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Iris Pigment Epithelium Expressing CD86 (B7-2) Directly Suppresses T Cell Activation In Vitro via Binding to Cytotoxic T Lymphocyte–associated Antigen 4

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Abstract

A monolayer of pigment epithelium (PE) lines the iris PE (IPE), ciliary body PE, and retina PE of the inner eye, an immune-privileged site. These neural crest-derived epithelial cells participate in ocular immune privilege through poorly defined molecular mechanisms. Murine PE cells cultured from different ocular tissues suppress T cell activation by differing mechanisms. In particular, IPE cells suppress primarily via direct cell to cell contact. By examining surface expression of numerous candidate molecules (tumor necrosis factor receptor [TNFR]1, TNFR2, CD36, CD40, CD47, CD80, CD86, PD-L1, CD95 ligand, and type I interferon receptor), we report that IPE cells uniquely express on their surface the costimulatory molecule CD86. When IPE were blocked with anti-CD86 or were derived from CD80/CD86 (but not CD80) knockout (KO) mice, the cells displayed reduced capacity to suppress T cell activation. IPE also failed to suppress activation of T cells in the presence of cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) immunoglobulin or if the T cells were obtained from CTLA-4 (but not CD28) KO mice. We conclude that iris pigment epithelial cells constitutively express cell surface CD86, which enables the cells to contact inhibit T cells via direct interaction with CTLA-4. Thus, ocular immune privilege is achieved in part by subversion of molecules that are usually used for conventional immune costimulation.

Key words: immune privilege • costimulatory molecules • T lymphocytes • inhibition • eye

Introduction

Ocular immune privilege is a multifactorial mechanism designed evolutionarily to prevent immunogenic inflammation from disrupting the visual axis and causing blindness (1–3). Components of this multifactorial mechanism that have been studied rather extensively include (a) the immunosuppressive properties of aqueous humor (4), and (b) the capacity of the anterior chamber of the eye to support induction of systemic immune deviation to antigenic material placed within the eye (5). Less experimental attention has been devoted to the ocular pigment epithelia, a component of inner eye tissues derived embryologically from the neural crest (6). A monolayer of these cells, laden with melanin pigment, forms a spherical, light-absorbing curtain within the eye, covering the posterior surface of the iris, lining the ciliary body that surrounds and extends posteriorly beyond the lens, and forming the layer just posterior to the neural retina. In fact, this curtain is only incomplete at the pupil within the iris, and it is through this aperture that focused light images pass to the retina where phototransduction takes place and the process of vision begins.

The diverse but related pigment epithelium (PE)* of the iris, the ciliary body, and the retina possess distinct properties related to their disparate anatomic locations, properties that are not obviously pertinent to ocular immune privilege. Nonetheless, various laboratories have demonstrated that the PE of iris, ciliary body, and retina display immunomodulatory features that suggest that this layer of cells contributes to ocular-immune privilege. Explanted and cultured iris and ciliary body, as well as PE cells derived from the ciliary body, have been reported to secrete factors that are immunosuppressive in vitro (7, 8). Freshly explanted retina PE (RPE), as well as immortalized cell lines derived from RPE, have been found to secrete immunosuppressive factors (9) and express surface molecules, such

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**Abbreviations used in this paper:* CBPE, ciliary body pigment epithelium; CD95L, CD95 ligand; CTLA-4, cytotoxic T lymphocyte–associated antigen 4; IPE, iris pigment epithelium; MMC, mitomycin C; PE, pigment epithelium; RPE, retina PE.

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as CD95 ligand (CD95L), which trigger apoptosis among $CD95^+$ T cells (10). If ocular PE contribute to ocular immune privilege, and if loss of privilege results in ocular inflammation and blindness, then understanding the contributions that ocular PE make to immune privilege has therapeutic implications.

Several years ago our laboratory began a systematic analysis of the immunoregulatory properties of PE cells cultured separately from iris, ciliary body, and retina of mouse eyes. We reported on the one hand that cultured PE from all three intraocular tissues were capable of suppressing T cell activation in vitro (11) and, on the other hand, that T cells exposed to cultured ocular PE were converted into regulatory cells capable of suppressing activation of bystander T cells in vitro (12). Recently, we discovered that PE cells cultured from the iris inhibited T cell proliferation in vitro by a contact-dependent mechanism, whereas PE cells cultured from ciliary body and retina inhibited T cell proliferation through soluble factors they secreted (13). In an effort to understand the molecular bases of the immunosuppressive properties of ocular PE, we have examined the expression of genes whose products are candidates for participating in the suppression. We confirm here that iris PE (IPE) cells differ from ciliary body PE (CBPE) and RPE by suppressing T cell activation in vitro, primarily by a cell to cell contact-dependent mechanism. Our experimental evidence indicates that CD86 (B7-2) is uniquely and constitutively expressed on the surface of IPE, and that IPE effect contact-dependent inhibition of T cells through interactions of CD86 with cytotoxic T lymphocyte–associated antigen 4 (CTLA-4).

Materials and Methods

Mice

Adult C57BL/6 and BALB/c mice were obtained from our domestic animal colony. Mice with disrupted genes for CD80, or both CD80 and CD86, were purchased from The Jackson Laboratory. Mice with disrupted genes for CD28 and CTLA-4 were obtained from The Jackson Laboratory and provided by J. Allison (University of California at Berkeley, Berkeley, CA), respectively. For most of the experiments described, pigmented C57BL/6 mice served as donors of ocular PE cells. For key experimental observations, parallel studies were also conducted with ocular PE cells obtained from albino BALB/c mice.

Culture Media

RPMI 1640 complete medium, used for primary cultures of IPE and CBPE, 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, 10% FBS (all from BioWhittaker), and 10^{-5} M 2-ME (Sigma-Aldrich). DMEM complete medium, used for primary cultures of RPE cells, 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 antibodies or in MLRs were grown in serum-free medium that is composed of complete medium, except for FBS, and supplemented with 0.1% BSA (Sigma-Aldrich) and 0.2% insulin, transferrin, selenium culture supplement (Collaborative Biochemical Products).

Preparation of Cultured PE from Iris, Ciliary Body, and Retina

PE cells of iris and ciliary body were cultivated separately as previously described (13). Eyes were enucleated from 6–8-wk-old male C57BL/6 or BALB/c mice, and cut into two parts (anterior and posterior) along a circumferential line just posterior to the ciliary processes. The lens was removed from the anterior eyecup and the entire iris and ciliary body tissues were gently peeled off with a fine forceps. Iris and ciliary body tissues were separated from each other surgically and incubated in PBS containing 1 mg/ml dispase and 0.05 mg/ml DNaseI (both from Boehringer) at 37°C for 1 h. Thereafter, iris and ciliary body tissues were triturated progressively, first through 21-gauge and then 23-gauge needles, to create a single cell suspension. Monodispersed iris and ciliary body cells were washed twice with RPMI complete medium, and then placed into 6-well plates and incubated at 37°C in a 5% CO_2 atmosphere.

RPE cells were obtained from the posterior part of eyes as described above. The neuronal retina was detached and removed, and the posterior eyecup comprised of RPE, choriocapillaris, and sclera was incubated in 0.2% trypsin (BioWhittaker) for 1 h at 37°C in a 5% CO_2 atmosphere. Thereafter, the eyecup was transferred to wash medium and RPE cells were peeled off gently as intact sheets with a fine forceps. RPE sheets were triturated as described above to create a single cell suspension, after which the cells were washed twice with medium, resuspended in DMEM complete medium, and seeded into 6-well plates and incubated at 37°C in a 5% CO_2 atmosphere for 13 d. At the end of this culture interval, the medium was removed. The RPE cells were washed with fresh medium devoid of calf serum and used for subsequent assays. PE cells cultured from iris, ciliary body, and retina were seeded directly into flat-bottomed 96-well culture plates (2×10^4 cells per well) or onto transwell membranes. After overnight incubation in serum-free medium, the cultured PE cells in flat-bottomed wells served as substrates for T cell activation cultures. Cultured PE cells of these types contain neither $CD45^+$ nor MHC class II^+ cells.

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

For MLRs, spleens were removed from responder (C57BL/6) and allogeneic stimulator (BALB/c) mice. Responder cell suspensions were pressed through nylon mesh (Immulan™ mouse T cell kit; Biotex Laboratories) to produce a single cell suspension of cells that were >95% $CD3^+$. Stimulator spleen cells were incubated for 20 min at 37°C with 0.05 mg/ml mitomycin C (MMC; Sigma-Aldrich), washed twice, and then added (2.5×10^5 cells/well) to wells containing PE cells or not (positive control). Responder T cells (2.5×10^5 cells/well) were then added and these cell mixtures were cultured in 200 μl medium at 37°C in a 5% CO_2 atmosphere for 96 h. In syngeneic control MLRs, counts per minute were always <3,000.

For anti–CD3-driven T cell activation, purified splenic T cells were added (2.5×10^5 cells/well) to culture wells containing PE cells or not. Anti-CD3 antibody (clone 2C11; BD Biosciences) was added to the wells and the cultures were maintained for 72 h.

For transwell membrane experiments, cultured PE cells were seeded onto FALCON cell culture inserts (0.4 μm pore size; Becton Dickinson) and cultured overnight in serum-free medium. Thereafter, the inserts were placed in 24-well culture plates already containing freshly prepared MLRs or anti–CD3-driven T cell reactions. After 72- or 96-h incubation the cultures were assayed for uptake of [^{3}H]thymidine (1 μC/ml for the terminal 8 h of culture), as a measure of cell proliferation. Thymi-

dine-pulsed T cells were harvested by automated cell harvester (Tomtec). Incorporated radioactivity was measured by liquid scintillation counter, and the amount was expressed in counts per minute.

Flow Cytometry with Anti-Cytokeratin and Anti-Costimulator Antibodies

Cultured IPE, CBPE, and RPE cells were stained with FITC-labeled anti-pan cytokeratin antibody (clone PCK-26; Sigma-Aldrich) at the completion of the 14-d primary culture. The cultured cells were fixed and permeabilized with 1× PermiaFix™ (Ortho Diagnostic Systems Inc.) for 40 min at room temperature, washed, and then blocked with PBS containing 5% FBS for 10 min, incubated with anti-pan cytokeratin antibody for 40 min at room temperature, and washed twice. >90% of the cells proved to be cytokeratin positive by flow cytometry (IPE = 99%, CBPE = 90%, and RPE = 95%). Similarly fixed and permeabilized cultured PE cells were also stained with FITC-labeled antibodies to CD80 (clone 16-10A1; BD Biosciences) and CD86 (clone GL1; BD Biosciences) for flow cytometric analysis.

Assay of Cytokine Content within Supernatants

To measure cytokine content (IFNγ) in supernatants of T cell cultures, quantitative capture ELISA was used. Culture supernatants were collected at 48 h after exposure of T cells to PE cells, and immediately frozen and stored at −20°C until used. ELISA was performed according to the manufacturer's instructions (BD Biosciences). Rat mAbs to mouse IFNγ (clone R4-6A2; BD Biosciences) were used as coating antibodies. Biotinylated rat mAbs to mouse IFNγ (XMG1.2; BD Biosciences) were used as detecting antibodies. The recombinant mouse IFNγ used to establish standards for the assays was purchased from BD Biosciences.

Detection of Transcripts within Ocular PE Cells for Pertinent Immunomodulatory Molecules

Cellular extracts were prepared from cultured PE cells and analyzed by RT-PCR as follows: Cultured PE cells were washed twice with PBS and then total treated with RNA STAT-60 (TEL-TEST, Inc.). Extracted RNA was treated with RNase-free DNase (Promega) for 15 min at 37°C, followed by chloroform extraction. RNA was precipitated by ethanol precipitation and dried. The RNA was then dissolved with RNase-free water, and cDNA was synthesized with M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. PCR was performed by the Hot Start PCR method with AmpliTaq and AmpliWax (Applied Biosystems). The following conditions were used: Denaturation at 94∼96.5°C for 30∼60 s, annealing at 51∼62°C for 60 s, and 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 to 5′-GGTGAAGGTCGGTGTGAACGGA-3′ and 5′-TGTTAGTGGGGTCTCGCTCCTG-3′, giving an amplification product of 245 bp. Primers for TNF-R1 (p55) were 5′-CGGGAGAAGAGGGATAGC-3′ and 5′-CAACAGCACCGCAGTACCTGA-3′, giving an amplification product of 537 bp. Primers for TNF-R2 (p75) were 5′-CCGCATCTGTAGCATCC-3′ and 5′-CCTTGGCATCTCTTTGTAGGC-3′, giving an amplification product of 350 bp. Primers for IFNα/β R were 5′-ACCAGGCTGCCAGCGGAGAC-3′ and 5′-GCCGGGAATCTCGTATTGTT-3′, giving an amplification product of 375 bp. Primers for CD36 were 5′-TCTACGCTGTGTTCGGATCT-3′ and 5′-CACTCCAATCCCAAGTAAGG-3′, giving an amplification product of 536 bp. Primers for CD47 were 5′-GCGAAGTGACAGAGTTATCC-3′ and 5′-CCGTCACTTCCCTTCACCTA-3′, giving an amplification product of 654 bp. Primers for CD95L were 5′-CATCACAACCACTCCCACTG-3′ and 5′-CCTCTTCTCCTCCATTAGCA-3′, giving an amplification product of 312 bp. Primers for CD40 were 5′-CGGCTTCTTCTCCAATCAGT-3′ and 5′-TAGAAACCCAGGGCGGATTA-3′, giving an amplification product of 1,072 bp. Primers for CD80 were 5′-GGTATTGCTGCCTTGCCGTT-3′ and 5′-TCCTCTGACACGTGAGCATC-3′, giving an amplification product of 546 bp. Primers for CD86 were 5′-TCCTGTAGACGTGTTCCAGA-3′ and 5′-TGCTTAGACGTGCAGGTCAA-3′, giving an amplification product of 521 bp. Primers for PD-L1 were 5′-CGAAGCTTCGCCCACCATGAGGATATTTGCTGGCATTATATTC-3′ and 5′-AGCGGCCGCTTACTTGTCATCGTCGTCCTTGTAGTCCGTCTCCTCGAATTGTGTAGTCCGTCTCCTCGAATTGTGTATCATT-3′, giving an amplification product of 850 bp (provided by W. Li, Bascon Palmer Eye Institute, Miami, FL). The PCR products were electrophoresed in 1, 1.5, and 2% agarose gel and visualized by staining with ethidium bromide.

Immunohistochemistry for Detection of CD80 and CD86 on Frozen Sections of Eye Tissues and Cells

Immunohistochemical Staining of Frozen Whole Eye Sections. Normal mouse eyes were removed and quick frozen in OCT embedding compound with dry ice. 10-μm sections were cut and mounted on microscope slides and then air dried for 60 min. After fixation in acetone for 10 min, sections were rinsed in 1× PBS three times and incubated for 1 h at room temperature. Sections were rinsed twice in 1× PBS and then blocked with 1% BSA in 1× PBS for 10 min at room temperature. Antibodies to CD80 (clone 16-10A1) and CD86 (clone GL1) were added to the sections at 1 μg/ml in antibody diluent with IHC (BD Biosciences) for 2 h at room temperature. Sections were washed three times for 2 min in 1× PBS, followed by the addition of biotin-conjugated mouse anti–hamster IgG for CD80 or biotin-conjugated donkey anti–rat IgG for CD86 at a 1:200 dilution for 1 h at room temperature. After washing three times for 2 min in 1× PBS, prediluted streptavidin/horseradish peroxidase (Jackson ImmunoResearch Laboratories) was applied to each slide and incubated for 30 min at room temperature. After washing three times for 2 min in 1× PBS, antibody location was determined with the addition of DAB substrate (DAB substrate kit; Vector Laboratories) for 5 min. Color development was stopped by washing in water for 5 min. After washing three times and fixing, the slides were examined by light microscopy.

Immunohistochemical Staining of Cultured PE Cells. Glass chamber slides (Nunc) were coated with collagen I. IPE, CBPE, and RPE cells were cultured on the glass chamber slides for 14 d and washed twice with 1× PBS. Some PE cells were treated with recombinant 100 U/ml IFNγ. PE cells were then fixed with acetone for 7 min and washed three times with 1× PBS. For Fc block, purified anti–mouse CD16/CD32 abs (1:200 dilution, Fcγ III/II receptor, clone 2.4G2; BD Biosciences) was added to the cells for 30 min. The slides were rinsed in 1× PBS three times and blocking was performed with 2% BSA in 1× PBS. FITC-conjugated antibodies CD80 (clone 16-10A1) and CD86 (clone GL1; each at a 1:50 dilution) were added to the cells for 2 h at room temperature. After washing three times and fixing, the slides were examined by fluorescent microscopy. Isotype controls included purified hamster IgG for CD80 (BD Biosciences) and purified rat IgG for CD86 (BD Biosciences) that used both staining.

Blockade of CD80 and/or CD86 During Exposure of T Cells to Cultured Ocular PE Cells

Anti–mouse CD80 and/or CD86 monoclonal antibodies were included in cultures in which cultured ocular PE cells were present during stimulation of T cells with anti-CD3 antibodies. Purified T cells (2.5×10^5 cells/well) were stimulated with 1 μg/ml anti-CD3 antibodies and cultured for 72 h with or without cultured IPE, CBPE, and RPE. As controls, purified hamster IgG (for CD80; BD Biosciences) and purified rat IgG (for CD86; BD Biosciences) were added to appropriate cultures. In some experiments, CTLA-4–Ig proteins were included in cultures in which cultured ocular PE cells were present during stimulation of T cells with anti-CD3 antibodies. Cultured PE cells were added in the presence of graded amounts (from 0 to 5 μg/ml) of CTLA-4–Ig in a dose-dependent manner. In MLRs, responder spleen cells (C57BL/6, 2.5×10^5 cells/well) were cultured with MMC-treated stimulator spleen cells (BALB/c, 2.5×10^5 cells/well) in the presence or absence of cultured PE cells for 96 h.

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 Capacity of Cultured Ocular PE to Inhibit T Cell Proliferation across Transwell Membranes. The diverse ocular PE cells exist in components of the eye (iris, ciliary body, and retina) with distinct functions, and in each location T cells from the blood pass between adjacent PE cells in monolayers to enter the inner eye. To assay the role of cell to cell contact in PE-dependent suppression of T cell activation, ocular PE cells from iris, ciliary body, and retina of eyes of C57BL/6 mice were cultured separately for 14 d in 24-well culture plates. Transwell inserts were then placed in these wells and the transwell contained C57BL/6 T cells plus anti-CD3, or MLRs composed of responder C57BL/6 T cells and MMC-treated BALB/c spleen cells as stimulators. T cell proliferation was assessed by [^{3}H]thymidine incorporation at 72 (anti-CD3) or 96 h (MLRs). As revealed in Fig. 1, RPE cells suppressed anti–CD3-driven T cell proliferation readily across a transwell membrane, indicating that soluble factors released from the PE cells in the insert were able to diffuse through the membrane and act on the responding T cells in the culture below (Fig. 1 C). By contrast, IPE cells largely failed to suppress T cell proliferation across the transwell membrane, indicating that cell to cell contact is important when IPE mediate suppression of T cell activation (Fig. 1 A). CBPE cells assumed an intermediate suppressing capacity, retaining a modest, but significant, capacity to suppress T cell activation across the transwell membrane (Fig. 1 B). The effects of PE cells on T cell proliferation was similar whether the T cell stimulation was mediated by anti-CD3 antibodies, or by exposure to allogeneic spleen cells (not depicted). Comparable results were obtained when PE cells were harvested from eyes of BALB/c mice (not depicted). These results strongly suggest that cultured IPE cells use a contact-dependent mechanism in suppressing T cell activation. The results do not exclude a similar mechanism for RPE and CBPE, however, because the capacity of these cells to secrete immunosuppressive soluble factors has the potential to eclipse our ability to detect a contact-dependent suppressive mechanism. The finding that CBPE suppressed less well when separated from target T cells by the transwell membrane suggests that CBPE probably use a cell contact inhibitory mechanism in addition to soluble immunosuppressive factors they secrete. The subsequent experiments describe our attempts to identify the interacting cell surface molecules that (a) are differentially expressed on IPE as they suppress T cell activation almost exclusively via a cell contact-dependent mechanism, and (b) are expressed on the inhibited T cells.

Expression in Cultured Ocular PE Cells of Genes Encoding Candidate Cell Surface Molecules That Could Mediate Contact-dependent Suppression of T Cell Activation. PE cells were cultured from iris, ciliary body, and retina of eyes of C57BL/6 mice. After 14 d, the cells were harvested and mRNA was extracted. Using RT-PCR, the mRNA of each cell type was probed for transcripts of the following candidate molecules: TNFR1, TNFR2, type I IFNR (IFNα/β receptor), CD95L, CD36, CD40, CD47, CD80

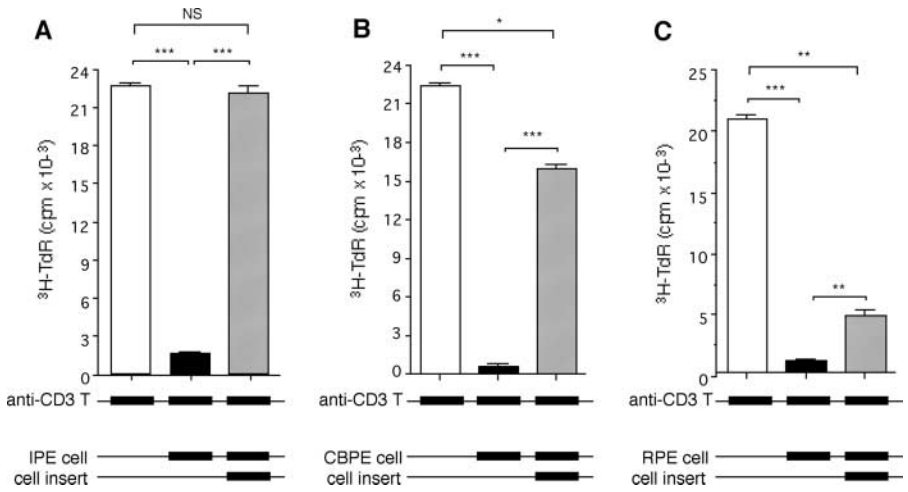

Figure 1. Influence of transwell membranes on capacity of cultured ocular PE to suppress anti-CD3 T cell activation. PE from C57BL/6 iris (A), ciliary body (B), and retina (C) were grown in 24-well culture plates. Transwell cell culture inserts were placed in these wells, and syngeneic T cells plus anti-CD3 antibodies were placed therein. [^{3}H]thymidine was added 8 h before the termination of culture at 72 h. Mean counts per minute for triplicate cultures are presented ± SEM. Differences of means of T cell proliferation in the presence or absence of PE cells are compared. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; NS, not significant.

(B7-1), CD86 (B7-2), and PD-L1 (B7-H1). The results of this experiment are presented in Fig. 2. None of the cultured PE cell types expressed the genes for CD95L, CD40, or PD-L1, whereas all of the cell types contained transcripts for TNFR1, TNFR2, type I IFNR, CD36, CD47, and CD80. Only in the case of CD86 did we detect differential expression of transcripts among the three ocular PE cell types. CD86 transcripts were easily detected in cultured IPE and to a lesser extent in CBPE, but not in RPE. Thus, CD86 emerged as a candidate molecule in the suppression of T cell activation achieved by contact exposure to IPE.

Influence of Antibodies to CD80 and CD86 on Capacity of Ocular PE Cells to Suppress T Cell Activation In Vitro. To determine whether CD80 and/or CD86 is involved in suppression of T cell activation by IPE cells, anti–CD3-driven T cell activation cultures were performed in wells containing a monolayer of cultured IPE, CBPE, or RPE as described above. In some wells, anti-CD80 or anti-CD86 antibodies were added alone or in combination. T cell proliferation was assessed as before, and the results are displayed in Fig. 3. In cultures containing IPE plus anti-CD86 antibodies alone, or anti-CD80/anti-CD86 antibodies, T cell proliferation was significantly greater than in cultures to which isotype control antibodies were added (Fig. 3 A). In cultures containing IPE plus anti-CD80 antibodies alone, a small increase in T cell proliferation was observed. By contrast, neither anti-CD80 nor anti-CD86 antibodies relieved the profound suppression observed in cultures in which T cells were exposed to CBPE (Fig. 3 B) or RPE (Fig. 3 C). Parallel experiments conducted with ocular PE harvested from BALB/c mice yielded similar results (not depicted). These findings support the hypothesis that IPE suppress T cell activation via a CD86-dependent mechanism. The results bearing on a comparable role for CD80 are less impressive, although CD80 cannot on the basis of these findings be excluded from participating in IPE-dependent T cell suppression. Anti-CD86 antibodies did not reverse completely the suppression mediated by IPE, even when higher antibody concentrations were used. We infer that IPE use surface molecules in addition to CD86 in mediating suppression of T cell activation.

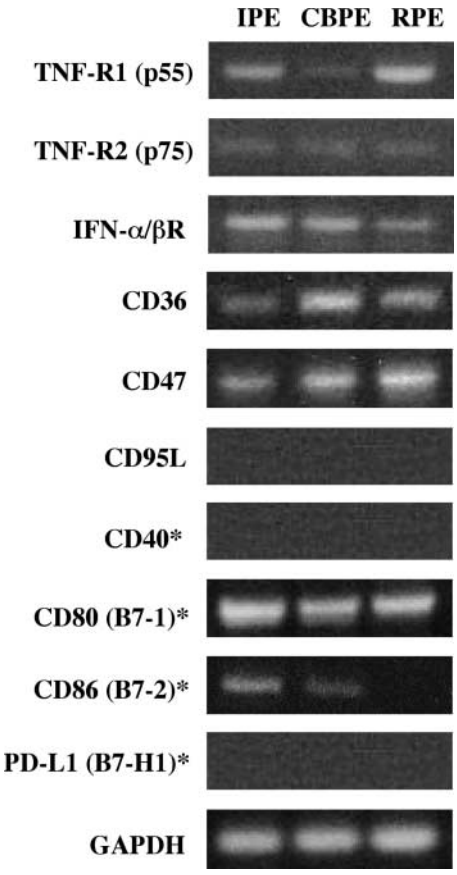

Figure 2. Survey of gene expression of candidate-suppressive cell surface molecules on PE cells cultured from iris, ciliary body, and retina. mRNA was extracted from IPE, CBPE, and RPE cells after 14 d of culture, reverse transcribed, and amplified by PCR. PCR products were electrophoresed in 1, 1.5, and 2% agarose gel and visualized by staining with ethidium bromide.

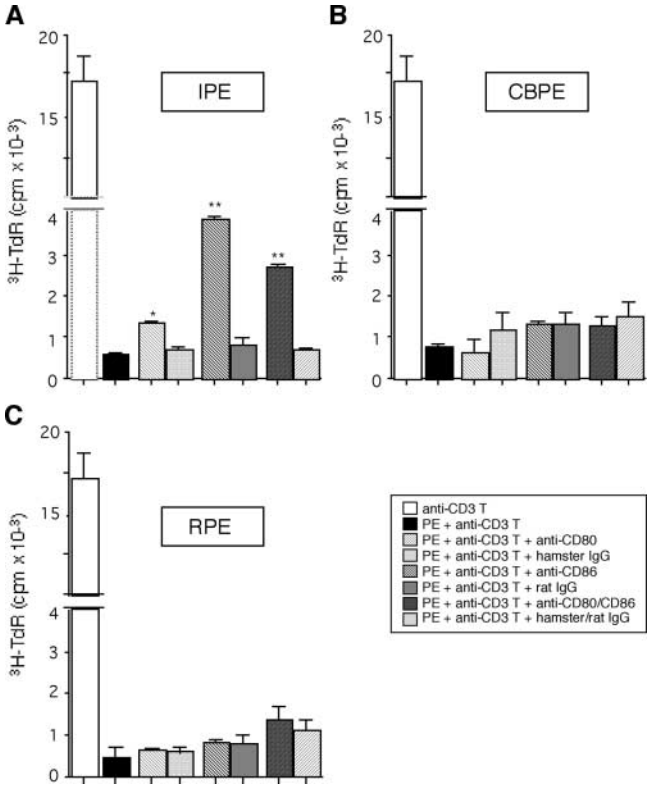

Figure 3. Capacity of neutralizing antibodies to CD80 and CD86 to prevent suppression of T cell activation by cultured PE cells. Anti-CD3 stimulation of purified C57BL/6 T cells was performed as described in the legend to Fig. 1. Cultured IPE (A), CBPE (B), or RPE cells (C) were added in the presence or absence of 1 μg/ml anti-CD80 and/or anti-CD86 antibodies. As isotype controls, purified hamster IgG for CD80 and purified rat IgG for CD86 were used. After 72 h of incubation, the cultures were assayed for uptake of [^{3}H]thymidine. Mean counts per minute for triplicate cultures are presented ± SEM. Differences of means of T cell proliferation in the presence of PE cells with and without anti-CD80 or anti-CD86 antibodies are compared. *, $P < 0.05$; **, $P < 0.005$.

CD80 and CD86 Expression on Cultured Ocular PE Cells Detected by Flow Cytometry. Because cultured PE cells expressed transcripts for CD80 and/or CD86, and because antibodies directed at CD86 partially alleviated the suppression mediated by IPE, we next examined by flow cytometry the extent to which cultured PE cells expressed CD80 and CD86. After 14 d of primary culture, IPE, CBPE, and RPE cells were harvested and labeled with FITC-conjugated antibodies directed at CD80 or CD86, and were analyzed by flow cytometry. The results of a representative experiment (of two) are presented in Fig. 4. CD80 was expressed on the majority of cultured IPE and CBPE, as well as on a large minority of RPE cells. By contrast, CD86 was expressed on a very high proportion of IPE cells, but on far fewer CBPE and RPE cells. The hypothesis that IPE cells mediate suppression of T cell activation via a CD86-dependent contact mechanism is supported by these data.

Expression of CD80 and CD86 Genes and Proteins in Freshly Procured Ocular PE and Tissues. Cells cultured from living tissues offer a convenient approach to the study of those tissues, but cultured cells often express molecules and adopt functional properties that are at variance with the intact tissue. We wished next to determine whether CD80 and CD86 are expressed on PE cells freshly obtained from iris, ciliary body, and retina. To that end, iris, ciliary body, and retina were harvested separately from eyes of adult mice. PE cells cultured from all three ocular tissues for 14 d were also prepared. mRNA was extracted from each of these tissue samples and from cultured cells, and then subjected to RT-PCR with primers for CD80 (Fig. 5 A), CD86 (B), PD-L1 (C), CD40 (D), or GAPDH (E). As revealed in Fig. 5, transcripts of CD80 and CD86 were detected in freshly obtained iris tissues. Transcripts for CD40 and PD-L1 were not detected in any ocular tissues containing PE or in cultured PE cells. However, transcripts for PD-L1 were inducibly detected in IFNγ-treated PE cells (Fig. 5 C). These results confirm that the genes for the costimulatory molecules CD80 and CD86 are open in both fresh and cultured PE cells. To further explore the expression of CD80 and CD86 in noncultured ocular tissues, frozen sections were prepared from eyes of C57BL/6 and BALB/c mice and subjected to immunohistochemical analysis using reagents that detect CD80 and CD86. As summarized in Table I, CD86 was detected in corneal epithelium (but not endothelium) in iris, ciliary body, lens, and RPE (but not in neural retina). A similar distribution pattern was observed for CD80, although the intensity of CD80 staining was much lower in epithelium of cornea and lens. The pattern of CD80 and CD86 expression on freshly obtained normal ocular tissues implies that expression of these costimulatory molecules is constitutive.

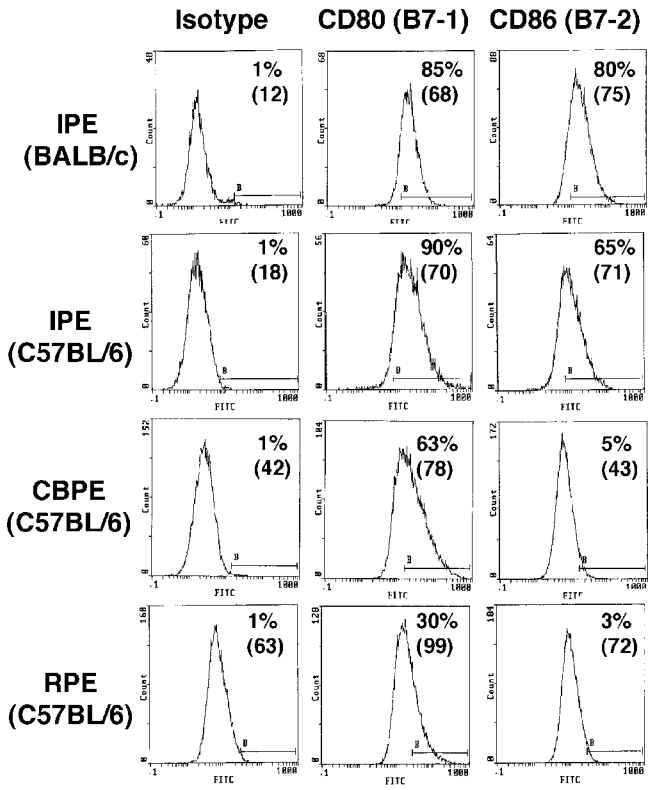

Figure 4. Detection of B7 molecules on cultured PE cells by flow cytometry. IPE (BALB/c and C57BL/6), CBPE (C57BL/6), and RPE cells (C57BL/6) were cultured on 6-well plates for 14 d. Thereafter, the confluent cells were triturated several times through 21-gauge and then 23-gauge needles to create a single cell suspension. PE cells were incubated with FITC-conjugated antibodies: anti-CD80, anti-CD86, or purified hamster IgG as isotype control for 1 h at room temperature. After washing and fixation, the cells were analyzed by flow cytometry. Percentages in upper right corners indicate positive cells. Number in parenthesis indicate mean fluorescence index.

Similar immunohistochemical studies were performed on primary cultures of PE cells from iris, ciliary body, and retina. As summarized in Table II, all three PE cell types expressed CD80. IPE cells expressed easily detectable CD86 that appeared to be diffuse within the cytoplasm and on the cell surface. Little CD86 expression was detected in or on CBPE. CD86 was expressed in RPE, but the distribution of label was restricted to cytoplasmic granules. The intracytoplasmic localization of CD86 in cultured RPE observed in these studies helps to explain why flow cytometric analysis of cultured RPE revealed so few cells with positive surface staining for this molecule. These results suggest further that it is surface expression of CD86 that is relevant to the ability of IPE cells to inhibit T cell activation by a contact-dependent mechanism.

Capacity of Cultured Ocular PE from Mice with Disrupted CD80 ± CD86 Genes to Inhibit Anti–CD3-driven T Cell Proliferation and Production of Cytokine In Vitro. If CD86 plays an important role in mediating suppression of T cell activation by IPE (and not CBPE and RPE), it is worthwhile to study mice in which the genes for CD80 and CD86 are disrupted. Two types of mice were available for our study: CD80 KO mice and mice in which both the CD80 and the CD86 genes had been disrupted. PE cells were cultured from iris, ciliary body, and retinas of these mice, as well as C57BL/6 wild-type mice. The cultured cells were then used as substrates for C57BL/6 T cells stimulated with anti-CD3 antibodies or with allogeneic spleen

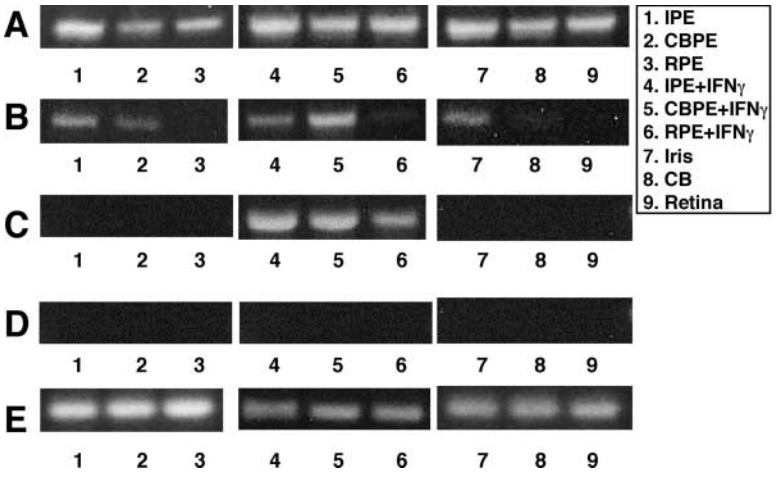

Figure 5. Detection of mRNA expression of costimulatory molecules in ocular PE. mRNA, extracted from cultured IPE, CBPE, and RPE cells with or without 100 U/ml recombinant IFN-γ (samples 1–6), and from freshly obtained mouse iris, ciliary body, and retina (samples 7–9), was reverse transcribed and amplified by PCR using primers for (A) CD80 (B7-1), (B) CD86 (B7-2), (C) PD-L1 (B7-H1), (D) CD40, and (E) GAPDH. PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide.

cells. T cell proliferation and IFNγ production were assayed as described above. T cells exposed to cultured IPE from CD80/CD86 double KO donors proliferated in response to anti-CD3 (Fig. 6 A) and allogeneic spleen cells (not depicted) to a much higher degree than T cells exposed to cultured wild-type IPE cells. No similar relief of suppression was observed when T cells were stimulated and exposed to cultured CBPE or RPE from double KO donors (Fig. 6, B and C). Very similar results were obtained when the concentration of IFNγ in supernatants was assayed. CD80/CD86 KO IPE cells suppressed IFNγ production by anti–CD3-stimulated T cells much less well than CBPE and RPE cells from the same KO donors (not depicted). T cells exposed to cultured IPE from CD80 KO donors displayed a modest, but significant increase in proliferation in comparison to T cells exposed to cultured wild-type PE cells (not depicted), but no comparable relief of suppression was observed in the presence of CBPE or RPE from CD80 KO donors. In previous experiments (13), ocular PE cells cultured from eyes of mice with defective genes encoding CD95L, TNFR1 (p55), and TNFR2 (p75) suppressed T cell activation as well as PE cells cultured from wild-type mouse eyes, excluding the products of these genes as relevant to the suppressive activities of ocular PE cells. It should be noted that IPE from CD80/CD86 KO donors did not completely fail to suppress T cell activation. Compared with the responses of the positive control T cells (stimulated in the absence of cultured PE cells), IPE from CD80/CD86 KO donors suppressed T cell activation to a much lesser extent than that achieved by IPE from wild-type donors (Fig. 6 A).

Capacity of CTLA-4–Ig to Interfere with Suppression of T Cell Activation by IPE. CD86 is a costimulation molecule that is expressed on APCs and through binding to CD28 provides a key activation signal to T cells. CD86 also binds

Table I. *Detection of CD80 and CD86 on Freshly Obtained Ocular Tissues*

Tissues	CD80 (B7-1)	CD86 (B7-2)
Corneal epithelium	+	+
Corneal stroma (keratocytes)	−	−
Corneal endothelium	−	−
Iris	+	+
Ciliary body	+	+
Lens epithelium	+	+
Lens fiber cells	−	−
Neural retina	−	−
RPE	+	+

Eyes of BALB/c mice were enucleated, snap frozen, sectioned, placed on glass slides, fixed with acetone, and stained with antibodies to CD80, CD86, and isotype IgG controls, followed by biotin-conjugated secondary anti-antibodies, and then developed with streptavidin/horseradish peroxidase and the DAB substrate. Specimens were examined by light microscopy. Staining of tissues and cells with DAB is indicated with +.

Table II. *Detection of CD80 and CD86 on Cultured Ocular PE Cells*

PE cell types	CD80 (B7-1)	CD86 (B7-2)
Iris	+	+
Iris, treated with IFNγ	++	++
Ciliary body	+	−
Ciliary body, treated with IFNγ	++	+
Retina	+	(+)[a]
Retina, treated with IFNγ	++	(+)[a]

[a]Staining within cytoplasmic granules without evidence of cell surface staining.
Extent of staining is presented semiquantitatively as −, +, or ++.
PE cells from iris, ciliary body, and retina were cultured for 14 d, washed, and then incubated overnight in serum-free medium, with or without 100 U/ml IFNγ. The cells were then removed, fixed with acetone, and stained with FITC-conjugated anti-CD80 or anti-CD86 antibodies, and examined by fluorescent microscopy.

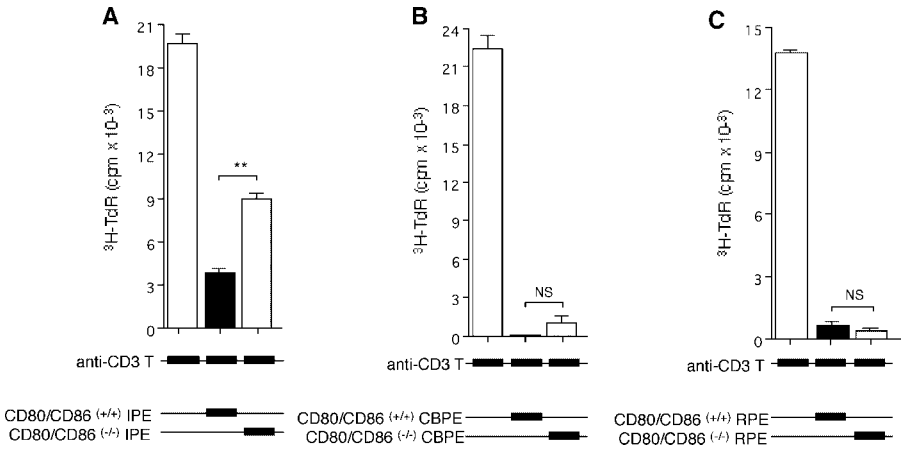

Figure 6. Capacity of cultured PE cells from donors deficient in CD80 plus CD86 expression to inhibit T cell proliferation. Purified C57BL/6 T cells (2.5×10^5 cells/well) were stimulated with 1 μg/ml anti-CD3 antibody and cultured for 72 h in the presence or absence of cultured IPE (A), CBPE (B), or RPE (C) obtained from eyes of CD80/CD86 KO or wild-type (C57BL/6) mice. After 72 h, the cultures were assayed for uptake of [^{3}H]thymidine. Mean counts per minute for triplicate cultures are presented ± SEM. Differences of means of T cell proliferation in the presence of wild-type or CD80/CD86 KO PE cells are compared. **, $P < 0.005$, compared with two groups. NS, not significant.

CTLA-4 on T cells, in which case the T cells are negatively signaled and acquire anergic and even regulatory properties. CTLA-4–Ig is a fusion protein that binds to CD86 with very high affinity and is able to thwart CD28-dependent T cell activation. In the following experiments, we tested the effect of CTLA-4–Ig on the activation of T cells exposed in vitro to IPE and stimulated with anti-CD3. As before, purified splenic T cells were placed in culture wells containing cultured IPE and stimulated with anti-CD3 antibodies in the presence of CTLA-4–Ig over a concentration range of 5.0–0.001 μg/ml. T cell proliferation was measured at 72 h by triturated thymidine uptake. As the results displayed in Fig. 7 reveal, above a concentration of 0.5 μg/ml CTLA-4–Ig, T cells exposed to IPE underwent significant levels of proliferation. In addition, IPE cells failed to suppress T cell activation with allogeneic spleen cells (not depicted). Similar experiments performed with anti–CD3-stimulated T cells exposed to CBPE or RPE in the presence of CTLA-4–Ig failed to reveal T cell proliferation (not depicted). These findings indicate that blocking of interactions between CD86 on IPE cells and coreceptors on T cells partially relieves the contact-dependent suppression mediated by IPE cells.

Identification of Costimulation Receptor on T Cells that Accounts for CD86-dependent Suppression. Next, we inquired into the nature of the costimulation receptor that is present on T cells and which upon binding to CD86 delivers a negative signal for activation. For these experiments we used mice with disrupted genes for CD28 or CD152 (CTLA-4). PE cells were cultured as before from iris, ciliary body, and retina and placed in culture wells. Purified splenic T cells were then prepared from CTLA-4$^{-/-}$, CD28$^{-/-}$, and wild-type control mice and added to these wells. The T cells were then stimulated with anti-CD3 antibodies at two different concentrations, 0.5 and 1.0 μg/ml, and proliferation was assessed by triturated thymidine uptake. CD28$^{-/-}$ T cells were as profoundly suppressed as wild-type T cells when exposed to IPE, CBPE, and RPE (Fig. 8). By contrast, CTLA-4$^{-/-}$ T cells proliferated when exposed to IPE, but not when exposed to CBPE and RPE. In fact, T cells from CTLA-4 KO mice that were stimulated with 0.5 μg/ml anti-CD3 in the presence of IPE cells proliferated almost to the same extent as did similar T cells exposed to anti-CD3 in the absence of IPE. Together, these results indicate that IPE constitutively express CD86 and this molecule binds directly to CTLA-4 on T cells, thereby changing the T cells' functional program and suppressing their susceptibility to activation through the T cell receptor.

Discussion

Recently published data (11–13) and our current results indicate that T lymphocytes that encounter PE of the inter-

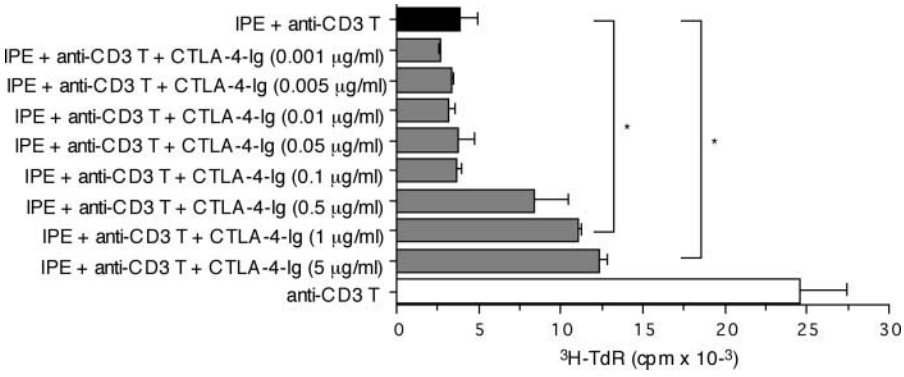

Figure 7. Influence of CTLA-4–Ig on suppression of T cell activation by ocular PE. PE cells were cultured from iris, ciliary body, and retina of C57BL/6 mice. IPE served as substrates for purified T cells stimulated with anti-CD3 antibodies in the presence of varying concentrations of CTLA-4–Ig. Cultures were terminated at 72 h and [^{3}H]thymidine incorporation was assessed. Mean counts per minute for triplicate cultures are presented ± SEM. Differences of means of T cell proliferation in the presence or absence of CTLA-4–Ig are compared. *, $P < 0.05$.

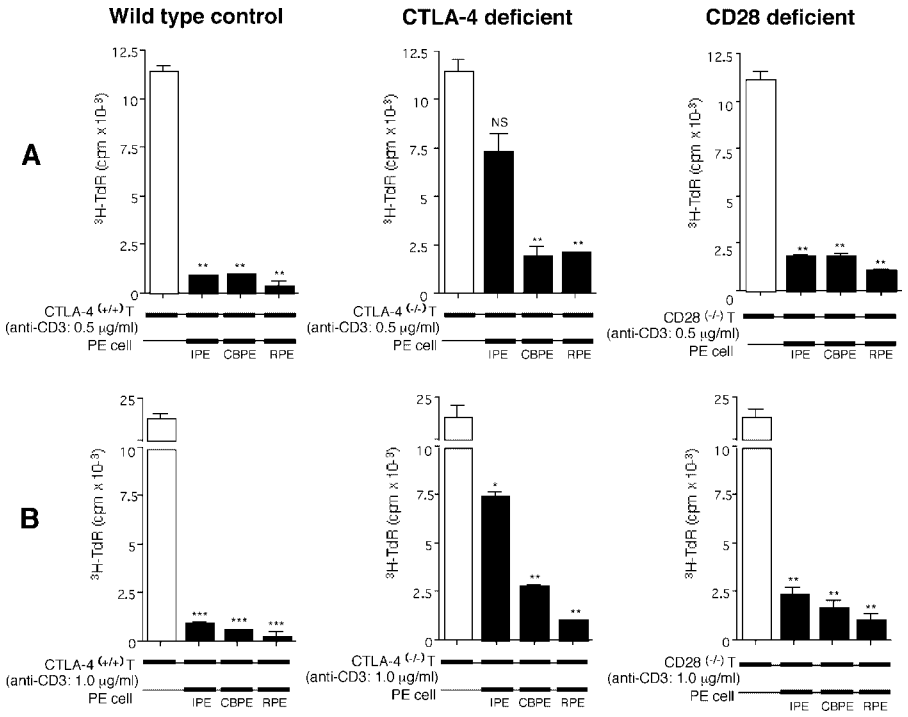

Figure 8. Capacity of ocular PE to suppress activation of T cells from mice with disrupted CD28 and CTLA-4 (CD152) genes. PE were cultured from iris, ciliary body, and retina of C57BL/6 mice and used as substrates for purified T cells prepared from the spleens of 22-d-old wild-type mice, 22-d-old CTLA-4$^{-/-}$ mice, and 6-wk-old CD28$^{-/-}$ mice. The T cells were stimulated with anti-CD3 antibodies at concentrations of (A) 0.5 and (B) 1.0 μg/ml. Cultures were terminated at 72 h and [^{3}H]thymidine incorporation was assessed. Mean counts per minute for triplicate cultures are presented ± SEM. Differences of means of T cell proliferation in the presence or absence of PE cells are compared. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; NS, not significant.

nal ocular compartments fail to respond to activation stimuli by differentiating into effector cells, i.e., the T cells fail to proliferate and secrete proinflammatory cytokines such as IFNγ. Instead, T cells that have experienced such an encounter differentiate into regulator cells that are capable of suppressing the activation of bystander T cells (12). The failure of T cells exposed to ocular PE cells to express effector functions, and their acquisition of a regulatory phenotype, support the view that ocular PE cells act locally on T cells to prevent the expression of immunogenic inflammation within the eye. In that regard, our results reveal that unique expression of the immune costimulation molecule CD86 (B7-2; reference 14) on IPE enables these cells to alter the functional properties of T cells they encounter, and that CD152 (CTLA-4) is the coreceptor on T cells with which CD86 interacts.

Activation of T lymphocytes by professional APCs occurs through the delivery of two distinctly different, but essential signals (15). Signal 1, presented by APCs as immunogenic peptides displayed on class I or II MHC molecules, is detected by the T cell receptor for antigen, and accounts for the antigen specificity of T cell activation. Signal 2, presented by APCs, provides costimulation that is mediated by cell surface molecules, such as CD80, CD86, and CD40, and/or by secreted cytokines, such as IL-12. Responding T cells receive Signal 2 via cell surface ligands such as CD28 and CTLA-4, and cytokine receptors, such as IL-12R. Nonprofessional APCs that express Signal 1 often fail to activate T cells properly because the former cells typically lack expression of costimulation molecules (Signal 2). Consequently, responding T cells display an anergic phenotype, or simply fail to be aroused (16).

Although ocular PE cells fit into the category of nonprofessional APCs, our results indicate that, paradoxically, ocular PE cells constitutively express molecules defined immunologically as costimulators: CD80 and CD86. We report that the genes encoding CD80 and CD86 are constitutively open in both freshly explanted as well as cultured PE cells of iris, ciliary body, and retina. Moreover, CD80 was found to be expressed where PE cells reside within intact iris, ciliary body, and retina, and on the surface of freshly prepared and cultured PE cells. CD86, by contrast, was expressed on a very high proportion of cultured IPE, but at much lower frequencies among cultured CBPE and RPE. This differential pattern of CD86 expression among the three types of ocular PE cells is relevant to the means by which these different cell types suppress T cell activation in vitro.

Whereas cultured CBPE and RPE secrete soluble factors that suppress T cell activation in vitro, IPE do so only very poorly. Instead, IPE suppress T cell activation primarily via a mechanism that implies direct contact between IPE and T cells, i.e., not across a transwell membrane. In our experiments, unique expression of CD86 on IPE correlated positively with the capacity of anti-CD86 antibodies to alleviate suppression of T cell activation mediated by IPE. Moreover, IPE derived from eyes of CD80/CD86 KO mice were far less able to suppress T cell activation in vitro than wild-type IPE. IPE from CD80 KO mice were almost as immunosuppressive for T cells as wild-type IPE. Importantly, CBPE and RPE from both CD80 KO and CD80/CD86 KO mice proved as effective at suppressing T cell activation in vitro as wild-type CBPE and RPE cells. This laboratory has reported elsewhere that antibodies capable of

neutralizing CD40, CD154, CD48, and ICAM-1 failed to relieve suppression mediated by ocular PE cells (13). In aggregate, the evidence presented here indicates that IPE cells suppress T cell activation largely, if not completely, through their surface expression of CD86.

Although CD86 expression on IPE cells accounts for much of their ability to suppress T cell activation, other molecules must also be involved. IPE from mice with a disrupted CD86 gene and IPE from wild-type mice in the presence of anti-CD86 antibodies still achieved partial suppression of T cell activation in vitro. In this regard, our results suggest that CD80 on IPE might also make a minor contribution to this suppression. Our survey of expression of candidate molecules and genes that might also mediate suppression tends to exclude from further consideration TNFR1, TNFR2, type I IFNR, CD36, CD40, CD47, CD95L, and PD-L1, an inhibitory member of the CD80/CD86 family of costimulation molecules (17). In an effort to discover the other molecules responsible for contact-dependent suppression by IPE, we are resorting to microchip gene array technology.

Because all mature T cells express CD28, this ligand was a prime suspect (18) for interacting with CD86 on IPE. Yet, the experiments reported here indicate that CTLA-4 rather than CD28 is the relevant coreceptor for CD86 that leads to suppression of T cell activation. This outcome is consistent with the view that activation of T cells through CTLA-4 delivers negative signals to the cells, resulting in anergy (19), apoptosis (20), and even acquisition of regulatory function (21–23). As mentioned in the Introduction, T cells that encounter cultured IPE cells in vitro are not only inhibited from proliferating, but they also acquire regulatory properties. Studies are in progress to determine whether interactions between CD86 on IPE and CTLA-4 on T cells also lead to the conversion of T cells into regulators.

It is worth pointing out that naive T cells that receive an activation stimulus (anti-CD3 or allogeneic spleen cells) in the presence of cultured PE cells do not differentiate into Th1 cells: they failed to secrete IFNγ. It is pertinent to thoughts of immunoregulation to point out that the responding T cells also fail to differentiate into Th2 cells: they failed to secrete IL-4 and IL-10 (24). Thus, T cells that encounter ocular PE cells are not merely deflected toward the Th2 pathway of differentiation. Rather, T cells that interact with ocular PE appear to take a third differentiation pathway, one in which they can regulate and inhibit the activation of bystander T cells in the local microenvironment. In a similar manner, T cells activated in the presence of aqueous humor differentiate into regulator cells that do not express the Th2 phenotype (25). Previous studies have indicated that T cells that have encountered cultured ocular PE cells in vitro secrete TGFβ (12). We are eager to understand the relationship between CD86-expressing IPE cells and responding T cells that use TGFβ to regulate bystander T cells.

The finding of constitutive expression of CD80 and CD86 on various tissues of the eye is unexpected, and the expression of these costimulation molecules on cells of nonhematopoietic lineage is, to our knowledge, unprecedented. In addition to their expression on neural crest-derived, ocular PE, CD80 and CD86 are also expressed on epithelia of cornea and lens, both of which are derived from nonneural ectoderm. Thus, embryologic origin cannot explain the distinctive and unexpected pattern of expression of CD80 and CD86 in the eye. A similar pattern of expression has been reported for CD95L, another molecule originally thought to be expressed exclusively on cells of the immune system (26). The expression of CD95L on certain cells of the testis (27), the eye (28), the placenta (29), and the brain (30), and our current demonstration that CD80 and CD86 are constitutively expressed on ocular PE lead us to propose that immune privileged tissues and sites hijack and subvert for their own special physiologic purposes molecules used otherwise by the immune system to carry out its normal physiologic functions. Our discovery of expression of CD80 and CD86 on certain cells of the eye extends the list of immune system–related molecules used for the creation and maintenance of tissue-restricted immune privilege.

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