B7+ Iris Pigment Epithelial Cells Convert T Cells into CTLA-4+, B7-Expressing CD8+ Regulatory T Cells

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PURPOSE. To determine whether iris PE (IPE) promotes the generation of regulatory T-cells (Tregs) with cell contact via B7-2/CTLA-4 interactions.

METHODS. T cells were cocultured with IPE cells obtained from eyes of normal and B7-deficient mice, x-irradiated, and used as regulators. IPE T regulator cells (IPE Tregs) of normal and CD28- or CTLA-4-deficient mice were established. Target bystander T cells were established from normal splenic T cells with anti-CD3 antibodies. T-cell activation was assessed for proliferation by [3H]-thymidine incorporation. Neutralizing anti-B7-1 and/or B7-2 antibodies, anti-CTLA-4 antibodies, CTLA-4-Ig fusion proteins were used to abolish regulatory function. IPE-exposed CD8+ T cells were evaluated for expression of B7, CTLA-4, and Foxp3 by using RT-PCR and flow cytometry.

RESULTS. T cells acquired T regulatory activity when exposed to cultured IPE. Ciliary body PE cells did not promote conversion of T cells into Tregs. IPE converted CD8+ T cells, but not CD4+ T cells into Tregs by direct cell contact. In the conversion, IPE and responding T cells must both express endogenously synthesized B7-1 and B7-2, and the T cells must also express CTLA-4. Expression of CD28 molecules was not necessary for Treg generation. In addition, the CD8+ Tregs that fully suppress activation of bystander T cells expressed Foxp3.

CONCLUSIONS. IPE cells promote conversion of T cells into Tregs solely through a contact-dependent mechanism. T cells exposed to IPE cells acquire full regulatory capacity. (Invest Ophthalmol Vis Sci. 2006;47:5376-5384) DOI:10.1167/iovs.05-1354

Immune privilege in the eye protects the delicate internal structures of the visual axis from the blinding consequences of innate and adaptive immune inflammation. Among the factors responsible for creating ocular immune privilege are the pigment-containing epithelia that line the posterior surface of the iris, the ciliary body, and the neural retina. Ocular pigment epithelia (PE) quench scattered unfocused light from degrading the focused visual images that reach the retina. Whereas retinal PE (RPE) provide specialized functions to support retinal photoreceptor cells, the intact RPE monolayer functions as an immune-privileged tissue, and RPE cells create an immunsuppressive microenvironment in the subretinal space. Both RPE and iris PE (IPE) contribute to the integrity of blood–ocular barriers and thereby secure immune privilege within the eye.

Cultured ocular PE have been found to be immunosuppressive in vitro, in part because they express cell surface molecules (CD95 ligand, B7-2 (CD86)), and secrete soluble factors (TGFβ, thrombospondin, PGE2) that can modulate both adaptive and innate immune effector mechanisms. We recently reported that cultured IPE cells inhibit T-cell activation by a cell contact–dependent mechanism resembling immune costimulation in which IPE cells express B7-2, which interacts with cocultured T cells that express cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4). Among ocular PE, only IPE constitutively express surface B7-2, and this accounts for why cell contact is essential for suppression of T-cell activation by IPE, but not by ciliary body PE (CBPE) and RPE.

It has been reported that T cells stimulated by anti-CD3 antibodies in the presence of iris and ciliary body PE acquire the capacity to regulate bystander T cells in secondary (subsequent) cultures. Having found B7 expression by IPE to be important in inhibiting T cell proliferation in primary cultures, we decided to determine whether B7 expression by IPE is also important in converting the T cells into T regulators (Tregs). To that end, we have conducted experiments designed to demonstrate whether T cells exposed to IPE in primary cultures acquire the capacity to suppress bystander T-cell activation in secondary cultures.

METHODS

Mice

Adult C57BL/6 mice, obtained from our domestic animal colony or purchased from Taconic Farms (Germantown, NY), served as donors of ocular PE cells and splenic T cells. Mice of the C57BL/6 background with disrupted genes for CD28, CD80, and/or CD86 were purchased from Jackson Laboratories (Bar Harbor, ME). James P. Allison (University of California at Berkeley, Berkeley, CA) provided CTLA-4 heterozygous mice from which we generated CTLA-4 homozygous progeny that were used at 3 weeks of age. All animal protocols were in accordance with NIH guidelines and approved by Schepens Animal Care and Use Committee. Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.


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Preparation of Cultured Ocular PE from Iris

PE cells of the iris and ciliary body were cultured as described previously.\textsuperscript{6,10} Eyes were enucleated from 6- to 8-week-old male C57BL/6 mice. Iris tissues were separated and incubated in PBS containing 1 mg/mL Dispase and 0.05 mg/mL DNAsel (both from Roche, Mannheim, Germany) for 1 hour. Single-cell suspensions were then incubated for 14 days. At the completion of the 14-day primary culture, more than 99% of the IPE cells were labeled with FITC anti-pan cytokeratin antibody (clone PCK-26; Sigma-Aldrich, St. Louis, MO).

The cultured IPE contained neither CD4\textsuperscript{+} nor MHC class II\textsuperscript{+} cells.\textsuperscript{8} The PE cells did not express F4/80 molecules by analysis of Western blots (Sugita S, unpublished data, 2006), and the IPE did not express transcripts for the molecules gene expression analysis (Gene Chip Expression Analysis; Affymetrix, Santa Clara, CA; manuscript in preparation).

Preparation of Purified T Cells and Description of Assays of T-Cell Activation

Responder T-cell suspensions were obtained by passing splenic cells through T-cell separation columns (Immune kit; Biotex Laboratories, Houston, TX, >90%-95% cells were CD3 positive). For anti-CD3-driven T-cell activation, purified splenic naive T cells were added (2.5 × 10\textsuperscript{5} cells/well) to culture wells containing IPE or γ-irradiated (2000 R) T cells exposed previously to IPE (IPE Tregs). Anti-CD3 antibody (clone 2C11; BD Pharmingen, San Diego, CA) was added to wells containing naive T cells and IPE or regulator T cells, and cultures were maintained for 72 hours, then assayed for uptake of [\textsuperscript{3}H]-thymidine (1 μCi/mL for the terminal 8 hours of culture). Thymidine-pulsed T cells were harvested by an automated cell harvester (Tomtec, Hamden, CT). Incorporative radioactivity was measured with a liquid scintillation counter (Betaplate; Wallac, Gaithersburg, MD), and the amount was expressed in counts per minute.

Exposure of T Cells to Cultured IPE

Enriched C57BL/6 T cells were placed in culture wells containing cultured iris, CB, or NIH 3T3 cells (fibroblasts; ATCC, Manassas, VA). After 48 hours, the T cells were harvested by gentle pipetting and washed twice with serum-free RPMI medium. The level of contamination of the harvested T cells with IPE was ≥0.97% cytokeratin positive.

Detection of Transcripts for B7 Costimulatory Molecules and Cytokines within T Cells Exposed to IPE

Enriched T cells, cultured with IPE (or CBPE) for 24 hours, were harvested, washed, and treated with an RNA extraction reagent (Stat-60; Tel-Test, Inc, Friendswood, TX). PCR was then performed (HotStart PCR method with AmpliTaq and AmpliWax; Applied Biosystems, Inc. [ABI], Foster City, CA). To examine B7 costimulatory molecules, the forward and reverse primers used for GAPDH, B7-1, and B7-2 were the same as described previously.\textsuperscript{8} To control for the nongenetic absorption of B7-2 onto the surface of T cells exposed to IPE, T cells from B7-1/B7-2 knockout (KO) mice were cultured with wild-type IPE. To examine mRNA for cytokines in T cells exposed to IPE, the forward and reverse primers used for IFNγ and IL-10 were the same as described previously.\textsuperscript{8} PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide. Photo- graphs of the gel were taken with a high-resolution camera, and the density of the band of negative image was analyzed by NIH image software. The expression level of mRNA was standardized to the expression of GAPDH as an internal control.

Detection of Transcripts for Foxp3 in T Cells Exposed to IPE

CD25\textsuperscript{+} and CD25\textsuperscript{-} T cells were harvested with a fluorescence-activated cell sorter (EPICS Cell Sorter; Beckman Coulter, Hialeah, FL). Of the selected cell suspensions injected, cells designated as CD4\textsuperscript{+} CD25\textsuperscript{-} contained >98% of cells of this phenotype, and cells designated as CD4\textsuperscript{+} CD25\textsuperscript{+} contained 90% to 95% of cells of this phenotype, as judged by flow cytometry. Total RNA was extracted from CD4\textsuperscript{+} CD25\textsuperscript{+} and CD4\textsuperscript{+} CD25\textsuperscript{-} T cells isolated from spleens of naive mice for 24 hours. In addition, total RNA was extracted from CD8\textsuperscript{+} CD25\textsuperscript{-} IPE Tregs or CD8\textsuperscript{+} CD25\textsuperscript{+} IPE Tregs established from T cells exposed to IPE. For PCR amplification, cDNAs were amplified using primers as follows: Foxp3, 5’-CAGCTGCTACAGTGCCCTAG-3’ and develop- ment by the 5’-CATTGGGCGAGTCGGTAG-3’, giving an amplification product of 382 bp. The PCR cycling condition was 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. After a 28-cycle amplification, the PCR products were separated by 1% agarose gel. Densitometric measurement of the bands was used to calculate a ratio of the gene of interest, GAPDH.

Flow Cytometry Analyses

The expression of CD80 (B7-1), CD86 (B7-2), and CD152 (CTLA-4) on CD8\textsuperscript{+} T cells exposed to IPE was assessed by flow cytometry. CD8\textsuperscript{+} T cells, purified by magnetic beads (CD8a\textsuperscript{-} T cell isolation kit, MACS system; Miltenyi Biotec, Auburn, CA), were cultured with IPE for 24 hours, harvested, and stained with Cy-Chrome-conjugated anti-CD8 mAbs (Ly2, clone 53-6.7) and either FITC-conjugated antibodies to CD80 (clone 16-10A1) or CD86 (clone GL1) for flow cytometric analy- sis. Before staining, the cocultured T cells were incubated with anti-CD16/CD32/CD82 Abs (Fcy III/II/Receptor, clone 2.4G2) for 15 minutes at 4°C. FITC-conjugated rat IgG isotype was used as the control. The expression of CTLA-4 on T cells exposed to IPE was analyzed as reported by Nakamura et al.\textsuperscript{17} In brief, purified T cells cultured with IPE blocked with anti-CD16/CD32 at 4°C for 15 minutes, washed, and stained with anti-CD152 mAbs (clone UC10-4F10-11) or control hamster IgG at 37°C for 2 hours. The cells were then stained with biotin- conjugated anti-hamster IgG at 4°C for 30 minutes. Then, cells were stained with Cy-Chrome-conjugated anti-CD8 mAbs and FITC-conju- gated streptavidin for 30 minutes at 4°C. The cells were washed and analyzed with flow cytometry. All the antibodies were purchased from BD PharMingen.

To determine whether cyclohexamide (CHX) inhibits de novo synthesis of protein, cells were analyzed for the detection of B7-2 on induction of IPE Tregs. CD8\textsuperscript{+} T cells that were stimulated with anti-CD3 Abs were incubated with CHX (20 μg/mL; Sigma-Aldrich), an inhibitor of protein synthesis, in the presence of IPE. B7-2 expression on untreated CD8\textsuperscript{+} T cells exposed to IPE or CHX-treated CD8\textsuperscript{+} T cells exposed to IPE was evaluated with flow cytometry. In other experi- ments CD8\textsuperscript{+} T cells were stimulated with anti-CD3 Abs cocultured with IPE in the presence or absence of CHX. As a control for CHX function of de novo synthesis, the CHX effect on IL-2 R expression was analyzed in CD8\textsuperscript{+} T cells from the same cultures.

To determine whether CD8\textsuperscript{+} T cells exposed to IPE express Foxp3, CD8\textsuperscript{+} T cells were cultured with IPE for 24 hours, harvested, and stained with PE-labeled anti-mouse Foxp3 Abs (eBioscience, San Diego, CA) or isotype (PE-labeled mouse IgG; BD Pharmingen) at 4°C for 30 minutes after they were permeabilized. The cells were washed and analyzed by flow cytometry.

CD8\textsuperscript{a} T cells were obtained from single cell suspension of the mouse spleen (MACS system; Miltenyi). The resultant cells were >95% pure CD8\textsuperscript{+} T cells. CD8\textsuperscript{+} T cells purified by this method contained ~14% CD4\textsuperscript{+} CD45\textsuperscript{+} T cells. For purification of CD8\textsuperscript{+} CD4\textsuperscript{+} Foxp3, and CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells were stained with anti-CD8-PE and anti-CD4-FITC and sorted on a fluorescence-activated cell sorter (EPICS Cell Sorter; Beckman Coulter). The sorted CD8\textsuperscript{+} CD4\textsuperscript{+} Foxp3 or CD8\textsuperscript{+} CD4\textsuperscript{+} Foxp3 T cells were >98% pure. For the assay, these three popula- tions and just CD8\textsuperscript{+} T cells as a control were cocultured with IPE.
FIGURE 1. Capacity of cultured IPE to convert T cells into regulators. Purified naïve syngeneic T cells were cultured with IPE (A) or CBPE (B) for 48 hours and used as Tregs. For control experiments, naïve T cells were cultured in the absence of PE cells. PE Tregs or Cont Tregs were then added to cultures containing naïve responder T cells plus anti-CD3 Abs. (C) Positive and negative control cultures containing naïve T cells alone ± anti-CD3. After 72 hours, the cultures were assayed for uptake of \[^{3}H\]\)-thymidine. Mean cpm for triplicate cultures are presented ± SEM. (C) Supernatants were harvested from 48-hour cultures and assayed by ELISA for IFN-\(\gamma\) and IL-2. Results of triplicate samples are presented as the mean ± SEM. **P < 0.005, compared with T resp ± anti-CD3. (D) IPE were plated on porous membranes and inserted into culture wells containing naïve T cells (Control Tregs, dark cross-hatched bars). Light cross-hatched bars: positive and negative control cultures containing naïve T cells alone ± anti-CD3. **P < 0.005, comparing IPE Tregs generated across porous membrane or not. Cont, Control Tregs (not exposed to IPE). (E) Preactivated T cells with anti-CD3 antibodies (concentration; 0, 0.25, 0.5, and 1.0 \(\mu\)g/mL) were cultured with IPE or CBPE for 48 hours and used as Tregs. \[^{3}H\]\)-thymidine uptake (mean cpm) for triplicate cultures are presented ± SEM. **P < 0.005. (F) Purified T cells were cultured with IPE for 48 hours, harvested, \(\gamma\)-irradiated, and used as Tregs. IPE Tregs were then added to cultures containing naïve CD4\(^+\) or CD8\(^+\) responder T cells plus anti-CD3. Also shown are results in positive control cultures containing naïve T cells alone (T resp) ± anti-CD3. The last 8 hours of a 72-hour incubation \[^{3}H\]\)-thymidine uptake (mean cpm) for triplicate cultures are presented ± SEM. **P < 0.005. (G) Control T cells were generated by naïve T cells in the presence of 1% IPE to confirm the contamination of cultured IPE. \[^{3}H\]\)-thymidine uptake (mean cpm) for triplicate cultures are presented ± SEM. **P < 0.005. (H) CD44 CD44\(^{low}\) ( naïve T cells) and...
Statistical Analysis
Each experiment was repeated at least twice with similar results. All statistical analyses were conducted with Student’s t-test. Results were considered statistically significant at P ≤ 0.05.

RESULTS
Capacity of Cultured IPE Cells to Convert T Cells into Regulators
We first determined whether cultured IPE could generate regulatory T cells in vitro and whether stimulation with anti-CD3 antibodies was essential for this conversion. PE cells were cultured separately from iris and ciliary body obtained from C57BL/6 eyes. Purified T cells were added to the PE cell cultures without anti-CD3 antibodies. After coculture with PE cells, the T cells are referred to as IPE T-regulators (IPE Tregs). Control T regulators (Cont Tregs), were generated by culturing naïve T cells in the absence of PE. After incubation, the T cells were harvested, γ-irradiated, and added to secondary cultures containing fresh naïve T cells plus anti-CD3. We observed that naïve T cells stimulated with anti-CD3 (referred to as bystander T-cell activation) in the presence of IPE Tregs proliferated significantly less well than did T cells similarly stimulated in the presence of Cont Tregs (Fig. 1A). By contrast, ciliary body PE failed to convert T cells into PE Tregs (Fig. 1B). Similarly, T cells first exposed to fibroblasts displayed no capacity to suppress bystander T-cell activation (data not shown). In addition, we determined that nonirradiated IPE Tregs suppressed the activation of bystander T cells. As did IPE cells, IPE Tregs significantly suppressed cytokine production (Th1-type cytokines, e.g., IL-2 and IFNγ) by activated T cells (Fig. 1C).

To determine whether the capacity of IPE to convert T cells into Tregs depends on direct cell-to-cell contact, IPE were plated separately into individual porous membranes (Transwells; Corning Costar, Corning, NY), and inserted into culture wells containing T cells. After incubation, T cells were removed, γ-irradiated, and added to secondary cultures containing naïve T cells and anti-CD3. IPE Tregs, cultured across a membrane from IPE, displayed significantly less capacity to suppress T-cell activation (Fig. 1D), suggesting that the ability of IPE to convert T cells into Tregs requires direct cell-to-cell contact, just as does the capacity of IPE to suppress the activation of T cells, as reported previously.18

Next, we determined whether IPE cells were able to modulate the function of preactivated T cells toward a regulatory phenotype. Our results indicate that both naïve and activated T cells (especially activated T cells) are able to acquire Treg function when exposed to IPE, but not when exposed to CBPE (Fig. 1E). Thus, IPE more efficiently converts preactivated T cells than naïve cells into Tregs.

To analyze the cellular target of the IPE Treg CD4+ and CD8+ T cells, PE cells were enriched from whole spleen cells exposed to activation by anti-CD3 Abs in the presence or absence of PE Tregs. IPE Tregs significantly suppressed CD4+ responder T cells, whereas they were virtually ineffective in suppressing anti-CD3 activation of CD8+ responder T cells (Fig. 1F). To confirm contamination of cultured IPE, a small population of IPE cells was added to the control T-cell suspension. The control T cells with 1% IPE or without IPE were unable to suppress the activation of bystander T cells, whereas T cells exposed to IPE (IPE Tregs) suppressed the activation (Fig. 1G).

Next, we determined whether IPE cells actually convert both CD4low (naïve T cells) and CD4high (memory T cells) populations into Tregs. In a naïve mouse spleen there are a certain number of antigen (Ag)-experienced T cells that result from exposure of the mouse to environmental Ags. As shown in Figure 1H, CD44high IPE Tregs significantly suppressed activation of bystander T cells, whereas CD44low IPE Tregs, as well as CD44 negative Tregs, poorly suppressed the activation of T cells. These results suggest that IPE can convert only preactivated, effector-memory phenotype (CD44high) into Tregs.

Capacity of Separated CD4+ and CD8+ T Cells to Become Tregs on Exposure to IPE
CD4+ or CD8+ T cells were enriched from dissociated spleen cell populations before their exposure to IPE. The IPE Tregs that were generated were then tested for their ability to interfere with anti-CD3 Ab-induced proliferation (Fig. 2A). Enriched CD8+ IPE Tregs suppressed T-cell activation in secondary cultures to the same extent, as did unfractionated Tregs obtained from similar cultures, whereas enriched CD4+ IPE Tregs displayed little capacity to suppress anti-CD3 induced T-cell activation. In complementary experiments, purified T cells were depleted of CD8+ cells cultured with IPE. When proliferation was measured after 72 hours, thymidine incorporation was suppressed in cultures containing undepleted IPE Tregs, but not with CD8+ depleted IPE Treg (Fig. 2B). However, cultures to which CD8-depleted IPE Tregs were added proliferated equally to anti-CD3 stimulated cultures containing responder T cells on Cont Tregs. Thus, CD8+ T cells must be present in T-cell suspensions exposed to IPE for the generation of the T-cell regulatory phenotype. CD4+ T cells appear to play little or no role in the development of IPE Tregs.

We examined whether the CD8+ IPE Tregs express Foxp3 transcripts. The transcription factor Foxp3 has been consistently linked to the regulatory functions of naturally arising CD4+CD25+ Tregs.19 To assess whether Foxp3 expression was associated with the induction of IPE Tregs, we compared the expression of Foxp3 transcript in CD4+CD25+ or CD4+CD25− T cells derived from naïve mice with the expression of Foxp3 in CD4+CD25+ or CD4+CD25− T cells that were exposed to cultured IPE. As presented in Figure 2C, freshly purified CD4+CD25+ T cells, not CD4+CD25− T cells, in naïve mice expressed Foxp3. Similarly, CD25+ T cells among CD8+ T cells exposed to IPE strongly expressed Foxp3 (Fig. 2C). Foxp3 was also detected in anti-CD3 Ab-treated CD8+ T cells (control) cultured without IPE. This suggests that signaling through the T-cell receptor may induce suppressor activity in CD8+ T cells.

The Role of Co-stimulation (B7-1 and B7-2) in Cultured IPE Conversion of T Cells into Regulators
Purified T cells were cultured for 48 hours in the presence of IPE with or without anti-CD80 and/or anti-CD86 antibodies before their addition to proliferation cultures containing naïve T cells and anti-CD3. IPE Tregs generated in the presence of either anti-CD80 or -CD86 alone suppressed T-cell activation in secondary cultures, whereas IPE Tregs generated in the presence of CD4low (naïve T cells) and CD4high (memory T cells) populations were separated with a cell-sorting system. These CD8+ T cells (CD4neg/ive or CD4high or CD4high) were cultured with IPE for 48 hours, harvested, γ-irradiated, and used as Tregs. As an experimental control, CD8+ T cells exposed to IPE were also used (■). The CD8+ IPE Tregs were then added to cultures containing responder T cells + anti-CD3. (□) Positive control cultures containing naïve T cells alone (T resp) + anti-CD3. *P < 0.05, **P < 0.005, comparing two groups.
isolated from naïve control mice and purified CD8+ T cells from CD8+ T cells freshly isolated from naïve mice (control results). Total RNA was also extracted from IPE. Foxp3 expression was determined by semiquantitative RT-PCR in cDNA samples obtained from CD4+CD25+ and CD4+CD25− T cells freshly isolated from naïve mice (control results). Total RNA was also extracted from CD8+ CD25+ IPE Tregs or CD8+ CD25− Tregs. The PCR products were electrophoresed in 1% agarose gel and stained with ethidium bromide. Normalized Foxp3 values were derived from the ratio of Foxp3 expression to GAPDH expression. Foxp3 expression was also determined by flow cytometry from CD4+CD25− (○) and CD4+CD25+ T cells (□) freshly isolated from naïve control mice and purified CD8+ T cells (Control T cells (■)). IPE-exposed T cells (△) stimulated with anti-CD3 Abs. These T cells were stained with PE-conjugated anti-mouse Foxp3 Abs. PE-conjugated mouse IgG1 Abs was used as the isotype control (▲). Number indicates percentage of Foxp3+ T cells.

**Figure 2.** CD4/CD8 phenotype of Tregs generated in the presence of cultured IPE. (A) T cells were added to cultured IPE (IPE Tregs) or cultured alone (Cont Tregs), removed, and negatively selected into enriched CD4+ and CD8+ populations, or used unselected. After 48 hours of culture, these enriched T cells were harvested, irradiated, and added to secondary cultures containing responding T cells (T resp) and anti-CD3 antibodies. (B) T cells were depleted (or not) of CD8+ T cells and cultured for 48 hours ± IPE. The T cells were harvested, irradiated, and added to cultures containing naïve T cells and anti-CD3 antibodies. (C) Expression of Foxp3 by CD8+ T cells exposed to IPE. Foxp3 expression was determined by RT-PCR in cDNA samples obtained from CD4+CD25− and CD4+CD25+ T cells freshly isolated from naïve mice (control results). Total RNA was also extracted from CD8+CD25+ IPE Tregs or CD8+CD25− Tregs. The PCR products were electrophoresed in 1% agarose gel and stained with ethidium bromide. Normalized Foxp3 values were derived from the ratio of Foxp3 expression to GAPDH expression. Foxp3 expression was also determined by flow cytometry from CD4+CD25− and CD4+CD25+ T cells freshly isolated from naïve control mice and purified CD8+ T cells (Control T cells (■)). IPE-exposed T cells (△) stimulated with anti-CD3 Abs. These T cells were stained with PE-conjugated anti-mouse Foxp3 Abs. PE-conjugated mouse IgG1 Abs was used as the isotype control (▲). Number indicates percentage of Foxp3+ T cells.

Expression of both CD80 and CD86 antibodies failed to suppress T-cell activation in secondary cultures (Fig. 3A).

In alternative experiments B7-2/− B7-1 receptor activation was blocked with mouse recombinant fusion protein, CTLA-4-Ig, or anti-CTLA-4 Abs during the primary culture of T cells and IPE. We observed that both CTLA-4-Ig (Fig. 3B) and anti-CTLA-4 Abs (Fig. 3C) significantly impaired the IPE-dependent generation of Tregs in primary cultures. Together, these findings indicate that IPE use the constitutively expressed B7-1 and B7-2 in the generation of Tregs from CD8+ T cells.

**Capacity of T Cells from CD28 or CTLA-4 KO Mice to Become Tregs When Exposed to IPE**

Because the ligand for B7-1/B7-2 includes CD28 (activator) and CTLA-4 (suppressor) ligand, the effect of IPE on T-cell donor mice with either the CD28 or the CTLA-4 genes disrupted was examined. IPE Tregs and Cont Tregs were generated from wild-type C57BL/6, CD28 KO, and CTLA-4 KO mice. IPE-exposed T cells from both wild-type and CD28 KO donors readily acquired the capacity to suppress T-cell activation in secondary cultures (Fig. 4A), whereas IPE-exposed T cells from CTLA-4 KO donors were almost devoid of Treg activity (Fig. 4B). These data support the postulate that the ligand for B7-1/B7-2 expressed by IPE cells is CTLA-4, not CD28, during the induction of IPE Tregs. Thus, T cells must express CTLA-4 for IPE to convert them into IPE Tregs.

**Capacity of T Cells and/or IPE Cells from B7-1/ B7-2 KO Mice to Generate Tregs**

Although B7-1 and B7-2 are universally acknowledged as costimulators of T cells when expressed on antigen-presenting cells (APCs),19–21 the meaning of the expression of these costimulatory molecules on T cells is less well understood.17,22–24 We next examined whether the requirement for the expression of B7 family molecules by T cells themselves is important in their conversion to regulatory cells. IPE and CD8+ T cells were obtained from mice with disrupted B7-1 and B7-2 genes, and from wild-type C57BL/6 donors. In one set of experiments, wild-type CD8+ T cells were exposed in primary cultures to wild-type or B7-1/B7-2 KO IPE (Fig. 5A). We observed that wild-type T cells first exposed to wild-type IPE acquired a strong capacity to suppress bystander T-cell activation. By contrast, T cells exposed to B7-1/B7-2 KO IPE were significantly less able to suppress bystander T-cell activation. These findings reveal that B7-1 and B7-2 expression on IPE is important in conversion of CD8+ T cells into Tregs. In a second set of experiments, primary cultures were established in which both CD8+ T cells and cultured IPE were obtained from B7-1/B7-2 KO mice. The T cells harvested from these cultures were tested for their ability to suppress T-cell activation in secondary cultures. Virtually no suppression was observed (Fig. 5B). Together these results support the notion that B7-1 and B7-2 expression on both IPE and T cells is required for their conversion to Tregs.

**Expression of B7-1 and B7-2 by T Cells Exposed to IPE**

Because it has been reported that T cells activated by B7-expressing APCs can passively acquire and express B7 molecules from the APCs (rather than synthesizing these costimulatory molecules),17,22–24 we next examined whether the requirement for the expression of B7 family molecules by T cells themselves is important in their conversion to regulatory cells. IPE and T cells is important in conversion of CD8+ T cells into Tregs. In a second set of experiments, primary cultures were established in which both CD8+ T cells and cultured IPE were obtained from B7-1/B7-2 KO mice. The T cells harvested from these cultures were tested for their ability to suppress T-cell activation in secondary cultures. Virtually no suppression was observed (Fig. 5B). Together these results support the notion that B7-1 and B7-2 expression on both IPE and T cells is required for their conversion to Tregs.
exposed to wild-type IPE. The T cells were then removed and assayed for B7-1/B7-2 mRNA. No detectable mRNA was found for these B7 genes, indicating that any potential contamination by IPE was below the level of resolution (data not shown). Thus, T cells that are exposed to IPE upregulate their own B7-1 and B7-2 genes rather than passively acquire them.

IPE Tregs and Cont Tregs were also examined for expression of CD8, CTLA-4, B7-1, and B7-2. Approximately 9.8% of CD8⁺ T cells exposed to IPE were positive for B7-1 (Fig. 6B), whereas only 2.5% B7-1-positive cells were present in the Cont Treg population. Similarly, 27.5% of IPE Tregs expressed B7-2, whereas only 11.1% of Cont Tregs expressed this costimulator. In addition, 8.5% of CD8⁺ T cells exposed to IPE were positive for CTLA-4, whereas only 1.1% of Cont Tregs were similarly positive. The expression of B7-1, B7-2, and CTLA-4 on naïve, uncultured CD8⁺ T cells were virtually identical with that of Cont Tregs (data not shown). Because B7-2 was synthesized de novo after exposure to IPE (Fig. 6 A-B), CHX was added (or not) to CD8⁺ T cells before their coculture with IPE. When the naïve CD8⁺ T cells were incubated with CHX (20 μg/mL for 12 hours) before exposure to IPE, the expression of B7-2 was blocked (data not shown). Thus, the detection of B7-2 on T cells exposed to IPE is the result of de novo B7-2 expression by the T cells. In other experiments, the metabolic blocker CHX was added (or not) directly to the cultures containing IPE, CD8⁺ T cells, and anti-CD3 Abs for 24 hours. The activated CD8⁺ T cells B7-2 were upregulated after coculture with IPE, and the expression of B7-2 was blocked if the CD8⁺ T cells were exposed to CHX during coculture (Fig. 6C). In control

**FIGURE 3.** Influence of anti-B7-1 and B7-2 mAbs on capacity of cultured IPE to convert naïve T cells into regulators. (A) CD8⁺ T cells were added to cultured IPE in the presence or absence of anti-mouse CD80 (B7-1) and/or CD86 (B7-2) monoclonal Abs (1 μg/mL). As controls, purified hamster IgG for CD80 and purified rat IgG for CD86 were used. Control Tregs were prepared as described in the legend to Figure 1. After 48 hours, the T cells were harvested, γ-irradiated, and added as IPE Tregs or Cont Tregs to fresh cultures containing naïve T cells (1 x 10⁶/well) plus anti-CD3 Abs. Also shown are positive and negative control cultures containing responder naïve T cells alone (T resp) ± anti-CD3. Mean cpm for triplicate cultures are presented ± SEM. **P < 0.005, comparing group F and Cont Tregs with IPE Tregs, group A. (B) CD8⁺ T cells were added to cultured IPE, with or without CTLA-4-Ig fusion protein (0.5, 1, 5 μg/mL). *P < 0.05, **P < 0.005, comparing all groups with IPE Tregs + T resp + anti-CD3 (A B). (C) Regulatory T cells were generated in similar cultures except that anti-mouse CTLA-4 mAbs (5 μg/mL) were used instead of CTLA-4-Ig fusion protein. **P < 0.005, comparing groups (B) and (C) with group (A).

**FIGURE 4.** Capacity of T cells from CD28 KO and CTLA-4 KO mice to become Tregs when exposed to IPE. T cells obtained from wild-type C57BL/6, CD28 KO (A), and CTLA-4 KO (B) donors were first cultured with or without IPE cells for 48 hours, then harvested, and γ-irradiated. The IPE Tregs were then added to fresh cultures of naïve responder T cells (1 x 10⁶/well) plus anti-CD3 Abs. Positive control cultures contained responder naïve T cells alone (T resp) + anti-CD3. Mean cpm for triplicate cultures incubated for 72 hours are presented ± SEM. **P < 0.005, comparing IPE Tregs with Cont Tregs.
The ability of IPE to convert T cells into Tregs depended solely on B7. B7-2. T cells into Tregs by IPE.

**Figure 5.** Capacity of T cells from wild-type or B7-1+B7-2 KO mice to differentiate into Tregs when exposed to wild-type or B7-1+B7-2 KO IPE. (A) CD8+ T cells (CD8+ IPE Tregs) first cultured for 48 hours in the presence of IPE obtained from wild-type (WT) or B7-1/B7-2 KO mice were harvested and γ irradiated. (B) T cells obtained from wild-type or B7-1/B7-2 KO mice were cultured for 48 hours with IPE from the same mice. Control Tregs were cultured in the absence of IPE. These various IPE Tregs and Cont Tregs were then added (1 × 10^5/well) to fresh cultures of naïve responder T cells (1 × 10^5/well) and anti-CD4 Abs. Positive control cultures contained responder naïve T cells alone + anti-CD4 Abs. Mean cpm for replicate cultures incubated for 72 hours are presented ± SEM. **P < 0.05, ***P < 0.005, comparing two groups.

experiments, CHX also blocked the expression of CD25, a molecule known to be upregulated by exposure to anti-CD3 Abs (data not shown). Thus, CD8+ T cells that are exposed to IPE (and that eventually acquire the capacity to suppress the activity of bystander T cells) upregulate their own B7-1 and B7-2 expression as well as CTLA-4 expression. Furthermore, the B7-2 expression is the result of de novo synthesis in the absence of IPE. These various IPE Tregs and Cont Tregs were then added (1 × 10^5/well) to fresh cultures of naïve responder T cells (1 × 10^5/well) and anti-CD4 Abs. Positive control cultures contained responder naïve T cells alone + anti-CD4 Abs. Mean cpm for replicate cultures incubated for 72 hours are presented ± SEM. **P < 0.05, ***P < 0.005, comparing two groups.

To test further the postulate that the suppressive activity of IPE Tregs is dependent on B7 and CTLA-4 interaction, CD8+ IPE Tregs were depleted of B7-2− and CTLA-4− T cells and assayed for suppressive activity by adding them to CD3-stimulated responder T cells (Fig. 6). CD8+ IPE Tregs significantly suppressed T-cell activation (Fig. 6C) but not B7-CTLA-4− depleted CD8+ IPE Tregs. Together these results indicate that B7−CTLA-4+ CD8+ IPE T regulators are dependent on B7−CTLA-4 interaction, to achieve the suppression of bystander T-cell activity.

**DISCUSSION**

Murine T cells were converted into regulatory T cells by in vitro exposure to PE cells harvested from the iris of the uninflamed eye. When added to secondary cultures, IPE Tregs suppressed the activation of bystander T cells by anti-CD3 Abs. The ability of IPE to convert T cells into Tregs depended solely on cell-to-cell contact. In the current studies, we identified some of the key molecules involved in the conversion of CD8+ T cells into Tregs by IPE. First, IPE must express either B7-1 or B7-2. Second, a subset of responding T cells must express CTLA-4. Third, the CTLA-4+ subset expresses CD8, rather than CD4. Fourth, the CD8+ IPE Tregs must also express B7-1 and/or B7-2. Our experimental results suggest that at least two concurrent pathways of cross-talk are established between IPE and responding T cells. One pathway is highlighted in this communication and resembles conventional costimulation in that B7 molecules expressed on IPE engage CTLA-4 on T cells. A second pathway resembles regulatory costimulation in that signaling through a TGFβ receptor on T cells is required as in our recent report.25

The cell-contact-dependent process by which IPE converted CD8+ T cells into Tregs resembles that described by Li et al.,26 who used human T cells and gut epithelium. In the latter case, an Ag-nonspecific interaction between CD8+ T cells and gp180 on intestinal epithelial cells was shown to be essential.27 The analogous interaction between CD8+ T cells and IPE involves CTLA-4 and B7-1. Li et al. reported that p56lck-dependent T-cell activation, presumably via the Tcr, is important when intestinal epithelial cells induce CD8+ Tregs. More recently, Allez et al.28 reported that diverse subpopulations of CD8+ T cells proliferate on exposure to intestinal epithelium, and that the subset that expresses CD101 and CD103, but not CD28, relies on cell contact to differentiate into Tregs. Despite the differences, our evidence and that of Allez et al. support the idea that certain specialized epithelia (intestinal, iris pigment) have the capacity to convert CD8+ T cells into Tregs by a cell-contact-dependent process. Because intestinal epithelium is believed to play a role in oral tolerance, and ocular pigment epithelia contribute to ocular immune privilege, both may do so by generating Tregs from T cells that enter these special epithelium-lined tissue sites.

The idea that IPE and CD8+ T cells establish cross-talk during Treg generation first emerged in our earlier studies evaluating the changes in CD4+ and CD8+ T cells exposed in primary cultures to IPE.16 The results of these published studies showed that anti-CD3 stimulation of T cells in the presence of IPE preferentially led to sustained proliferation of CD8+, rather than CD4+, T cells. Moreover, the responding CD8+ T cells upregulated their own B7 molecules, and this was required for global suppression of both CD4+ and CD8+ T-cell activation in the primary cultures. In the present report, we provide additional evidence for IPE cross talk with CD8+ T cells. IPE exposed to T cells upregulate their expression of B7-1 and B7-2, implying that IPE are responding to signals coming from the T cells. Conversely, T cells exposed to IPE upregulate expression of their own B7-1 and B7-2 genes. Our evidence suggests that the most efficient development of Tregs in these cultures depends on this bilateral upregulation of these potent costimulators. At present, we are not able to identify the nature of the T-cell-derived signal that induces IPE to upregulate B7 expression, but we suspect that upregulation of B7-1 and B7-2 by responding T cells is related to IPE-dependent signaling via CTLA-4. We are aware that T cells stimulated via CTLA-4 begin to synthesize and secrete TGFβ.17,29 and we are mindful of our finding that IPE Tregs are not able to suppress bystander T-cell activation if the latter are obtained from dominant negative TGFβ receptor II donors.25 Because IPE-exposed T cells also upregulate B7-1 and B7-2, we wonder whether these molecules are used by Tregs to interact with other CTLA-4+ T cells in their environment, and whether this type of interaction is sufficient to convert these “bystander” T cells into Tregs.

To date, all our experiments demonstrating the critical importance of B7 expression on ocular PE in promoting the emergence of B7-bearing T cells have addressed the in vitro regulatory properties of these cells. It is of considerable interest that Taylor et al.24 reported that T cells that regulate the expression of graft-versus-host disease in vivo also express B7, and that this expression follows ligation of CTLA-4. These
findings are complementary to our findings in vitro, because IPE-derived B7 interactions with CTLA-4 on CD8+ T cells in the primary cultures is shown to be essential for the generation of Tregs in this system. Experiments to examine the potential in vivo functions of in vitro–generated IPE Tregs are now under way.

Because TGFβ promotes the upregulation of the expression of CTLA-4 on responding T cells, we propose that intraocular TGFβ produced by ocular PE cells facilitate CTLA-4 expression on PE-exposed T cells. In fact, ocular PE cells are a major source of the TGFβ that is found to be constitutively present in ocular fluids.

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