

# Human Corneal Endothelial Cells Expressing Programmed Death-Ligand 1 (PD-L1) Suppress PD-1<sup>+</sup> T Helper 1 Cells by a Contact-Dependent Mechanism

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**PURPOSE.** This study was designed to determine whether human corneal endothelial (HCE) cells could regulate the activation of bystander T cells in vitro.

**METHODS.** HCE cell lines were established from primary HCE cells. Target-activated T cells were used allogeneic T cells and Jurkat T-cell lines. As an additional target, T-cell clones from uveitis patients were established from aqueous humor via a limiting dilution. T-cell activation was assessed for proliferation by [<sup>3</sup>H]-thymidine incorporation, carboxyfluorescein succinimidyl ester incorporation, or IFN $\gamma$  production. Expression of co-stimulatory molecules on IFN $\gamma$ -treated corneal endothelial and non-treated cells was evaluated by flow cytometry, RT-PCR, or immunohistochemistry. Expression of co-stimulatory receptors on target T cells was evaluated by flow cytometry. Blocking antibodies was used to abolish the HCE-inhibitory function.

**RESULTS.** HCE cells suppressed both in vitro proliferation and IFN $\gamma$  production by CD4<sup>+</sup> T cells via a cell contact-dependent mechanism. HCE constitutively expressed co-stimulatory molecules programmed death-ligand 1 (PD-L1) and PD-L2, and their expression was enhanced by IFN $\gamma$ . HCE efficiently inhibited the proliferation of Th1 cells that overexpressed PD-1 among various activated T-cell lines and clones established from patients with uveitis or corneal endotheliitis. A neutraliz-

ing mAb for PD-L1, but not PD-L2, blocked the suppressive effect of HCE on Th1 cells.

**CONCLUSIONS.** HCE can impair the effector functions and activation of Th1 infiltrating CD4<sup>+</sup> T cells via the PD-1/PD-L1 interaction. The data support the hypothesis that corneal endothelium may contribute to maintenance of the privileged immune status of the anterior chamber of the eye by inducing peripheral immune tolerance. (*Invest Ophthalmol Vis Sci.* 2009;50:263–272) DOI:10.1167/iovs.08-2536

To avoid the adverse consequences of intraocular inflammation such as blindness, the eye possesses an extensive array of mechanisms by which innate and adaptive immune effectors can be regulated or even silenced. These mechanisms include an intraocular microenvironment (aqueous humor, etc.) that is rich in soluble immunomodulatory factors,<sup>1,2</sup> the blood-ocular barrier, and constitutive expression by resident ocular T cells of CD95 ligand,<sup>3</sup> CD86 co-stimulatory molecules,<sup>4,5</sup> membrane-bound transforming growth factor-beta (TGF $\beta$ ),<sup>6</sup> prostaglandin E<sub>2</sub>,<sup>7,8</sup> and thrombospondin-1,<sup>8,9</sup> which triggers apoptosis or inactivation of effector T cells.<sup>1–9</sup> It is important to understand the processes by which the eye normally regulates immune mechanisms because this provides insight into regulation of the immune system that may have more general applicability.

Corneal endothelial (CE) cells are in contact with the aqueous humor as a part of the inner surface of the anterior chamber of the eye. Human CE (HCE) cells do not proliferate throughout life. To compensate for their lack of regenerative capacity, CE cells possess immune protection systems that inhibit IL-2 production by T cells<sup>10</sup> and also constitutively express CD95 ligand.<sup>11</sup> In patients with corneal diseases like herpetic keratitis, endotheliitis, and corneal allograft rejection, and in patients with iritis (including uveitis and endophthalmitis), inflammatory cells can attack CE cells directly or indirectly via the aqueous humor. In these inflammatory conditions, keratic precipitates are often created by clusters of cells that adhere to the CE cells,<sup>12,13</sup> but the immunologic role of CE cells in inflammation of the anterior chamber remains unknown.

We have previously reported that cultured iris pigment epithelial cells established from the anterior segment of the eye suppress anti-CD3 mAb-driven T-cell activation (the signal consists of interaction with the T-cell receptor) in vitro by cell-to-cell contact, rather than by secretion of immunosuppressive soluble factors.<sup>14</sup> To achieve such suppression, another signal that involves engagement of co-stimulatory receptors on T cells by ligands expressed on the other of cells is also required.<sup>4,5</sup> Cultured iris pigment epithelial cells uniquely express B7-2 and suppress CTLA-4<sup>+</sup> effector bystander T cells. Molecular homologs of B7-like ligands such as programmed death-ligand 1 (PD-L1/B7-H1) have been identified.<sup>14–16</sup> These co-stimulatory molecules have been shown to downregulate T-cell activation through PD-1 similar to CTLA-4 (both are negative signals).<sup>17</sup>

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PD-1 is expressed by activated CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, NKT cells, B cells, and monocytes. Cellular expression of PD-L1 is promoted by IFN $\gamma$ . Therefore, PD-L1 may interact with PD-1<sup>+</sup> cells to achieve the suppression of inflammation.

In the present study, we demonstrated that our newly established HCE cell line could negatively regulate CD4<sup>+</sup> T-cell proliferation via a cell contact mechanism that was dependent on interaction of PD-1 and PD-L1. The CD4<sup>+</sup> T cells inhibited by HCE cells were exclusively of the Th1 type. We discuss the possibility that the PD-1/PD-L1 interaction has a critical role in maintenance of the privileged immune status in the cornea and anterior chamber of the eye.

## MATERIALS AND METHODS

### Preparation of Cultured HCE Cell Lines from Donor Human Corneas

We established an HCE cell line transduced with hTERT and the large T gene. Primary cultures of HCE cells were prepared as described previously.<sup>18</sup> The retroviral vectors BABE-hygro-hTERT (for hTERT) and MFG-tsT-IRES-neo (for SV40 large T antigen) were used in these experiments.<sup>19</sup> Briefly, viral supernatants were produced from the ecotropic packaging cell line BOSC23 by transfection of 8  $\mu$ g of plasmid DNA in a transfection reagent (Lipofectamine; Invitrogen-Life Technologies, Tokyo, Japan). The viral supernatant from BOSC23 was used to infect the amphotropic packaging cell line CRIP-P131, and was selected by incubation with 0.1 mg/mL hygromycin for 8 days (BABE-hygro-hTERT) or with 1 mg/mL G418 for 4 days (MFG-tsT-IRES-neo). The titer of viral supernatant generated by producer CRIP-P131 cells was  $1.75 \times 10^5$  CFU/mL (BABE-hygro-hTERT) and  $5 \times 10^5$  CFU/mL (MFG-tsT-IRES-neo). Viral supernatants were passed through a 0.45- $\mu$ m filter to remove cellular debris before use. In the presence of 8  $\mu$ g/mL polybrene (Sigma Chemical Co., St Louis, MO),  $2 \times 10^5$  HCE cells (passage 2) in a 10-cm dish were exposed for 8 hours to viral supernatant containing both retroviruses at an approximate multiplicity of infection of 1 to ensure single-copy integration. After washing with PBS, the transduced HCE cells were incubated for 48 hours and selected with 0.1 mg/mL hygromycin (BABE-hygro-hTERT) and 1 mg/mL G418 (MFG-tsT-IRES-neo).

For assay, cultured HCE cells were triturated to obtain a single cell suspension, and then were re-suspended in Dulbecco's modified Eagle's medium (DMEM; 0.1 mM nonessential amino acids, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 10% fetal bovine serum [FBS]), placed into 24-well plates, and incubated for several days at 37°C. At the end of culture, the HCE cells were washed with serum-free fresh medium and added to the wells of culture plates. The established HCE cell line expressed Na(+)-HCO(3)(-) cotransporter and ATP-dependent sodium-potassium ion pump (Na[+]/K[+]-ATPase) genes, which were closely associated with transport activity of *in vivo* CE cells. Extracellular matrix and components constituting cell-cell junctions in the *in vivo* CE cells were also detected in the cell line (Yamagami S, unpublished observation, 2007). Passage-7 to -10 HCE cells were used for experiments.

### 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 and incubated overnight. Pan-T cells, CD4<sup>+</sup> T cells, or CD8<sup>+</sup> T cells were prepared separately from the peripheral blood mononuclear cells (PBMCs) of healthy volunteers using cell isolation kits (MACS; Miltenyi Biotec, Auburn, CA). These cells were purified by a single immunomagnetic depletion step using magnetic beads (MACS MicroBeads; Miltenyi Biotec), and were found to be >95% CD3-, CD4-, or CD8-positive.

Th1 cell lines were established by culture with human rIL-12 (Wako, Osaka, Japan), human rIL-2, anti-CD3 mAb (Clone 2C11; BD

PharMingen, San Diego, CA), X-irradiated (50 Gy) allogeneic PBMCs (feeder cells), and phytohemagglutinin-p (Difco Laboratory Inc., Detroit, MI). Purified CD4<sup>+</sup> T cells were prepared from the PBMCs of healthy volunteers, and then were cultured for 2 weeks in RPMI 1640 medium with 10% FBS. Feeder cells were added to each well along with rIL-2 every 5 days until outgrowth of cells was observed. We used the cells as IL-12-induced Th1 cells if the T cells showed high production of IFN $\gamma$ .

T-cell clones (TCCs) were established in our laboratory by the limiting dilution method as described elsewhere.<sup>20,21</sup> To establish ocular TCCs, approximately 0.1 mL of aqueous humor was drawn into a tuberculin syringe from each patient. The cells obtained were CD4<sup>+</sup> T cells (Th1 or non-Th1), while the patients with uveitis had Behçet's disease, sarcoidosis, or Vogt-Koyanagi-Harada (VKH) disease. TCCs were also established from aqueous humor of a patient with corneal endotheliitis. All samples were harvested after informed consent was obtained from the patients. This research was done according to the Declaration of Helsinki and was approved by the Institutional Review Board of Tokyo Medical and Dental University.

### Evaluation of T-Cell Activation

Purified T cells ( $0.5 \times 10^6$ /well) were stimulated with anti-CD3 mAb and incubated for 48 hr (assessment of IFN $\gamma$  production) or 72 hr (assessment of proliferation). The concentration of soluble anti-CD3 mAb in these cultures was 0.01, 0.1, or 1.0  $\mu$ g/mL, as indicated. IFN $\gamma$  levels in the supernatant of cultured T cells exposed to HCE were measured by ELISA (R&D Systems, Minneapolis, MN). In some experiments, purified blocking anti-human PD-L1/B7-H1 mAb (MIH1; eBiosciences, San Diego, CA) or anti-human PD-L2/B7-DC mAb (MIH1 8; eBiosciences) was added to the cells. After incubation, the uptake of [<sup>3</sup>H]-thymidine was measured to assess cell proliferation. Incorporated radioactivity was measured with a liquid scintillation counter and was expressed in cpm. T cells stimulated with anti-CD3 mAb were grown in serum-free medium (complete medium except for FBS) that was supplemented with 0.1% BSA (Sigma Chemical Co.) and with 0.2% insulin, transferrin, selenium (ITS) + culture supplement (Collaborative Biochemical Products, Bedford, MA).

### Flow Cytometric Analysis

Flow cytometry was used to analyze the expression by HCE cells and T cells of various costimulatory or cell-surface molecules using the following mAbs: FITC-conjugated anti-CD70 mAb (L01.1, IgG2a) and anti-CD40 mAb (5C3, IgG1); PE-conjugated anti-CD80 mAb (L307.4, IgG1), anti-CD86 mAb (IT2.2, IgG2b), anti-4-1BBL mAb (C65-485, IgG1), anti-ICOS-L mAb (2D3/B7-H2, IgG2b), anti-PD-L1/B7-H1 mAb (MIH1, IgG1), anti-PD-L2/B7-DC mAb (MIH1 8, IgG1), anti-PD-1 mAb (J116, IgG1), and anti-OX40L mAb (ANC10G1, IgG1); and mouse isotype IgG as the control. For FACS analysis of the surface expression of various molecules by HCE cells, subconfluent cells were stimulated with 500 U/mL of human rIFN $\gamma$  (BD Pharmingen) for 48 hr. Single-cell suspensions of the HCE cells were then obtained by treatment with 0.5% trypsin/EDTA (Sigma Chemical Co.) and were washed twice in PBS, after which  $1 \times 10^6$  cells were incubated with FITC- or PE-labeled mAbs. After washing twice in PBS, the stained cells (live-gated on the basis of the forward and side scatter profile and propidium iodide exclusion) were analyzed with a flow cytometer (FACSCalibur; BD Biosciences). Data were processed using commercial software (CellQuest; BD Biosciences), and results are expressed as the mean fluorescence intensity (MFI).

In companion experiments, anti-CD3 mAb stimulated CD4<sup>+</sup> T cells or Jurkat T-cell lines were harvested after 24 hr of culture, and stained for PD-1 (J116, IgG1). rIL-12- induced Th1 cells and intraocular TCCs were also stained. Before staining, the co-cultured cells were incubated with human Fc-block (Miltenyi Biotec) for 15 minutes. As an isotype control for PD-1, we used PE-conjugated mouse IgG (BD Pharmingen). Cultured HCE cells were analyzed with a PE-labeled mAb for PD-L1/B7-H1 (MIH1, IgG1). Before flow cytometry, HCE cells were exposed

to the culture supernatants of rIL-12-induced Th1 cells or human rIFN $\gamma$ . Then the cells were harvested and stained with anti-PD-L1 mAb. PE-conjugated mouse IgG (BD Pharmingen) was used as the control.

### CFSE Labeling of T Cells

Labeling of CD4<sup>+</sup> T cells (Jurkat T cells) with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) was performed as described elsewhere.<sup>5</sup> Briefly,  $1 \times 10^7$  T cells were diluted in 1 mL of serum-free HBSS, 1  $\mu$ M/ml CFSE was added, and the cell suspension was incubated for 8 minutes at room temperature. Then labeling of the cells was stopped by quenching unlabeled CFSE through adding an excess of HBSS containing FBS. Subsequently, cells were washed three times and used for the experiments, while unlabeled cells were used as the control. Purified T cells ( $1 \times 10^7$ ) were labeled with CFSE at a final concentration of 1  $\mu$ M or not labeled (controls), and then were added ( $0.5 \times 10^6$ /well in 24-well plates) to wells containing anti-CD3 mAb at a final concentration of 1.0  $\mu$ g/mL plus cultured HCE cells (or not). After 72 hr, the T cells exposed to HCE cells were washed and analyzed by flow cytometry.

### Apoptosis Flow Cytometric Assay

Flow cytometry was applied to analyze whether HCE-exposed T cells could undergo apoptosis. Annexin V-PE staining (Annexin V:PE Apoptosis Detection Kit I; BD Pharmingen) was used for detection of annexin V-positive cells. We prepared three types of T cells as follows: (1) anti-CD3 stimulated T cells (control); (2) anti-CD3 stimulated T cells with camptothecin (12  $\mu$ M, positive control); and (3) anti-CD3 stimulated T cells exposed to HCE cells. These cells were incubated with annexin V-PE in a buffer containing 7-amino-actinomycin D (7-AAD) or FITC-labeled CD4 (BD Pharmingen) and analyzed by flow cytometry.

### Reverse Transcription–Polymerase Chain Reaction

Cellular extracts were prepared from cultured HCE cells and analyzed by RT-PCR. Extracts of HCE cells that had been exposed to culture supernatants of rIL-12-induced Th1 cells were also prepared. First, rIL-12-induced Th1 cells were incubated with serum-free medium for 24 hr in the presence of anti-CD3 mAb and then the supernatants were harvested for use. Cultured HCE cells were washed twice with PBS, and total RNA was isolated with a reagent (Trizol; Invitrogen-Life Technologies, Carlsbad, CA). After cDNA synthesis, PCR was carried out by the standard method. The PCR conditions and primer sequences for human PD-L1 have been described in detail elsewhere,<sup>22</sup> but included denaturation at 94°C for 60 seconds, annealing at 60°C for 60 seconds, and extension at 72°C for 120 seconds. PCR amplification was done for 35 cycles with primers for PD-L1 (5'-GACCTATATGTGGTAGATATG GTAGC-3' and 5'-TTCAGCTGTATGGTTTTCCTCAGGATC-3') that yielded a product of 568 bp. PCR products were electrophoresed on 1.5% agarose gel and visualized by staining with ethidium bromide. Then the level of mRNA expression was standardized by that of  $\beta$ -actin as an internal control.

### Immunohistochemistry

Cultured HCE cells were grown in a 4-well chamber slide (cell culture slide; BD Biosciences) for 5 days. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, followed by permeabilization with 0.1% Triton X. Then cells were incubated for 1 hour with anti-human PD-L1/B7H1 mAb (1:100 dilution, eBiosciences) or mouse IgG (1:100) as an isotype control, followed by washing with PBS and incubation with a fluorescent-labeled secondary Abs (Alexa Fluor 488; Invitrogen) for 1 hour. Fluorescence signals were detected by confocal microscopy (Radiance 2000; Bio-Rad Laboratories, Hercules, CA).

### Statistical Analysis

Each experiment was repeated at least twice. All statistical analyses were conducted with Student's *t*-test and differences were considered significant at  $P < 0.05$ .

## RESULTS

### Inhibition of T-cell Proliferation by Cultured HCE Cells

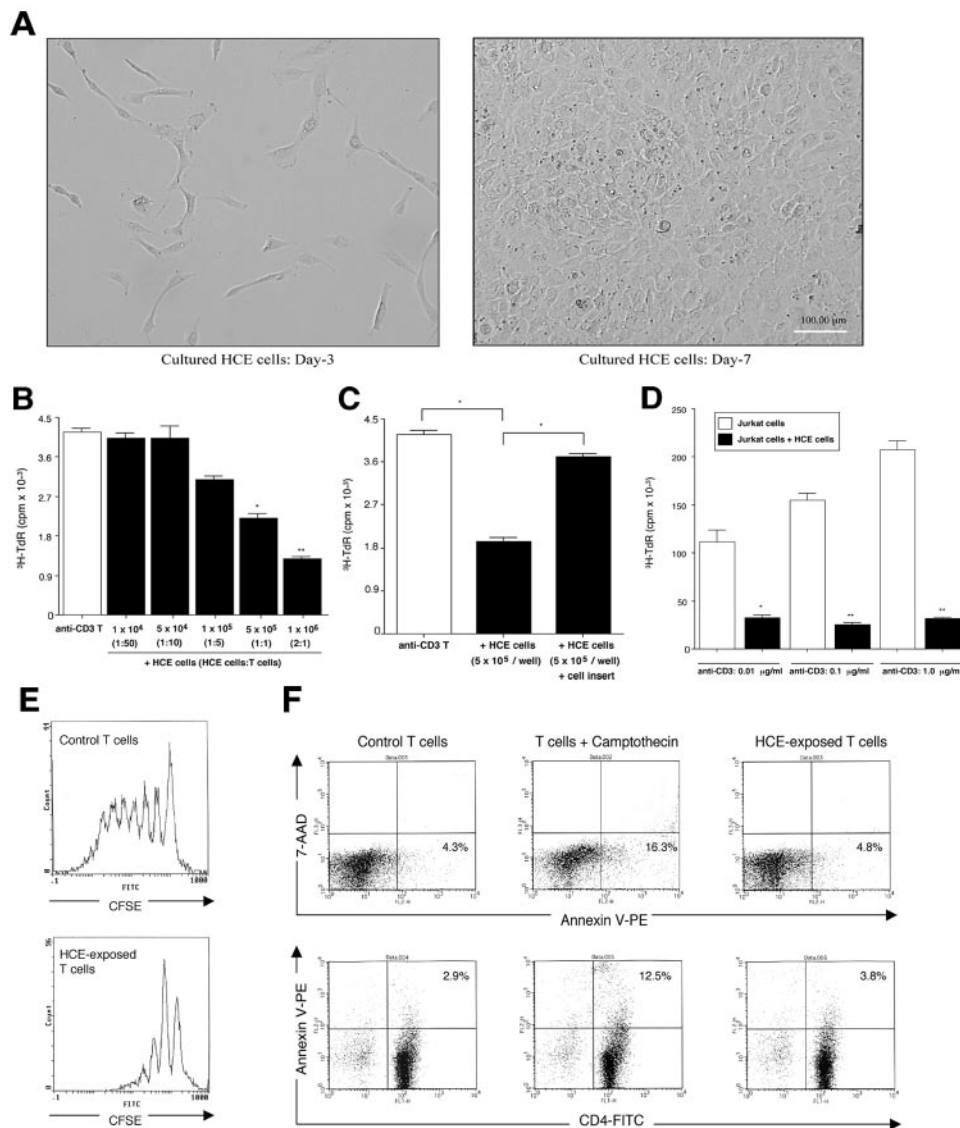
We first tested whether the HCE cell line could suppress bystander T-cell proliferation and whether the extent of suppression was dependent on HCE numbers. HCE cells were cultured for 7 days, as shown in Figure 1A, and then were co-cultured for 72 hours with CD4<sup>+</sup> T cells in the presence of anti-CD3 mAb. T-cell proliferation was assessed by [<sup>3</sup>H]-thymidine incorporation at 72 hours. In the presence of  $1 \times 10^4$  to  $1 \times 10^5$  HCE cells, anti-CD3 mAb-stimulated T-cell proliferation was not suppressed, whereas the activated T cells were significantly suppressed by  $5 \times 10^5$  or  $1 \times 10^6$  HCE cells (Fig. 1B). Therefore, we used  $5 \times 10^5$  or  $1 \times 10^6$  HCE cells in further experiments.

To elucidate the role of cell-to-cell contact in the suppression of T-cell activation by HCE cells, these cells were first cultured separately in 24-well plates. Transwell cell inserts were then placed in these wells and each transwell contained CD4<sup>+</sup> T cells plus anti-CD3 mAb. Anti-CD3 mAb-driven CD4<sup>+</sup> T-cell proliferation was significantly suppressed in the absence of transwell cell inserts, as shown in Figure 1C. In contrast, HCE cells did not suppress T-cell proliferation across the transwell membrane. These findings imply that direct contact of T cells and HCE cells was essential for the suppression of T-cell proliferation, suggesting the involvement of an interaction between surface molecules on both cells in the suppression of T-cell proliferation.

To test whether proliferation of T cells was suppressed in cultures with or without HCE cells, we exposed Jurkat cells (a T-cell line) to HCE cells in the presence of various concentrations of anti-CD3 mAb. Jurkat cells were co-cultured with HCE cells for 72 hours and then cell proliferation was evaluated. We found that when Jurkat cells were stimulated with anti-CD3 mAb (0.01–1.0  $\mu$ g/mL), their proliferation was significantly suppressed by exposure to HCE cells (Fig. 1D). We also examined whether HCE cells could suppress the proliferation of CFSE-labeled T cells in vitro. Anti-CD3 stimulated CD4<sup>+</sup> T cells were stained with CFSE and stimulated with anti-CD3 mAb in the presence or absence of HCE cells. The cultured T cells were harvested at 72 hours, and flow cytometry was used to evaluate the extent of progressive cell division. As shown in Figure 1E, up to 6 to 7 rounds of T-cell division were evident in positive control cultures without the HCE cells (upper panel). When HCE cells were present, the anti-CD3-stimulated T cells underwent only 2 to 3 rounds of division (lower panel in Fig. 1E). These results obtained by using CD4<sup>+</sup> cells were consistent with the finding that HCE cells suppressed activation of bystander CD4<sup>+</sup> T cells in vitro.

We next tested whether HCE-exposed T cells could undergo apoptosis. Apoptotic cells were determined by binding of annexin V with flow cytometry. As shown in Figure 1F, anti-CD3 stimulated T cells with camptothecin that can induce apoptosis to T cells expressed a higher level of annexin V (16.3% positive) than untreated control T cells (no camptothecin, 4.3% positive, Fig. 1F). When HCE cells were present in the culture wells, the anti-CD3-stimulated T cells did not express a higher level of annexin V compared with untreated control T cells (4.8% positive). Lower histograms in Figure 1F showed that annexin V-positive cells were almost CD4 positive. CD4<sup>+</sup> T cells exposed to HCE, however, did not up-regulate the expression level of annexin V compared with control CD4<sup>+</sup> T cells unexposed to HCE. These results indicate that HCE cells do not promote apoptosis to bystander T cells in vitro, suggestive of low or no expression of functional Fas ligand (CD95 ligand) or apoptosis-inducing molecule(s) in con-





**FIGURE 1.** Ability of HCE cells to suppress activation of T cells. (A) Our established HCE cell line was grown in 24-well culture plates. HCE cells were seen as non-confluent fibroblast-like cells on day 3 of culture by phase-contrast microscopy. On day 7 of culture, HCE cells were confluent and had a hexagonal CE cell-like morphology. Scale bar = 100  $\mu\text{m}$ . (B) Purified CD4<sup>+</sup> T cells were stimulated with 1  $\mu\text{g}/\text{mL}$  anti-CD3 mAb and cultured with (black bars) or without (open bar) HCE cells at  $1 \times 10^4$  to  $10^6$ /well. Culture with HCE cells at  $>5 \times 10^5$ /well significantly suppressed [<sup>3</sup>H]-thymidine uptake. \* $P < 0.05$ , \*\* $P < 0.005$  compared with anti-CD3 mAb-stimulated T cells without HCE cells (anti-CD3 T, positive control). Mean cpm for triplicate cultures are presented ( $\pm$ SEM). (C) HCE cells were grown in 24-well culture plates ( $5 \times 10^5$ /well). Transwell cell culture inserts were placed in the wells to prevent cell-to-cell contact, and CD4<sup>+</sup> T cells ( $5 \times 10^5$ /well) plus anti-CD3 mAb were added. After 72 hr, the cells were assayed for uptake of [<sup>3</sup>H]-thymidine. Mean cpm for triplicate cultures are presented ( $\pm$ SEM). \* $P < 0.05$ . (D) Jurkat cells were cultured with HCE cells in the presence of anti-CD3 mAb. Jurkat cell proliferation increased in a CD3-dependent manner, and was suppressed by coculture with HCE cells. \* $P < 0.05$ , \*\* $P < 0.005$ . (E) Jurkat cells, labeled with CFSE, were added ( $5.0 \times 10^5$ /well) to 24-well plates. These cells were cultured in medium containing anti-CD3 mAb (final concentration: 1  $\mu\text{g}/\text{mL}$ ) with (lower image) or without (upper image, control) HCE cells. After 72 hr, the T cells were harvested for flow cytometry. (F) Anti-CD3 stimulated CD4<sup>+</sup> T cells exposed to

HCE cells were incubated with annexin V-PE in a buffer containing 7-AAD or CD4-FITC and analyzed by flow cytometry to detect apoptotic cells. For controls, anti-CD3 stimulated CD4<sup>+</sup> T cells with (middle histograms, positive control) or without camptothecin (left histograms, untreated control cells). After 5 hr incubation, these T cells were harvested and stained with annexin V-PE and 7-AAD or annexin V-PE and CD4-FITC. There were primarily two populations of cells: Cells that were viable and not undergoing apoptosis (annexin V-PE and 7-AAD negative); cells undergoing apoptosis (annexin V-PE positive and 7-AAD negative). Upper histograms, annexin V-PE and 7-AAD. Lower histograms, annexin V-PE and CD4-FITC.

trast to positive expression of these molecules in mouse corneas.<sup>3,11,23</sup>

### Suppression of Various Activated T Cells by HCE Cells

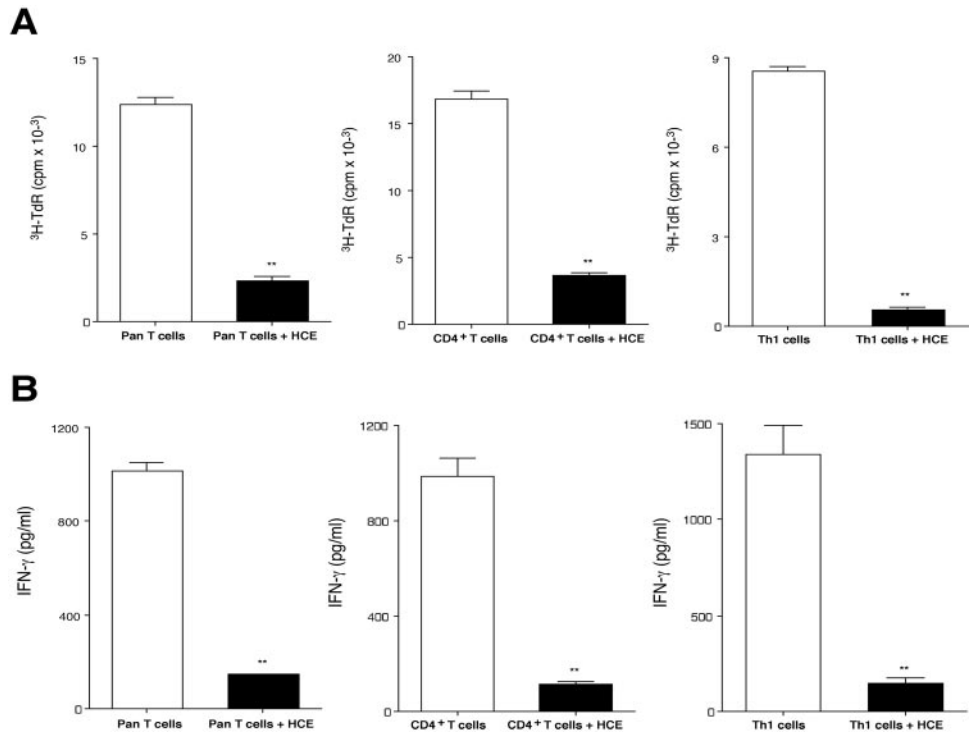
We next examined whether HCE cells could suppress the proliferation of various T cells, including pan-T cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and rIL-12-induced Th1 cells. We also examined the extent to which T cells stimulated by anti-CD3 mAb in the presence of HCE cells produced IFN $\gamma$ , an effector cell-associated cytokine. T cells were stimulated with anti-CD3 mAb in the presence or absence of HCE cells. Supernatants were removed after 48 hours and analyzed by ELISA to measure the IFN $\gamma$  concentration, while cultured T cells were harvested for evaluation of proliferation after 72 hours, Figure 2A shows that the proliferation of pan-T cells, CD4<sup>+</sup> T cells, and Th1 CD4<sup>+</sup> T cells was significantly reduced compared with the

positive control when T cells were stimulated by anti-CD3 mAb in the presence of HCE cells. Moreover, production of IFN $\gamma$  was profoundly reduced when these activated T cells were co-cultured with HCE cells (Fig. 2B). Similarly, HCE cells significantly suppressed the activation of CD8<sup>+</sup> T cells (data not shown). These results indicate that HCE cells suppressed anti-CD3-driven T-cell proliferation and cytokine production such as Th1 cytokine IFN $\gamma$ .

### Ability of HCE Cells to Suppress Ocular Infiltration by T Cells

T cells, especially CD4<sup>+</sup> T cells, have the most important role in the pathogenesis of ocular inflammation. Accordingly, we tested whether HCE cells could suppress intraocular TCCs established in our laboratory from the aqueous humor of patients with active uveitis or corneal endotheliitis.<sup>24,25</sup> We found that HCE cells significantly suppressed the proliferation

**FIGURE 2.** Effect of HCE cells on proliferation and IFN $\gamma$  production by T cells activated with anti-CD3 mAb. Purified T cells ( $5.0 \times 10^5$ /well) were stimulated with 1  $\mu$ g/mL anti-CD3 mAb and cultured for 48 hr (B) or 72 hr (A) in the presence or absence of HCE cells ( $5 \times 10^5$ /well). (A) Anti-CD3 mAb-stimulated T cells were harvested after 72 hr of culture with HCE cells. Isolated pan-T cells, CD4<sup>+</sup> T cells, and rIL-12-induced Th1 cells were assayed for uptake of [<sup>3</sup>H]-thymidine. Proliferation of these T cells was suppressed by culture in the presence of HCE cells. (B) Supernatants were harvested and assayed by ELISA for measurement of the IFN $\gamma$  concentration. IFN $\gamma$  production was significantly reduced when these T cells were incubated in the presence of HCE cells. Mean cpm for triplicate cultures are presented ( $\pm$ SEM). \*\**P* < 0.005 compared with positive control cultures (no HCE cells, open bars).

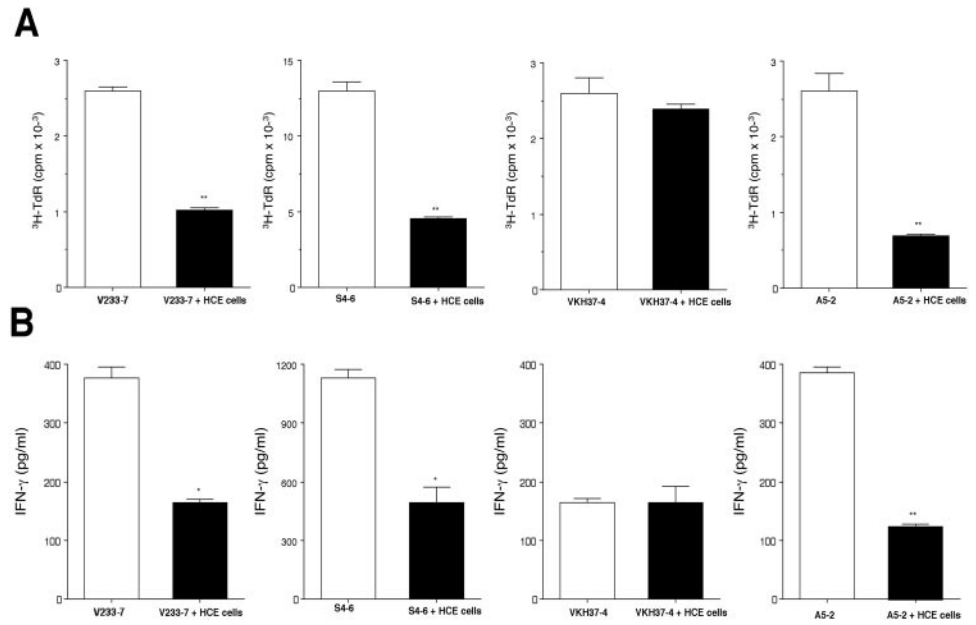


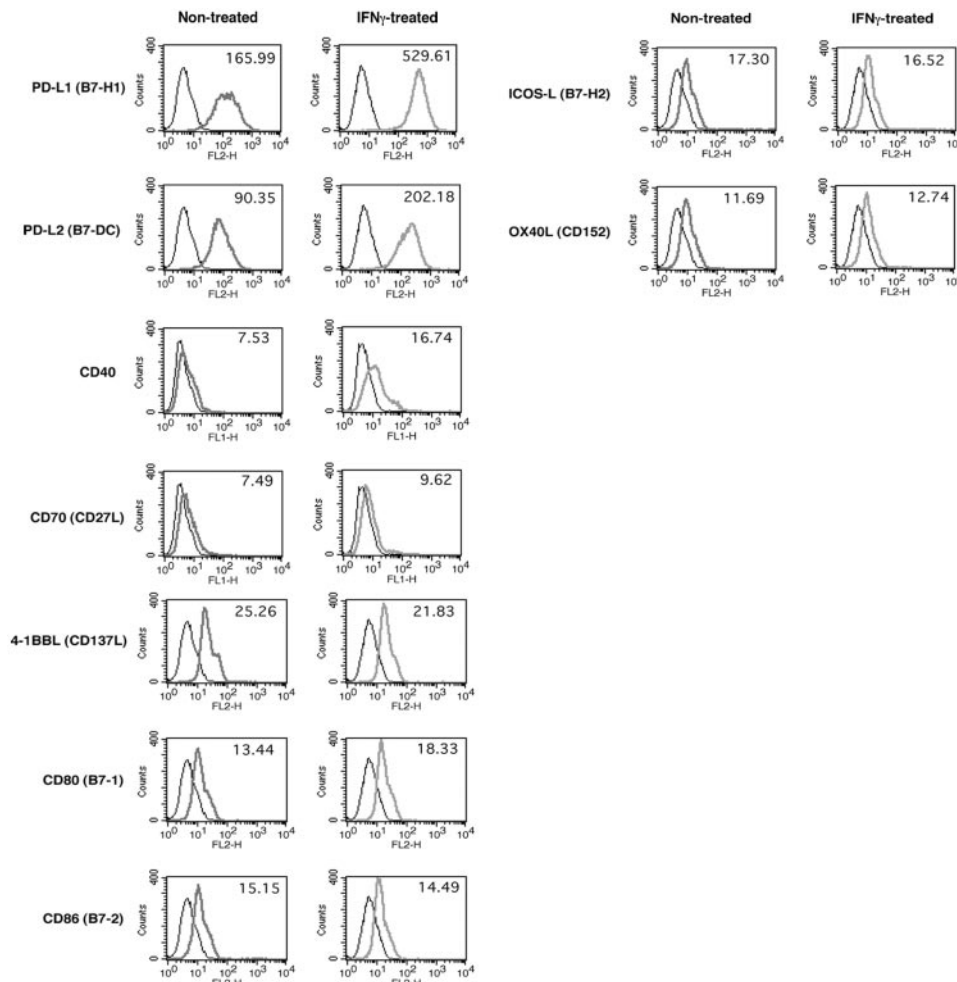
of Th1 CD4<sup>+</sup> TCCs (Behçet's disease: V233-7; sarcoidosis: S4-6; corneal endotheliitis: A5-2), but not the proliferation of non-Th1 TCCs (VKH disease: VKH37-4; sarcoidosis: S3-8; corneal endotheliitis: A5-6; data not shown), as shown in Figure 3A. Similarly, IFN $\gamma$ -production by Th1 CD4<sup>+</sup> TCCs (V233-7, S4-6, A5-2), but not that by non-Th1 TCCs (VKH37-4, S3-8, A5-6, data not shown), was significantly suppressed by co-culture with HCE cells (Fig. 3B). These findings demonstrate that HCE cells could actively suppress the proliferation of Th1 TCCs, but not non-Th1 TCCs, from patients with ocular inflammation and suggest that HCE cells modify T-cell function by modulating production of the effector cytokine IFN $\gamma$ . In fact, the aqueous humor of patients with active ocular inflammation contains high levels of Th1 effector cytokines.<sup>24,25</sup>

**HCE Cell Expression of Candidate Surface Molecules That Could Mediate Contact-Dependent Suppression of Th1 Activation**

Based on the finding that HCE cells exclusively suppress activation of Th1 cells producing IFN $\gamma$  via a contact-dependent mechanism, we hypothesized that molecules expressed on the surface of HCE cells were involved in the suppression of activated T cells. To detect cell surface molecules, IFN $\gamma$ -pre-treated or untreated HCE cells were subjected to flow cytometry after being stained with specific mAbs for the following candidate molecules: PD-L1 (B7-H1), PD-L2 (B7-DC), CD40, CD70 (CD27 ligand), 4-1BBL (CD137 ligand), CD80 (B7-1), CD86 (B7-2), ICOS-L (B7-H2), and OX40L (CD152). HCE cells

**FIGURE 3.** Effect of HCE cells on proliferation and IFN $\gamma$  production by ocular infiltrating T cells. TCCs were established from the aqueous humor of patients with active uveitis, including Behçet's disease (clone V233-7), sarcoidosis (clone S4-6), and VKH disease (clone VKH37-4), as well as from a patient with corneal endotheliitis (clone A5-2). TCCs ( $5.0 \times 10^5$ /well) were stimulated with 1  $\mu$ g/mL anti-CD3 mAb and cultured for 48 hr (B, IFN $\gamma$  production by T cells) or 72 hr (A, T-cell proliferation) in the presence or absence of HCE cells. HCE cells significantly suppressed proliferation and IFN $\gamma$  production by Th1 CD4<sup>+</sup> TCCs (V233-7, S4-6, and A5-2), but not non-Th1 TCCs (VKH37-4). Mean cpm for triplicate cultures are presented ( $\pm$ SEM). \**P* < 0.05, \*\**P* < 0.005 compared with TCCs alone (no HCE cells, open bars).





**FIGURE 4.** Expression of candidate cell surface molecules by HCE cells. For FACS analysis of the expression of immunosuppressive molecules by HCE cells, subconfluent cells were stimulated with rIFN $\gamma$  (500 U/mL) for 48 hr. Data were processed using commercial software (CellQuest; BD Biosciences) and expressed as MFI. PD-L1, PD-L2, and 4-1BBL were expressed by HCE cells. In addition, PD-L1 and PD-L2 were induced on IFN $\gamma$ -treated HCE cells.

did not express CD40, CD70, CD80, CD86, ICOS-L or OX40L, but the cells expressed PD-L1, PD-L2, and 4-1BBL (Fig. 4). Moreover, PD-L1 and PD-L2 (but not 4-1BBL) were induced on IFN $\gamma$ -treated HCE cells (Fig. 4). Thus, the co-stimulatory molecules PD-L1 and PD-L2 seemed to be candidates for a role in the suppression of T-cell activation by contact with HCE cells. It is assumed that the major cellular interactions involved in HCE-suppression are not B7-CTLA-4 pathway, because HCE cell lines poorly express B7-1 and B7-2 costimulatory molecules.

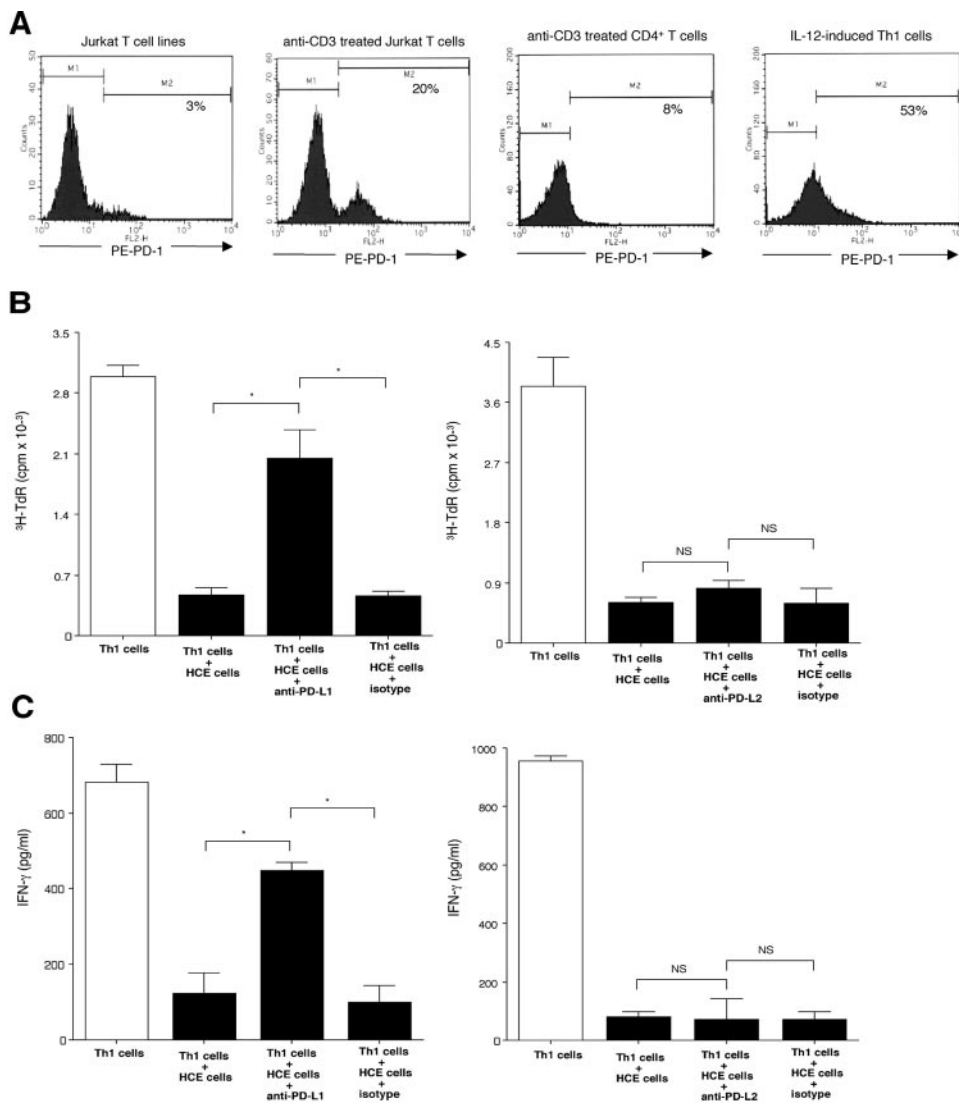
#### PD-1 Expression by T Cells and Functional Analysis of HCE-Dependent Suppression of Activated T Cells

Among the family of ligands that bind to B7-like co-stimulators, PD-L1 and PD-L2 are both ligands for PD-1.<sup>14-16</sup> This homologue of CTLA-4 transmits negative signals to T cells when it binds with PD-L1 or PD-L2 expressed on APCs.<sup>17</sup> Therefore, we evaluated PD-1 expression on T cells activated by anti-CD3 mAb. Jurkat cells (a T-cell line), CD4<sup>+</sup> T cells, and Th1 cells were harvested after 24 hours of culture with anti-CD3 mAb. Then the cells were stained with anti-human PD-1 mAb and analyzed by flow cytometry. Basal PD-1 expression by Jurkat cells was very low (3% positive, Fig. 5A), but Jurkat cells stimulated with anti-CD3 mAb showed up-regulation of PD-1 expression (20% positive). CD4<sup>+</sup> T cells stimulated with anti-CD3 mAb showed low PD-1 expression (8% positive). In contrast, rIL-12-induced Th1 cells stimulated with anti-CD3 mAb showed high PD-1 expression (53% positive, Fig. 5A). In addition,

Th1-type TCCs (V233-7) established from active uveitis of Behçet's disease greatly expressed PD-1, whereas the expression by non-Th1-type TCCs (VKH37-4) from VKH disease was very low (data not shown). These findings suggested that PD-1 on responding Th1 cells was the functional receptor for PD-L1 or PD-L2 when HCE cells suppressed T-cell activation.

To investigate whether interactions between costimulatory molecules had a role in HCE-dependent suppression of activated T cells, we conducted functional analyses with blocking mAbs for human PD-L1 or PD-L2. rIL-12-induced Th1 cells were stimulated with anti-CD3 mAb in the presence or absence (positive control) of HCE cells. One set of cultures was stopped at 72 hours for evaluation of [<sup>3</sup>H]-thymidine uptake. In a companion set of cultures, supernatants were harvested at 48 hours for measurement of the IFN $\gamma$  concentration. Suppression of Th1 cell proliferation by HCE cells was blocked in the presence of anti-PD-L1 mAb, but not isotype control IgG, whereas HCE cells significantly suppressed Th1 cell proliferation in the presence of anti-PD-L2 (B7-DC) blocking mAb (Fig. 5B). As shown in Figure 5C, HCE cells significantly suppressed IFN $\gamma$  production by Th1 cells incubated with anti-CD3 mAb, whereas suppression of IFN $\gamma$  production was prevented when HCE cells were co-cultured with anti-PD-L1 blocking mAb. In contrast, anti-PD-L2 blocking mAb did not affect the suppression of IFN $\gamma$  production by Th1 cells (Fig. 5C). These data clearly demonstrated that the suppression of Th1 CD4<sup>+</sup> T-cells by HCE cells was predominantly dependent on the interaction of PD-1 with PD-L1, but not PD-L2, as a result of cell-to-cell contact.





**FIGURE 5.** PD-1 expression on T cells and effect of PD-L1 and PD-L2. (A) T cells cultured in the presence or absence of anti-CD3 mAb (1  $\mu$ g/mL) were stained with PE-labeled anti-human PD-1 mAb, and examined by flow cytometry. Jurkat cells, CD4<sup>+</sup> T cells, and rIL-12-induced Th1 cell lines were tested. Percentages in the upper right corner indicate PD-1-positive cells. rIL-12-induced Th1 cells showed the highest prevalence of PD-1-positive cell among the tested T cells. (B) For the in vitro assay, rIL-12-induced Th1 cells were stimulated with 1  $\mu$ g/mL of anti-CD3 mAb antibody and cultured for 48 hr (C, IFN $\gamma$  production by T cells) or 72 hr (B, T-cell proliferation) in the presence or absence of cultured HCE cells. Anti-human PD-L1 neutralizing abs (1  $\mu$ g/mL), anti-human PD-L2 neutralizing abs (1  $\mu$ g/mL), or isotype mouse IgG (1  $\mu$ g/mL) were added to some wells. Mean cpm for triplicate cultures are presented ( $\pm$ SEM). \* $P$  < 0.05 compared with Th1 cell alone (*open bar*). NS, not significant.

### PD-L1 Expression by Th1 Cell-Exposed HCE Cells

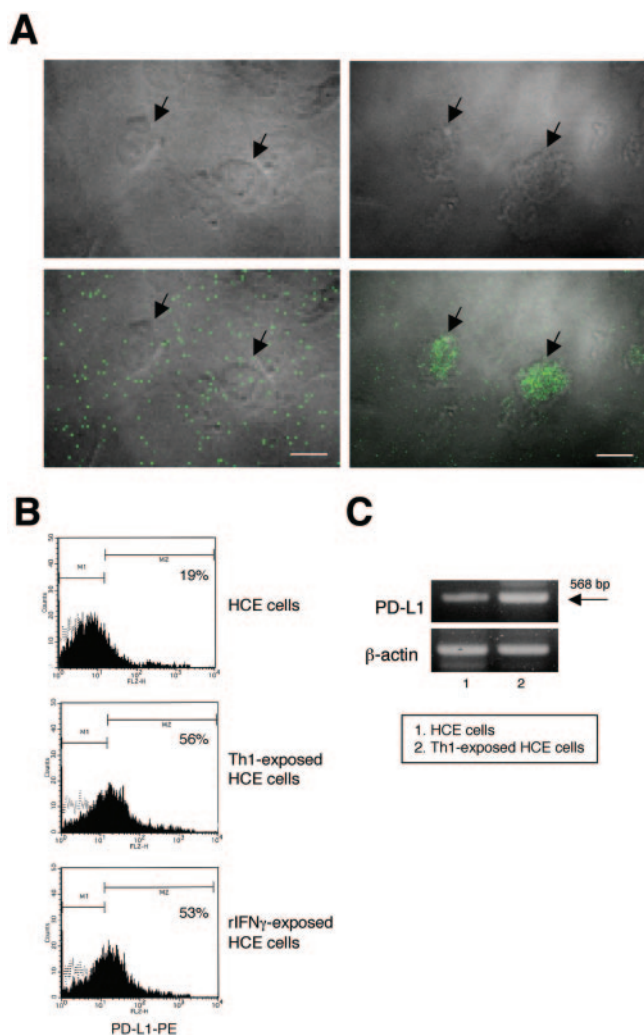
HCE cells efficiently suppressed the activation of Th1 cells via the interaction between PD-1 on T cells and PD-L1 on HCE cells. To determine whether Th1 cells bearing PD-1 could affect PD-L1 expression by HCE cells, we evaluated PD-L1 expression on HCE cells cultured with supernatant from Th1 cells. To avoid contamination by PD-L1-expressing T cells, only culture supernatants of Th1 cells were used for this assay. Immunohistochemistry showed that PD-L1 expression was prominent on the surface of HCE cells cultured with supernatants of Th1 cells (Fig. 6A), while PD-L1 expression was fairly low on HCE cells cultured without Th1 supernatant (data not shown). According to flow cytometric analysis, supernatant-exposed HCE cells, as well as recombinant IFN $\gamma$ -treated cells, had much greater PD-L1 expression than non-exposed HCE cells (Fig. 6B). Similarly, supernatant-exposed HCE cells had much greater PD-L1 expression by RT-PCR (Fig. 6C). These results imply that soluble factors including IFN $\gamma$  derived from Th1 cells promoted PD-L1 expression by HCE cells.

### DISCUSSION

We demonstrated that our HCE cell line could suppress proliferation and IFN $\gamma$  production by CD4<sup>+</sup> T cells in a contact-

dependent manner and constitutively expressed the co-stimulatory molecule PD-L1, with its expression being enhanced by IFN $\gamma$  or soluble factors produced by Th1 cells. HCE cells selectively inhibited proliferation of Th1 cells bearing PD-1 among various T-cell lines and TCCs derived from patients. A neutralizing mAb for PD-L1, but not PD-L2 blocked the suppression of Th1 cells by HCE cells. These findings suggest that HCE cells with no regenerative capacity in vivo can protect themselves against other cells infiltrating the anterior chamber of the eye via the PD-1/PD-L1 interaction and that these cells contribute to maintenance of the privileged immune status of the cornea.

PD-1 is expressed by activated T and B cells and by a subset of thymocytes.<sup>26</sup> PD-1 contains an ITIM sequence in its cytoplasmic tail that negatively regulates T-cell Ag receptor signaling through interaction with specific ligands.<sup>27,28</sup> PD-L1 (B7-H1) and PD-L2 (B7-DC) both belong to the B7 family, and have been cloned<sup>15,16</sup> and identified as potential ligands for PD-1.<sup>17</sup> The PD-L1/PD-1 costimulatory pathway has been demonstrated to play a role in regulation of the immune response. Although PD-L1 expression has been detected in lymphoid cells, including activated T cells, APCs, monocytes, and B cells,<sup>15</sup> PD-L1 is also expressed in non-lymphoid tissues such as the heart, lung, placenta, kidney, and liver.<sup>15</sup> PD-1-deficient mice have been



**FIGURE 6.** PD-L1 expression on HCE cells cultured with Th1 supernatants. (A) HCE cells cultured in the presence of Th1 cell supernatants were stained with anti-human PD-L1 mAb and observed by confocal fluorescence microscopy. *Upper panels* show bright-field images of cultured HCE cells. *Lower panels* are merged bright-field and PD-L1 stained images. On the *lower right*, PD-L1 staining (*green*) is clearly detected on the surface of HCE cells. The *lower left panel* shows control staining with isotype-matched control antibody. Positive staining on the surface of HCE cells is not detected. Scale bar = 20  $\mu$ m. (B) HCE cells cultured in the presence (*middle histogram*) or absence (*left histogram*) of Th1 supernatants were stained with PE-labeled anti-human PD-L1 mAb and analyzed by flow cytometry. HCE cells cultured in the presence of recombinant IFN $\gamma$  (100 U/mL) were also stained (*right histogram*). Percentages in upper right corners indicate positive cells rates. Th1 cell supernatants and recombinant IFN $\gamma$  increased the percentage of PD-L1-positive cells. (C) PD-L1 mRNA expression was compared in HCE cells. mRNA was extracted from HCE cells cultured with or without Th1 cell supernatant, and was reverse-transcribed and amplified by PCR using primers for PD-L1 and  $\beta$ -actin. PCR products were electrophoresed on 1.5% agarose gel and visualized by staining with ethidium bromide. Expression of PD-L1 mRNA by HCE cultured with supernatants of Th1 cells was higher than by cells cultured without Th1 supernatants.

shown to spontaneously develop systemic autoimmune disease.<sup>29</sup> PD-L1 has been reported to have a role in peripheral immune tolerance as a negative regulator of T-cell responses via PD-1 in a mouse corneal transplantation model,<sup>23,30</sup> although it is still controversial whether mouse CE expresses PD-L1, as shown by the findings of the positive<sup>23</sup> and negative<sup>30</sup> expression of PD-L1 in mouse CE. Since engagement of

PD-1 on T cells by either of its ligands (PD-L1 or PD-L2) leads to inhibition of both CD4<sup>+</sup> and CD8<sup>+</sup>-T-cell proliferation in mice,<sup>31–33</sup> further investigation of the relation between PD-L1 and human CD8-T cell is required and is ongoing at our laboratory.

Ocular cells have already been shown to inhibit T-cell proliferation, especially cells in tissues that form other parts of the anterior chamber. Ciliary body cells are known to have inhibitory effects, at least partly as a result of producing prostaglandin E, because their effect is blocked by indomethacin.<sup>34</sup> Cultured iris pigment epithelial cells markedly suppress the activation of T cells<sup>4,5</sup> and can convert CD8<sup>+</sup> T cells to CD25<sup>+</sup>Foxp3<sup>+</sup> T regulators in vitro.<sup>6,35</sup> The aqueous humor that fills the anterior chamber contains a substantial amount of TGF $\beta$ , produced by ciliary body cells.<sup>36,37</sup> Similarly to anterior segment tissues/cells, those from the posterior segment of the eye also have inhibitory effects. Retinal Müller cells can inhibit T-cell responses, and this effect is abolished by trypsinization and fixation of the Müller cell monolayer.<sup>38</sup> Retinal pigment epithelial cells are reported to modulate lymphocyte proliferation.<sup>7,8</sup> In the cornea, relatively little is known regarding the immunologic activity of CE cells, despite a potentially important role in contributing to the privileged immune status of the anterior chamber and the high success rate of corneal transplantation. CD95 ligand (FasL) is constitutively expressed on CE cells, and induces Fas-mediated apoptosis of infiltrating cells.<sup>11</sup> In addition, cultured rat CE cells suppress in vitro lymphocyte proliferation by affecting cytokine production,<sup>10,39,40</sup> but the molecular mechanisms underlying the inhibitory activity of CE cells are still unclear. We demonstrated that under inflammatory conditions, human CE cells express the co-stimulatory molecule PD-L1 and inhibit effector T cells via a contact-dependent mechanism.

In intraocular inflammation, keratic precipitates that adhere to the HCE are a common finding and an important marker for evaluating the severity of inflammation. Such precipitates are mainly composed of monocytes, neutrophils, macrophages, and lymphocytes.<sup>41</sup> In addition to cell adhesion molecules, e.g., CD54/intercellular adhesion molecule-1 (ICAM-1),<sup>42</sup> vascular cell adhesion molecule-1, and E-selectin,<sup>43,44</sup> human CE cells abundantly produce chemoattractants for neutrophils, monocytes, and lymphocytes, such as CXCL8 (IL-8), CCL2 (MCP-1), and CXCL2 (GRO $\beta$ ), which may orchestrate and promote the formation of keratic precipitates on the posterior surface of the cornea.<sup>45</sup> These findings suggest that keratic precipitates are actively rather than passively formed on the CE in inflamed eyes by expression of chemokines and cell adhesion molecules. Moreover, our finding of a suppressive effect of the PD-1/PD-L1 interaction on activated T cells suggests that keratic precipitates are not a simple indicator of inflammation, but are the result of a protective mechanism against inflammation in the anterior chamber.

In summary, cultured HCE cells expressing the co-stimulatory molecule PD-L1 selectively suppressed the activation of IFN $\gamma$ -producing Th1 cells that showed high expression of PD-1. Ocular infiltrating cells appear in the anterior chamber of the eye when a patient has uveitis or corneal endotheliitis. Effector T cells are converted to inactivated cells during their migration through the HCE or by adhesion to HCE cells. When exposed to IFN $\gamma$ , HCE cells show marked induction of PD-L1 expression, and T cells exposed to HCE cells can be inactivated since effector CD4<sup>+</sup> T cells express high levels of PD-1. Eventually, the response of infiltrating T cells to the influence of HCE cells impairs T-cell activation. Our data suggest that PD-L1 expression by HCE cells contributes to the immune privileged status of the anterior chamber of the eye and helps to avoid blindness by suppressing inflammation. In present study, we found human CE cells are able to suppress CD4<sup>+</sup> T cells in



vitro. Nonetheless, at the present time, immune privileges in human cornea are as of yet unknown. In an effort to understand the molecular bases of the immunosuppressive properties of the human CE in immune privileges, we are now conducting the experiments whether human CE cells can convert T-cells into T regulators that have a suppressive phenotype (CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells). In addition, we are going to identify the immunosuppressive factors except PD-L1 costimulatory molecules that might be important for understanding immune suppressive mechanisms on human CE. The major cellular interactions involved in the suppression are at least costimulatory pathway (e.g., PD-1:PD-L1) and the CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells.

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