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B7+ Iris Pigment Epithelium Induce CD8+ T Regulatory Cells; Both Suppress CTLA-4+ T Cells1,2

Sunao Sugita,* Tat Fong Ng,* Philip J. Lucas,† Ronald E. Gress,† and J. Wayne Streilein*‡

Ocular pigment epithelia contribute to immune privilege by suppressing T cell activation and converting T cells into regulatory T regulatory cells (Tregs) that inhibit bystander T cell activation. Iris pigment epithelium (IPE) does so through direct cell-cell contact with naïve T cells, and this suppressive contact is via interactions between B7 expressed constitutively on IPE cells and CTLA-4 expressed on a subpopulation of CD8+ T cells. We have now examined whether TGFβ is required in this process. We report that IPE produces both soluble and membrane-bound active TGFβ, but that only the latter is actually delivered to CD8+ T cells. In turn, these T cells become IPE Tregs by up-regulating their own expression of B7-1/B7-2 and soluble and membrane-bound TGFβ. IPE Tregs through their expression of B7 are able to engage CTLA-4+ bystander T cells, and thus precisely, target delivery of membrane-bound TGFβ. We propose that this mechanism of suppression via TGFβ ensures that soluble active TGFβ is not released into the ocular microenvironment where it can have unregulated and deleterious effects, including elevation of intraocular pressure and development of glaucoma. The Journal of Immunology, 2006, 176: 118–127.

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mmune privilege is an evolutionary adaptation of the eye that minimizes the ability of intraocular inflammation to disrupt the visual axis and cause blindness (1, 2). Immune privilege is achieved within the eye through the complementary activities of two related mechanisms: 1) soluble immunosuppressive and anti-inflammatory factors within the ocular microenvironment (TGFβ2, neuropeptides) (3) and 2) surface molecules expressed on ocular parenchymal cells, especially the pigment epithelium (4–8), the corneal epithelium (9), and the corneal endothelium (10). Ocular pigment epithelium (PE) lines the posterior surface of the iris, the ciliary body, and neural retina, thereby surrounding partially the privileged sites of the anterior chamber, the vitreous cavity, and the subretinal space, respectively. Freshly prepared, as well as cultured, PE cells from iris, ciliary body, and retina share the property of suppressing TCR-dependent activation of naive and primed T cells with which they are cultured (4, 5, 7). Moreover, T cells that have encountered ocular PE in vitro are spared from TCR-induced apoptosis and, instead, differentiate into regulatory T cells (6). We suspect that the ability of ocular PE to suppress T effector cell activity and to convert responding T cells into regulators is an important immune privilege strategy to limit immunogenic inflammation in the eye while promoting immune privilege.

Iris PE (IPE) are of particular interest because these cells, unlike ciliary body PE (CBPE) and retina PE (RPE), use a cell surface contact-dependent mechanism exclusively to suppress T cell activation in vitro, i.e., soluble factors released from IPE fail to suppress activation of cocultured T cells, nor do they induce the responding T cells to become regulators (4). We have begun to identify the molecules involved in the cell contact process and to unravel the mechanism of suppression: IPE, both fresh and cultured, constitutively express B7-1 and B7-2 on their surface, and these molecules are required to be expressed by IPE if they are to suppress naive splenic T cells and then convert them into regulators (4). A subpopulation of CD8+ T cells among the splenic T cells in these cultures expresses CTLA-4, and interactions between B7 on IPE and CTLA-4 on CD8+ T cells leads to impaired T cell activation and conversion into IPE T regulators (Tregs) (5).

TGFβ is an immunomodulatory cytokine (11–13) that has been found to be constitutively present in ocular fluids (3, 14) and to be associated with regulatory T cells of different types (15–23). Suspecting that TGFβ might be involved in the process by which IPE generate Tregs, we have recently reported that as the CTLA-4+CD8+ T cells cultured with IPE become B7-expressing IPE Tregs, they secrete enhanced amounts of both latent and active TGFβ (5). Moreover, neutralizing anti-TGFβ Abs permitted naive T cells to be activated by anti-CD3 Abs in cultures containing IPE Tregs (6). Both of these findings suggest that IPE Tregs may use secreted TGFβ to suppress bystander T cells. However, we have also determined that neutralizing anti-TGFβ Abs are unable to prevent IPE from suppressing the activation of naive T cells by anti-CD3 Abs (8). It is relevant that there are several recent reports to the effect that a membrane-bound form of active TGFβ exists, and that this form may be used by other types of regulatory T cells (15).

The present experiments were designed to determine the extent to which IPE and T cells exposed to IPE 1) up-regulate their TGFβ and TGFβ receptor genes, 2) convert the latent TGFβ they produce into the active form, and 3) use membrane-bound or soluble TGFβ to suppress bystander T cells. The results indicate that both IPE and B7+ CTLA-4+ CD8+ IPE Tregs 1) produce enhanced amounts of active TGFβ and 2) use predominantly the membrane-bound form of TGFβ to suppress T cell activation. The evidence strongly
suggests that surface interactions between B7 and CTLA-4 function to target the delivery of membrane-associated TGFβ to the appropriate T cells.

**Materials and Methods**

**Mice**

Adult C57BL/6 mice, purchased from Taconic Farms, served as donors of ocular PE cells and splenic T cells. Dr. 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 wk of age (4, 5). Mice of the C57BL/6 background with disrupted TGFα genes for TGFα were purchased from The Jackson Laboratory. Dominant-negative TGFβ type II receptor (DN TGFβ RII) transgenic mice were generated by Drs. R. E. Gress and P. J. Lucas (24, 25).

**Culture media**

Primary cultures of pigmented epithelial cells from the iris (IPE) and ciliary body (CBPE) were cultured in RPMI 1640 complete medium composed of RPMI 1640, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FBS (all from BioWhittaker), and 10−5 M 2-ME (Sigma-Aldrich). DMEM complete medium, used for primary cultures of retinal PE (RPE) cells, contained 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20% FBS. Serum-free medium was used in cultures and in assays involving T cells stimulated with anti-CD3 Abs to mimic as closely as possible the intraocular microenvironment behind the blood–ocular barrier. Serum-free medium is composed of RPMI 1640 with 0.1% BSA (Sigma-Aldrich) and 0.2% insulin, transferrin, and selenium culture supplement (ITS) (Collaborative Biochemical Products).

**Preparation of cultured pigment epithelium from iris**

PE cells of iris were isolated and cultured as described previously (4, 5, 7). In brief, iris tissue was dissected out of C57BL/6 eyes, incubated (1 h) in PBS containing 1 mg/ml Dispase and 0.05 mg/ml DNase I (both from Boehringer Mannheim). The single-cell suspensions from four iris tissues were cultured for 14 days at 37°C in 5% CO2 and air. The primary cultures were found to be >99% cytokeratin positive (Clone PCK-26; Sigma-Aldrich) by flow cytometry. The CBPE and RPE used as controls were obtained from C57BL/6 mice as described in previous studies (4, 5, 7) and were also found to be 95% cytokeratin positive after 14 days of incubation.

**Culture preparation and activation assays of T cells**

The CD3+ T cells were enriched using an Immulon mouse T cell kit from Biotex Laboratories that yielded >95% CD3+ cells—positive cells by flow cytometric analysis. The CD3− T cells (106 cells/well) were placed into the IPE culture wells for 24–48 h. The level of IPE contamination of the harvested T cells was <0.97% by flow cytometry using anti-cytokeratin Abs. For anti-CD3-driven T cell activation, purified T cells (wild type or DN TGFβ RII donors) were added (2.5 × 105 cells/well) to culture wells containing gamma-irradiated (2000 rad) IPE or not. Anti-mouse CD3e Ab (clone 2C11; BD Pharmingen) was added to the wells and the cultures were maintained for 72 h. Purified T cells were stimulated with the Abs at different concentrations, 0.1, 0.25, 0.5, and 1.0 μg/ml. After 72 h of incubation, the cultures were assayed for uptake of [3H]thymidine (1 μCi/ml) added during the terminal 8 h of culture cell proliferation. In some experiments, CD8+ T cells were enriched using MACS beads (MACS cell isolation kits; Miltenyi Biotec) where >95% of cells expressed CD8+ by flow cytometric analysis.

**ELISA for cytokines and bioassay for TGFβ**

The concentration of IFN-γ in supernatants of the T cell cultures was measured by sandwich ELISA according to the manufacturer’s instructions (BD Pharmingen) using a rat mAb to mouse IFN-γ (clone R4-6A2; BD Pharmingen) as the detecting Ab. Mouse rIFN-γ (clone R4-6A2; BD Pharmingen) was used at 3 wk of age (4, 5). Mice of the C57BL/6 background with disrupted TGFα genes for TGFα were purchased from The Jackson Laboratory. Dominant-negative TGFβ type II receptor (DN TGFβ RII) transgenic mice were generated by Drs. R. E. Gress and P. J. Lucas (24, 25).

**Detection of TGFβ and TGFβ receptor transcripts within ocular PE and in T cells exposed to IPE**

Cellular extracts were prepared from the cultured primary ocular PE or from purified T cells exposed to IPE cultured as described above. Enriched naïve T cells (column-purified splenic T cells) obtained from wild-type, DN TGFβ RII, or CTLA-4 knockout (KO) donors were added to cultures of primary or purified IPE as described above, but were cultured for 24 h. The cultured PE and T cells were washed twice with PBS, then treated with RNA STAT60. PCR was conducted by the Hot-start PCR method with AmpliTaq and AmpliWax (Applied Biosystems). The products were subjected to 35–40 cycles of PCR amplification. Primers for TGFβ1 were 5′-CAAGGAGACGGAATACAGGGCT-3′ and 5′-CCGACACAG CGTTCTTCCTCGT-3′, giving an amplification product of 260 bp. Primers for TGFβ2 were 5′-CACCAAGAACAGGAACCTG-3′ and 5′-GGCAAGAGAGCATATTCTCGC-3′, giving an amplification product of 327 bp. Primers for TGFβ receptor II were 5′-GCCGAAACA CATCAACACC-3′ and 5′-CAGGCAACGTAATCGTGC-3′, giving an amplification product of 439 bp. The forward and reverse primers used for GAPDH, CD80 (B7-1), and CD86 (B7-2) were the same as described previously (4). The PCR products were electrophoresed in 1% or 1.5% agarose gel and visualized by staining with ethidium bromide. The expression level of mRNA was standardized by the expression of GAPDH as an internal control.

**Flow cytometry**

CD8+ T cells were enriched by magnetic MACS beads as described above. The purified CD8+ T cells from wild-type, CD28 KO, or DN TGFβ RII mice were stimulated with anti-CD3 in the presence or absence of IPE and were incubated for 24 h. The T cells were collected and stained with PE-conjugated anti-CD152 mAb (clone UC10-4F10-11; BD Pharmingen) or control mouse IgG isotype at 4°C for 30 min. The bound primary Abs 1/100 (BD Pharmingen) were detected with a biotin-conjugated anti-mouse IgG (BD Pharmingen) and an Ab against one of the TGFβ receptors (I, II, and III) (all from BD Pharmingen) using a rat mAb to mouse IFN-γ (clone R4-6A2; BD Pharmingen) using a hamster IgG isotype (BD Pharmingen) as the isotype control for CTLA-4. The expression of CD152 was also analyzed on T cells treated with TGFβ RII (R&D Systems) at its ocular physiological concentration of 5 ng/ml.

Flow cytometry was also used to analyze the expression of membrane-bound (cell surface) TGFβ on cultured IPE or CD8+ T cells exposed to IPE. Enriched CD8+ T cells were incubated with IPE blocked with mouse Fc block (Fc-y/III/II receptor, clone 2.4G2; BD Pharmingen) for 15 min and incubated with intracellular staining materials (BD Cytofix/Cytoperm kits; BD Pharmingen). We used PE-conjugated hamster IgG isotype (BD Pharmingen) as the isotype control for CTLA-4. The expression of CD152 was also analyzed on T cells treated with TGFβ RII (R&D Systems) at its ocular physiological concentration of 5 ng/ml.

Flow cytometry was also used to analyze the expression of membrane-bound (cell surface) TGFβ on cultured IPE or CD8+ T cells exposed to IPE. Enriched CD8+ T cells were incubated with IPE blocked with mouse Fc block and stained with monoclonal anti-TGFβ1/2 (R&D Systems) or control mouse IgG isotype at 4°C for 30 min. The bound primary Ab was detected with a biotin-conjugated anti-mouse IgG (BD Pharmingen) Ab and FITC-conjugated streptavidin (BD Pharmingen). The cells were double stained with CyChrome-conjugated anti-CD8 mAbs (BD Pharmingen). Cultured IPE cells were stained with anti-TGFβ Abs (R&D Systems) or control mouse IgG using the same methods. We also examined intracellular TGFβ2 with the same Abs (BD Pharmingen Cytofix/Cytoperm kits).

**Detection of surface TGFβ and TGFβ receptors on IPE and T cells exposed to IPE in immunohistochemistry**

Colocalization of both TGFβ2 and the receptors was done by double immunohistochemical labeling. IPE was harvested from the iris of C57BL/6 mice as described above and cultured on coverslips for 14 days. Anti-CD3-stimulated T cells were either cultured on coverslips or added to the culture. The cultured cells were fixed with acetone directly on the coverslips and then rinsed with PBS treated with Fc blocks for 30 min. The cultures were double labeled with an anti-TGFβ2 Ab (1/50; Santa Cruz Biotechnology) and an Ab against one of the TGFβ receptors (I, II, and III) (all Abs 1/100; Santa Cruz Biotechnology). The bound anti-TGFβ2 Abs were visualized with Cy3-conjugated secondary Abs, anti-rabbit IgG (for receptor III, donkey Abs were used) (Jackson ImmunoResearch Laboratories). The bound anti-TGFβ receptor (I, II, and III) Abs were visualized with Cy2-conjugated secondary Abs (Jackson ImmunoResearch Laboratories). The images were visualized and photographed on an epifluorescence microscope (Nikon E800).

**In vitro assays of Treg cell activity**

**Proliferation.** Naïve T cells were exposed to cultured IPE as described above, were harvested, gamma-irradiated (2000 rad), and then added (105...
We first examined whether pigment epithelial cells and tissues irradiated CD8* H11003 found to be RT-PCR for mRNA. The level of IPE contamination of harvested T cells was depleted of mTGF* H9252. Each experiment was repeated at least twice with similar results. All statistical analyses were conducted with control, undepleted CD8* H9252/Purified enriched CD8* H9252/mAbs (anti-TGF* H9252/2, 1 Ab was used to stain IPE Tregs followed by biotin conjugated anti-mouse IgG. MACS beads separated the population into mTFB* H9252 or mTGF* H9252 IPE Tregs before staining with FITC-conjugated CD80 (B7-1; BD Pharmingen), CD86 (B7-2; BD Pharmingen), CD152 (CTLA-4) Abs, or control hamster IgG (15). The cells were washed and analyzed by flow cytometry for protein and RT-PCR for mRNA. The level of IPE contamination of harvested T cells was found to be <0.97% with anti- cytokeratin Abs by flow cytometry.

Depletion of mTFB* T cell population. The CD8* H9252 T cells were depleted of mTFB* T cells by using anti-TGF* H9252/2 and biotin-conjugated anti-mouse IgG and anti-biotin MACS beads as described above. As a control, undepleted CD8* H9252 IPE T regulators were used in these experiments.

Statistical evaluation of results. Each experiment was repeated at least twice with similar results. All statistical analyses were conducted with Students t test. Values were considered statistically significant if p ≤ 0.05.

Results Detection of TGFβ and TGFβ receptor expression by ocular pigment epithelial cells and tissues

We first examined whether TGFβ1 and TGFβ2, as well as TGFβ RII genes were expressed by cultured PE obtained from normal C57BL/6 mice. Each PE cell type (IPE, CBPE, RPE) expressed easily detectable mRNA for TGFβ1 and TGFβ2 (Fig. 1A). We also searched for similar transcripts in excised whole ocular tissues (iris, ciliary body, retina) freshly obtained from eyes of normal C57BL/6 mice (Fig. 1B). In results for TGFβ RII genes, these tissues all expressed these same transcripts, although expression of the TGFβ RII gene was barely detected in IPE (Fig. 1C).

We examined cultured IPE for evidence of surface expression of TGFβ2, the dominant isoform of TGFβ in the eye, using immunostaining and bright field microscopy or flow cytometry. Cultured IPE were placed on coverslips and immunostained with anti-TGFβ2 Abs. Cultured IPE were readily identified on bright field microscopy by the presence of cytoplasmic melanin granules (Fig. 1D). By fluorescence microscopy, these same cells expressed membrane-associated TGFβ2. The pattern of TGFβ2 expression was punctate, suggesting either that the cytokine was present in relatively large cell surface patches or perhaps in cytoplasmic granules subjacent to the cell surface. Similarly, cultured IPE expressed surface TGFβ (46.4% positive; Fig. 1E) and intracellular TGFβ (89.7% positive; Fig. 1E). Together these results imply that IPE under the culture conditions we used produced active TGFβ2 in two phases: membrane-associated and soluble.

Detection of TGFβ and TGFβ receptor expression by T cells in contact with IPE

Our next goal was to determine the extent to which naive T cells, T cells activated by anti-CD3, and T cells expressed to IPE TGFβ and its receptors. As revealed in Fig. 2A, uncultured naive T cells as well as anti-CD3-stimulated T cells after 72 h expressed the TGFβ RII gene. Similarly, T cells exposed to IPE clearly expressed the TGFβ RII gene (data not shown). Naive splenic T cells contained no identifiable transcripts for either TGFβ1 or TGFβ2 (data not shown). Anti-CD3-stimulated T cells expressed easily detectable transcripts for TGFβ1 but little, if any, TGFβ2 transcripts (Fig. 2B). In companion experiments, naive T cells were cultured with IPE for 24 h; transcripts of TGFβ1 and TGFβ2 were readily detected in these T cells (IPE Tregs; Fig. 2C), as were transcripts of the TGFβ RII gene (data not shown).

In separate experiments, T cells cultured for 24 h in the presence (IPE T cells; Fig. 2D) or absence (Cont T cells; Fig. 2D) of IPE were harvested. The supernatants of T cells were removed and...
assayed for TGFβ protein. No TGFβ was detected in supernatants of Cont T cells; by contrast, we readily detected significant amounts of TGFβ in the culture supernatants of IPE T cells (Fig. 2D). Similarly, CD8+ IPE T cells expressed more intracellular TGFβ (92.9%) than CD8+ control T cells (12.2%; Fig. 2E). Neither T cell population bound the control mouse IgG (<5%; data not shown). Together these results indicate that T cells exposed to IPE acquire the capacity to secrete active TGFβ, unlike T cells cultured in the absence of IPE. Moreover, IPE Tregs express receptors for TGFβ, as do conventional T cells.

Patterns of expression of TGFβ and TGFβ RII on cocultured IPE cells and T cells

We next examined surface expression of TGFβ and TGFβ receptors (RI, RII, RIII) on IPE and T cells that were cultured together for 24 h. Glass coverslips on which these cells were cultured were immunostained with Abs to TGFβ (Fig. 3A and B), and staining for TGFβ RII was evident in both bright field and fluorescence microscopy. Representative photomicrographs are presented in Fig. 3. Melanin-positive cells (Fig. 3A and B) were stained with Abs to TGFβ and GAP DH; PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide. D. Supernatants were harvested from naive T cells that were cultured for 24 h in the presence (IPE T cells) or absence (Cont T cells) of cultured IPE. Supernatants were assayed in the TGFβ bioassay as described in Materials and Methods. Results of triplicate samples are presented as mean ± SEM. E, Detection of intracellular TGFβ by IPE T cells. Purified CD8+ T cells cultured with IPE for 24 h were stained with anti-TGFβ1/2. Then cells were stained with secondary Abs of biotin-conjugated anti-mouse IgG and then stained with PE-conjugated streptavidin. As controls, naive T cells were cultured for 24 h in the absence of IPE (Cont T cells). Number indicates percentage positive cells for TGFβ.

Influence of signaling via TGFβ RII on CTLA-4 expression by T cells exposed to IPE

We postulated that IPE-derived TGFβ was important in inducing cocultured T cells to up-regulate CTLA-4. To test our hypothesis, we took advantage of genetically manipulated mice in which a DN TGFβ RII expressed under a human CD2 promoter/enhancer is present only on T cells (24, 25) where it successfully competes with the T cells’ own signaling TGFβ RII. Purified CD8+ T cells were obtained from wild-type and DN TGFβ RII donors, then cultured with or without IPE in the presence of anti-CD3. In control experiments, CD8+ T cells from CD28 KO mice were used in place of the DN TGFβ RII donors. CTLA-4 expression was detected by flow cytometry. CTLA-4 was up-regulated on anti-CD3-stimulated T cells in the time course (24-h culture, 12%; 48-h culture, 50%), whereas little up-regulation of CTLA-4 was observed if the responding T cells in these cultures were derived from DN TGFβ RII mice (24-h culture, 2%; 48-h culture, 14%; Fig. 4A). Similarly, CTLA-4 was up-regulated on anti-CD3-stimulated T cells in the absence of IPE (8%); when T cells were similarly stimulated in the presence of IPE, the proportion of T cells expressing CTLA-4 rose dramatically to 51% (Fig. 4B). When the responding T cells in these cultures were derived from DN TGFβ RII mice, very little up-regulation of CTLA-4 was observed (Fig. 4B), implying that the capacity of anti-CD3 to induce CTLA-4 expression and the capacity of IPE to amplify that expression is
dependent on TGFβ signaling. We observed that the up-regulation of CTLA-4 on T cells obtained from CD28 KO mice was comparable to that achieved by wild-type T cells stimulated with anti-CD3 in the presence of IPE. To confirm this point, expression of CTLA-4 was examined by flow cytometry on T cells stimulated with anti-CD3 in the presence of recombinant TGFβ. A large proportion (48%) of anti-CD3-stimulated T cells cultured for 24 h in the presence of recombinant TGFβ expressed CTLA-4, compared with anti-CD3-stimulated T cells cultured without recombinant TGFβ (6%) (data not shown). These results indicate that up-regulation of CTLA-4 expression on anti-CD3-stimulated T cells is enhanced in the presence of active TGFβ, further implying that active TGFβ is produced by cultured IPE.

Expression of TGFβ gene in IPE-exposed T cells from DN TGFβ RII and CTLA-4 KO mice

Many laboratories have reported that a functional link exists between CTLA-4 signaling and T cell production of TGFβ (15, 17, 18, 23); i.e., T cells fail to express CTLA-4 in the absence of TGFβ signaling. To explore whether a similar link prevails when IPE induce T cells to convert into IPE Tregs, T cells were obtained from wild-type and genetically manipulated mice: DN TGFβ RII, CTLA-4 KO. These cells were cultured for 24 h in the presence (or absence) of IPE, then examined for expression of mRNA for TGFβ1 and TGFβ2. IPE Tregs obtained from wild-type donors up-regulated mRNA expression of both TGFβ1 and TGFβ2 (Fig. 5, A and B). By contrast, neither IPE-exposed T cells from DN TGFβ RII donors (Fig. 5A) nor CTLA-4 KO donors (Fig. 5B) contained significant levels of transcripts of either TGFβ isoform. Importantly, the CTLA-4 KO mice were unable to express the TGFβ2 isoform as shown in Fig. 5B. These results support the postulate that T cells destined to become IPE Tregs receive at least two obligatory signals from IPE: one signal is delivered via TGFβ RII (and is presumably triggered by TGFβ derived from the IPE); the other signal is delivered via CTLA-4 and is triggered by B7 molecules expressed constitutively by IPE.

Role of TGFβ RII in enabling IPE to suppress anti-CD3-driven T cell activation in coculture

To test the hypothesis that signaling through TGFβ RII was required for IPE suppression of T cell activation by anti-CD3, we...
used T cells that lacked the ability to receive a signal via TGFβ RI. Purified splenic T cells obtained from wild-type (A and B), DN TGFβ RI (A), and CTLA-4 KO donors (B), and then cultured with or without IPE for 24 h. RT-PCR was performed as described in Materials and Methods. To estimate PCR products semiquantitatively, the density of the band of negative image was analyzed by NIH image software. The expression level of mRNA was standardized by the expression of GAPDH as an internal control, TGFβ1/GAPDH, TGFβ2/ GAPDH. PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide.

**Importance of membrane-bound TGFβ and TGFβ RI in enabling IPE Tregs to suppress bystander T cell activation**

In an attempt to validate the hypothesis that IPE Tregs use cell contact and a membrane-associated form of active TGFβ to suppress bystander T cells, we made use of Transwell inserts that permit soluble molecules, but not cells, to pass between segregated cell populations. CD8⁺ IPE Tregs (anti-CD3 pretreated or not) were seeded into cell inserts, and bystander naive T cells were seeded below the cell inserts onto the bottoms of the culture wells in the presence of anti-CD3. In control cultures, IPE Tregs were seeded directly into the wells containing naive T cells and anti-CD3. The results of this experiment demonstrated that the efficiency with which IPE Tregs suppressed bystander T cell proliferation was significantly impaired if the Tregs and the bystander T cells were separated by a Transwell membrane (Fig. 7A). When IPE Tregs in the Transwell were treated with anti-CD3 Ab, they suppressed activation of bystander T cells; again the efficiency was more significantly impaired if the Tregs and bystander T cells were separated by a Transwell membrane. These data indicate that direct cell-cell contact optimizes the capacity of IPE Tregs to suppress bystander T cells. We next compared the capacity of IPE Tregs and control T cells to suppress proliferation when added to secondary cultures containing naive T cells, anti-CD3 Abs, and neutralizing anti-TGFβ Abs (or not). As expected, IPE Tregs profoundly suppressed T cell proliferation, whereas control T cells did not (Fig. 7B).
This suppression was partially relieved in a dose-dependent manner in the presence of anti-TGFβ Abs. These results are compatible with the hypothesis that IPE Tregs bearing membrane-associated TGFβ lose their regulatory effect if the TGFβ is neutralized.

The availability of DN TGFβ RII made it possible to determine whether IPE can convert naive T cells into IPE Tregs if TGFβ RII signaling is curtailed and whether IPE Tregs derived from wild-type donors were capable of inhibiting bystander T cells if the latter were from DN TGFβ RII donors. T cells from DN TGFβ RII and wild-type donors were cultured with IPE and added to secondary cultures containing naive T cells plus anti-CD3. When proliferation of bystander T cells was assessed (Fig. 7C), only regulatory T cells created by exposing naive T cells to IPE were inhibitory. In fact, bystander T cells in cultures containing IPE-exposed DN TGFβ RII T cells displayed enhanced proliferation in response to anti-CD3. We then prepared IPE Tregs, using wild-type T cells, and added these cells (after gamma-irradiation) to secondary cultures containing anti-CD3 plus naive T cells from wild-type or DN TGFβ RII donors. The results indicated that wild-type T cells were capable of suppressing bystander T cell activation only when the T cells could accept a TGFβ signal (Fig. 7D). Bystander T cells bearing DN TGFβ RII were not suppressed and actually outperformed the positive controls. Together these results indicate that T cell signaling via TGFβ RII is required 1) when naive T cells are converted into regulators by IPE and 2) when bystander T cells are inhibited by IPE Tregs.

**Evidence that active TGFβ is membrane associated in CD8+ B7+ CTLA-4+ IPE Tregs**

IPE achieve suppression of T cell activation, and convert the T cells into regulators of bystander T cells, exclusively via a cell-cell contact mechanism in which interaction of costimulation molecules (B7 and CTLA-4) is required (4, 5). Because supernatants of cultured IPE and IPE Tregs contained active TGFβ, we wondered whether soluble active TGFβ or its membrane-bound form was the more effective mechanism of signaling via TGFβ RII. We analyzed the expression of membrane-associated TGFβ on IPE Tregs using flow cytometry. The results indicated that CD8+ IPE Tregs expressed TGFβ on their cell surfaces, whereas control T cells (cultured in the absence of IPE cells) expressed little or no surface TGFβ (Fig. 8A). We next determined whether membrane-bound TGFβ-positive IPE Tregs express B7-1, B7-2, and CTLA-4 costimulatory molecules. After being cocultured with IPE, CD8+ IPE Tregs were stained with anti-TGFβ Abs, then separated into mTGFβ or mTGFβ- T cell subpopulation. Flow cytometry analyses showed that mTGFβ-CD8+ IPE Tregs expressed greater levels of CD80 (B7-1-positive cells, 69.8%), CD86 (B7-2-positive cells, 59.7%), and CD152 (CTLA-4-positive cells, 32.1%), whereas mTGFβ-CD8+ IPE Tregs poorly expressed these molecules on their surface (Fig. 8B). Similarly, mTGFβ-CD8+ IPE Tregs expressed greater levels of mRNA for CD80 and CD86, whereas mTGFβ- subsets slightly expressed mRNA for these molecules (data not shown). Thus, IPE-exposed CD8+ T cells up-regulate the expression of B7-1, B7-2, CTLA-4, and mTGFβ.
FIGURE 8. Expression of mTGFβ by T cells exposed to IPE. A, TGFβ and CD8 staining of T cells cultured with IPE for 24 h. The upper panel is the double staining of IPE Treg cells for TGFβ and CD8. The lower panel is the histogram comparing the expression of TGFβ on CD8+ IPE Tregs (black line) to CD8+ control T (Cont T) cells (T cells not cultured with IPE, dotted line). B, Comparison of mTGFβ− CD8+ IPE Tregs to mTGFβ+ CD8+ IPE Treg expression of costimulatory molecules CD80 (B7-1), CD86 (B7-2), and CD152 (CTLA-4) by flow cytometry. Number indicates percentage-positive cells for each molecule. C, To demonstrate that the suppressive activity of IPE Treg is with the mTGFβ+ cells, CD8+ IPE Tregs were depleted of mTGFβ+ cells (■) and assayed for suppressive activity by adding them to CD3-stimulated T resp cells as described in Materials and Methods. The mTGFβ−-depleted CD8+ IPE Treg cell suppression of T resp cell proliferation was compared with the suppression of proliferation by, undepleted CD8+ IPE Tregs (cross-hatched bar) and to the proliferation by a positive control culture containing only CD3-stimulated T resp cells (□). Presented are the mean cpm for triplicate cultures incubated for 72 h ± SEM. **, p < 0.005, comparing two groups.

Finally, we examined whether mTGFβ-depleted CD8+ IPE Tregs were able to suppress bystander T cell activation. Similar to previous experiments, CD8+ IPE Tregs significantly suppressed bystander T cell activation, whereas mTGFβ-depleted CD8+ IPE Tregs failed to suppress the T cell activation (Fig. 8C). Together these results indicate that B7− CTLA-4− CD8+ IPE T regulators predominately use the mTGFβ to achieve the suppression of bystander T cells.

Discussion
There are many examples of T cell inhibitory mechanisms in which soluble molecules are implicated in suppression. Our interest in the regulatory properties of IPE stems from its remarkable dependence on direct cell-cell contact for T cell suppression to be achieved. Moreover, T cells exposed by direct contact to IPE in vitro are converted into regulatory T cells with the capacity to suppress activation of bystander T cells. Previously, we reported that one set of costimulatory molecules, B7 and CTLA-4, were required for suppression by IPE Tregs (5). In this communication, we have identified another required set of ligands and receptors, TGFβ and TGFβ RI, the signaling receptor. Based on the evidence presented here, as well as our recently published data, we have formulated the following hypothesis: IPE make use of one of the immune system’s powerful costimulation strategies to target T cells (B7-1 and B7-2 on IPE, CTLA-4 on a subpopulation of CD8+ T cells), and when thus engaged, IPE membrane-associated active TGFβ is delivered precisely to these T cells. In turn, these CD8+ T cells (IPE Tregs) up-regulate expression of B7-1/B7-2 and TGFβ1/TGFβ2. This enables the IPE Tregs to target bystander T cells, again, via direct cell contact, to deliver a membrane-associated TGFβ signal that suppresses bystander cell activation. We suggest that the capacity of IPE to contribute to immune privilege within the eye depends on their using a novel and highly targeted mechanism to suppress T cell activation, and that the responding T cells themselves adopt this mechanism to suppress other T cells. We speculate that modifying T cell behavior through membrane-associated, rather than soluble, active TGFβ is important for maintaining the integrity of the eye from the deleterious nonimmune consequences of active, soluble TGFβ.

TGFβ is a pleiotropic cytokine/growth factor and its capacity to suppress aspects of immunity is only one dimension of its activities (11–13). In the absence of TGFβ, mice develop a massive, multifocal inflammatory disease, suggesting a major role for this factor in regulation of both adaptive and innate immunity (26). Mice in which T cells express a DN TGFβ RII develop hyperproliferation of CD8+ T cells, implying a role for TGFβ in maintaining CD8+ T cell homeostasis. CD8+ T cells that express CTLA-4 have regulatory properties and secrete TGFβ as one of the mediators of suppression (5). It is within this context that the capacity of IPE to suppress T cell activation and convert T cells into regulators takes on meaning. The evidence presented here demonstrates that cultured IPE, along with their counterpart PE from ciliary body and retina, secrete soluble active TGFβ. In the case of IPE, some of this active molecule was displayed on the cell surface as membrane associated. Cultured IPE expressed very low levels of TGFβ RII on their surface, implying that autocrine effects are minimal. Moreover, IPE-derived TGFβ proved to be essential in order for IPE to suppress T cell activation triggered by anti-CD3 Abs. That is, T cells incapable of receiving a TGFβ signal (DN TGFβ RII) were not suppressed when exposed to IPE. This latter finding is important in the context of histologic evidence that distribution of surface TGFβ on IPE was uneven and was localized to punctate areas on adjacent T cells where expression of TGFβ RII was also colocalized. This provides circumstantial evidence that the TGFβ delivered to T cells by IPE may be, if not exclusively, membrane-associated and that the delivery of TGFβ is precisely targeted.

Our results suggest that IPE-exposed T cells, become regulatory, use a similar mechanism to mediate suppression of bystander T cells in secondary cultures. CD8+ T cells exposed to IPE up-regulated expression of TGFβ1 and TGFβ2, and these cells proved capable of converting latent TGFβ into the active forms. Moreover, T cell:T cell contact was observed by microscopic examination of primary cultures comprised of IPE and T cells. In these T...
cell aggregates, membrane-associated TGFβ was unevenly distributed on one T cell and localized to the point of contact with an adjacent T cell; moreover, the adjacent T cell membrane was enriched for TGFβ RII expression at the same point of contact. Since IPE Tregs proved incapable of suppressing the activation of bystander T cells that expressed the DN TGFβ RII and since the efficiency with IPE Tregs suppressed bystander T cells was markedly impaired when the Tregs and the target T cells were separated by a Transwell membrane, we conclude that CD8+ IPE Tregs use primarily a contact-dependent mechanism to suppress bystander T cell activation and that the IPE Tregs deliver active, membrane-associated TGFβ to the target T cells at the point of contact.

When we determined the CD4/CD8 phenotype of IPE Tregs, we observed that enriched CD8+ IPE Tregs significantly suppressed T cell activation, whereas enriched CD4+ IPE Tregs showed little capacity to suppress T cell activation. In contrast, although both CD4+ and CD8+ T cells were needed to establish RPE Tregs, it was the CD4+ RPE Treg population that was suppressive after exposure to RPE cells obtained from the posterior segment of the eye.

It was interesting to determine that up-regulation of CTLA-4 on T cells destined to become IPE Tregs was dependent on TGFβ signaling. We believe this to be important because CTLA-4 is the T cell surface molecule that interacts with the costimulatory molecule B7 that is constitutively expressed by IPE. We speculate that the contact between CD8+ T cells and IPE, which is required for the generation of IPE Tregs, is mediated by B7-CTLA-4 interactions. Thus, IPE-derived TGFβ initiates the regulatory process by inducing CTLA-4 up-regulation on CD8+ T cells. Then CTLA-4 ligation provides physical stability of IPE-T cell interactions from which IPE Tregs eventually emerge. In turn, IPE Tregs up-regulate surface expression of B7 and active TGFβ, and a similar two-step process enables these cells to engage bystander CTLA-4+ T cells that express TGFβ RII and then to suppress their activation.

There are many recent reports that indicate that regulatory T cells express surface TGFβ and/or the secreted soluble form (15, 17, 19–23). Included in this list are natural regulatory CD4+CD25+ T cells (15, 17, 19, 20), CD4+CD25- T cells (21), and CD8+CD25+ T cells (22). However, this is by no means the rule because it has been reported that CD4+CD25+ Tregulators suppress in the absence of TGFβ (27). Nakamura et al. (15) have reported evidence that cell-cell contact is important when natural CD4+CD25+ regulatory T cells inhibit bystander T cells with mTGFβ, but the molecular basis for “mTGFβ remains obscure.”

In addition to its immunosuppressive and anti-inflammatory effects, TGFβ displays properties that have been considered to be deleterious to vision (28–30). In wound healing, TGFβ promotes the healing response, in part by recruiting macrophages and when excessive TGFβ can push wound healing toward scarring (31). Whereas scarring in many somatic tissues is not a particularly devastating outcome of wound healing, in the eye, scar formation in the corneal stroma often causes visual impairment and even blindness (32). Gliosis is a prominent feature of optic nerve head pathology in the late stages of glaucoma, and this reactive gliosis contributes to the blinding neuropathy; TGFβ has been suspected of promoting gliosis in this situation (30). Pertinent to the findings reported in this communication, TGFβ has been implicated in the changes in the outflow pathway of the eye that lead to elevated intraocular pressure in primary open angle glaucoma (29). A recent, definitive study supporting this view was reported to have produced increased resistance in the outflow path (trabecular meshwork) by infusing active TGFβ, especially the β1 isoform, into the anterior segment of the eye (28). We interpret these several examples to mean that soluble, active TGFβ can be deleterious to the eye. However, since TGFβ has been demonstrated to play a central role in conferring immune privilege upon the eye (1–3), a conundrum exists: how can TGFβ promote the dual consequences of preserving vision and yet of threatening quality vision?

Under normal circumstances, levels of total TGFβ2 in aqueous humor are in the nanogram per milliliter range, whereas the levels of the active isoform are in the low picogram range (14) even with TSP-1, a powerful converter of latent TGFβ to its active form, is present in aqueous humor (33). We suspect that in the eye IPE succeed in suppressing T cell activation in the anterior chamber, and in converting the T cells into regulators, by delivering active, mTGFβ to immigrating T cells that are targeted by B7-CTLA-4 interactions. Suppression of sight-threatening T cell effector mechanisms within the anterior chamber is then extended and made pervasive by B7-expressing CD8+ T cells that can deliver their own membrane-bound active TGFβ to targeted bystander T cells in the ocular microenvironment. In this manner immunogenic inflammation within the anterior segment of the eye is suppressed, i.e., immune privilege is present, and vision is preserved. It is pertinent to this consideration that elevated intraocular pressure and glaucoma are frequent complications of chronic inflammation of the uveal tract (iris, ciliary body, retina, and chorio-capillaris) (34). In animal models where inflammation has been induced in the eye, the bulk of the TGFβ that is present in ocular fluids is in the active rather than latent form, and this TGFβ is soluble (35). This line of reasoning suggests that under normal circumstances the ocular microenvironment avoids the generation of active, soluble TGFβ and achieves delivery of the active form through membrane-dependent interactions between cells.

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