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 responders. 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 cultured for expression of B7+, CTLA-4+, and Foxp3 by using RT-PCR and flow cytometry. CD8+ IPE-Tregs were depleted of B7-2 and CTLA-4+ T cells and assayed for suppressive activity by adding them to bystander T cells.

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+, 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

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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.8,10 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.
Preparation of Cultured Ocular PE from Iris

PE cells of the iris and ciliary body were cultured as described previously.5,10 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 DNase (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+ nor MHC class II+ cells.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 (Immunal mouse T cell 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 × 10^3 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 [3H]-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). Incorporation of radioactivity was measured with a liquid scintillation counter (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 [3H]-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). Incorporation of radioactivity was measured with a liquid scintillation counter (2000 R) T cells exposed previously to IPE (IPE Tregs).

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.8 To control for the nongeneric 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.16 PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide. Photographs 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+ and CD25− 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+CD25− contained >98% of cells of this phenotype, and cells designated as CD4+CD25+ contained 90% to 95% of cells of this phenotype, as judged by flow cytometry. Total RNA was extracted from CD4+CD25− and CD4+CD25+ T cells isolated from spleens of naive mice for 24 hours. In addition, total RNA was extracted from CD8α1CD25+ IPE Tregs or CD8α1CD25− IPE Tregs established from T cells exposed to IPE. For PCR amplification, cDNAs were amplified using primers as follows: Foxp3, 5′-CAGCTGCTACAGTGGCCCTAG-3′ and reverse primers used for IFNγ, 5′-CATTTGCCAGCAGTGGGTAG-3′. The expression of Foxp3 was analyzed by reverse transcription PCR in 18-cycle amplification. The PCR products were separated by 1% agarose gel.

Flow Cytometry Analyses

The expression of CD80 (B7-1), CD86 (B7-2), and CD152 (CTLA-4) on CD8+ T cells exposed to IPE was assessed by flow cytometry. CD8+ T cells, purified by magnetic beads (CD8α+ 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 analysis. Before staining, the cocultured T cells were incubated with anti-CD16/CD32 mAbs (Fcγ 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.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-conjugated 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. CD8α+ 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 CD8α+ T cells exposed to IPE or CHX-treated CD8α+ T cells exposed to IPE was evaluated with flow cytometry. In other experiments CD8α+ 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 Rα expression was analyzed in CD8α+ T cells from the same cultures.

To determine whether CD8α+ T cells exposed to IPE express Foxp3, CD8α+ 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.

CD8α+ T-Cell Purification and Sorting

CD8α+ T cells were obtained from single cell suspension of the mouse spleen (MACS system; Miltenyi). The resultant cells were >95% pure CD8α+ T cells. CD8α+ T cells purified by this method contained ~14% CD4+Foxp3+ T cells. For purification of CD8αCD4+Foxp3+, CD8αCD4+, and CD4+ T cells, CD8α+ 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 CD8αCD4+Foxp3+ or CD8αCD4+ T cells were >98% pure. For the assay, these three populations and just CD8α+ 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 [3H]-thymidine. Mean cpm for triplicate cultures are presented ± SEM. (C) Supernatants were harvested from 48-hour cultures and assayed by ELISA for IFNγ 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 μg/mL) were cultured with IPE or CBPE for 48 hours and used as Tregs. [3H]-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, 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 [3H]-thymidine uptake (mean cpm) for triplicate cultures are presented ± SEM. **P < 0.005. (G) 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 \( P \leq 0.05 \).

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 naive T cells in the absence of PE. After incubation, the T cells were harvested, \( \gamma \)-irradiated, and added to secondary cultures containing fresh naive T cells plus anti-CD3. We observed that naive 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 IFN\( \gamma \)) 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, \( \gamma \)-irradiated, and added to secondary cultures containing naive 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.\(^\text{8}\)

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 naive 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 naive cells into Tregs.

To analyze the cellular target of the IPE Treg CD4\(^+\) and CD8\(^+\) T cells were enriched from whole spleen cells exposed to anti-CD3 Abs in the presence or absence of IPE 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 CD4\(^{\text{high}}\) (naive T cells) and CD4\(^{\text{high}}\) (memory T cells) populations into Tregs. In naive 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, CD4\(^{\text{high}}\) IPE Tregs significantly suppressed activation of bystander T cells, whereas CD4\(^{\text{low}}\) IPE Tregs, as well as CD4\(^{\text{negative}}\) Tregs, poorly suppressed the activation of T cells. These results suggest that IPE can convert only preactivated, effector-memory phenotype (CD4\(^{\text{high}}\)) 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 splenic 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 circumstantially linked to the regulatory functions of naturally arising CD4\(^+\)CD25\(^+\) Tregs.\(^\text{18}\) 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 naive mice with the expression of Foxp3 in CD8\(^+\)CD25\(^+\) or CD8\(^+\)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 naive 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 Costimulation (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 naive 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...
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\(^+\) Tregs.

**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 also 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 for 48 hours ± IPE. The T cells were harvested, irradiated, and added to secondary cultures in which both CD8\(^+\) and CD4\(^+\) T cells were depleted. T-cell activation in secondary cultures (Fig. 3A).

**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 costimulators endogenously),\(^{17}\) we tested IPE-exposed T cells for endogenous expression of the B7-1 and B7-2 genes. T cells were cultured with or without wild-type IPE, harvested, and assayed by semiquantitative RTPCR for content of mRNA of B7-1, B7-2, and GAPDH. Cont Tregs expressed small or trivial amounts of B7-1 and B7-2 mRNA (Fig. 6A), whereas IPE Tregs expressed significantly greater levels of both B7-1 and B7-2 mRNA. In contrast, T cells exposed to CBPE failed to upregulate the B7 expression (data not shown). To examine whether the mRNA for B7 found in T cells was from contaminating IPE cells, T cells were harvested from B7-1/B7-2 KO mice and
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<sup>+</sup> 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<sup>+</sup> 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 naive, uncultured CD8<sup>+</sup> 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<sup>+</sup> T cells before their coculture with IPE. When the naive CD8<sup>+</sup> 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<sup>+</sup> T cells, and anti-CD3 Abs for 24 hours. The activated CD8<sup>+</sup> T cells B7-2 were upregulated after coculture with IPE, and the expression of B7-2 was blocked if the CD8<sup>+</sup> T cells were exposed to CHX during coculture (Fig. 6C). In control
CD8+ T cells and IPE Tregs is dependent on B7 and CTLA-4 interaction, CD8+ B7-2 expression as well as CTLA-4 expression. Furthermore, IPE (and that eventually acquire the capacity to suppress the proliferation of CD8+ T cells that enter these special epithelium-lined tissue sites. Because intestinal epithelium is believed to play a role in oral tolerance, and ocular pigment epithelium contribute to ocular immune privilege, they both may do so by generating Tregs from T cells to synthesize and secrete TGF-β,17,29 and we are mindful of 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 B7-1.2 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 epithelium contribute to ocular immune privilege, they 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 upregulated 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 upregulated 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 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,13 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.9–11

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