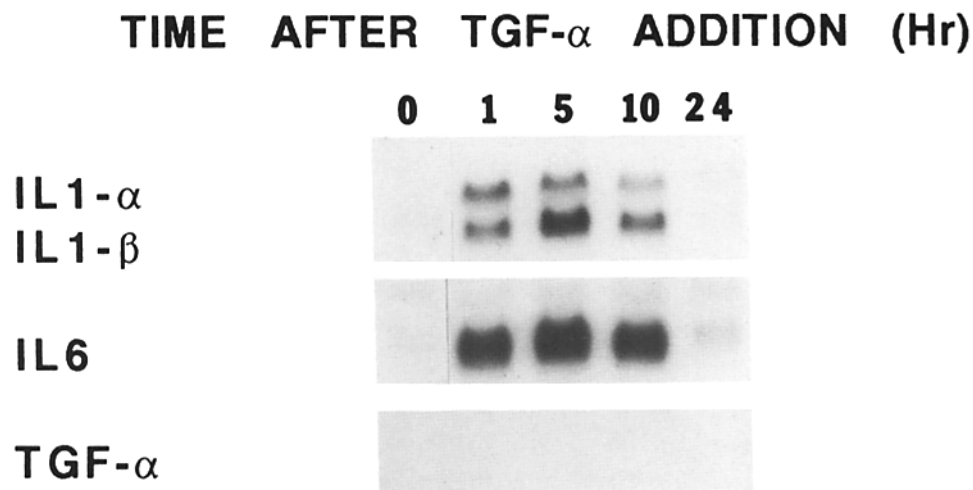
TIME AFTER TGF-α ADDITION (Hr)

Figure 8. TGF-α increased the levels of IL-1α, IL-1β, and IL-6 but not TGF-α in transient fashion. TE cells were treated with 100 ng/ml of human recombinant TGF-α for 5 h and total RNA analyzed for cytokine mRNA levels.
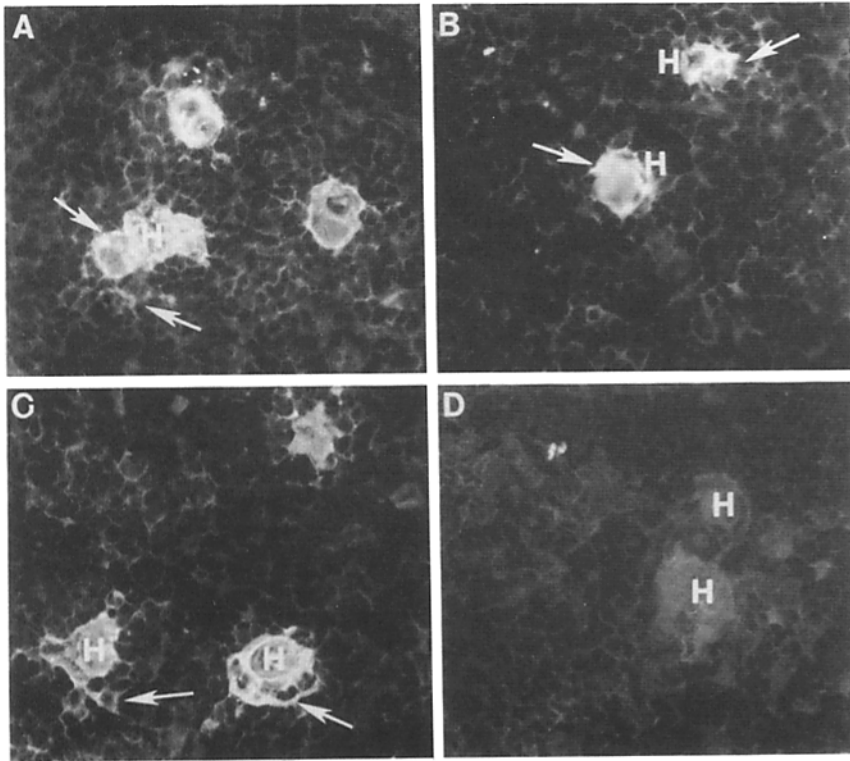
Figure 9. Detection of TGF-α in human thymus by indirect immunofluorescence assay. Acetone-fixed frozen human thymic sections were stained with a purified polyclonal chicken anti–human TGF-α antibody at 30 μg/ml (IgG) (*A*, *B*, and *C*) or a purified IgG fraction from normal chicken serum at the same concentration (*D*). *Arrows* indicate cells stained for TGF-α. *H* indicates Hassall's body.

TRANSCRIPTION RATE OF

CONTROL   EGF TREATED   TGF-α TREATED
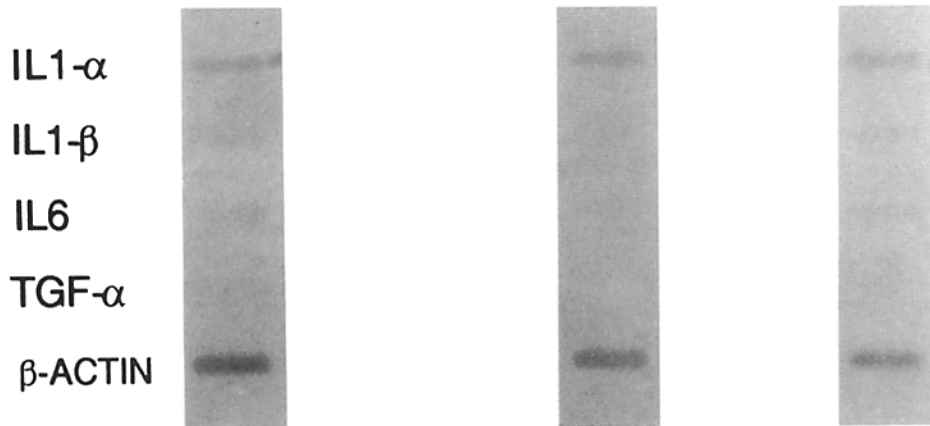

IL1-α
IL1-β
IL6
TGF-α
β-ACTIN

Figure 10. Effect of EGF and TGF-α on transcription rates of IL-1α, IL-1β, IL-6 and TGF-α genes. Nuclei were isolated from untreated TE cells or cells treated with EGF (5.0 ng/ml) or TGF-α (100 ng/ml) for 1 h and used in an in vitro nuclear transcription assay. An excess amount of plasmid (5.0 μg/slot) containing cDNA for IL-1α, IL-1β, IL-6 and TGF-α was used.
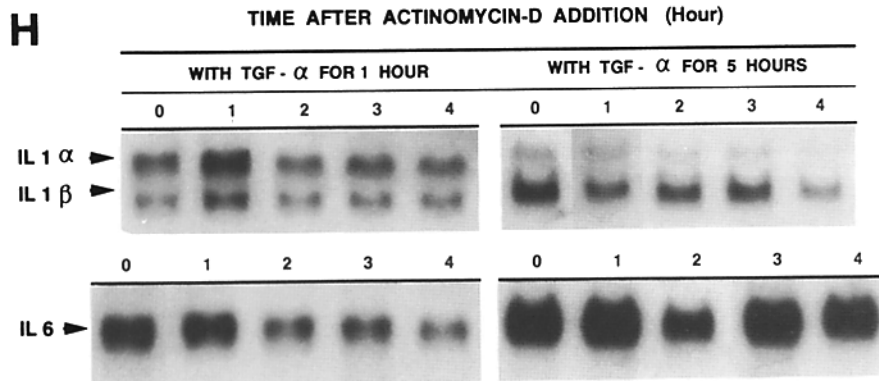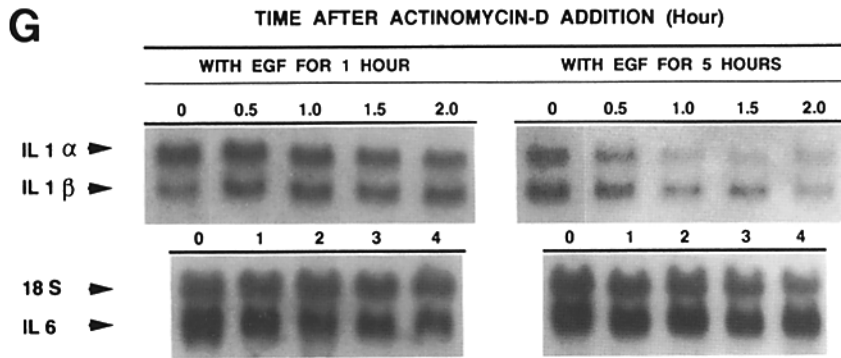
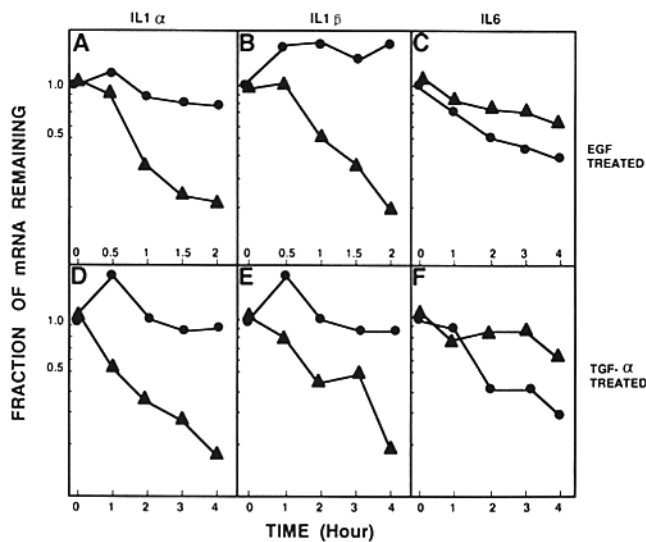**Figure 11.** EGF and TGF-α affected stability of IL-1α, IL-1β and IL-6 mRNA. TE cells were either treated with EGF (5.0 ng/ml) (*A*, *B*, *C*, and *G*) or TGF-α (100 ng/ml) (*D*, *E*, *F*, and *H*) for 1 (●) or 5 (▲) h followed by the addition of 10 μg/ml of actinomycin-D. The cells were lysed and total RNA was isolated at 0, 0.5, 1, 1.5, 2, 3, and 4 h after the addition of actinomycin-D and analyzed by RNA blot. *A–C* and *D–F* were graphic presentation of *G* and *H*, respectively. The Y-axis represented the ratios of the levels of mRNA at 0.5, 1, 1.5, 2, 3, and 4 h over the levels of mRNA at 0 h after the addition of actinomycin-D.

observed between 1 and 5 h after the addition of EGF or TGF-α. Since Marzluff and Pandy (49) reported that increases in stability of a number of mRNA species correlate with cell cycle, we compared the concentration of EGF required to induce TE cell proliferation with the concentration of EGF that affected the stability of cytokine mRNA. The minimal concentration of EGF (1.0 ng/ml) that affected the level of cytokine mRNAs was 10-fold less than that which induced TE cell proliferation (10 ng/ml) (data not shown). Thus, the

data strongly suggest that the effect of EGF on the levels of cytokines is not related to the cell cycle or TE cell growth.

Differences in the effect of EGF and TGF-α on IL1α, IL1β and IL6 mRNA levels were observed in the presence of the RNA synthesis inhibitor actinomycin-D. A superinduction effect was observed with IL1α and IL1β when TE cells were treated with either EGF or TGF-α plus actinomycin-D; however, the combination of actinomycin-D plus EGF or TGF-α treatment did not show a superinduction effect for IL6. The

superinduction phenomenon has been shown for interferon (44) and IL1 (45) by actinomycin-D and cycloheximide treatment. Cycloheximide alone has also been shown to affect mRNA stability (46, 48, 50, 51). It has been postulated that cycloheximide affects cytoplasmic mRNA levels by inhibiting protein synthesis of labile factors that promote mRNA degradation (48, 50, 52). The similar effect of EGF or TGF-α and cycloheximide on cytoplasmic mRNA stability suggests that EGF or TGF-α may also target similar labile regulatory factors that are involved in the regulation of IL-1α, IL-1β and IL-6 mRNA stability, possibly by inhibiting the mRNA stability regulatory function of these labile factors. Since EGF and TGF-α required 1 h to induce mRNA stability for IL-1α and IL-1β and 5 h for IL-6, the time course difference in inducing mRNA stability suggests that IL-1α, IL-1β and IL-6 are independently regulated by at least two different factors; a labile factor that affects IL-1α and IL-1β mRNA stability, and a relatively more stable factor that affects IL-6 mRNA stability.

Control of mRNA stability involves both RNA specific sequence and RNA binding proteins (53). A conserved A+U rich sequence in the 3' untranslated region (UTR) has been identified among labile mRNA for cytokines, lymphokines and proto-oncogenes (27, 54). It has been shown that for IL-6 and GM-CSF, the A+U rich region is required for mRNA stability (27, 55, 56). Recently, Malter has identified a cytoplasmic protein that binds specifically to the AUUUA element (56). The resultant RNA-protein complexes have been proposed to target the susceptible mRNA for rapid cytoplasmic degradation (56). Whether EGF and TGF-α mediate cytokine mRNA stability by affecting the function of an AUUUA

binding protein is not known. However, phosphorylation by a protein kinase has been shown to increase stability of TNF mRNA (57). Since both EGF and TGF-α can induce tyrosine phosphorylation via the EGF-R protein tyrosine kinase, it is conceivable that EGF and TGF-α regulate TE cell-derived cytokine mRNA stability by inducing tyrosine phosphorylation of an RNA-binding protein, thereby inhibiting the formation of the RNA-protein complexes and preventing rapid mRNA degradation.

The demonstration in this study that both EGF and TGF-α can affect TE cell-derived IL-1α, IL-1β and IL-6 mRNA and protein levels implies a critical role for EGF and TGF-α in regulating intrathymic T cell growth and development. In humans, IL-1 synergizes with GM-CSF in stimulating proliferation of immature human thymocytes (58). In both humans and mice, IL-1 together with IL-6 preferentially induces CD4$^+$ T cell proliferation (59, 60). IL-6 by itself selectively induces only CD8$^+$ T cell proliferation (61), and in combination with IFN-γ and IL-2 induces differentiation of cytotoxic T cells from immature thymocytes (62). TE cells express EGF-R in vivo, and TGF-α is present in vivo in medullary TE cells. We were unable to detect EGF in thymus by indirect immunofluorescence assay using a monoclonal antibody raised against human EGF (data not shown). Since TGF-α is present in human thymus, and EGF is present in rat thymus (63), and both TGF-α and EGF regulate the production of important cytokines for T cell proliferation, we postulate that both EGF and TGF-α may play a critical regulatory role in the production of cytokines by TE cells in vivo during intrathymic T cell development.

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