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CTLA-4⁺CD8⁺ T Cells That Encounter B7-2⁺ Iris Pigment Epithelial Cells Express Their Own B7-2 to Achieve Global Suppression of T Cell Activation

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Pigment epithelial (PE) cells cultured from the eye possess the novel property of suppressing TCR-dependent activation of T cells in vitro. Iris PE (IPE) cells accomplish this suppression by a direct cell contact mechanism in which B7-2 expressed by the PE cells interacts with CTLA-4 on responding T cells. Because CTLA-4 expression is constitutively expressed on a very small proportion of naive splenic T cells and since exposure of splenic T cells to IPE leads to global T cell suppression, we have inquired into the mechanism by which suppression is achieved. Using splenic T cells and IPE from donor mice with disrupted genes for CD80 (B7-1), CD86 (B7-2), CTLA-4, and/or CD28, we report that B7-2⁺ IPE in the presence of anti-CD3 supported selectively the activation of CTLA-4⁺CD8⁺ T cells that express their own B7-2 and secrete enhanced amounts of active TGFβ. By contrast, activation of CTLA-4-negative T cells, especially CD4⁺ cells, in these cultures was profoundly suppressed. Because global suppression of T cell activation in these cultures was obtained only when both IPE and T cells possessed B7-2 genes and expressed the costimulators as surface molecules, we propose that T cells activated in the presence of parenchymal cells from the eye (an immune privileged site) express B7-2 in a manner that equips them to suppress bystander T cells. Thus, B7-2 expression on T cells participates in their eventual ability to function as regulators in vitro. The Journal of Immunology, 2004, 172: 4184–4194.

To avoid the blinding consequences of intraocular inflammation, the eye expresses an extensive array of mechanisms by which innate and adaptive immune effectors can be regulated, even silenced. Mechanisms that have been revealed to date include 1) an intraocular microenvironment (aqueous humor) that is rich in soluble immunomodulatory factors (1, 2), 2) blood-tissue barriers that limit the ingress of blood-borne cells and molecules capable of mediating immunogenic inflammation (3), and 3) constitutive expression on ocular parenchymal cells of CD95 ligand that triggers apoptosis of effector T cells otherwise destined to reject orthotopic corneal allografts (4, 5). These and other mechanisms help to explain why the eye has been shown experimentally to be an immune privileged site (6, 7), i.e., the eye accepts foreign tissue grafts for extended, often indefinite intervals, whereas conventional body sites reject such grafts summarily. It is important to study and understand the processes by which the eye normally regulates immune effector mechanisms because insights into immune regulation can be gained that may have more general applicability and usefulness.

Ocular pigment epithelia (PE) of the iris, ciliary body, and retina have been identified as important participants in creating and maintaining ocular immune privilege (8–13). Ocular PE, which are derived from the neuroectoderm, have a primary vision-related function to serve as a light sink, quenching unfocused light that might otherwise enter the eye and compete with the focused images that pass through the visual axis to the retina (8). Ocular PE also have secondary functions, related in part to the region of the eye in which they are located. For example, retinal PE (RPE) provide nutritive and biochemical support for the proper functioning of rod and cone photoreceptor cells of the retina (9). In addition, the pigment epithelium-lined iris and ciliary body have been demonstrated to suppress T cell activation in vitro, and it has been proposed that this immunoregulatory property of ocular PE is related to intracellular suppression of immunogenic inflammation (10, 11).

We have recently reported that cultured iris PE cells (IPE) suppress anti-CD3-driven T cell activation in vitro by direct cell contact, rather than by secretion of immunosuppressive factors (12). Moreover, cultured IPE constitutively express the immune costimulation molecule B7-2 (13). Interaction between B7-2 on IPE and CTLA-4 on T cells has been demonstrated to mediate the contact-dependent inhibition that IPE exert on T cells. IPE that lack B7-2 expression are unable to suppress T cell activation, and T cells deficient in CTLA-4 are incapable of being suppressed by cultured IPE. B7-1 and CD28 are irrelevant to this suppression. Thus, B7-2 binding of CTLA-4 is the contact event that enables IPE to suppress T cell activation.

IPE are capable of suppressing anti-CD3-driven activation of both previously primed and naive T cells (10). Whereas CTLA-4 expression is enhanced on primed T cells, only a small percentage of naive T cells express CTLA-4. Nonetheless, cultured IPE still achieve global suppression of activation of naive T cells in vitro. This outcome suggests that global T cell suppression arises from the initial interaction of B7-2⁺ IPE cells with a small subpopulation of CTLA-4⁺ T cells, and it implies that secondary effects lead to global T cell suppression. In the studies reported here, we searched for the secondary effects through which IPE achieve global suppression of T cell activation, including the majority of T cells that lack CTLA-4 expression. Our results indicate that initial...
cross-talk between a small number of CTLA-4−CD8+ T cells and B7-2− IPE at culture inception results in further up-regulation of B7-2 on IPE and, more importantly, induces B7-2 and CTLA-4 expression on expanding numbers of T cells. These latter cells acquire the capacity to secrete high levels of active TGFβ, which in turn accounts for the profound suppression that overtakes the entire T cell population in these cultures.

Materials and Methods

Mice

We used as donors of lymphoid cells and ocular PE adult C57BL/6 mice obtained from our domestic animal colony or purchased from Taconic Farms (Germantown, NY). C57BL/6 background mice with disrupted genes for CD28 and for both CD80 and CD86 or only CD80 were purchased from The Jackson Laboratory (Bar Harbor, ME). Dr. J. P. Allison (University of California, Berkeley, CA) provided us with C57BL/6 background CTLA-4−/+ heterozygote mice, from which we generated CTLA-4−/− mice that served as donors of lymphoid cells at 3 wk of age as described previously (13).

Culture media

RPMI 1640 complete medium was used for primary cultures of IPE and ciliary body PE cells (CBPE). It was 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, and 10% FBS (all from BioWhittaker, Walkersville, MD), and 1 × 105 M 2-ME (Sigma-Aldrich, St. Louis, MO). RPMI 1640 complete medium was used for primary cultures of RPE cells. It was composed of DMEM, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20% FBS. Cultures of T cells stimulated with anti-CD3 Abs were grown in serum-free medium, which contains complete medium except for FBS, and which is supplemented with 0.1% BSA (0.1% BSA; Sigma-Aldrich) and 0.2% insulin, transferrin, selenium plus culture supplement (Collaborative Biochemical Products, Bedford, MA).

Preparation of cultured PE from iris, ciliary body, and retina

IPE and CBPE were cultivated separately as described in previous studies (12, 13). Eyes were enucleated from 6- to 8-wk-old male C57BL/6 mice, cut into two parts along a circumferential line just posterior to the ciliary process. The lens was removed from the anterior eye cup, and whole iris and ciliary body tissue was gently peeled off. The iris and ciliary body tissues were separated from each other by careful dissection and then incubated separately in PBS containing 1 mg/ml Dispase and 0.05 mg/ml DNase I (both from Boehringer-Mannheim, Mannheim, Germany) at 37°C for 1 h. Thereafter, iris and ciliary body tissues were triturated with 21-gauge and 23-gauge needles to make single-cell suspensions. Monodisperse iris and ciliary body cells were washed and then placed in six-well plates and incubated at 37°C for 2 wk. RPE cells were also cultivated as described previously (12, 13). Eyes were enucleated from 6- to 8-wk-old male C57BL/6 mice, and cut into two parts along a circumferential line posterior to the ciliary process, creating a ciliary body-deficient posterior eyecup. The neuronal retina was detached and removed from the posterior eyecup, and then the eyecup was incubated in 0.2% trypsin (BioWhittaker) for 1 h. Thereafter, the eyecup was transferred to wash medium, and RPE cells were pelleted off as intact sheets. RPE were triturated to make a single-cell suspension and then resuspended in DMEM complete medium, placed in six-well plates, and incubated for 2 wk at 37°C. At the end of this culture interval, the medium was removed, and the PE cells were washed with fresh and twice-devoided calf serum and added to wells of culture plates. As controls for cultured PE cells, the murine fibroblast cell line (NIH 3T3 cell) was used in some experiments.

Preparation of purified T cells and assay of proliferation

As described previously (13), separately cultured, cytokerin-positive PE cells (1.0 or 2.0 × 106 cells/well) were seeded in flat-bottom 96-well culture plates and incubated overnight. For stimulation with anti-CD3 Abs, T cells were prepared by pressing spleen fragments through nylon mesh (Imumulv mouse T cell kit; Biotex Laboratories, Houston, TX) to produce a single-cell suspension that was >90–95% CD3+. In some experiments, CD4+ T cells or CD8+ T cells were prepared separately from donor spleens, using MACS cell isolation kits (Miltenyi Biotec, Auburn, CA). These cells, which were purified by a single immunomagnetic depletion step using MACS magnetic beads, proved to be >94% CD4+ or CD8+. Purified T cells (2.5 × 105 cells/well) were stimulated with anti-CD3 Ab (clone 2C11; BD Pharmingen, San Diego, CA) and incubated for 24, 48, and 72 h. The concentration of soluble anti-CD3 Abs in these cultures ranged between 0.5 and 1 μg/ml as indicated in each experiment. After incubation, the cultures were assayed for uptake of [3H]thymidine (NEN, Boston, MA), as a measure of cell proliferation. Thymidine-pulsed T cells were isolated by automated cell harvester (Tomtec, Hamden, CT). Incorporation of radioactive was measured by liquid scintillation counting (Beta- plate; Wallac, Gaithersburg, MD), and the amount was expressed in counts per minute.

Flow cytometry

Flow cytometry was used to analyze expression on PE cells and T cells of various costimulatory molecules: CD86 (B6-2; CD86, programmed death (1 PDL); PE-conjugated CD80 (CTLA-4−)), PE-conjugated CD152 (CTLA-4+), PE-conjugated CD154 (clone UC10-4F10-11), and PE-conjugated anti-CD28 mAbs (clone 37.51) were used to stain purified T cells; 72 h after activation with anti-CD3 Abs, the T cells were harvested, washed twice, and stained with anti-CD28 mAbs or anti-CD152 mAb or both. Unstimulated, purified naive T cells were also stained directly with these mAbs. In companion experiments, anti-CD3-stimulated T cells or naive T cells in the presence or absence of IPE cells were harvested for 24-h culture and stained for cell surface or intracellular CTLA-4 (BD Cytofix/Cytoperm Kits; BD Pharmingen). Before staining, the cocultured cells were incubated with mouse Fc block (FcγRIII/IIR, clone 2.4G2; BD Pharmingen) for 15 min. As isotype control for CTLA-4, we used PE-conjugated hamster IgG isotype (BD Pharmingen). Cultured IPE cells were analyzed with FITC-labeled Abs to CD86 (clone GL1, BD Pharmingen) for flow cytometric analysis. IPE cells exposed to anti-CD3-stimulated T cells or IPE cells without T cells were harvested and stained with anti-CD68 mAbs. FITC-conjugated rat IgG isotype (BD Pharmingen) was used as control. Other mAbs used for T cell staining included FITC-conjugated anti-CD3 Abs (CD3ε, clone145-2C11), FITC-conjugated anti-CD4 mAbs, PE-conjugated anti-CD4 mAbs (both L3T4, clone RM4-5, and Cy-Chrome-conjugated anti-CD8 mAbs (both Ly2, clone 53-6.7), and PE-conjugated anti-IFN-γ mAbs (clone J43), all purchased from BD Pharmingen.

CFSE labeling of T cells

Labeling of purified T cells with CFSE (Molecular Probes, Eugene, OR) was conducted as described previously (14). Briefly, 1 × 107 T cells were diluted in 1 ml of serum-free HBSS, and 1 μM CFSE per ml was added. The cell suspension was incubated for 8 min at room temperature. Cell labeling was stopped by quenching unlabeled CFSE by adding excess amounts of HBSS containing FBS. Subsequently, cells were washed three times and used for experiments. As controls, CFSE unlabeled cells were used. 5 × 105 T cells (1 × 107) were labeled with CFSE (final concentration, 1 μM) or not (controls) and then added (2.5 × 105 cells/well in 96-well plate) to culture wells containing anti-CD3 Abs (final 1 μg/ml) and cultured T cells or PE cells. After 72 h, the T cells were harvested, washed twice, and incubated with anti-CD16/CD32 Abs for 15 min at 4°C. The CFSE-labeled T cells were stained with PE-conjugated anti-CD4 mAb (L3T4) and Cy-Chrome-conjugated anti-CD8 mAbs (Ly2) in flow cytometry.

ELISA for cytokines and bioassay for TGFβ

To measure cytokine content (IFN-γ, IL-2, IL-4, IL-10) in supernatants of T cell cultures, quantitative capture ELISA was used. Culture supernatants were collected at 48 h after exposure of T cells (from CTLA-4− knockout (KO), CD83 KO, CD80/CD86 KO and wild-type control donors) to PE cells (from CD80 KO, CD80/CD86 KO, and wild-type control donors), and immediately frozen and stored at −20°C until used. ELISA was conducted according to manufacturer’s instructions. Rat mAbs to mouse IFN-γ (clone R4-6A2), IL-2 (JES-6-1A2), IL-4 (11B11), or IL-10 (JES-2A5; all from BD Pharmingen) were used as coating Abs. Biotinylated rat mAbs to mouse IFN-γ (XMG1.2), IL-2 (JES6-5H4), IL-4 (BVD6-24A2), and IL-10 (SXC-1; all from BD Pharmingen) were used as detecting Abs. All recombinant cytokines used to establish standards for the assays were purchased from BD Pharmingen.

To determine amounts of mature (active) and total (active plus latent) TGFβ, supernatants of T cells exposed for 48 h to cultured PE cells were collected and then added to cultured Mv1Lu cells (CCL-64 cell line; from American Type Culture Connection, Manassas, VA). To detect mature TGFβ1 and TGFβ2, the supernatants were diluted 1/4 with Eagle’s MEM (BioWhittaker), which consisted of 2 mM l-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5% FBS. Diluted supernatants (100 μl) were added to 96-well flat plates. To measure total TGFβ, supernatants were pretreated with 1 N HCl (1:10) for 1 h and then neutralized with...
mixture of 1 N NaOH, 1 M HEPES (1:5). These acidified supernatants were diluted 1/10 with complete Eagle’s MEM (0.5% FBS), and then 100 μl were added to 96-well flat plates. Mv1Lu cells (1 × 10^5/100 μl) were added to each well and cultured for 24 h at 37°C. Cultures were pulsed with 1 μCi [3H]thymidine 4 h before termination. Thymidine-pulsed cells were harvested by automated cell harvester, and incorporated radioactivity was measured by liquid scintillation counter. These results are expressed as picograms per milliliter. TGFβ concentrations of these samples were calculated by the suppression of Mv1Lu cell proliferation in comparison with the suppression in proliferation by known amounts of TGFβ1 or TGFβ2 (R&D Systems, Minneapolis, MN). This bioassay had a limit of 3 pg/ml TGFβ.

Detection of cytokine transcripts within T cells exposed to ocular PE cells

Cellular extracts were prepared from purified anti-CD3-stimulated T cells exposed to cultured PE cells and analyzed by RT-PCR. Purified naïve T cells were cultured with PE cells for 48 h in the presence of anti-CD3 Abs. Thereafter, T cells were harvested (<0.1% of the harvested cells stained positive for cytokeratin, a marker for PE cells). The T cells were washed twice with PBS and then treated with RNA STAT-60 (TEL-TEST, Friendswood, TX). RNA was precipitated with ethanol and dried. The RNA was then dissolved with RNase-free water, and cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) according to manufacturer’s instruction. PCR was conducted by the Hot start PCR method with AmpliTaq and AmpliWax (Applied Biosystems, Foster City, CA). The following conditions were used: denaturation at 94°C for 60 s; annealing at 54°C (IFN-γ) or 57°C (IL-10, TGFβ2) for 60 s; extension at 72°C for 60 s. The products were subjected to 40 cycles of PCR amplification. The forward and reverse primers used for GAPDH were 5′-GCTCTTCCGGTGTGGAACGGA-3′ and 5′-TGGCAG-TGGTGTGAACGGA-3′, giving an amplification product of 245 bp. Primers for IFN-γ were 5′-TGGGTTGCGTCTCGTGTCC-3′, giving an amplification product of 287 bp. The following conditions were used: denaturation at 94°C for 60 s; annealing at 54°C (IFN-γ) or 57°C (IL-10, TGFβ2) for 60 s; extension at 72°C for 60 s. The products were subjected to 40 cycles of PCR amplification. The forward and reverse primers used for GAPDH were 5′-GCGAAGGCAGCAATTATCCTGCAC-3′ and 5′-GTCATCTCGGTGTAGTACACA-3′, giving an amplification product of 686 bp. Primers for IL-10 were 5′-CAGCGCGGAAGACATAACT-3′ and 5′-TTGCATGCGCCTGTGAACACC-3′, giving an amplification product of 408 bp. Primers for TGFβ2 were 5′-CACCACAAAGACGAAAC-CCTG-3′ and 5′-GGAGAAGGGACATATCTCCTGCAC-3′, giving an amplification product of 327 bp. The PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide. To evaluate the semiquantitative PCR, photographs of the gel were taken with a high resolution camera, and the density of the band of negative image was analyzed by National Institutes of Health image software. The expression level of mRNA was standardized by the expression of GAPDH as an internal control.

Detection of B7-2 and B and T lymphocyte attenuator (BTLA) transcripts within T cells exposed to IPE cells

Naive C57BL/6 spleen T cells were purified as described above and placed in culture wells containing cultured IPE cells. After 24 h, the T cells were harvested by gentle pipetting from the culture and were used for additional experiments after being washed twice with serum free RPMI medium. The level of contamination of the harvested T cells with IPE cells was 0.97% or less by flow cytometry using anti-cytokeratin Abs. Cellular extracts were prepared from purified naive T cells exposed to cultured IPE cells plus anti-CD3 Abs (0.5 μg/ml) and were analyzed by RT-PCR. RNA extraction, cDNA synthesis, and PCR (40 cycles of amplification) for B7-2 were conducted by the same methods as described previously (13). 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 KO mice were cultured with wild-type IPE. In some experiments, we examined for the presence of transcripts on BTLA molecules. The selected primers for BTLA were 5′-TGGCCAG-GAAAGGACACTA AC-3′ and 5′-GGCTCACTGGCACGTTAAC-3′, giving an amplification product of 287 bp. The following conditions were used: denaturation at 94°C for 60 s; annealing at 55°C for 60 s; extension at 72°C for 60 s. Analysis of semiquantitative RT-PCR was performed as described above.

Immunohistochemistry

IPE was harvested from the irises of C57BL/6 mice and cultured on glass coverslips (diameter, 12 mm) for 14 days. T cells and anti-CD3 Abs were added to these coverslip PE cultures for 24 h. Thereafter, the entire coverslip containing cocultured PE cells and T cells was fixed with acetone. After rinsing with PBS, the coverslip was incubated with mouse Fc block (anti-CD16/CD32 mAbs) for 30 min, washed with PBS, and then double-labeled with the first primary Ab against CTLA-4 (1/100, clone UC10-4F10-11; BD Pharmingen), visualized by Cy3 (1/500, Jackson ImmunoResearch, West Grove, PA), and finally labeled with FITC-conjugated B7-2 (1/100, clone GL1). Fluorescence images were obtained using a confocal microscope (TCS SP2; Leica Microsystems, Bannockburn, IL).

Statistical evaluation of results

Each experiment was repeated at least twice with similar results. All statistical analyses were conducted with Student’s t test. Values were considered statistically significant if p < 0.05.

Results

Differential vulnerability of CD4 and CD8 T cells to IPE-mediated suppression of proliferation

PE cells were cultured from irises of normal eyes of C57BL/6 mice. After 14 days, when the cultures contained a virtually pure population of cytokeratin+ cells, the culture medium was discarded and replaced with fresh serum-free medium. As an alternative tissue culture, cultured murine fibroblasts (NIH 3T3 cells) were substituted for IPE cells. T cells were harvested and column purified from splens of normal C57BL/6 mice. CD4+ T cells and CD8+ T cells were selected using MACS beads. Unfractionated T cells, CD4+ T cells or CD8+ T cells were layered onto cultured IPE substrates and stimulated with anti-CD3 Abs. [3H]Thymidine was added 8 h before the cultures were terminated at 72 h. The amount of radioisotope incorporated was then measured. The results of a representative experiment (of three) are presented in Fig. 1. As reported previously, unfractionated purified naïve T cells failed to proliferate when stimulated with anti-CD3 Abs in the presence of cultured IPE. NIH 3T3 cells failed to similarly suppress activation of these T cells (data not shown). IPE profoundly suppressed proliferation of purified CD4+ T cells. By contrast, a small but significant amount of proliferation was detected among purified CD8+ T cells. These results raised the possibility that CD4+ and CD8+ T cells might be differentially vulnerable to suppression by IPE.

To gain insight into this possibility, naïve T cells were stained with CFSE and stimulated with anti-CD3 Abs in the presence of

![FIGURE 1. Relative capacity of cultured IPE cells to suppress activation of CD4+ and CD8+ T cells as separate populations. Purified naive C57BL/6 T cells (2.5 × 10^5 cells/well, respectively) were fractionated into CD4-enriched or CD8-enriched populations. These populations or unfractionated T cells were stimulated with 1 μg/ml anti-CD3 Ab and cultured with □ or without □ IPE cells. After 72 h, the cultures were assayed for uptake of [3H]thymidine. Mean counts per minute for triplicate cultures are presented as SEM. *p < 0.05, **p < 0.0005, compared with anti-CD3-treated T cells.](http://www.jimmunol.org/ Downloaded from)
cultured IPE or in the absence of these cells (positive control). The cultures were harvested at 72 h, and the T cells were evaluated by flow cytometry for the extent to which CD4+ and CD8+ T cells underwent progressive rounds of cell division. As revealed in Fig. 2A, up to six rounds of cell division were evident in control cultures without IPE. CD4+ T cells participated in four rounds, whereas CD8+ T cells participated in all six rounds. In the presence of IPE (Fig. 2B), anti-CD3-stimulated CD4+ T cells underwent only two rounds of division, whereas a subpopulation of CD8+ T cells still managed five to six rounds of division in response to anti-CD3. For example, as shown in Fig. 2B5, the CFSE-positive T cell population contained predominantly CD8+ T cells (76% of the T cells were CD8+, and 10% were CD4+). These results reveal that in the absence of cultured IPE anti-CD3 stimulates CD4+ T cells to a limited number of cell divisions (approximately four in these experiments), whereas CD8+ T cells achieved a larger number of divisions (six in these experiments). When T cells were stimulated with anti-CD3 in the presence of IPE, this difference was exaggerated. Beyond the third round of replication, only CD8+ T cells actually participated. Thus, IPE were more efficient at suppressing proliferation of CD4+ T cells than CD8+ T cells.

**Differential vulnerability of CD4 and CD8 T cells to IPE-mediated suppression of cytokine production**

Proliferation is only one manifestation of anti-CD3-driven T cell activation; production of cytokines is another. Since cytokines can cross-regulate bystander T cells, we next examined the extent to which T cells stimulated with anti-CD3 Abs in the presence of ocular PE produced the effector cell-associated cytokines IL-2, IFN-γ, IL-4, and IL-10. Naive T cells were stimulated with anti-CD3 in the presence (or absence) of cultured IPE, CBPE, and RPE. After 48 h, supernatants were removed and analyzed by ELISA for content of cytokines, and the cultured T cells were examined by semiquantitative RT-PCR for expression of the same cytokine genes. The results displayed in Fig. 3A indicate that production of all four cytokines was profoundly reduced (compared with positive controls) in T cell cultures stimulated with anti-CD3 in the presence of IPE. Moreover, the results displayed in Fig. 3B indicate that reduced IFN-γ production correlated with markedly reduced gene expression, whereas IL-10 gene expression was unchanged in T cells cultured with IPE.

Activated T cells can also produce the immunosuppressive cytokine, TGFβ, and TGFβ has been implicated in certain forms of T cell-mediated suppression (15, 16). Accordingly, we examined the influence of ocular PE on the content of TGFβ in culture supernatants, and on the expression of TGFβ transcripts within T cells stimulated with anti-CD3 in the presence of the diverse ocular PE. For this experiment, 48-h supernatants were evaluated for content of total and active TGFβ1 and TGFβ2, and T cells were examined for their content of TGFβ-mRNA by semiquantitative RT-PCR. As revealed in Fig. 4, A and B, supernatants from cultures in which T cells were stimulated with anti-CD3 in the presence of diverse ocular PE contained comparable amounts of total TGFβ, and small but significantly more TGFβ than supernatants from cultures with anti-CD3-stimulated T cells in the absence of ocular PE. However, supernatants of cultures containing ocular PE, T cells and anti-CD3 contained much larger amounts of active TGFβ than did similar cultures without PE. When T cells were harvested from similar cultures and subjected to semiquantitative RT-PCR analysis of TGFβ mRNA transcripts (Fig. 4C), all harvested T cell populations contained comparable amounts of TGFβ1 and TGFβ2 mRNA. For comparison, T cells cultured alone in the absence of anti-CD3 contained only trace levels of TGFβ mRNA (data not shown). These results indicate 1) that anti-CD3 stimulation promotes TGFβ gene transcription in T cells and 2) that stimulation of

**FIGURE 2.** Relative capacity of IPE cells to suppress activation of CD4+ and CD8+ T cells contained within CFSE-labeled T cell suspensions. Purified C57BL/6 T cells (1 × 10^5) were labeled with CFSE (final concentration, 1 μM) or not (controls) and then added (2.5 × 10^5 cells/well in a 96-well plate) to culture wells containing anti-CD3 Abs (final concentration, 1 μg/ml) plus cultured IPE cells (B) or not (A). After 72 h, the T cells were harvested and stained with PE-conjugated anti-CD4 mAbs and Cy-Chrome-conjugated anti-CD8 mAbs in flow cytometry. Histograms: A1, CFSE (FITC); A2, CFSE (FITC); A3–A6, CD4-PE/CD8-Cy-Chrome. Arrows with numbers 6, 5, 4, and 3 above A1, A2, B1, and B2, individual replicating populations of cells stained with CFSE. Each number refers to population displayed in detail in A3–A6 and B3–B6.
FIGURE 3. Effect of cultured ocular PE cells on Th1 and Th2 cytokine production by T cells activated by anti-CD3. Purified C57BL/6 T cells (2.5 x 10^5 cells/well) were stimulated with 1 μg/ml anti-CD3 Ab and cultured for 48 h in the presence or absence of cultured syngeneic IPE, CBPE, or RPE. A, At culture completion, supernatants were harvested and assayed by ELISA for content of IFN-γ, IL-2, IL-4, and IL-10. Mean counts per minute for triplicate cultures are reported ± SEM. B, At culture completion, anti-CD3-stimulated T cells exposed to PE cells were harvested after 48 h of culture. RNA was extracted from these cells, and cDNA was synthesized by RT-PCR. To estimate PCR products semiquantitatively, the density of the band of negative image was analyzed by National Institutes of Health image software. The expression level of mRNA was standardized by the expression of GAPDH as an internal control: IFN-γ/GAPDH; IL-10/GAPDH. PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide.

**Influence of IPE on T cell expression of CTLA-4 and B7-2 during anti-CD3 stimulation**

The results above reveal that IPE have a differential capacity to suppress activation of CD4⁺ and CD8⁺ T cells, and that certain T cells respond to anti-CD3 stimulation in the presence of IPE by acquiring an efficient mechanism for converting TGFβ into its active form. We have recently reported that IPE, through constitutive expression of B7-2, suppress T cell activation by engaging CTLA-4 on the T cells (13). The next experiments explored the possibilities that a subpopulation of CTLA-4⁺CD8⁺ T cells is the first to encounter B7-2⁺ IPE and that these T cells eventually cross-regulate bystander CD4⁺ T cells in the culture, thereby accounting for the observed global T cell suppression. Purified splenic T cells from naive C57BL/6 mice were stimulated with anti-CD3 Abs in the presence (or absence, control) of IPE. At 72 h, the T cells were harvested and examined for expression of CD28, CTLA-4, CD4, and CD8. The results of a representative experiment are presented in Fig. 5. Only a tiny proportion of anti-CD3-unstimulated CD4⁺ and CD8⁺ T cells expressed CTLA-4 after 72 h in cultures without IPE (Fig. 5A). By contrast, a much higher proportion of responding T cells up-regulated CTLA-4 expression in the presence of IPE (Fig. 5B). This was particularly true for CD8⁺ T cells, in which 23% of the anti-CD3-stimulated cells were CTLA-4⁺ after 72 h exposure to IPE. The enhanced expression of CTLA-4, especially on CD8⁺ T cells in IPE-containing cultures, correlates positively with enhanced expression of the TGFβ gene in cultured T cells.

To quantitify this effect, T cells were stimulated with anti-CD3 (or not) in the presence or absence of IPE. T cells were removed at 24 h and examined by flow cytometry for CTLA-4 expression. As displayed in Fig. 6A, naive T cells exposed to IPE in the absence of anti-CD3 stimulation showed a small increase in both surface and intracellular expression of CTLA-4. However, T cells stimulated with anti-CD3 in the presence of IPE showed a much greater increase in CTLA-4 expression, and this was especially true for intracellular CTLA-4 content. Thus, IPE and anti-CD3 stimulation appear to act synergistically to enhance significantly CTLA-4 expression by T cells. In companion cultures maintained for 48 h, T cells were discarded, and the IPE were removed and assayed by flow cytometry for surface expression of B7-2. As revealed in Fig. 6B, IPE cultured with anti-CD3-stimulated T cells significantly up-regulated their own surface expression of B7-2. To determine whether the B7-2 expressed by T cells in these cultures...
was captured from IPE cells or synthesized de novo by the T cells, cultures were established containing naive T cells, IPE cells, and anti-CD3. After 24 h, the T cells were separated from the IPE and subjected to RT-PCR analysis for expression of B7-2. For controls, naive T cells or T cells stimulated with anti-CD3 in the absence of IPE were used. Several controls were performed in an effort to

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confirm that B7 expression by T cells exposed to IPE arose from their own endogenous B7 genes. First, naive T cells or T cell stimulated with anti-CD3 in the absence of IPE were used. Second, T cells from B7-1/B7-2 KO mice, as naive, anti-CD3 stimulated, or following exposure to wild-type IPE were used. The results displayed in Fig. 6C indicate that mRNA levels for B7-2 are significantly up-regulated in T cells that had been stimulated with anti-CD3 in the presence of wild-type IPE. Moreover, B7-1/B7-2 KO T cells failed to express detectable mRNA for these B7 genes whether tested as naive cells, after stimulation with anti-CD3, or when cultured with wild-type IPE (data not shown). These results imply that cross-talk emerges between T cells and IPE in these cultures, leading to enhanced expression of B7-2 by IPE and enhanced expression of CTLA-4 by responding T cells.

In an attempt to gain morphologic insight into this cross-talk, we next examined by immunohistochemistry the morphologic features of cultures of anti-CD3-stimulated T cells layered onto cultured IPE. Tagged Abs to B7-2 and CTLA-4 were used, and specimens were examined by bright field and fluorescence microscopy after 24 h of culture. Representative images are presented in Fig. 7. Bright field examination enabled us to identify the location of pigment containing IPE cells. Both near and at a distance from these cells are B7-2+ cells that are smaller and not associated with pigment in the cytoplasm (Fig. 7A). When these cultured cells were also stained for CTLA-4, the large majority of the small B7-2+ cells also stained heavily for CTLA-4 (Fig. 7B). This indicates that CTLA-4 expression is briskly up-regulated (within 24 h) on T cells stimulated with anti-CD3 in the presence of IPE. Unexpectedly, many of these same T cells also appeared to express B7-2-independent of IPE cells (Fig. 7B). Thus, B7-2-expressing IPE not only up-regulate their own B7-2 expression in these cultures but also induce responding T cells (presumably CTLA-4-bearing) to express de novo their own B7-2. This finding raised the possibility that novel B7-2 expression on T cells in these cultures might be contributing to the global T cell suppression observed.

Influence of IPE on T cell expression of PD-1 and BTLA
Among the family of ligands that bind to B7-like costimulators is PD-1 (17). This homolog of CD28 and CTLA-4 delivers negative signals to T cells when it encounters B7-like molecules on APCs (18, 19). We therefore examined T cells activated by anti-CD3 in the presence of IPE for change in expression of PD-1. T cells were harvested after 24 h of culture with anti-CD3 and IPE. The cells were stained with anti-mouse PD-1 Abs plus anti-CD4 or anti-CD8 Abs, and analyzed by flow cytometry. Among unstimulated naive splenic T cells, PD-1 expression on CD4 and CD8 T cells was very low (Fig. 8A). T cells stimulated with anti-CD3 Abs up-regulated PD-1 to a limited degree on both CD4+ and CD8+ cells (9.9 and 2.7%; data not shown). However, similarly stimulated T cells in the presence of IPE up-regulated PD-1 to a slightly smaller amount on both CD4+ and CD8+ cells (Fig. 8B). As found in the CFSE experiments described above, there were significantly more CD8+ T cells than CD4+ T cells in these cultures at the end of 24 h of incubation (compared Fig. 8A with Fig. 8B). We have also examined by semiquantitative RT-PCR expression of BTLA another member of the CD28 family of coreceptors for B7 molecules.
BTLA is an inhibitory receptor on lymphocytes that resembles CTLA-4 and PD-1 and is up-regulated in Th1, but not Th2, cells (20). We found no difference in BTLA gene expression in T cells activated with anti-CD3 alone or in the presence of IPE (data not shown). Together these findings argue against the possibility that either PD-1 or BTLA is the ligand for B7-2 on responding T cells when suppression of T cell activation occurs in the presence of IPE cells.

**Influence of B7-2 and CTLA-4 expression by T cells on magnitude of IPE-dependent suppression of T cell activation**

Naïve T cells were stimulated with anti-CD3 in the presence or absence (positive control) of cultured IPE. IPE cells were cultured from irises of wild-type mice and of mice with disrupted B7-1 genes alone, or with both B7-1 and B7-2 disrupted genes. T cells were obtained from wild-type C57BL/6 mice or from mice with disrupted CTLA-4 or CD28 genes. After 48 h, the supernatants of these cultures were harvested and assayed by ELISA for content of IFN-γ. The results presented in Fig. 9A indicate that wild-type and B7-1 KO IPE profoundly suppressed IFN-γ production by anti-CD3, whereas IPE from B7-1/B7-2 KO mice suppressed T cell proliferation much less effectively. Moreover, C57BL/6 IPE profoundly suppressed wild-type and CD28 KO T cells, but they were less successful at suppressing CTLA-4 KO T cells (Fig. 9B). Thus, CTLA-4 expression by T cells and B7-2 expression by IPE are both important to achieving suppression of T cell activation in cultures containing IPE. Whether B7-2 expression by T cells also contributes to the magnitude of suppression was revealed in the next experiment.

**FIGURE 7.** Photomicrographs of IPE cells cocultured with anti-CD3-stimulated T cells. Purified C57BL/6 T cells were stimulated with anti-CD3 Abs in the presence of IPE cells for 24 h. The cocultured cells were then fixed with acetone, stained with anti-CD86 (B7-2) and anti-CD152 (CTLA-4) Abs, and examined by bright field and fluorescence microscopy. A, Examination by bright field (top), fluorescence (middle), and combined (bottom) microscopy of the same image. IPE cells (top) are identified by dense content of melanin granules. B7-2 positive T cells (middle) are numerous, small, and round. B7-2 + small round cells are distinct from melanin-containing IPE cells (bottom). B, Examination by fluorescence microscopy of the same sample of B7-2 + cells (top) and CTLA-4 + cells (bottom). The same small round cells (T cells) express both B7-2 and CTLA-4. Bar, 20 μm.

**FIGURE 8.** Attempt to identify expression of PD-1 on T cells exposed to IPE. Naïve T cells were cultured with anti-CD3 Abs (B) or not (A) in the presence (B) or absence (A) of IPE. After 24 h, the T cells were harvested and analyzed by flow cytometry for expression of PD-1 (PE-conjugated anti-PD-1 mAbs), CD4 (FITC-conjugated anti-CD4 mAbs), and CD8 (Cy-Chrome-conjugated anti-CD8 mAbs). PE-conjugated hamster IgG isotype was used as control for anti-PD-1 Abs.
proliferation was largely, but not completely, abolished when both T cells and IPE were obtained from mice lacking B7-2 expression (compare Fig. 10A with Fig. 10B). T cells from B7-1/B7-2 KO mice secreted significantly less IFN-γ when stimulated by anti-CD3 than did their wild-type counterparts (Fig. 10C). However, when T cells from B7-2-deficient mice were stimulated with anti-CD3 in the presence of IPE that were also deficient in B7-2 expression, no suppression of IFN-γ production was observed. Together, these results indicate that global suppression of T cell activation by cultured IPE requires that at least some of the participating T cells express CTLA-4 and B7-2.

Discussion
IPE constitutively express B7-2, and these cells use this molecule in a contact-dependent manner to suppress activation of naive T cells in vitro. CTLA-4 expression by target T cells is essential to the suppression observed (13). Because few T cells among naive splenocytes express CTLA-4, the mechanism by which IPE achieve global T cell suppression is obscure. The results of the experiments reported here reveal that T cells themselves participate in the global suppression achieved by IPE and that novel expression of B7-2 on a subpopulation of IPE-exposed T cells is essential to reaching maximum suppression. The experimental evidence in favor of these conclusions is manifold.

First, a high frequency of T cells activated by anti-CD3 for 72 h in the presence of IPE expressed CTLA-4, predominantly, but not exclusively, within the cytoplasm. The increase in CTLA-4 expression was particularly strong among CD8+/H11001 T cells where the percentage of positive cells rose from 1% to 23%, and was not observed among T cells stimulated with anti-CD3 in the absence of IPE. T cells exposed to IPE without anti-CD3 stimulation showed only a modest up-regulation in CTLA-4 expression. Thus, a subpopulation of CD8+ T cells that encounter B7-2+/H11001 IPE cells become B7-2+ suppressors.

Second, CD8+ T cells were less vulnerable to IPE-mediated suppression of proliferation than were CD4+ T cells, and a subset of CD8+ T cells exposed to IPE even sustained their proliferation through five to six rounds of division (when the cultures were

![FIGURE 9](http://www.jimmunol.org/)

Capacity of B7-1/B7-2 KO IPE cells to suppress IFN-γ production by anti-CD3 stimulated wild-type or CTLA-4 KO T cells. A, Purified wild-type C57BL/6 T cells were stimulated with 1 μg/ml anti-CD3 Ab and cultured for 48 h in the presence or absence of IPE cells obtained from eyes of B7-1/B7-2 KO, B7-1 KO, or wild-type mice. B, Purified T cells harvested from wild-type control (22 days old), CTLA-4 KO (22 days old), and CD28 KO (6 wk old) mice were stimulated with anti-CD3 in the presence of wild-type C57BL/6 IPE. After 48 h, culture supernatants were harvested and assayed by ELISA for IFN-γ content. *, p < 0.05; **, p < 0.005; ***, p < 0.0005, compared with anti-CD3-treated T cells. ND, Not detected.

![FIGURE 10](http://www.jimmunol.org/)

Capacity of B7-1/B7-2 KO IPE cells to suppress activation of B7-1/B7-2 KO T cell. Cocultures containing purified splenic T cells, cultured IPE cells, and anti-CD3 Abs were established as described above. Cultured IPE cells and purified splenic T cells were obtained from C57BL/6 wild-type controls (A and C) or from B7-1/B7-2 double KO mice (B and C). One set of cultures was terminated at 72 h to assay proliferation by terminal addition of [3H]thymidine (A and B). From another set of cultures supernatants were harvested after 48 h and assayed for IFN-γ content by ELISA (C). Mean cpm for triplicate cultures are reported ± SEM. *, p < 0.05; **, p < 0.005; ***, p < 0.0005, comparing the indicated groups. NS, Not significant.
terminated at 72 h). By contrast, very few CD4+ T cells treated with anti-CD3 in the presence of IPE proceeded beyond one round of cell division, and many of these cells failed to replicate at all. Therefore, B7-2+ IPE single out a subset of CD8+ T cells for sustained activation via interactions with CTLA-4, whereas activation of the remaining T cells in the culture is suppressed.

Third, the subset of T cells that expressed CTLA-4 after anti-CD3 stimulation in the presence of IPE for 24 h also expressed B7-2, and B7-2 gene expression by these T cells proved to be critical to global suppression. Naïve T cells harvested from mice with disrupted B7-2 genes were significantly less vulnerable to suppression by exposure to IPE than were T cells from genetically intact mice. Thus, global suppression of T cell activation in the presence of IPE is dependent in part on expression of both CTLA-4 and B7-2 by participating T cells.

Fourth, the subset of T cells that was activated to express CTLA-4 and B7-2 in these cultures lacked the capacity to secrete effector cytokines, such as IFN-γ, IL-2, and IL-4, because the genes encoding these molecules were suppressed. These T cells also failed to secrete IL-10, even though IL-10 gene expression was undiminished. Instead, T cells that expressed CTLA-4+ and B7-2+ on exposure to IPE significantly up-regulated their expression of the TGFβ gene, and the supernatants of cultures containing these cells, anti-CD3, and ocular PE cells contained high levels of active TGFβ. Thus, the subset of T cells preferentially targeted for activation by B7-2+ IPE express CD8, CTLA-4, and B7-2, and interactions between these T cells and PE cells generate enhanced amounts of active TGFβ.

We propose that the global suppression achieved when TCR-stimulated T cells are exposed in vitro to IPE cells involves a unique collaboration between IPE and a subpopulation of T cells in which the latter become equipped to secrete the immunosuppressive molecule TGFβ. Whether this T cell-dependent suppression is mediated by TGFβ alone or in concert with other immunosuppressive factors is the subject of ongoing experiments.

The expression of B7-2 by IPE-exposed CD8+ T cells in our experiments is worthy of comment. Several laboratories have reported that activated T cells can express B7-1 and B7-2, although the evidence is somewhat controversial. Hakamada-Taguchi et al. (21) described expression of B7-2 on memory, but not naive, T cells, although they failed to find B7-1 expression. They suggested that B7-2 expression on memory CD4+ T cells functioned as co-stimulation for the activation of naive CD4+ T cells. Tatar-Calderone et al. (22) reported in 2002 that activated human T cells acquired CD80 expression and also implicated this expression in T cell-T cell interactions. However, these authors provided evidence that the CD80 expressed by activated T cells had been acquired from the APCs with which they were stimulated, whereas Hakamada-Taguchi et al. reported B7 gene expression in memory T cells, implying that the B7 had been endogenously produced. Whereas this line of investigation implicates T cell expression of B7 in activation toward effector and memory cell function, another line of investigation implicates B7 expression on T cells in activation toward acquisition of regulatory function. Nakamura et al. (23) have reported that naturally occurring CD4+CD25+ T regulators express cell surface CD80, CD86, and CTLA-4, and that expression of these molecules is linked to the ability of these T cells to suppress bystander T cells by a contact-dependent mechanism. The results of our experiments are similar to this line of inquiry. Although we cannot rule out the possibility that some of the B7-2 expressed by T cells exposed to IPE was captured from the latter, our results strongly support the conclusion that T cells exposed to IPE and stimulated with anti-CD3 up-regulate their own B7-2 gene and express on their surface the protein thus encoded. Moreover, in a manner similar to that of natural CD4+CD25+ T regulators, B7-2-expressing T cells stimulated with anti-CD3 in the presence of IPE also express CTLA-4 and promote the suppression of bystander T cells in the same cultures. TGFβ-generated T regulators, however, appear to be derived from CD8+, rather than CD4+, T cells. The link between cell contact and regulation by surface-bound TGFβ, as reported by Nakamura et al. (23), is also reminiscent of the need for contact between IPE and T cells for activation of the latter cells to be suppressed. Whether a similar mechanism of activation of TGFβ within the plane of the contacting cell membranes exists when IPE cells encounter T cells is now under investigation.

The capacity of naïve T cells to begin to express B7-2 maximally in response to exposure to IPE is dependent on the delivery of two distinct signals. The first signal is transmitted via the TCR. In the present experiments, anti-CD3 Abs were used, and we have shown previously that TCR-dependent activation also suffices when IPE are present in mixed lymphocyte reactions and when recognition of OVA peptide 423–339 on I-Aα molecules by transgenic DO11.10 T cell is arranged. We believe that the second signal is delivered when B7-2, constitutively expressed on IPE, engages CTLA-4 on T cells, especially CD8+ T cells. We have ruled out several other costimulators as substitutes for B7-2 and CTLA-4: in the absence of B7-2, IPE fail to suppress T cell activation even though IPE express CD95 ligand, B7-1, and galectin-1 on their surface (12, 13). Thus, there is something special about B7-2 expression on IPE in the process by which CD8+ T cells lose effector functions and acquire the capacity to secrete active TGFβ.

What is interesting is that global T cell suppression is promoted by B7-2 expressed on T cells. Because virtually the same population of T cells that expressed B7-2 on exposure to IPE in our experiments also expressed CTLA-4, and because signaling via CTLA-4 has been demonstrated to divert T cells toward production of TGFβ (23, 24), we suspect that a link exists among B7-2, CTLA-4, and TGFβ production in T cells. Although we cannot identify the molecular basis of this link, we strongly suspect that this link promotes the conversion of activated T cells into regulators, rather than effectors.

We have previously shown that IPE constitutively express B7-2 (13). Our present results indicate that B7-2 expression on IPE is also dynamic. In particular, IPE that were exposed to anti-CD3-driven T cells significantly up-regulated their own expression of B7-2. We do not know whether this is a direct consequence of B7-2 interaction with CTLA-4 on T cells or whether B7-2 expression is enhanced on IPE by other signals arising from the responding T cells. In any event, we take this as evidence that cross-talk is established between B7-2+ IPE and CTLA-4+ T cells. Our evidence suggests that the initial interaction (cross-talk) involves IPE and a relatively tiny fraction of T cells that express CTLA-4. As this exposure proceeds temporally, we found that the level of B7-2 expression on IPE escalated, as did the number of T cells that expressed CTLA-4. The cross-talk then evolved to include B7-2+ IPE, B7-2+ T cells, and ever increasing numbers of CTLA-4+ T cells. We propose that this rising tide of B7-2/CTLA-4 interactions eventuates in profound suppression of bystander T cells (predominantly CD4+). Our results implicate active TGFβ in this suppression, but other mechanisms may also be involved.

We have reported previously that T cells that are stimulated via the TCR in the presence of pigment epithelial cells lose effector functions and acquire the capacity to regulate other T cells (13). This was shown by harvesting T cells from cultures containing anti-CD3-stimulated T cells and IPE, then x-irradiating the harvested T cells and placing them in coculture with fresh naïve T
cells and anti-CD3 Abs. Proliferation in these cultures was markedly curtailed. It is possible that the subset of CD8⁺ T cells that we found in the present experiments to express CTLA-4 and B7-2 and to secrete active TGFβ after exposure to IPE and anti-CD3 is the source of the regulatory T cells observed previously. Experiments to test this possibility are now under way.

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References