

# Mechanisms of Immune Suppression for CD8<sup>+</sup> T Cells by Human Corneal Endothelial Cells via Membrane-Bound TGFβ

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**PURPOSE.** To determine whether CD8<sup>+</sup> T cells in vitro are suppressed or converted into regulators (Treg cells) by human corneal endothelial (HCE) cells.

**METHODS.** HCE cell lines were established from primary HCE cells, and allogeneic CD8<sup>+</sup> T cells were isolated from peripheral blood mononuclear cells of healthy donors. T-cell activation was assessed for proliferation by [<sup>3</sup>H]-thymidine incorporation. Expression of TGFβ on HCE cells was evaluated by flow cytometry, RT-PCR, and immunohistochemistry. Anti-TGFβ-neutralizing antibodies or TGFβ siRNA was used to abolish the HCE-mediated inhibitory function. CD8<sup>+</sup> T cells exposed to HCE cells were used as Treg cells, to induce Treg cells in vitro by exposure to HCE cells. Expression of CD25 or Foxp3 on Treg cells was evaluated by flow cytometry.

**RESULTS.** Cultured HCE cells produced the membrane-bound active TGFβ isoform 2. HCE surface TGFβ2 was necessary, to inhibit CD8<sup>+</sup> T-cell activation via direct cell contact. In addition, although HCE cells were found to constitutively express costimulatory molecules, such as programmed death-ligand 1 (PD-L1) and PD-L2, and secreted thrombospondin-1, only membrane-bound TGFβ2 was actually delivered to the CD8<sup>+</sup> T cells. HCE cells were also found to convert some CD8<sup>+</sup> T-cell populations into Treg cells via their membrane-bound TGFβ. These HCE-induced Treg cells produced soluble forms of TGFβ1, but not of TGFβ2, and they also acquired a regulatory phenotype that expressed CD25<sup>high</sup> and Foxp3.

**CONCLUSIONS.** Ocular resident tissue-exposed T cells can be suppressed or induced to become regulators within the human peripheral microenvironment. (*Invest Ophthalmol Vis Sci* 2010;51:2548–2557) DOI:10.1167/iovs.09-4233

Complementary activities of two related mechanisms have been proposed as the method by which the eye achieves immune privilege. The first mechanism involves the surface

molecules that are expressed on the ocular parenchymal cells, especially those on the corneal endothelium<sup>1–3</sup> and on the ocular pigment epithelium,<sup>4–8</sup> whereas the second mechanism involves the soluble immunosuppressive factors that are found within the aqueous humor (TGFβ2, neuropeptides).<sup>9–11</sup> Corneal endothelial (CE) cells are in contact with the aqueous humor, as they are part of the inner surface of the anterior chamber of the eye.<sup>5</sup> CE cells can contribute to peripheral immune tolerance in the human eye, as human corneal endothelial (HCE) cells are able to inhibit the intraocular effector T cells in a cell-contact-dependent manner.<sup>3</sup> Infiltrating inflammatory cells, especially activated T cells, bind to the CE. Once these T cells are exposed to the CE, they eventually fail to acquire any effector T functions.<sup>1–3</sup> In our newly established HCE cell line, we found that the activation of the Th1 type CD4<sup>+</sup> T cells is suppressed by a cell-contact mechanism that is dependent on the interaction of PD-1 and PD-L1 co-stimulatory molecules in vitro.<sup>3</sup> In addition, we found that primary cultured human iris pigment epithelium (IPE) constitutively expresses TGFβ2 on the cell surfaces and that human IPE cells fully suppress the activation of intraocular T cells established from patients with uveitis.<sup>8</sup> These findings indicate that ocular parenchymal cells from the anterior segment of the eye may create an immune privileged site to avoid the adverse consequences of intraocular inflammation such as blindness. However, as of yet, the immunologic role of the human cornea in the inflammation of the anterior chamber remains unknown. For example, although it is known that the established HCE cell line suppresses the activation of Th1 type CD4<sup>+</sup> T cells,<sup>3</sup> whether HCE cells suppress CD8<sup>+</sup> T cells has not been determined.

The present experiments were designed to examine the extent to which cultured HCE cells (1) suppress the activation of CD8<sup>+</sup> T cells in vitro, (2) convert CD8<sup>+</sup> T cells into T regulatory cells (Treg cells), and (3) use immunosuppressive factor(s) such as TGFβ or costimulatory molecules to suppress responder CD8<sup>+</sup> T cells. Results indicated that cultured HCE cells produced enhanced amounts of active TGFβ2 and predominantly used the membrane-bound form of TGFβ to suppress T-cell activation and convert the cells into Treg cells. This evidence strongly suggests that the constitutive expression of membrane-associated immunosuppressive molecules in the eye protect the delicate tissues from inflammatory effector cell attacks.

## MATERIALS AND METHODS

### HCE Cell Lines

We established an HCE cell line transduced with hTERT and the large T gene. Primary cultures of HCE cells were prepared, as has been described.<sup>12</sup> The retroviral vectors BABE-hygro-hTERT (for hTERT) and

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MFG-tsT-IRES-neo (for SV40 large T antigen) were used in these experiments,<sup>13</sup> according to a published method.<sup>3</sup> For the assay, cultured HCE cells were triturated to obtain a single-cell suspension and then were resuspended in DMEM+10% FBS, placed into 24-well plates, and incubated for several days at 37°C. At the end of culturing, the HCE cells were washed with serum-free fresh medium and then added to the wells of the culture plates. Information on the DMEM and serum-free medium has been reported.<sup>3</sup>

### Preparation of Purified T Cells and Assay of Proliferation

Cultured HCE cells ( $1.0 \times 10^4$ – $1.0 \times 10^6$ /well) were seeded into 24-well culture plates, as described previously.<sup>3</sup> CD8<sup>+</sup> T cells were prepared separately from the PBMCs of healthy volunteers by using cell isolation kits (MACS; Miltenyi Biotec, Auburn, CA). These cells were purified by a single immunomagnetic depletion step with magnetic beads (MACS; Miltenyi Biotec), and were found to be >94% CD8-positive. For the assay, target T cells ( $1.0 \times 10^5$ /well in a 96-well plate or  $0.5 \times 10^6$ /well in a 24-well plate) were stimulated with anti-CD3 mAb (clone 2C11; BD PharMingen, San Diego, CA) and incubated for 72 hours for assessment of T-cell proliferation. The concentration of soluble anti-CD3 mAb in these cultures was 0.01, 0.1, or 1.0  $\mu$ g/mL. As described in one of our published reports,<sup>3</sup> we established CD8<sup>+</sup> T-cell clones from a patient with corneal endotheliitis for use as alternative targets. The T cells were cultured with anti-human CD3 along with recombinant IL-2, which was used for the purpose of activation. After incubation, the uptake of [<sup>3</sup>H]-thymidine was measured to assess cell proliferation (in counts per million). T cells stimulated with anti-CD3 mAb were grown in serum-free medium.

All samples were harvested after informed consent was obtained from the healthy donors. This research was performed according to the Declaration of Helsinki and was approved by the Institutional Review Board of Tokyo Medical and Dental University.

### In Vitro Assays of Treg Cell Activity

**Proliferation.** CD8<sup>+</sup> (or CD4<sup>+</sup>) T cells in the presence of anti-CD3 Abs (0.5  $\mu$ g/mL) were exposed to HCE cells for 48 hours. The T cells were harvested and added ( $10^5$  cells/well) to 96-well plates containing target syngeneic responder pan-T cells (at  $10^5$  cells/well) plus anti-CD3 antibody (1.0  $\mu$ g/mL). Proliferation was assessed by [<sup>3</sup>H]-thymidine incorporation, as described earlier.

**Effect on Proliferation of Blocked Interactions between TGF $\beta$  and TGF $\beta$  Receptors.** Anti-human TGF $\beta$  monoclonal antibodies (1  $\mu$ g/mL anti-TGF $\beta$ 1, -2, and -3 mAbs; clone 1D11; R&D Systems, Minneapolis, MN) were added to cultures containing anti-CD3-stimulated target T cells plus CD8<sup>+</sup> HCE-induced Treg cells. As a control, purified mouse IgG was added to the appropriate cultures. In companion experiments, TGF $\beta$ 2-siRNA transfected HCE cells were used for Treg cell induction. In other experiments, TGF $\beta$ 1-siRNA transfected or anti-TGF $\beta$ -neutralizing Abs-pretreated CD8<sup>+</sup> T cells (HCE-induced Treg cells) were used for suppression of the target T cells.

The siRNA for human TGF $\beta$ 1 and -2 was designed with siDirect (developed by a consortium and provided in the public domain at <http://design.RNAi.jp/>). Highly effective siRNA sequences were selected with the novel guidelines for siDirect. Duplex siRNA targeted the TGF $\beta$ 1 coding sequence at nucleotide positions 1092-1114 (sense, 5'-CGGUGCUCGCUUUGUACAACA-3'; antisense, 5'-UUGUACAAAGC-GAGCACC GCC-3') and 1163-1185 (sense, 5'-GAAGCGGACUAC-U AUGCUAAA-3'; antisense, 5'-UAGCAUAGUAGUCCGCUUCGG-3'). The methods for the transfection and siRNA sequence for the targeted TGF $\beta$ 2 have been described.<sup>8</sup> The T cells were removed after 48 hours and added to a secondary culture containing target pan-T cells plus anti-CD3 antibodies.

**Production of TGF $\beta$  by Treg Cells.** The concentration of active TGF $\beta$ 1 or TGF $\beta$ 2 in the supernatants of the HCE-induced Treg cells was measured by ELISA (Human TGF $\beta$  Immunoassay; R&D Systems).

### Blocking Antibodies and Recombinant Proteins

Purified anti-TGF $\beta$  mAb (clone 1D11; R&D Systems), anti-TSP-1 polyclonal Abs (clone sc-14013; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-human PD-L1/B7-H1 mAb (clone MIH1; eBiosciences, San Diego, CA), or anti-human PD-L2/B7-DC mAb (clone MIH1 8; eBiosciences) was added to the HCE cells. Mouse IgG (R&D Systems) or rabbit IgG (BD PharMingen) was used as the isotype control. Recombinant proteins such as human TGF $\beta$ 2 (R&D Systems) and human TSP-1 (Hematologic Technologies Inc., Essex Junction, VT) were added to the CD8<sup>+</sup> T cells.

### ELISA or Cytometric Bead Array Assay for Cytokines

The concentration of cytokines in supernatants of the HCE cultures (HCE cell lines or primary cultured HCE cells) was measured by a cytometric bead array (CBA) assay kit that included IL-2, IL-4, IL-5, IL-10, TNF $\alpha$ , or IFN $\gamma$ . The assay was performed according to the manufacturer's instructions (Human Th1/Th2 Cytokine Kit; BD PharMingen). The concentration of thrombospondin-1 (TSP-1; American Research Products, Belmont, MA) or TGF $\beta$ 2 (active TGF $\beta$ 2; R&D Systems) in the supernatants of the HCE cell cultures was also measured by ELISA.

### Flow Cytometric Analysis

Flow cytometry was used to analyze membrane-associated TGF $\beta$ 2 expression on the HCE cell surfaces with the mAbs, anti-human TGF $\beta$ 2 Abs, or mouse isotype IgG used as the control. Single-cell suspensions of the HCE cells were obtained by treating the cells with 0.5% trypsin/EDTA (Sigma-Aldrich, St. Louis, MO), washing them twice in PBS, with  $1 \times 10^6$  cells, and incubating them with FITC-labeled mAbs. Before they were stained, the co-cultured cells were incubated with human Fc-block (Miltenyi Biotec) for 15 minutes. In companion experiments, HCE cells were also stained with FITC-labeled anti-human TGF $\beta$  receptor II (RII) Abs (clone 25508; R&D Systems). The harvested cells were stained for intracellular TGF $\beta$  RII after permeabilization (Cytofix/Cytoperm Kits; BD PharMingen).

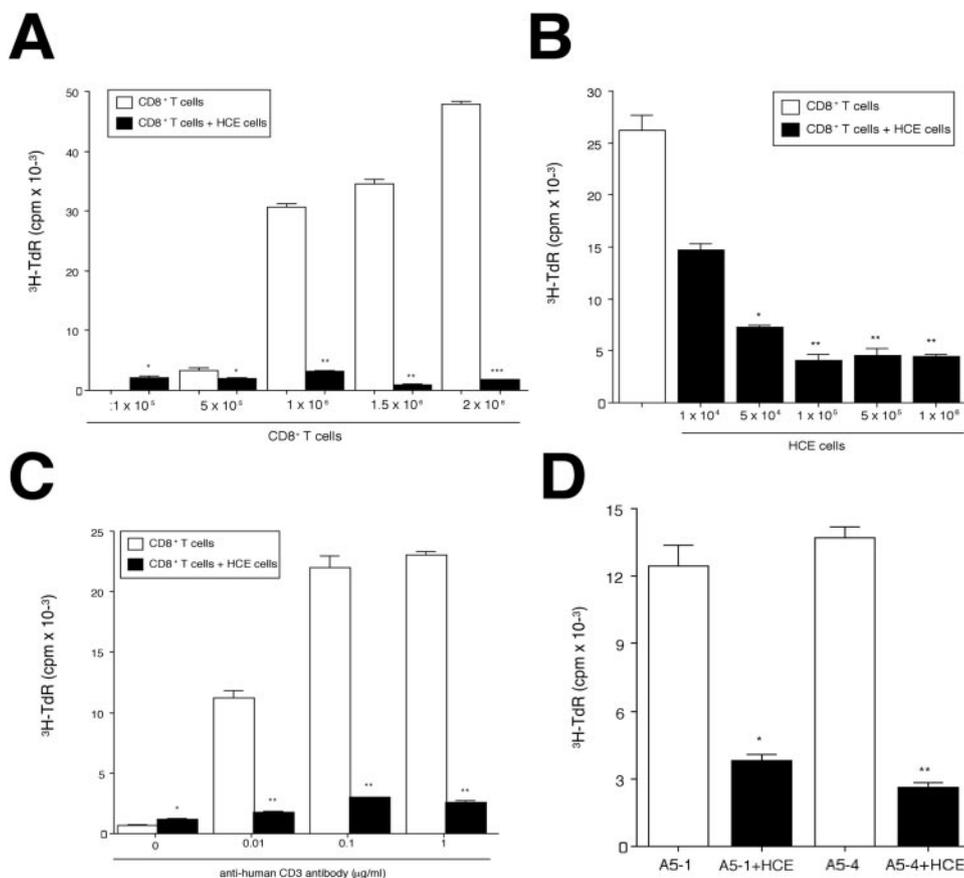
HCE-exposed or HCE supernatant-exposed CD8<sup>+</sup> T cells were stained with phycoerythrin (PE)-conjugated anti-human CD25 (IL-2R $\alpha$ ; eBioscience) and fluorescein isothiocyanate (FITC)-conjugated antibody to anti-CD8 antibody (BD PharMingen). Flow cytometry was used to analyze the expression of Foxp3 by the HCE-induced Treg cells. Before staining, the cocultured cells were incubated with Fc-receptor block for 15 minutes. After permeabilization, the cells were stained with FITC-conjugated anti-human Foxp3 antibody (clone PCH101; eBioscience) or rat IgG (eBioscience) at 4°C for 30 minutes. After they were washed twice in PBS, the stained cells (live-gated on the basis of the forward- and sidescatter profiles and propidium iodide exclusion) were analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA).

### Immunohistochemistry

Cultured HCE cells were grown in a four-well chamber slide (BD Biosciences). After they were washed with PBS, the cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, followed by permeabilization with 0.1% Triton X. The cells were then incubated for 1 hour with anti-human TGF $\beta$ 2 mAb (1:100 dilution, clone 8607; R&D Systems) or mouse IgG (1:100) as an isotype control, followed by washing with PBS and incubation with a fluorescence-labeled secondary Ab (Alexa Fluor 488; Invitrogen-Life Technologies, Carlsbad, CA) for 1 hour. Fluorescence signals were detected by confocal microscopy.

### PCR Analysis

Cellular extracts were prepared from HCE or TGF $\beta$ -siRNA-transfected HCEs. The cultured cells were washed twice with PBS, and then total RNA was isolated (TRIzol; Invitrogen-Life Technologies). After cDNA synthesis, RT-PCR for human TGF $\beta$ 2 was performed by using a standard PCR method. The products were subjected to 35 cycles of PCR



**FIGURE 1.** Capacity of HCE cell lines to suppress activation of effector CD8<sup>+</sup> T cells. (A) In a target T-cell number-dependent manner, HCE cells ( $5 \times 10^5$ /well) were co-cultured for 72 hours with CD8<sup>+</sup> T cells (range,  $1 \times 10^5$ – $2 \times 10^6$ /well) in the presence of 1.0  $\mu$ g/mL anti-human CD3 mAb. (B) In an HCE cell number-dependent manner, HCE cells ranging in number from  $1 \times 10^4$  to  $1 \times 10^7$ /well were co-cultured for 72 hours with CD8<sup>+</sup> T cells ( $1.5 \times 10^6$ /well) in the presence of anti-human CD3 mAb (1.0  $\mu$ g/mL). (C) In a dose-dependent manner for T-cell activation by anti-CD3 stimulation, HCE cells ( $5 \times 10^5$ /well) were co-cultured with CD8<sup>+</sup> T cells ( $1.5 \times 10^6$ /well) in the presence of anti-human CD3 mAb (0, 0.01, 0.1, and 1.0  $\mu$ g/mL). (D) HCE cells were co-cultured with preactivated T cells such as CD8<sup>+</sup> T-cell clones that were established from the aqueous humor of a patient with corneal endotheliitis. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ , compared with the anti-CD3 mAb-stimulated CD8<sup>+</sup> T cells without HCE (positive control). Data expressed as the mean results of triplicate cultures  $\pm$  SE of mean (SEM).

amplification. Primers for TGF $\beta$ 2 that resulted in an amplification product of 279 bp were used as the primer-pair kits (R&D Systems). The forward and reverse primers for  $\beta$ -actin are described elsewhere.<sup>8</sup> The PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide.

### Statistical Analysis

Each experiment was repeated at least twice with similar results. All statistical analyses were conducted by Student's *t*-test. Differences were considered statistically significant at  $P \leq 0.05$ .

## RESULTS

### Capacity of Cultured HCE Cells to Suppress CD8<sup>+</sup> Effector T Cells In Vitro

We first examined whether cultured HCE cell lines suppress the proliferation of CD8<sup>+</sup> T cells and whether the extent of suppression is dependent on the number of T cells and HCE cells or the extent of T-cell activation by anti-CD3 stimulation. HCE cells were cocultured for 72 hours with CD8<sup>+</sup> T cells in the presence of 1  $\mu$ g/mL anti-human CD3 antibodies. T-cell proliferation was assessed by [<sup>3</sup>H]-thymidine incorporation at 72 hours. Cultured HCE cells significantly suppressed cell proliferation of CD8<sup>+</sup> T cells when the HCE cells were cocultured with  $5 \times 10^5$  to  $2 \times 10^6$  T cells for 72 hour (Fig. 1A). In the presence of  $5 \times 10^4$  to  $1 \times 10^6$  HCE cells, CD8<sup>+</sup> T-cell proliferation (number of T cells,  $1.5 \times 10^6$ /well) was significantly reduced (Fig. 1B). HCE cells ( $10^4$ ) also reduced CD8<sup>+</sup> T-cell proliferation, but the reduction was not statistically significant in comparison to the control cultures. HCE cells ( $5 \times 10^5$ ) significantly suppressed T-cell ( $1.5 \times 10^6$ ) proliferation at all concentrations of anti-CD3 antibody stimulation (0.01, 0.1,

and 1.0 mg/mL) tested (Fig. 1C). As shown in Figure 1D, the HCE cells also significantly suppressed proliferation of CD8<sup>+</sup> T cell clones (cornea endotheliitis: A5-1 and A5-4). The overall findings in the present study indicate that HCE cells suppressed both the activated CD8<sup>+</sup> T cells and CD4<sup>+</sup> cells, which was similar to the results we reported in a previous study.<sup>3</sup>

### Survey for Candidate Molecules Produced by HCE to Suppress CD8<sup>+</sup> T Cells

We found that HCE cells exclusively suppress activation of Th1 type CD4<sup>+</sup> cells producing IFN $\gamma$ , via a cell-contact-dependent mechanism involving PD-L1 (B7-H1) costimulatory molecules.<sup>3</sup> Among the cell-surface candidate molecules, the HCE cells constitutively expressed PD-L1 and -L2 (B7-DC). However, there was less or no expression noted for CD40, CD70 (CD27 ligand), 4-1BBL (CD137 ligand), CD80 (B7-1), CD86 (B7-2), ICOS-L (B7-H2), and OX40L (CD152) in the HCE cells.<sup>3</sup> In addition to surveying the cell-surface candidate molecules in the present study, we examined the cytokines that were produced by HCE cells. To measure the production of the various cytokines, we collected supernatants of the HCE cell lines. As a control, we used the supernatants of primary cultured HCE cells ( $n = 3$ ). As shown in Table 1, the supernatants of the HCE cell lines contained TGF $\beta$ 2 and TSP-1. Similarly, the supernatants of primary cultured HCE cells, which were used as the control, also contained these cytokines. However, IL-2, IL-4, IL-5, IL-10, TNF $\alpha$ , and IFN $\gamma$  were at undetectable levels in the both supernatants (Table 1). Since the cultured HCE cells can produce TGF $\beta$ 2 and TSP-1 cytokines, in addition to expressing PD-L1 and -L2 cell surface molecules, we further examined whether these candidate molecules were relevant with regard to the HCE-dependent suppression of the effector CD8<sup>+</sup> T cells.

**TABLE 1.** Cytokine Production in the Supernatants of Cultured HCE Cells

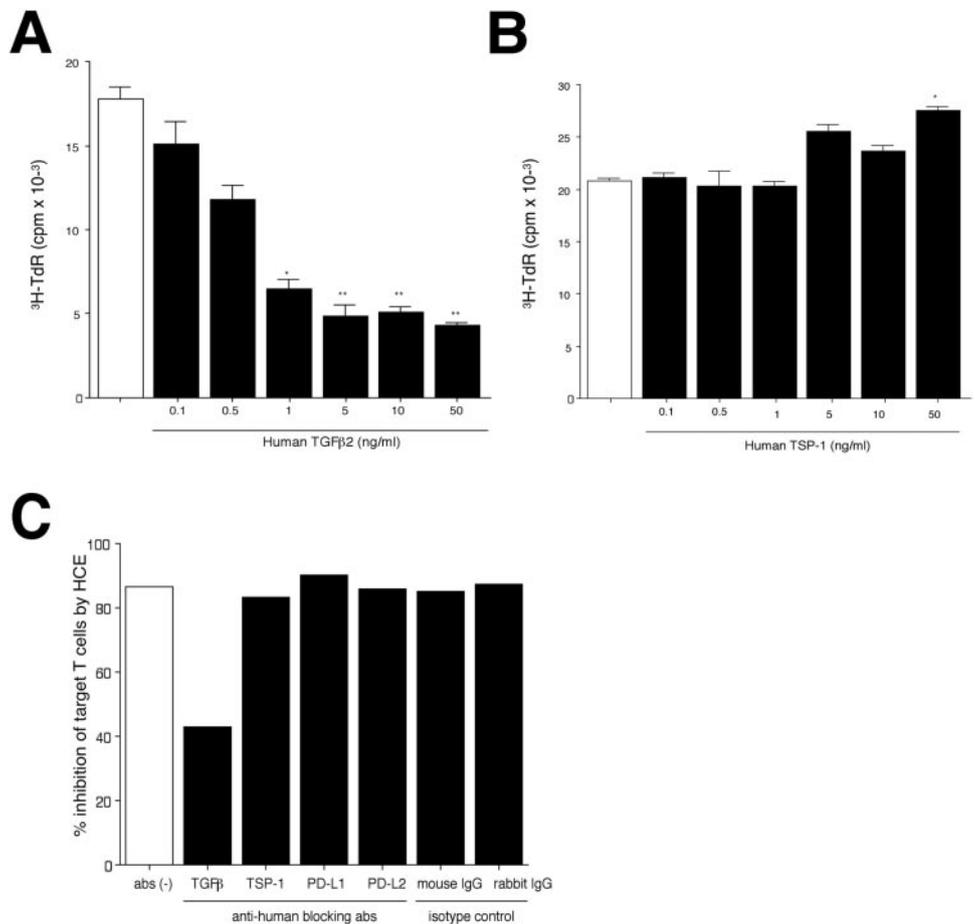
Cytokines	Cell Lines	Primary Cultured Cells
IL-2	ND	ND
IL-4	ND	ND
IL-5	ND	ND
IL-10	ND	ND
TNF- $\alpha$	ND	ND
IFN- $\gamma$	ND	ND
TGF $\beta$ 2, pg/mL	521.0 $\pm$ 20.0	1443.0 $\pm$ 93.0
TSP-1, ng/mL	39.1 $\pm$ 0.3	24.1 $\pm$ 0.3

The supernatants were harvested from HCE cell lines ( $1 \times 10^6$  cells/well in a 24-well culture plate) and primary cultured HCE cells ( $1 \times 10^5$  cells per culture dish). Before the supernatants were collected, the media were removed and replaced by serum-free medium, with the cultures then incubated for 24 hours at 37°C. ND, not detected.

### Use of Recombinant Proteins and Neutralizing Antibodies in HCE-Dependent Suppression

We examined whether human recombinant proteins for TGF $\beta$ 2 or TSP-1 can suppress activation of CD8<sup>+</sup> T cells in vitro. As expected, recombinant TGF $\beta$ 2 significantly suppressed the T-cell activation in a dose-dependent manner (Fig. 2A). While concentrations ranging from 1 to 50 ng/mL rTGF $\beta$ 2 caused a significant suppression, concentrations of TGF $\beta$  at 0.1 to 0.5 ng/mL caused only modest reductions in T-cell proliferation that were not significantly different from those in control cultures. On the other hand, rTSP-1 exhibited no suppression of T-cell activation (Fig. 2B).

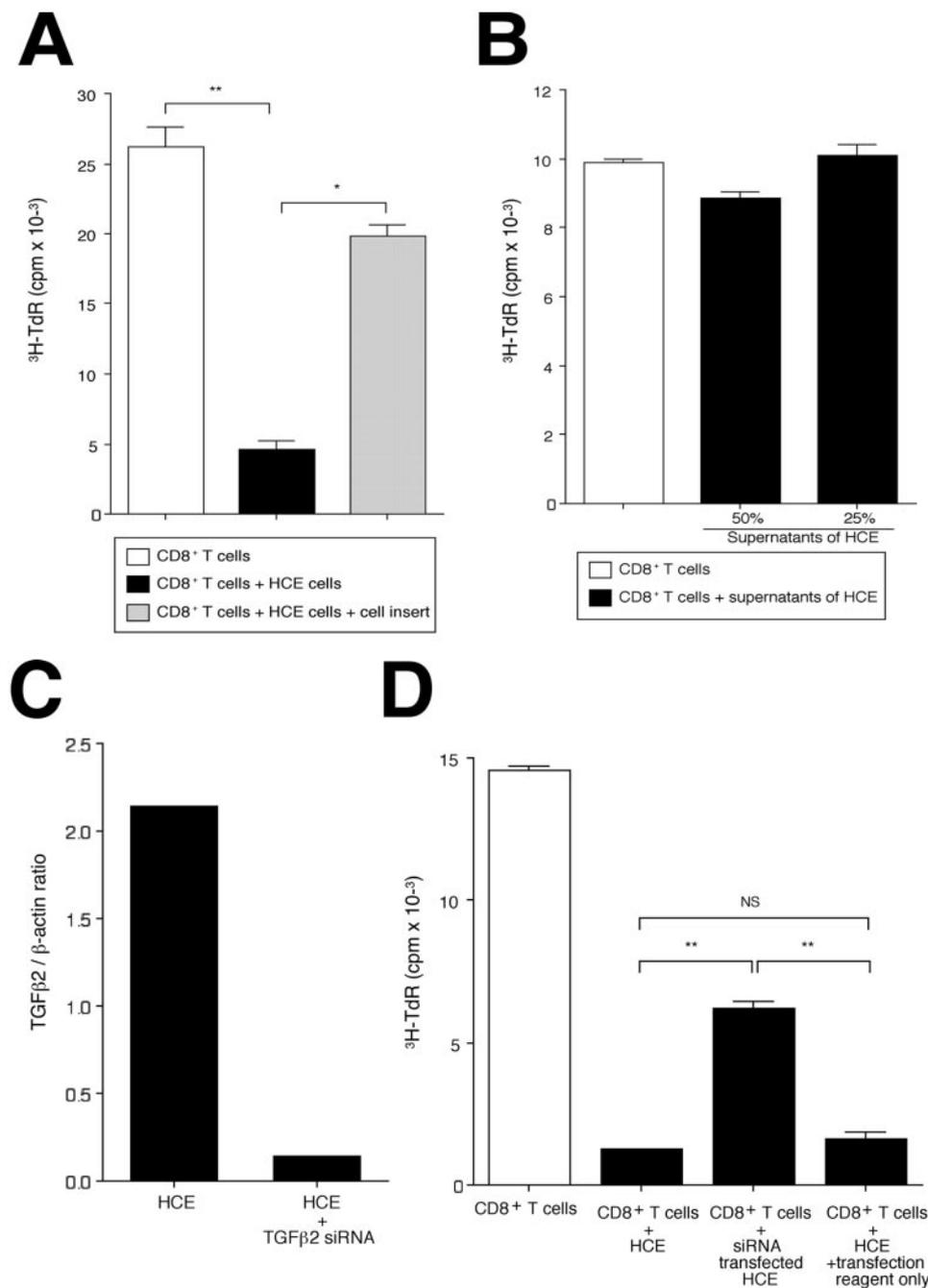
**FIGURE 2.** Effect of recombinant proteins and neutralizing antibodies in HCE-dependent T-cell suppression. Human recombinant proteins for TGF $\beta$ 2 (A) and TSP-1 (B) at 0, 0.1, 0.5, 1, 5, 10, and 50 ng/mL were co-cultured with purified CD8<sup>+</sup> T cells in the presence of anti-CD3 Abs. After 72 hours, the cultures were assayed for the uptake of [<sup>3</sup>H]-thymidine. Results are expressed as the mean ( $\pm$ SEM) results for triplicate cultures. (C) For the in vitro assay, CD8<sup>+</sup> T cells were stimulated with 1  $\mu$ g/mL of anti-CD3 mAb antibody for 72 hours in the presence of cultured HCE cells. Neutralizing Abs, anti-human TGF $\beta$  (1  $\mu$ g/mL), anti-human TSP-1 (1  $\mu$ g/mL), anti-human PD-L1 (1  $\mu$ g/mL), anti-human PD-L2 (1  $\mu$ g/mL), isotype mouse IgG (1  $\mu$ g/mL), or isotype rabbit IgG (1  $\mu$ g/mL) was added to some of the wells. After incubation for 2 hours, the Ab-pre-treated HCE cells were rinsed with PBS and then added to wells containing the responder target T cells. Bars, percentage of inhibition of the target CD8<sup>+</sup> T cells by the HCE cells in vitro. Abs (-), target CD8<sup>+</sup> T cells + HCE without Abs. \* $P$  < 0.05, \*\* $P$  < 0.005, as compared with the positive control (open bar). Data expressed as the mean results of triplicate cultures  $\pm$  SEM.



We also examined whether blocking antibodies impair HCE cell-induced suppression. For this experiment, we conducted functional analyses with neutralizing antibodies for TGF $\beta$ 2, TSP-1, PD-L1, and PD-L2. Cultured HCE cells in the presence of anti-human TGF $\beta$  Abs failed to suppress proliferation of the activated CD8<sup>+</sup> T cells, whereas HCE cells in the presence of anti-human TSP-1, PD-L1, PD-L2, or two isotype controls (mouse or rabbit IgG) significantly suppressed the T-cell proliferation (Fig. 2C). These results imply that TGF $\beta$ 2, which is the dominant isoform of TGF $\beta$  in the eye, may play an important role in the HCE-dependent suppression of activated CD8<sup>+</sup> T cells. In addition, the PD-1/PD-L1 interactions were not involved in the suppression, and in fact, PD-1 was found to be poorly expressed in the target CD8<sup>+</sup> T cells that were established from healthy donors (data not shown).

### T-Cell Suppression by HCE Cells Using Membrane-Bound TGF $\beta$ 2

In an attempt to validate the hypothesis that HCE cells use cell contacts and a membrane-associated form of active TGF $\beta$  to suppress bystander T cells, we made use of permeable inserts (Transwell; Corning Costar, Corning, NY), which permit soluble molecules, but not cells, to pass between the segregated cell populations. Anti-CD3-treated CD8<sup>+</sup> T cells were seeded into the cell inserts, whereas the HCE cells were seeded below the cell inserts and on the bottom of the culture wells. In suppression control cultures (no cell insert was present), CD8<sup>+</sup> T cells were seeded directly into the wells that contained HCE and anti-CD3. The results of this experiment demonstrated that the efficiency of HCE in suppressing bystander CD8 T-cell proliferation was significantly impaired if the HCE and the bystander T



**FIGURE 3.** Capacity of T-cell suppression by membrane-associated TGFβ<sub>2</sub><sup>+</sup> HCE cells. **(A)** HCE cells were grown in 24-well culture plates (5 × 10<sup>5</sup>/well). Porous cell culture inserts were placed in the wells to prevent cell-to-cell contact, with CD8<sup>+</sup> T cells (1 × 10<sup>6</sup>/well) and anti-CD3 mAb added. **(B)** Supernatants of HCE cells (50% or 25%) were added to the anti-CD3 stimulated CD8<sup>+</sup> T cells. **(C)** RNA was extracted from TGFβ<sub>2</sub> siRNA-transfected HCE or nontransfected cells (only the transfection reagent was added) and analyzed for mRNA expression of TGFβ<sub>2</sub>. **(D)** TGFβ<sub>2</sub> siRNA-transfected HCE cells were co-cultured with target CD8<sup>+</sup> T cells. After 72 hours, the cells were assayed for the uptake of [<sup>3</sup>H]-thymidine. Results are expressed as the mean (±SEM) for triplicate cultures; \**P* < 0.05, \*\**P* < 0.005. NS, not significant.

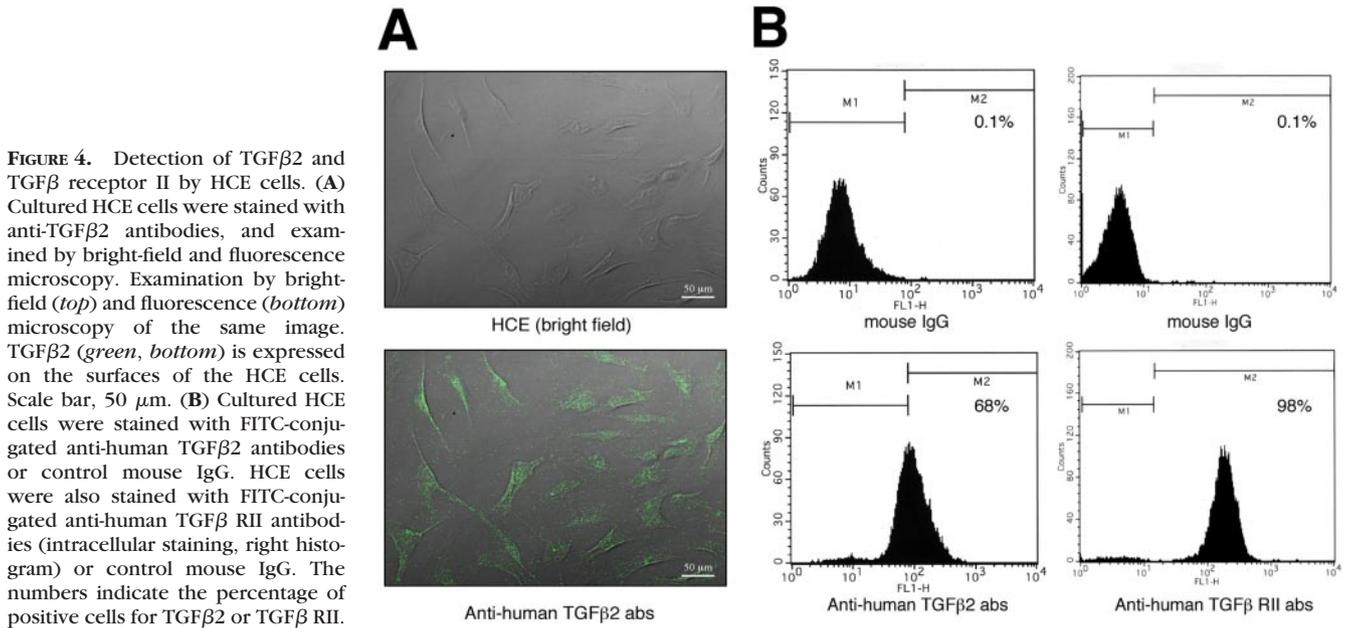
cells were separated by the permeable membrane (Fig. 3A). As shown in Figure 3B, similar results were seen in the supernatants of the HCE cells (final concentration, 50% or 25%) for which the bystander CD8 T-cell proliferation was not suppressed. This suggests that direct cell-to-cell contact optimizes the HCE cells' capacity to suppress effector CD8<sup>+</sup> T cells.

In a subsequent experiment, we examined whether the membrane-associated TGFβ<sub>2</sub> that is expressed by HCE cells would suppress the proliferation of CD8<sup>+</sup> T cells. To evaluate this possibility, we used TGFβ<sub>2</sub>-siRNA transfection or anti-TGFβ<sub>2</sub> blocking Abs. TGFβ<sub>2</sub>-siRNA-transfected HCE cells did not express the mRNA for human TGFβ<sub>2</sub>, whereas nontransfected control HCE cells clearly expressed the mRNA (Fig. 3C). It is important to note that TGFβ<sub>2</sub>-siRNA-transfected HCE cells partially restored CD8 T-cell proliferation (Fig. 3D). In contrast, nontransfected HCE cells and those treated with transfection

reagents alone comparably suppressed CD8 T-cell proliferation (Fig. 3D). In addition, this HCE-mediated suppression was partially relieved in the presence of anti-TGFβ<sub>2</sub> blocking antibodies (data not shown). When taken together, these results suggest that the membrane-associated TGFβ contributes to HCE-dependent suppression of activated CD8<sup>+</sup> T cells in a cell-contact-dependent manner.

#### Expression of Membrane-Bound TGFβ<sub>2</sub> by Cultured HCE Cells

Our results led us to speculate that HCE cells can efficiently suppress the activation of CD8<sup>+</sup> T cells via the interaction between TGFβ<sub>2</sub> on HCE cells and the receptor (e.g., TGFβ receptor II) on the T cells. To further clarify this, we examined cultured HCE cells for evidence of surface expression of



TGFβ2 by using immunostaining and bright-field microscopy or flow cytometry. Cultured HCE cell lines were placed on coverslips and immunostained with anti-human TGFβ2 antibodies. Representative photomicrographs are presented in Figure 4A. Immunohistochemistry showed that TGFβ2 expression was clearly prominent on the HCE cell culture (Fig. 4A). The pattern of TGFβ2 expression was diffuse and punctate, suggesting that the cytokine was present in relatively large cell surface patches. On the other hand, the expression was not detected on HCE cells staining with control mouse IgG isotype (data not shown). According to our flow cytometric analysis, there were greater levels of TGFβ2 expression found on the surface of the HCE cells (68% positive; Fig. 4B). Similarly, the HCE cells expressed TGFβ RII (98% positive; Fig. 4B, right histogram). In addition, the target responder CD8<sup>+</sup> T cells clearly expressed TGFβ RII (data not shown). Immunohistochemistry suggested that TGFβ2 may be localized in large cell surface patches (Fig. 4A), and flow cytometric analysis (Fig. 4B) confirmed cell surface expression, which may be mediated by TGFβ RII expression on the surface of HCE cells.

### Importance of Membrane-Bound TGFβ in HCE Cells in the Conversion to Treg Cells

Cultured ocular resident cells from the anterior segment of the eye (e.g., iris pigment epithelium) can convert CD8<sup>+</sup> T cells into Treg cells in vitro.<sup>6,7</sup> Since both corneal endothelium and iris pigment epithelium are located in the anterior segment and both of these tissues are in contact with the aqueous humor, we examined the capacity of the HCE-exposed CD8<sup>+</sup> T cells (HCE-induced Treg cells) to suppress the bystander T-cell proliferation after being added to secondary cultures that contained effector T cells plus anti-CD3 antibodies. CD8<sup>+</sup> HCE-induced Treg cells suppressed T-cell proliferation by target pan-T cells, provided the CD8<sup>+</sup> T cells were exposed to HCE cells in the absence of anti-CD3 in the primary culture (Fig. 5A, left). On the other hand, when CD8<sup>+</sup> T cells were exposed to HCE cells in the presence of anti-CD3 in the primary culture, the CD8<sup>+</sup> HCE-induced Treg cells fully and profoundly suppressed T-cell proliferation (Fig. 5A), suggesting that anti-CD3 activation may enhance the conversion to regulatory T cells. Of note, HCE-exposed CD4<sup>+</sup> T cells also significantly suppressed the activation of bystander target T cells (data not shown),

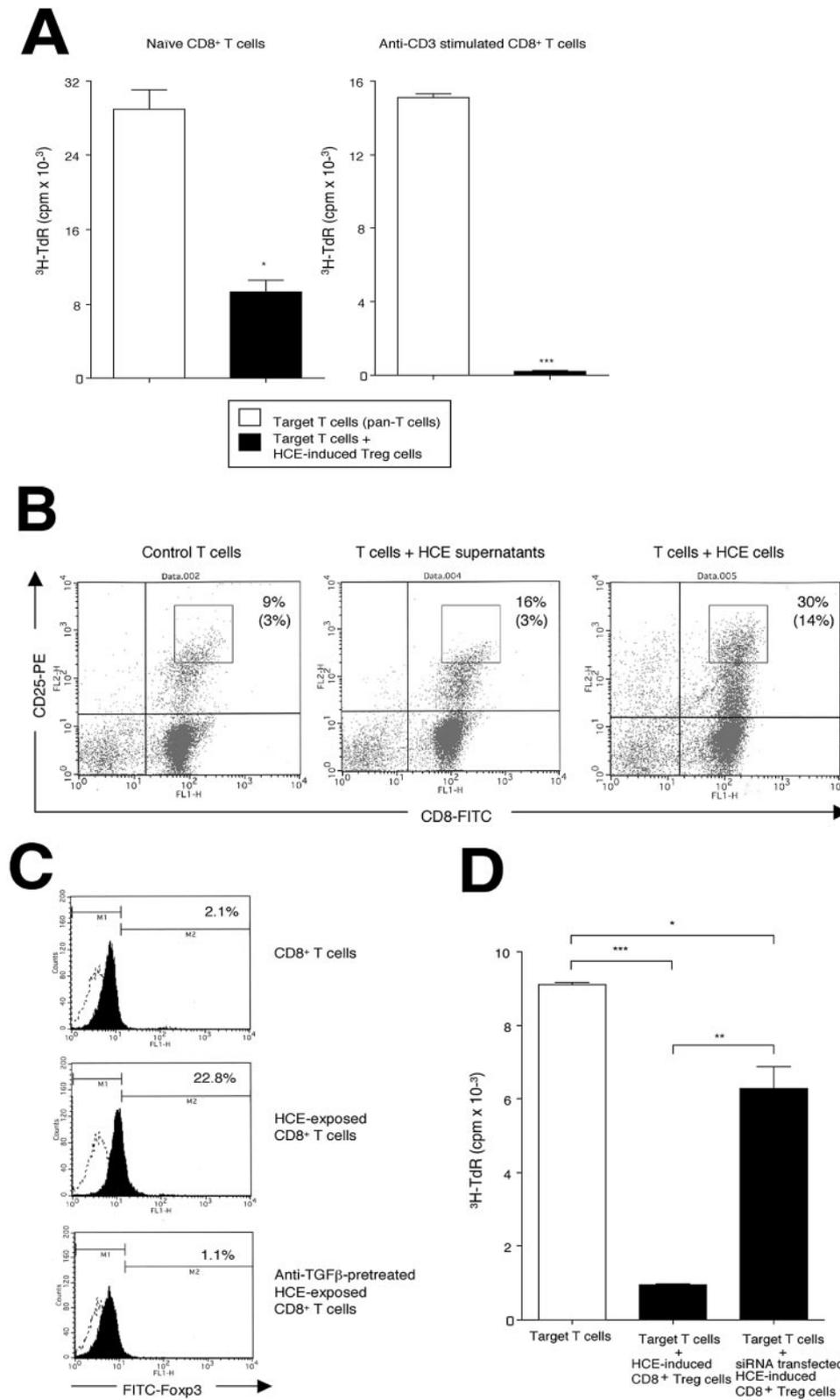
which suggests that the HCE cells can convert CD8<sup>+</sup> (and/or CD4) T cells into Treg cells in vitro.

We then examined whether HCE-exposed CD8<sup>+</sup> T cells express CD25 and Foxp3, which is one of the best Treg cell markers that has been found so far. As shown in Figure 5B, CD8<sup>+</sup> T cells exposed to HCE greatly expressed CD25, especially CD25<sup>high</sup> (14% positive), compared with the CD8<sup>+</sup> T cells that were exposed to the HCE supernatants (3% positive for CD25<sup>high</sup>) or the control CD8 T cells (3% positive for CD25<sup>high</sup>). In flow cytometric analysis for Foxp3, CD8<sup>+</sup> T cells exposed to HCE cells expressed much higher levels of the intracellular Foxp3 molecule (22.8% positive) than did the control CD8<sup>+</sup> T cells (2.1% positive, Fig. 5C). More important, the CD8<sup>+</sup> T cells that were exposed to HCE poorly expressed Foxp3 (1.1% positive) when the HCE cells were pretreated with anti-human TGFβ-neutralizing Abs.

We then attempted to confirm whether HCE cells convert CD8<sup>+</sup> T cells into Treg cells by using the membrane-associated TGFβ2. To examine this possibility, we used TGFβ2-siRNA transfected HCE cells or HCE cells in the presence of anti-TGFβ2 blocking Abs. Although TGFβ2-siRNA-transfected HCE cells failed to convert CD8<sup>+</sup> T cells into Treg cells, the non-transfected control HCE cells were converted into Treg cells (Fig. 5D). This conversion was partially inhibited in a dose-dependent manner by the presence of anti-TGFβ antibodies (data not shown). These results are compatible with the hypothesis that states that cultured HCE cells bearing membrane-associated TGFβ lose their regulatory effect if the TGFβ is either neutralized or lost.

### Capacity of HCE-Induced Treg Cells to Suppress Effector T Cells

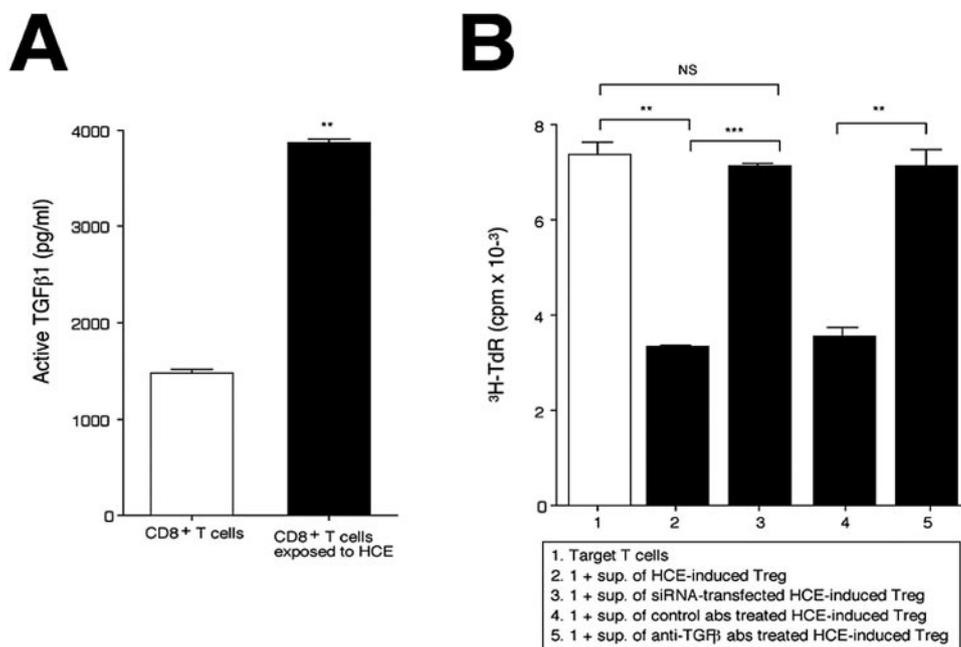
Naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells suppress effector T cells via cell contact<sup>14,15</sup> and Th3 cells, which can produce soluble TGFβ, suppress the bystander T cells via a soluble inhibitory factor(s).<sup>16,17</sup> Thus, we further examined whether HCE-induced Treg cells produce TGFβ. Compared with the control CD8<sup>+</sup> T cells, HCE-induced Treg cells significantly produced soluble forms of active TGFβ1 (Fig. 6A), but not TGFβ2 (data not shown). To block the TGFβ production by HCE-induced Treg cells, we used supernatants of Treg cells that were transfected with TGFβ1-siRNA,



**FIGURE 5.** Capacity of HCE to convert CD8<sup>+</sup> T cells into regulators. **(A)** Purified CD8<sup>+</sup> T cells ( $1 \times 10^6$ /well in a 24-well plate) were cultured with HCE for 48 hours in the presence (anti-CD3 stimulated T cells, *right*) or absence (naïve T cells, *left*) of anti-CD3 antibody (0.5  $\mu$ g/mL), harvested, and then used as Treg cells (HCE-induced Treg cells). The HCE-induced Treg cells were added to cultures containing responder target pan-T cells plus anti-CD3. **(B)** After the HCE-induced CD8<sup>+</sup> Treg cells were stained with phycoerythrin (PE)-conjugated anti-CD25 and FITC-conjugated CD8 antibodies, they were analyzed by flow cytometry. As a control, CD8<sup>+</sup> T cells in the presence of anti-CD3 without HCE cells (*left* histogram) or with supernatants of the HCE cells (*middle* histogram) were also stained. The numbers in the histograms indicate the percentage of positive cells. The numbers in the parenthesis indicate the percentage of CD25<sup>high</sup>-positive cells. **(C)** HCE-exposed CD8<sup>+</sup> T cells were stained with FITC-conjugated anti-human Foxp3 Abs or isotype rat IgG. CD8<sup>+</sup> T cells exposed to anti-TGFβ blocking Abs (5  $\mu$ g/mL)-pretreated HCE cells also stained (*bottom* histogram). CD8<sup>+</sup> T cells without HCE (upper histogram) were used as the control. Dot histogram indicates the isotype control of rat IgG. **(D)** TGFβ2-siRNA transfected (or not) HCE cells were cultured with CD8<sup>+</sup> T cells in the presence of anti-CD3 for 48 hours. The harvested and x-irradiated T cells were co-cultured with target pan-T cells plus anti-CD3. After 72 hours, the cells were assayed for uptake of [<sup>3</sup>H]-thymidine. The samples are presented as the mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ , when comparing two groups.

and we added anti-human TGFβ antibodies to cultures in which the supernatants of the HCE-induced Treg cells were present during the stimulation of the target T cells. Control mouse IgG was also added to the appropriate cultures. Supernatants of the HCE-induced Treg cells that were pre-treated with siRNA of TGFβ1 impaired the T-cell suppression caused by the HCE-induced Treg cells (Fig. 6B). Simi-

larly, anti-TGFβ-neutralizing antibodies impaired the suppression of T cells by the supernatants of the HCE-induced Treg cells, whereas the supernatants in the presence of control mouse IgG significantly suppressed T-cell stimulation (Fig. 6B). These findings indicate that secretion of TGFβ1 by the HCE-induced Treg cells is necessary for the suppression of effector T cells to occur.



**FIGURE 6.** Capacity of TGFβ-producing HCE-induced Treg cells to suppress effector T cells. (A) Supernatants of CD8<sup>+</sup> T cells exposed to HCE (HCE-induced Treg cells) were harvested for production of soluble forms of active TGFβ1. As a control, supernatants of control CD8<sup>+</sup> T cells were also collected. (B) TGFβ production by HCE-induced Treg cells was neutralized by transfecting the T cells were transfected with TGFβ1-siRNA. Supernatants of HCE-induced Treg cells in the presence of anti-TGFβ blocking antibodies were present during the stimulation of the target T cells. Control mouse IgG was also added to the appropriate cultures. Data are presented as the mean results for triplicate cultures ± SEM. \*\**P* < 0.005, \*\*\**P* < 0.0005, when comparing two groups. NS, not significant.

## DISCUSSION

In patients with uveitis and corneal inflammatory disorders such as corneal keratitis, corneal endotheliitis, and corneal allograft rejection, infiltrating inflammatory cells can attack the CE directly via the aqueous humor in the anterior chamber. However, the immunologic mechanisms and immunologic role of the CE cells in the inflammation of the anterior chamber remains unknown. In the present study, HCE cells inhibited the activation of effector CD8<sup>+</sup> T cells in vitro. Flow cytometry, RT-PCR, and immunohistochemical analysis all showed that HCE cells constitutively expressed membrane-bound active TGFβ2.

HCE cells efficiently suppressed T-cell activation via membrane-bound TGFβ. Direct cell contact optimized the capacity of HCE cells to suppress the activated CD8<sup>+</sup> T cells. Moreover, the HCE cells converted CD8<sup>+</sup> T cells into Treg cells that expressed Foxp3 and CD25<sup>high</sup>. In addition, for the suppression of the bystander T cells to occur, soluble active TGFβ1 via the HCE-induced Treg cells was essential. Although the cultured HCE cells produced TGFβ2 and TSP-1 and expressed PD-L1 and -L2 cell surface molecules, only TGFβ2 was found to be essential for the HCE-dependent immune tolerance (e.g., the T-cell suppression and regulatory T-cell induction). Thus, our findings indicate that HCE cells that contain active membrane-bound TGFβ2 have a regulatory effect.

Under normal circumstances, levels of the total TGFβ2 in the aqueous humor are within the nanogram per milliliter range, whereas the level of the active isoform of TGFβ2 is within the low picogram per milliliter range.<sup>18</sup> Our established HCE cell lines produced ~0.5 ng/mL active TGFβ2 in the culture supernatants (Table 1). However, we found that 0.5 ng/mL recombinant TGFβ2 did not suppress the activation of CD8<sup>+</sup> T cells (Fig. 2B). Because of this, we suspect HCE cells can only suppress T-cell activation in the anterior chamber and in converting the T cells into regulators if they deliver active, membrane-bound TGFβ, but not soluble TGFβ, to migrating T cells that are being targeted by the TGFβ-TGFβ receptor interactions. Thus, immunogenic inflammation within the anterior segment of the eye is suppressed (i.e., immune privilege is present), which leads to the preservation of vision. In immune-privileged sites such as the eye, TGFβ expression on the

corneal endothelium contributes to this immune tolerance and ultimately prevents blindness.

Investigators have reported that cultured human CE cells constitutively express TGFβ2 on their surfaces and that HCE can profoundly produce cytokines and chemokines such as MIP-1 (CCL2), IL-8 (CXCL8), MIP-2α (CXCL2), and IL-6. This especially occurs through stimulation of the proinflammatory cytokines.<sup>19</sup> We have reported that the PD-L1 cell surface costimulatory molecules via HCE cells suppress activation of effector Th1 cells. In addition, the expression of this molecule is enhanced by stimulation of the Th1 cytokine IFNγ.<sup>3</sup> These results imply that inflammatory conditions involving inflammatory cytokines and chemokines often cause cell clusters that adhere to the CE cells to create keratic precipitates.<sup>19</sup> We have found that cultured HCE cell lines constitutively express CD54 (ICAM-1) cell adhesion molecules and that this expression was enhanced when the HCE cells were treated with recombinant IFNγ in vitro (SS, unpublished observations, 2009). In the present study, we found that membrane-associated active TGFβ2 via the HCE cells suppressed CD8<sup>+</sup> effector T cells. In addition, some populations of CD8<sup>+</sup> T cells exposed to HCE acquired a regulatory phenotype that produced active TGFβ1 and expressed CD25<sup>high</sup> and Foxp3.

Naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> Treg cells mediate immune suppression to limit the immunopathogenesis that is associated with chronic inflammation, persistent infections, and autoimmune diseases. Their mode of suppression is contact-dependent and antigen-nonspecific and involves a nonredundant contribution from the cytokine TGFβ. Treg cells are both anergic at least in vitro and immunosuppressive. The absence of Treg cells results in the breakdown of tolerance and the development of autoimmune diseases.<sup>20</sup> The use of cell culture experiments has led to a rapid advancement in our understanding of the functional domain of these cells. In vitro, in order for CD4<sup>+</sup>CD25<sup>+</sup> Treg cells to suppress responder T-cell proliferation and cytokine production, these cells must be activated, a process that is both dependent on cell contact and antigen-nonspecific.<sup>21-23</sup> The membrane-associated TGFβ has been identified as a pivotal regulator.<sup>15,23,24</sup> In vitro stimulation with anti-CD3 and antigen-presenting cells (APCs) enhances membrane-bound active TGFβ, which is consistent

with the finding that Treg activation promotes their suppressive potential.<sup>23,25</sup> TGF $\beta$  receptor type II (TGF $\beta$  RII) was detected at elevated levels on the responder T cells once they were activated via their T-cell receptor. Thus, this provided a molecular bridge by which TGF $\beta$  on the Treg cells orchestrated suppression of the responder cells. As shown in the present study, some populations of CD8<sup>+</sup> T cells that were exposed to HCE were converted into Treg cells via interactions with membrane-associated TGF $\beta$  (via the CE) and receptor (via the T cells).

TGF $\beta$  is a pleiotropic cytokine that has the capacity to suppress aspects of immunity, although this is only one dimension of its overall activities.<sup>18,26–28</sup> In the absence of TGF $\beta$ , mice develop a massive, multifocal inflammatory disease, suggesting a major role for this factor in the regulation of both adaptive and innate immunity.<sup>29</sup> Mice in which T cells express a dominant negative TGF $\beta$  RII develop hyperproliferation of the CD8<sup>+</sup> T cells, implying a role for TGF $\beta$  in maintaining CD8<sup>+</sup> T-cell homeostasis.<sup>30</sup> TGF $\beta$  is an immunomodulatory cytokine that has been found to be constitutively present in ocular fluids,<sup>9–11,18</sup> and it is associated with Treg cells of various types.<sup>6,7,10,15,23,24</sup> We have reported that culturing CD8<sup>+</sup> T cells with iris pigment epithelium (IPE) causes the cells to become B7-expressing IPE-induced Treg cells that secrete enhanced amounts of active TGF $\beta$ .<sup>5,6</sup> In addition, the CD8<sup>+</sup> IPE-induced Treg cells use soluble TGF $\beta$  to achieve the suppression of responder T cells.<sup>31</sup> More recently, we have shown that CD4<sup>+</sup> retinal PE (RPE)-induced Treg cells can produce and secrete active TGF $\beta$ .<sup>32</sup> The RPE- or IPE-induced Treg cells express high levels of Foxp3 via this TGF $\beta$  signal. Several investigators have reported that the TGF $\beta$  signal can promote the expression of Foxp3.<sup>33–36</sup> Moreover, in vitro-inducing Treg cells from eye tissue also produce active soluble TGF $\beta$ 1. The present study also confirmed that the HCE-induced Treg cells produce and secrete active TGF $\beta$ 1. Neutralizing anti-TGF $\beta$  antibodies permitted T cells to be activated by anti-CD3 antibodies in cultures that contain HCE-induced Treg cells. All these findings suggest that eye-specific Treg cells that are induced by the TGF $\beta$  signal may use the secreted TGF $\beta$  to suppress the effector T cells.

In conclusion, surface-associated immunoregulatory molecules on the intraocular tissues may contribute to eye-dependent suppression of the inflammatory effector cells in a cell-contact-dependent manner. Thus, ocular resident tissue-exposed T cells can be induced to become regulators within the peripheral microenvironment. We are now conducting further experiments to determine whether the anterior chamber-infiltrating cells that are found in patients with intraocular inflammations such as uveitis or corneal endotheliitis have the ability to express Foxp3 and CD25<sup>high</sup> and thus produce active forms of TGF $\beta$ . Moreover, when examining mechanisms of in vivo inflammation, our findings suggest that we should consider that inflammatory cytokines such as IL-6 could potentially be acting as inhibitors of TGF $\beta$ <sup>37</sup> or as promoters of Th17-type effector T-cell induction in the presence of TGF $\beta$ .<sup>38</sup> To further explore such possibilities, we are currently in the process of conducting experiments to determine whether ocular infiltrating Treg cells that produce TGF $\beta$  can suppress effector T cells such as Th17 cells.

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