Induction of T Regulatory Cells by Cytotoxic T-Lymphocyte Antigen-2α on Corneal Endothelial Cells

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PURPOSE. To determine whether murine corneal endothelial (CE) cells can promote the generation of T regulatory (Treg) cells in vitro.

METHODS. To induce Treg cells in vitro by CE cell lines, T cells exposed to CE cells were used as Treg cells. T cells exposed to CE cells in the presence of anti–mouse CD3 antibody were harvested and added to target bystander T cells in vitro. T-cell activation was assessed for proliferation by [³H]-thymidine incorporation. Expression of CD25 or Foxp3 on Treg cells was evaluated by flow cytometry. Expression of cytotoxic T-lymphocyte antigen-2 alpha (CTLA-2α) on CE cells was evaluated by flow cytometry, RT-PCR, immunohistochemistry, or in situ hybridization. Anti–CTLA-2α neutralizing antibodies, CTLA-2α siRNA, or pro-cathepsin L blocking proteins were used to abolish the CE-inhibitory function.

RESULTS. Cultured CE cells produced CTLA-2α on their surfaces, thereby enabling bystander CD4$^+$ T cells to be converted to Treg cells by TGFβ promotion. CE-induced Treg cells had immunosuppressive capacities by highly expressing CD25^{high} and Foxp3. When mRNA downregulation (siRNA transfection), neutralizing antibodies, or blocking proteins were used to block CTLA-2α expression on CE cells, CE-induced Treg cells failed to acquire Treg function.

CONCLUSIONS. These findings indicate that cell surface CTLA-2α contributes to the CE-dependent suppression of bystander T cells. Thus, ocular resident tissue-exposed T cells can be induced to become regulators within the peripheral microenvironment. (*Invest Ophthalmol Vis Sci.* 2011;52:2598–2605) DOI:10.1167/iovs.10-6322

Cysteine proteinases are widely distributed in a variety of biological tissues/cells and fluids, where they are involved in the process of intracellular and extracellular protein degradation and turnover and, therefore, in disease-related tissue remodeling.[1,2] Cytotoxic T-lymphocyte antigen-2 alpha (CTLA-2α), which is a cysteine proteinase inhibitor, was originally discovered and expressed in mouse activated T cells and mast cells.[1] Structurally, CTLA-2α is highly similar to the proregions of cysteine proteinase on mouse cathepsin L.[3,4] In addition, CTLA-2α has been shown to exhibit selective inhibition of mouse cathepsin L-like cysteine proteinases.[4] The propeptide cysteine proteinase proregion is part of the enzyme, but CTLA-2α is expressed independently and is designated a member of the 129 proteinase inhibitory family (MEROPS database, assigned code 129.002; http://merops.sanger.ac.uk/). Thus, though CTLA-2α functions as a cathepsin L inhibitor, little is known of its other functions.

Recently, we reported that retinal pigment epithelial (RPE) cells from the posterior segment in the eye convert CD4$^+$ T cells to T regulatory (Treg) cells in vitro and in vivo.[5–7] RPE cells produce and secrete CTLA-2α, thereby enabling bystander T cells to be converted to Treg cells by TGFβ promotion.[6,7] RPE CTLA-2α functions as a TGFβ promoter and cathepsin L inhibitor in the eye. Cathepsin L-inhibited T cells eventually convert to Treg cells that acquire regulatory functions. Thus, eye-derived immunosuppressive factors, such as CTLA-2α and TGFβ, are upregulated when intraocular inflammation occurs in immunologically privileged sites such as the eyes. In fact, CTLA-2 is most strongly expressed in the brain, the placenta, and the eyes.[6,8,9] These organs and tissues are considered immune-privileged sites.[10,11]

Little is known of the tissue locations and physiological functions of CTLA-2α proteins in the eye. In our previous report,[6] protein expression on immunohistochemistry was distributed on the corneal endothelium, which contributed to immune tolerance in the anterior segment of the eye. In addition, corneal endothelial (CE) cell lines from human CE primary cultures suppress T-cell activation by cell-surface molecules.[12] Moreover, T cells exposed to human CE cells (human CE-induced Treg cells) can suppress bystander effector T cells through the production of TGFβ.[13] However, the actual immunosuppressive mechanism of murine CE cells and CE-induced Treg cells remains unclear. Therefore, the present study was designed to clarify the role of the murine corneal endothelium-derived immunosuppressive factor CTLA-2α and to clarify how it converts T cells to regulatory T cells. We investigated whether CE-induced Treg cells express CD25 and Foxp3, and we also determined whether CTLA-2α produced by CE cells is able to promote intraocular TGFβ activation.

MATERIALS AND METHODS

Preparation of CE Cell Lines

Simian virus 40 (SV40)-transformed mouse CE cell lines were kindly provided by J. Wayne Streilein (Harvard Medical School).[14] CE cells were cultured in complete Dulbecco's modified Eagle's medium plus

10% fetal bovine serum.[15,16] The Institutional Animal Research Committee of Tokyo Medical and Dental University approved all the experiments, and animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Target Cells

Adult C57BL/6 mice purchased from CLEA Japan Inc. (Tokyo, Japan) were used for obtaining lymphoid cells. Pan-T cells, CD4^{+} T cells, and CD8^{+} T cells were prepared separately using cell isolation kits (MACS; Miltenyi Biotec, Auburn, CA). These cells were found to be >94% CD3^{+}, CD4^{+}, or CD8^{+}. CE cells were cultured separately in 96-well plates (1×10^3 to 1×10^5 cells/well) or in 24-well plates (2×10^5 cells/well). After stimulating these target T cells (2.5×10^5 cells/well) with anti–mouse CD3 (1 μg/mL, clone 2C11; BD PharMingen, San Diego, CA) for T cells, the cells were incubated for 72 hours. After incubation, the cultures were assayed for cell proliferation by measuring the uptake of [³H]-thymidine.

Induction of CE-Induced Treg Cells

CD4^{+} or CD8^{+} T cells exposed to CE (CE-induced Treg cells) in the presence of anti–mouse CD3 antibody (0.1 μg/mL) were harvested, γ-irradiated (20 Gy), and added (1×10^5 cells/well) to 96-well plates containing fresh pan-T cells (1×10^5 cells/well) and anti–CD3 antibody (1.0 μg/mL). To evaluate Treg cell suppression, target T-cell proliferation in the presence of CE-induced Treg cells was measured by the uptake of [³H]-thymidine. In separate experiments, anti–mouse CTLA-2α neutralizing antibody, anti–TGFβ neutralizing antibody (clone 1D11; R&D Systems, Minneapolis, MN), or pro-cathepsin L (proCathL) blocking proteins was used for the Treg assay, as previously reported.[6]

Flow Cytometry

Flow cytometry was used to analyze the expression of Foxp3 on T cells exposed to CE cells. Before staining, cells were incubated with mouse Fc blocking reagent (Fcγ III/II receptor; BD PharMingen) at 4°C for 15 minutes. After permeabilization, the cells were stained with fluorescein isothiocyanate (FITC)-labeled anti–mouse Foxp3 (eBioscience, San Diego, CA) or with isotype rat control antibody and phycoerythrin (PE)-conjugated anti–mouse CD4 antibodies (BD PharMingen) at 4°C for 30 minutes. To examine the expression of CD25 on T cells exposed to CE, CD4^{+} T cells were added to culture wells in the presence or absence (control T cells) of cultured CE cells. T cells were harvested and stained with FITC-conjugated antibody to CD25 (IL-2Rα; eBioscience) and PE-conjugated antibody to CD4.

Flow cytometry was also used to analyze the expression of CTLA-2α or TGFβ on CE cells. To establish an anti–mouse CTLA-2α antibody, antiserum against CTLA-2α was obtained by immunizing rabbits with purified rCTLA-2α, as described previously.[6] Before staining, CE cells were incubated with a mouse Fc block at 4°C for 15 minutes. After permeabilization, cells were stained with rabbit anti–mouse CTLA-2α antibody or rabbit IgG (isotype control) at 4°C for 30 minutes. Cells were washed, and bound primary antibody was detected by incubation with biotin-conjugated anti–rabbit IgG (BD PharMingen) at 4°C for 30 minutes, followed by FITC-conjugated streptavidin (BD PharMingen) at 4°C for 15 minutes. Similarly, CE cells were stained with anti–TGFβ (R&D Systems) or isotype mouse IgG (BD PharMingen), followed by biotin-conjugated secondary antibodies (anti–mouse IgG) and FITC-conjugated streptavidin.

RT-PCR and qRT-PCR

Cellular extracts were prepared from cultured CE cells. Total RNA from CE cells was isolated using reagent (TRIzol; Invitrogen Life Technologies, Carlsbad, CA). Forward and reverse primers for CTLA-2α and GAPDH and PCR conditions were as described previously.[6] PCR products were separated by electrophoresis on a 2%

agarose gel and were visualized by staining with ethidium bromide. CTLA-2α mRNA levels were normalized against GAPDH. qRT-PCR analysis was conducted using different primers for CTLA-2α. Expression of CTLA-2α was analyzed by qRT-PCR using an RT-PCR kit (SYBR ExScript; Takara, Tokyo, Japan) on a real-time PCR instrument (LightCycler; Roche, Basel, Switzerland), and primers were as described previously.[6]

Immunohistochemistry

Cultured CE cells were grown on coverslips for 3 days. After washing, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and were permeabilized with 0.1% Triton X-100. Cells were then incubated with 1:100 anti–CTLA-2α or isotype control antibody (rabbit IgG) for 1 hour, followed by Alexa Fluor 488–conjugated anti–rabbit antibody (Invitrogen Life Technologies). For TGFβ, CE cells were incubated with 1:200 anti–TGFβ or isotype control antibody (mouse IgG) on overnight culture, followed by Alexa Fluor 488-conjugated anti–mouse antibody (Invitrogen Life Technologies). Fluorescence signals were detected by confocal microscopy.

Transfection of CTLA-2α with siRNA

siRNA for murine CTLA-2α was designed with siDirect (http://design.RNAi.jp/) and was used as reported previously.[6] Transient transfection was carried out on day 1 of culture in 500 μL transfection medium, which consisted of serum-free medium, 1.5 μL siRNA transfection reagent (INTERFERin; Funakoshi, Tokyo, Japan), and 1 or 10 nM siRNA. On day 3, cells were harvested and examined by qRT-PCR for CTLA-2α mRNA expression.

Measurements of TGFβ

Concentrations of the active form of TGFβ in CE cell supernatants (CTLA-2α-siRNA–transfected, CTLA-2α-overexpressing, or anti–CTLA-2α abs-treated CE cells) were measured with mouse TGFβ1 ELISA (R&D Systems). For CTLA-2α overexpression in cultured CE cells, mouse CTLA-2α-pIRES-eGFP vector was synthesized and used with transfection reagent, as reported previously.[6]

In Situ Hybridization

Albino ICR or black C57BL/6 mice were perfused with Bouin's solution through the left ventricle. Sampled eyes were dehydrated in a graded series of ethanol, incubated in xylene, and embedded in paraffin wax. Transverse sections (4 μm) were mounted onto poly-L-lysine–coated slides. Sections were fixed as reported previously[6] and were hybridized overnight at 45°C in 50% formamide, 25% 20× SSC, 2% 50× Denhardt's solution, 100 U/mL heparin sodium, 10% dextran sulfate, and 1.5 μg/mL CTLA-2 sense or antisense cRNA probe. RNA signals were detected with an alkaline phosphatase-conjugated anti–digoxin antibody using a nucleic acid detection kit (DIG; Roche Diagnostics, Tokyo, Japan).

Western Blot Analysis

CE cells and control RPE cells were washed in PBS twice and suspended in RIPA buffer (Thermo-Scientific, Rockford, IL) for 5 minutes at 4°C and then centrifuged at 15,000 rpm for 15 minutes. Protein concentration in the supernatant was detected using the DC protein assay kit (Bio-Rad, Hercules, CA). Total protein (20 μg) in the samples was separated on 15% SDS-polyacrylamide gels and then transferred to PVDF membranes. Immunodetection was carried out using anti–mouse CTLA-2α antibodies diluted 1:100 in 2% enhanced chemiluminescence (ECL) blocking solution. For detection assay, 5% ECL blocking agent in TBS-T for 1 hour at room temperature was used, followed by washing of the membrane in TBS-T and incubation with primary antibody at 4°C overnight. After incubation, the membrane was washed for 10 minutes three times with TBS-T followed by incubation with ECL HRP-conjugated anti–mouse secondary antibody (GE Healthcare, Buckingham-

shire, UK) diluted 1:5000 in TBS-T for 90 minutes at room temperature. The membrane was washed for 30 minutes three times in TBS-T and then processed for detection with chemiluminescent substrate (West Pico; Thermo-Scientific) using a charge-coupled device camera (LAS-4000; Fuji-Film, Tokyo, Japan).

Statistical Analysis

Each experiment was repeated at least twice with similar results. Statistical analyses for parametric data were performed using Student's *t*-test. Nonparametric data were analyzed by the Mann-Whitney U test. Values were considered statistically significant at $P < 0.05$.

RESULTS

Induction of Treg Cells by CE Cells

Cultured human CE cells are able to convert T cells to Treg cells in vitro.[13] We examined the capacity of T cells exposed to murine CE cells (CE-induced Treg cells) to suppress bystander T-cell proliferation after they were added to secondary cultures containing target T cells plus anti–CD3 antibodies. CD4^{+} CE-induced Treg cells were found to profoundly suppress T-cell proliferation by target pan-T cells (Fig. 1A). Similarly, CE-exposed CD8^{+} T cells significantly suppressed the activation of bystander target T cells (Fig. 1A). In contrast, control T cells
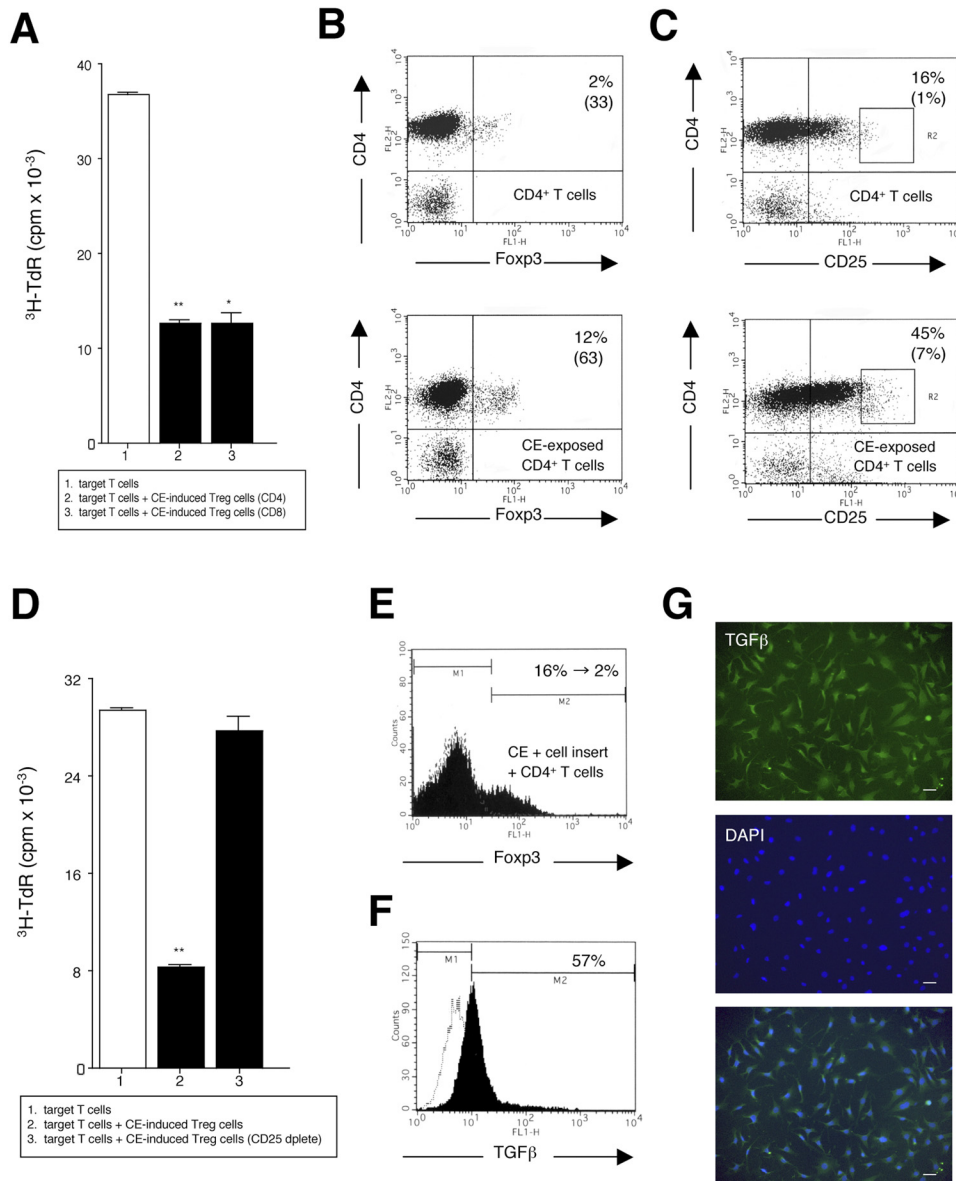

FIGURE 1. Capacity of CE cells to induce Treg cells. (**A**) Purified CD4^{+} or CD8^{+} T cells (1×10^6/well in a 24-well plate) were cultured with CE cells for 24 hours in the presence of anti–CD3 (0.1 μg/mL), harvested, and then used as Treg cells (CE-induced Treg cells). CE-induced Treg cells were added to cultures containing responder target pan-T cells plus anti–CD3 (1.0 μg/mL). *Open bars*: target T cells alone (positive control). After 72 hours, cultures were assayed for the uptake of [³H]-thymidine. *$P < 0.05$, **$P < 0.005$, compared with the positive control. (**B**) After CE-induced Treg cells (CE-exposed CD4^{+} T cells) were stained with FITC-conjugated anti–mouse Foxp3 and PE-conjugated anti–mouse CD4, they were analyzed by flow cytometry. As a control, CD4^{+} T cells in the presence of anti–CD3 without CE were also stained (*top histogram*). Numbers in histograms indicate the percentage of positive cells, and numbers in parentheses indicate mean fluorescence intensity. (**C**) CE-exposed CD4^{+} T cells (*bottom histogram*) or control CD4^{+} T cells (*top histogram*) were stained with FITC-conjugated anti–mouse CD25 and PE-conjugated anti–CD4 antibodies. The R2 gate in the right corner indicates the population of CD25^{high}. Numbers in histograms indicate the percentage of positive cells, and numbers in parentheses indicate the population of CD25^{high} cells. (**D**) Effect of CD25^{+} cell depletion on Treg generation in the presence of cultured CE cells. The CD25 population was depleted using a CD4^{+}CD25^{+} regulatory T-cell isolation kit after culture with CE cells, and cells were added to secondary cultures containing target T cells and anti–CD3. After 72 hours, cultures were assayed for uptake of [³H]-thymidine. Mean ± SEM cpm for triplicate cultures is presented. Asterisks indicate significant differences (**$P < 0.005$) comparing positive control cultures (target T cell only; *open bar*). (**E**) CE cells were first cultured separately in 24-well culture plates, and transwell cell inserts were then placed into these wells. Each transwell contained CD4^{+} T cells plus anti–CD3. After the T cells were harvested, the expression of Foxp3 on T cells was examined. CE-exposed T cells were stained with FITC-labeled anti–Foxp3 abs (*black histogram*), and the CE-exposed T cells in the presence of the cell insert membrane were also stained with anti–Foxp3 abs (*open histogram*). Values on the *left* indicate the percentage of Foxp3-positive cells by CE-exposed T cells (16%), and values on the *right* indicate the percentage of positive cells by CE-exposed T cells in the presence of the cell insert membrane (2%). (**F**) CE cells were analyzed by flow cytometry for surface expression of TGFβ. Cells were stained with anti–TGFβ or an isotype mouse IgG (*dotted histogram*), followed by biotin-conjugated secondary antibodies and FITC-conjugated streptavidin. Numbers indicate percentage-positive cells for TGFβ. (**G**) CE cells were stained with anti–TGFβ and were examined by fluorescence microscopy. CE cells strongly expressed TGFβ on the cell surface. Examination by fluorescence (*green*; *top*), DAPI nuclear staining (*blue*; *middle*), and combined (*bottom*) microscopy of the same image. Scale bar, 40 μm.

without CE cells did not suppress this activation (data not shown). These results suggest that murine CE cells are able to convert these T cells into Treg cells in vitro.

On flow cytometric analysis of Foxp3, CD4$^+$ T cells exposed to CE cells expressed much higher levels of intracellular Foxp3 (12% positive) than did control CD4 T cells (only 2% positive; Fig. 1B). These results indicate that T cells exposed to CE cells express Foxp3, which is one of the best Treg cell markers found to date.

We next examined whether CE-induced Treg cells, which suppress target bystander T cells, express CD25 molecules. As shown in Figure 1C, CD4$^+$ T cells exposed to CE cells strongly expressed CD25 (45% positive), particularly CD25^{high} (7% positive), compared with control CD4$^+$ T cells; control T cells were 16% CD25 positive and only 1% CD25^{high} positive (Fig. 1C). For the in vitro assay, we prepared CD25-depleted CE T cells by culturing CD4$^+$ T cells with CE in primary culture, harvesting them, and then removing CD25$^+$ cells. We found that CD4$^+$ CE-induced Treg cells significantly suppressed the proliferation of target T cells, but CD25-negative CE-induced Treg cells (CD25$^+$ cell depletion) did not (Fig. 1D). These results suggest that CD4$^+$CD25$^+$ Treg cells assist T cells exposed to CE cells to suppress bystander T-cell activation.

To elucidate the role of cell-to-cell contact in CE-mediated Treg cell induction, cells were first cultured separately in 24-well culture plates. Transwell cell inserts were then placed in these wells, and each transwell contained CD4$^+$ T cells plus anti–mouse CD3 mAb. After harvesting the T cells, the expression of Foxp3 on T cells was examined. CE-exposed T cells expressed Foxp3 in the absence of transwell cell inserts (16% positive; Fig. 1E). In contrast, CE-exposed T cells in the presence of transwell membranes poorly expressed Foxp3 (only 2%). These results suggest that direct cell contact between T cells and CE is essential for the induction of Treg cells.

In previous experiments, we demonstrated that cell-to-cell contact between CE and bystander T cells is required for the induction of Treg cells. We also showed that human CE cells promote Treg cell induction through TGFβ signals.[13] Therefore, we attempted to confirm whether murine CE cells express TGFβ using mouse CE cell lines and anti–TGFβ antibodies. As shown in Figure 1F, mouse CE cells constitutively expressed membrane-bound TGFβ on their surfaces. On immunohistochemistry, CE cells strongly expressed TGFβ on the cell surface (Fig. 1G). Thus, membrane-associated TGFβ on CE cells appears to be required for the induction of Treg cells.

Detection of CTLA-2α by CE Cells

RPE cells from the posterior segment of the eye primarily use soluble inhibitory factors to promote the induction of Treg cells.[5–7] We recently showed that RPE-derived cytotoxic T lymphocyte antigen-2α (CTLA-2α), a novel immunosuppressive factor in the eye, induces Foxp3-positive regulatory T cells.[6,7] Therefore, we examined whether cultured CE cells that can convert T cells to Foxp3$^+$ Treg cells express CTLA-2α. We used immunohistochemistry, flow cytometry, Western blot analysis, quantitative (q)RT-PCR, and in situ hybridization to confirm the expression of CTLA-2 by CE cells. As expected, CTLA-2α was expressed in a patch-like pattern on the surfaces of CE cells, and positive staining was not observed with the isotype control antibody (rabbit IgG; Fig. 2A). CTLA-2α was strongly expressed in CE cells by flow cytometry, yet the expression was poor when the control rabbit isotype was used (Fig. 2B). Western blot analysis clearly detected CTLA-2α protein in CE cells and in control retinal pigment epithelial (RPE) cells (Fig. 2C).

We also examined the localization of CTLA-2α expression in the anterior segment of the eye using in situ hybridization (Fig.
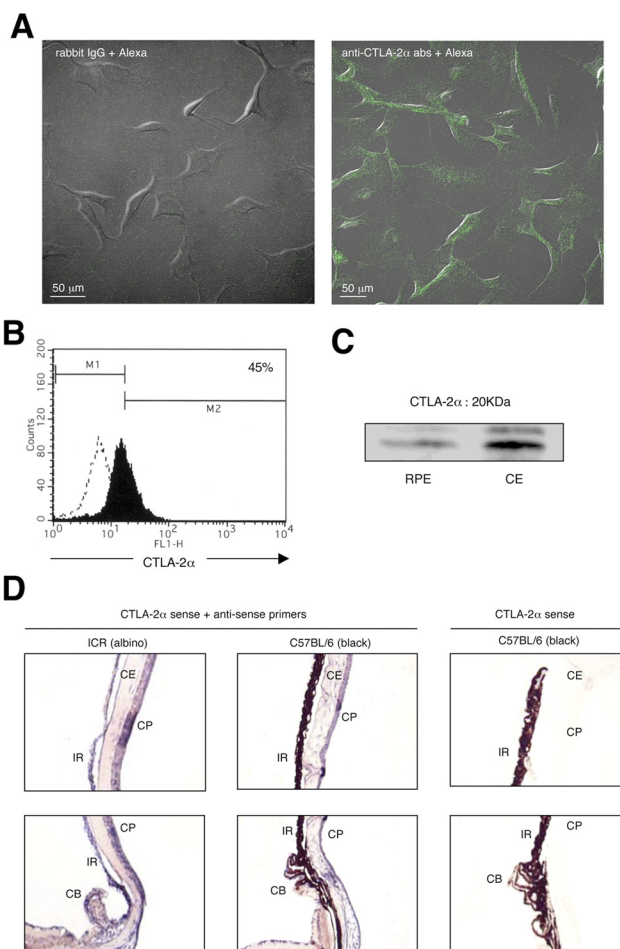

FIGURE 2. Expression of CTLA-2α by CE cells. (**A**) CE cells were stained with anti–CTLA-2α and examined by fluorescence microscopy. CE cells expressed CTLA-2α (*green*) in a discontinuous, patch-like manner on the cell surface (*right*). However, CE cells did not express these molecules when stained with isotype rabbit IgG plus secondary antibody (Alexa anti–rabbit 488, *left*). Scale bar, 50 μm. (**B**) CE cells were analyzed by flow cytometry for intracellular expression of CTLA-2α. Cells were stained with anti–CTLA-2α or an isotype rabbit IgG (*dotted histogram*), followed by biotin-conjugated secondary antibodies and FITC-conjugated streptavidin. (**C**) Western blot analysis was conducted to determine the expression of CTLA-2α proteins in CE cells and in primary cultured retinal pigment epithelium (RPE), which served as the positive control. (**D**) Location of CTLA-2α in the eye as determined by in situ hybridization with specific primer pairs (*left* and *middle*). *Left*: results for albino ICR mice; *middle*: results for C57BL/6 mice. As a control, in situ hybridization with specific sense primer alone was performed (*right*). CB, ciliary body; CE, corneal endothelium; CP, corneal epithelium; IR, iris.

2D). Intense staining was observed on the corneal endothelium and epithelium, iris, and ciliary body. Staining for CTLA-2 was found in both albino ICR and black C57BL/6 mice at similar levels, whereas specific staining was not found in black mice for the negative controls (sense primer only; Fig. 2D). These findings indicate that the murine corneal endothelium constitutively expresses CTLA-2α.

Promotion of TGFβ Production by CTLA-2α on CE Cells

We next examined whether CTLA-2α from CE cells is able to promote TGFβ production because TGFβ is the most important factor for converting Treg cells in the periphery.[17,18] To

investigate the importance of the relationship between CTLA-2α and TGFβ for Treg cell induction by CE cells, we upregulated CTLA-2α expression in CE cells using CTLA-2α overexpression systems. For CTLA-2α overexpression in CE cells, mouse CTLA-2α-pIRES-EGFP vector was synthesized and used with transfection reagent, as reported previously.[6,7] To confirm the expression of CTLA-2α, RT-PCR was used. As shown in Figure 3A, on quantitative PCR, the targeted mRNA was upregulated in CTLA-2α–overexpressing CE cells. Similar results were obtained on semiquantitative RT-PCR using different primers for mouse CTLA-2α (data not shown). Moreover, CTLA-2α–overexpressing CE cells significantly produced TGFβ1 (Fig. 3B). Therefore, CTLA-2α produced by corneal endothelium promotes the activation of TGFβ.

Capacity of CTLA-2α siRNA-Transfected CE Cells to Induce Treg Cells

We recently reported that the production of CTLA-2α in the eye is necessary to mediate the induction of Treg cells.[6,7] To establish whether CTLA-2α produced by CE cells also mediates the induction of eye-specific Treg cells, we examined the effects of downregulating the expression of CTLA-2α mRNA using siRNA. When compared with untreated cells, CTLA-2α siRNA-transfected CE cells expressed less TGFβ1 at the mRNA (Fig. 4A) and protein levels (Fig. 4B). Similarly, anti–CTLA-2α neutralizing antibodies impaired the production of TGFβ1 by RPE compared with the isotype control (Fig. 4B). As shown in Figure 4C, CTLA-2α siRNA-transfected CE cells failed to convert CD4+ T cells to Treg cells. Similarly, anti–CTLA-2α antibodies pretreated with CE cells failed to convert CD4+ T cells to Treg cells (Fig. 4C). We then tested whether CE cells were able to convert T cells to Treg cells if the production of both CTLA-2α and TGFβ by CE cells was blocked. For this assay, CTLA-2α siRNA-transfected CE cells in the presence of anti–TGFβ blocking antibody were used. As expected, the CE cells failed to convert CD4+ T cells to Treg cells in vitro (Fig. 4D), thus suggesting that the CTLA-2α/TGFβ interaction plays an important role in the induction of Treg cells by CE cells.
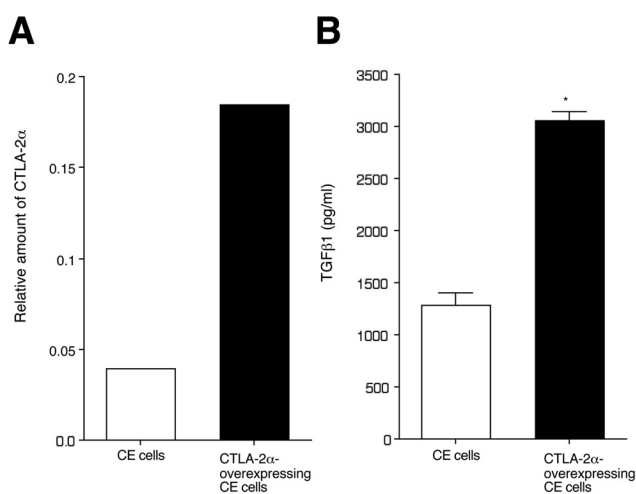
FIGURE 4. Effect of downregulating CTLA-2α expression by siRNA-transfected CE cells. (**A**) CTLA-2α siRNA-transfected CE cells were harvested on day 3 and were examined for the expression of CTLA-2α mRNA by RT-PCR. CE cells were treated with 1 or 10 nM CTLA-2α siRNA. *Top image*: qRT-PCR results; *bottom image*: semiquantitative RT-PCR results with GAPDH. (**B**) CTLA-2α siRNA-transfected (control, transfection reagent only) or anti–CTLA-2α neutralizing abs (control, isotype rabbit IgG)-treated CE cells were cultured for 72 hours. Supernatants from these cells were harvested and examined for the production of TGFβ1 by ELISA. **P < 0.005, compared with two groups. (**C**) CD4+ T cells cultured for 24 hours in the presence of anti-CD3 and CTLA-2α siRNA-transfected CE cells were used as Treg cells (*right*). CE cells were treated with 1 or 10 nM CTLA-2α siRNA. CE-induced Treg cells (*black bars*) were added to secondary cultures with target T cells (*open bar*, responder pan-T cells plus anti-CD3). Anti-CTLA-2α abs-treated CE-induced CD4+ Treg cells were also added to the secondary cultures. As a control, rabbit isotype was used. *P < 0.05, **P < 0.005, compared with two groups. (**D**) CTLA-2α siRNA-transfected CE cells in the presence or absence of anti–TGFβ neutralizing antibody (10 μg/mL) were used for the Treg inhibition assay. Before assay, CE cells were transfected with CTLA-2α siRNA (10 nM). Target T cells indicate responder pan-T cells plus anti-CD3. Error bars represent SEM. *P < 0.05 and **P < 0.005, compared with two groups.

FIGURE 3. Promotion of TGFβ production by CTLA-2α–overexpressing CE cells. (**A**) CTLA-2α–overexpressing CE cells were harvested and examined for the expression of CTLA-2α by qRT-PCR. (**B**) ELISA was performed to measure TGFβ1 in the supernatants of CTLA-2α–overexpressing CE cells. As a control, CE cells were pretreated with the plasmid only, and cells and supernatants were harvested (*open bars*). Error bars represent the SEM. *P < 0.05 vs. control cultures (*open bars*).
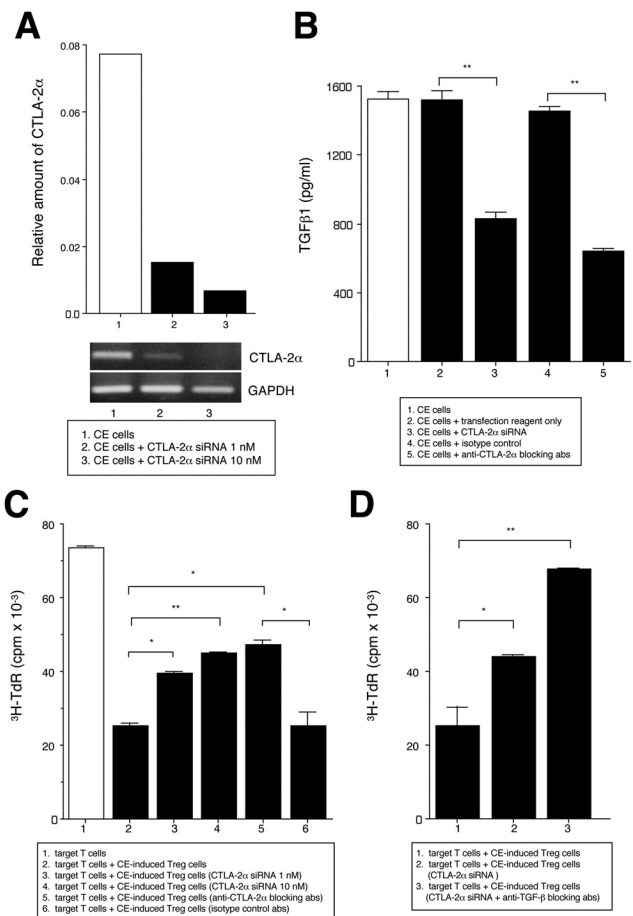
Ability of Cathepsin L Blocking Protein-Treated CE Cells to Induce Treg Cells

We previously reported that CTLA-2α, which is a cathepsin L inhibitor, controls cathepsin L activities in infiltrating T cells in the inflamed eye.[6,7] In addition, pro-cathepsin L (pro-CathL) proteins impaired the generation of Treg by retinal pigment epithelium.[6] We thus aimed to determine whether CE cells can convert CD4+ T cells to Treg cells in the presence of pro-CathL blocking proteins. First, we examined whether CE cells can produce TGFβ in the presence of pro-CathL proteins. The

supernatants of CE cells in the presence of pro-CathL proteins contained less TGFβ1 when compared with CE cells incubated in the absence of pro-CathL proteins (Fig. 5A). CE-exposed CD4$^+$ T cells expressed Foxp3, whereas CD4$^+$ T cells exposed to CE cells in the presence of pro-CathL proteins poorly expressed Foxp3 (Fig. 5B). As expected, CE-induced Treg cells significantly suppressed the activation of bystander target T cells, whereas pro-CathL proteins impaired Treg cell suppression (Fig. 5C). We conclude from these results that the CTLA-2α effect on Treg cell induction occurs through TGFβ signaling because CTLA-2α promotes TGFβ activation and controls cathepsin L activity in corneal immune tolerance.

DISCUSSION

Immune-privileged tissues have recently led to increased interest in the local mechanisms of tolerance. These tissues allow sites to function passively by creating immune privilege, and the molecules that are expressed either as soluble factors or cell surface molecules actively maintain this privileged status.
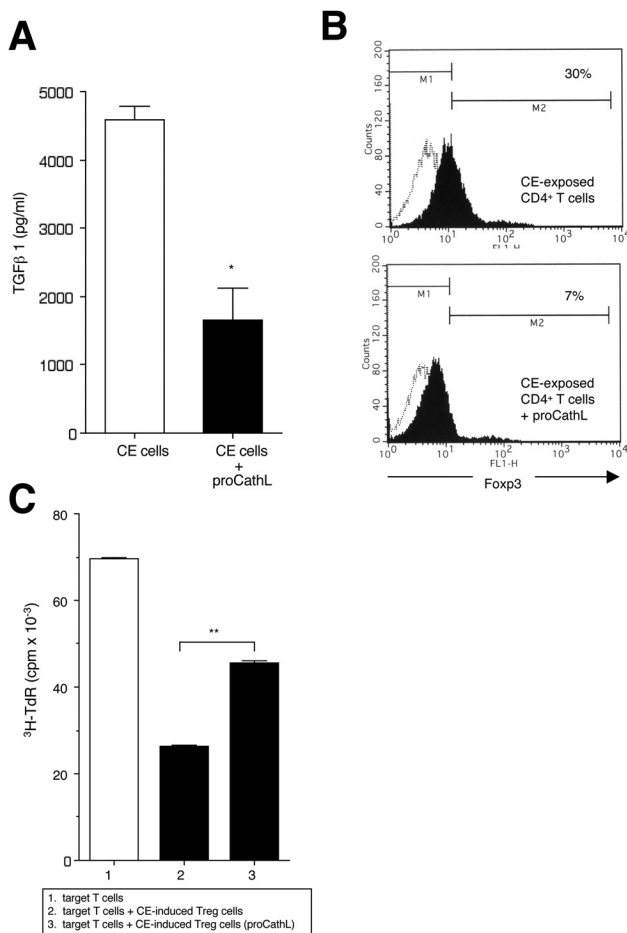

FIGURE 5. Ability of cathepsin L blocking proteins pretreated with CE cells to induce Treg cells. (**A**) Cultured CE cells were treated with pro-CathL proteins (100 ng/mL) for 24 hours, and supernatants were harvested to measure TGFβ1. Error bars represent SEM. *P < 0.05, compared with CE cells without proteins. (**B**) CE-exposed CD4$^+$ T cells in the presence of pro-CathL proteins were stained with FITC-conjugated anti–Foxp3 (*black histogram*). *Open histogram*: isotype control of rat IgG. (**C**) CE-exposed T cells in the presence of pro-CathL proteins (*black bars*) were added to secondary cultures with target T cells (*open bar*, responder pan-T cells plus anti-CD3). Error bars represent SEM. **P < 0.005, compared with two groups.

For instance, constitutive cell surface expression of CD95 ligand on CE is critical to its immune-privileged status.[19,20] CD95 ligand induces programmed cell death (apoptosis) in cells bearing its receptor (CD95/Fas). In this study, we provide evidence that the function of corneal endothelium-derived CTLA-2α is to induce Foxp3$^+$ T cells to acquire a regulatory phenotype. Cultured CE cell lines express CTLA-2α on their surfaces and convert CD4$^+$ T cells to Treg cells. We show that these newly generated Treg cells display immunoregulatory activity and suppress responder T-cell activation. Thus, the corneal endothelium has an immunosuppressive capacity to induce regulatory T cells when CE cells encounter infiltrating cells in the inflamed eye.

The cornea is a transparent window in the eye, and corneal transparency is essential for good vision. Inflammatory reactions within the cornea result in tissue destruction and scar formation. Inflammatory-associated processes interfere with corneal transparency and cause blindness. During such inflammatory conditions, the cornea has evolved to develop mechanisms that prevent and modulate inflammatory reactions because it is an immune-privileged tissue and site.[10,21] Studies of ocular immune privilege have revealed several mechanisms, including the presence of immunosuppressive factors in aqueous humor[22,23]; the presence of regulatory T cells in the eye[5–7,24,25]; and the presence of surface immunosuppressive molecules on the ocular tissues.[12,26–28] TGFβ expression by many ocular cells/tissues has been shown to promote immune privilege.[29–31] Uncharacterized immunoregulatory activities have also been attributed to several nonlymphoid cells of the eye, including retinal Müller cells,[32] retinal pigment epithelial cells,[5–7,26–28,31] ciliary body pigment epithelial cells,[15,31] and iris pigment epithelial cells.[15,16,24,27] CE cells form part of the boundaries of the anterior chamber, also an immune-privileged site, and might therefore contribute to immune privilege. Kawashima et al.[33,34] reported that cultured CE cells suppress in vitro T-cell proliferation in response to antigen or mitogen. Given that aqueous humor contains immunosuppressive factors such as TGFβ and CE cells form part of the anterior chamber, it is possible that CE cells produce immunosuppressive factors that lead to these inhibitory effects.

In this study, we show that CD4$^+$ T cells exposed to CE cells express CD25^{high} and Foxp3 and that these T cells significantly suppress the activation of target T cells in vitro. For the induction of Treg cells, the production of CTLA-2α by CE cells is required. CTLA-2α inhibits cathepsin L activity in T cells, which enhances Treg cell differentiation. When we blocked CTLA-2α expression on CE cells using siRNA and neutralizing antibodies against this molecule, the Treg cells failed to acquire Treg function. Similarly, CE-induced Treg cells failed to suppress the activation of bystander target T cells when pro-cathepsin L blocking proteins against CTLA-2α were added to the culture. Importantly, CE cells produce less TGFβ when the cells are pretreated with blocking materials such as CTLA-2α-siRNA transfection, anti–CTLA-2α neutralizing antibody, and pro-cathepsin L blocking proteins. However, as shown in Figure 4C, T cells exposed to CE in the presence of CTLA-2α-siRNA still had a suppressive effect on target T cells. This suggested that CE cells also produced TGFβ and other suppressive factors. On the other hand, the CE cells failed to convert CD4$^+$ T cells to Treg cells in vitro when the production of both CTLA-2α (CTLA-2α siRNA-transfected CE cells) and TGFβ (anti–TGFβ blocking antibody) by CE cells was blocked (Fig. 4D). Thus, intraocular TGFβ is also required for the induction of eye-specific Treg cells. TGFβ is a critical factor that induces "induced Treg cells (iTreg cells)" in the periphery.[17,18,35–38] TGFβ converts CD4$^+$CD25$^-$ naive T cells to CD25$^+$ Treg cells by inducing the transcription factor *Foxp3*.[18] We recently showed that intraocular Treg cells inducibly express Foxp3 through induction of the intraocular TGFβ signal.[6,39] In fact,

TGFβ is expressed by a variety of ocular cells and tissues, including corneal endothelium.

In murine studies by Hori et al.,[40] programmed cell death 1 ligand 1 (PD-L1/B7-H1) cell surface molecules expressed on CE cells provide negative costimulation for the effector T cells, helping to inhibit corneal allograft rejection. We recently showed that human CE cells constitutively express these cell surface molecules and suppress Th1-type T cells that express PD-1 in a cell contact-dependent mechanism.[12] Retinal pigment epithelial cells convert $CD4^+$ T cells to $Foxp3^+$ Treg cells through soluble TGFβ and membrane-associated TGFβ.[6,7] Membrane-associated TGFβ plays critical roles in the immunosuppressive mechanisms mediated by iris pigment epithelium located on the anterior segment in the eye.[24,27] These previous reports suggest that ocular pigment epithelium has immunosuppressive capacities by cell-contact and through the secretion of immunosuppressive factors. However, murine CE cells and human CE cells, as described in our previous report,[12,13] exclusively exhibit these immunosuppressive properties by cell-to-cell contact. When we used a cell insert transwell membrane in culture, the CE cells did not induce Foxp3-positive Treg cells, but when CE cells were used as a control (i.e., the cell insert was not present), there was significant expression of these T cells (Fig. 1E). Similarly, $CD4^+$ T cells exposed to CE cells expressed Foxp3, but $CD4^+$ T cells exposed to the supernatants poorly expressed Foxp3 (Sugita S, unpublished observation, 2009). These results indicate that CE cells are capable of inducing regulatory T cells in vitro, similar to what has been seen for murine iris pigment epithelium. Both CE and iris pigment epithelium face the aqueous humor as part of the inner side of the anterior chamber of the eye. These intraocular tissues and aqueous humor create immune tolerance in the anterior segment of the eye.

In conclusion, cultured CE cells established from the anterior segment in the eye have been demonstrated to convert Treg cells in vitro. The corneal endothelium-derived immunosuppressive factor CTLA-2α promotes the induction of regulatory T cells that are able to suppress bystander effector cells. Thus, the immunoregulatory property of corneal endothelium is related to the intraocular suppression of immunogenic inflammation.

Acknowledgments

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