Participation of Pigment Epithelium of Iris and Ciliary Body in Ocular Immune Privilege. 1. Inhibition of T-Cell Activation In Vitro by Direct Cell-to-Cell Contact

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PURPOSE. To determine by what mechanism(s) iris and ciliary body (I/CB) pigment epithelial (PE) cells inhibit T-cell activation in vitro.

METHODS. Pure cultured I/CB PE cells were obtained from eyes of normal and CD95 ligand (CD95L)-deficient mice and tested for their capacity to suppress T-cell activation in three different T-cell receptor (Tcr) ligand systems: mixed lymphocyte reactions, stimulation of Tcr transgenic T cells (D011.10) by specific antigen (ovalbumin), and ligation of the Tcr-associated CD3 molecule by anti-CD3 antibodies. Proliferation and secretion of cytokines (interferon [IFN]-γ, interleukin [IL]-2, IL-4, and IL-10) were assessed as measures of T-cell activation. Suppressive influences of I/CB PE cells were determined on the basis of RT-PCR–detected cytokine genes expressed by I/CB PE cells, immunosuppression mediated by supernatants of cultured I/CB PE cells, direct contact between I/CB PE cells and T lymphocytes, and promotion of apoptosis among responding T cells. Attempts to reverse I/CB PE–dependent suppression of T-cell activation included the use of neutralizing antibodies to IL-10, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β, and the addition of exogenous IL-2 and IL-12.

RESULTS. Cultured mouse I/CB PE cells (including CD95L-deficient cells), which were more than 95% keratin positive, suppressed T-cell proliferation and secretion of IFN-γ, IL-2, IL-4, and IL-10 in a dose-dependent fashion in all three Tcr ligand systems. Supernatants of cultured I/CB PE cells displayed little suppression activity, whereas cultures in which I/CB PE cells contacted responding T cells directly were profoundly immunosuppressive. Cultured I/CB PE cells expressed mRNA for TGF-β1, TGF-β2, IL-6, IL-10, and TNF-α, but not IL-4, IFN-γ, proopiomelanocortin (POMC), and CD95L (Fas L). Antibodies to TGF-β, IL-10, and TNF-α failed to reverse suppression mediated by I/CB PE cells. Moreover, neither exogenous IL-2 or IL-12 relieved the suppression.

CONCLUSIONS. Cultured I/CB PE cells, through direct cell-to-cell contact, prevent T cells from proliferating and secreting cytokines when stimulated through the Tcr for antigen by a mechanism that does not involve CD95 or apoptosis. (Invest Ophthalmol Vis Sci. 2000;41:811–821)

Although it is well established that immune privilege exists in the anterior chamber, vitreous cavity, and subretinal space of the eye, our knowledge of the mechanisms responsible for privilege is incomplete. Physical isolation of these ocular compartments from the systemic blood circulation partially explains immune privilege, in that blood–tissue barriers exist at the level of the retinal pigment epithelium and within the microvasculature of the retinal vessels and the vessels supplying the iris. These physical barriers limit access of potentially deleterious blood-borne cells and molecules into the eye. In addition, intraocular fluids, especially aqueous humor, have been found to contain a variety of immunosuppressive factors that, on the one hand, inhibit the expression of T-cell–directed immunogenic inflammation in oculi, and, on the other hand, alter the antigen-presenting properties of indigenous ocular cells. When local antigen-presenting cells capture antigen and escape into the systemic circulation, they induce anterior chamber–associated immune deviation (ACAI D). Finally, parenchymal cells that line the inner cavities of the eye display on their surfaces molecules that regulate immune effector function, ranging from membrane-bound inhibitors of complement activation to CD 95 ligand (CD95L), which triggers apoptosis among CD95-bearing T cells and other leukocytes. In the aggregate, these physical, cellular, and biochemical factors help to explain why foreign tissues grafted into the eye enjoy prolonged, often indefinite, survival.

Several laboratories have studied the immunomodulatory properties of iris and ciliary body (I/CB), in part because these tissues line the anterior and posterior chambers of the eye and because they are involved in secretion of aqueous humor. Explants of I/CB placed in vitro have been found to be directly immunosuppressive to T cells. Specifically, when lymphoid cells are cultured with allogeneic stimulator cells in vitro in the presence of I/CB explants, lymphocyte proliferation is profoundly suppressed. Supernatants obtained from cultured I/CB tissue display similar immunoinhibitory properties, due in
part to their content of transforming growth factor (TGF)-
\(\beta\).\(^{8,9,27}\) However, the immunosuppressive properties of super-
natants of cultured I/CB cannot be totally explained by the
presence of TGF-\(\beta\), implying that other modulatory soluble
factors must also be present.\(^{23,24}\) Moreover, cell lines derived
from ciliary body epithelium, as well as retinal Müller cells,
have been found to suppress T-cell activation by a direct,
contact-dependent process.\(^{22,28}\) Some, but not all, of the inhib-
itory activity is due to the production of prostaglandins.\(^{23,26,29}\)

In an effort to learn with more precision the manner in
which the I/CB contributes to ocular immune privilege, we
developed homogeneous populations of nontransformed pig-
ment epithelial (PE) cells from normal I/CB of mouse eyes. In
this communication, we report that these cells are potent
inhibitors of T-cell activation, largely through a direct, cell-to-
cell contact-dependent mechanism, and that the mechanism
of suppression is not dependent on CD95 or promotion of
apoptosis among responding T cells.

**MATERIALS AND METHODS**

**Animals**

Normal male BALB/c, C57BL/6, C3H/HeJ (wild-type), and C3H/
HeJ.gld/gld (CD95L-deficient) mice at 6 to 8 weeks of age were
obtained from our domestic breeding facility or purchased
from Taconic Farms (Germantown, NY). DO11.10 TCR trans-
genic mice were maintained in our colony (original parents
were a generous gift of Dennis Loh, Washington University, St.
Louis, MO). DO11.10 mice express the DO11.10 TCR, which is
specific for the peptide fragment of ovalbumin (OVA), 323-
339, in the context of I-Ad.\(^{30,31}\) Animals were treated in accord-
ance with the ARVO Statement for the Use of Animals in
Ophthalmic and Vision Research.

**Culture Media**

RPMI 1640 complete medium was used for primary cultures of
I/CB PE cells, and mixed lymphocyte reactions (MLRs). It was
composed of RPMI 1640, 10 mM HEPES, 0.1 mM nonessential
amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100
\(\mu\)g/ml streptomycin, 10% fetal bovine serum (FBS; all from
Biowhitaker, Walkersville, MD), and 0.2% insulin, transferrin,
selenium (ITS) from DO11.10 mouse spleens and lymph nodes, as previously
expressed in counts per minute (cpm).

**Culture of I/CB PE Cells**

PE cells of I/CB (I/CB PE) were cultivated as follows. Eyes were
enucleated from 6- to 8-week-old male BALB/c, C3H/HeJ wild-
type, or CD95L-deficient mice. The eyes were cut into two
parts along a circumferential line just posterior to the ciliary
process. The lens was removed from the anterior eyecup, and
whole I/CB tissue was gently peeled off and collected by fine
forceps. I/CB tissue was incubated in phosphate-buffered sa-
line (PBS) containing 1 mg/ml Dispase and 0.05 mg/ml DNAseI
(both from Boehringer–Mannheim, Mannheim, Germany) at
37°C for 1 hour. Thereafter, I/CB tissue was triturated several
times with 21-guage and 23-guage needles to make a single-cell
susension. Monodispersed I/CB cells were washed twice with
RPMI complete medium, and then placed into 35-mm cell
culture dishes and incubated at 37°C in a 5% CO\(_2\) atmosphere.

**Flow Cytometry with Anti-keratin Antibody Staining**

I/CB cells were stained with fluorescein-isothiocyanate–labeled
anti-pan keratin antibody (Clone PCK-26, Sigma) on days 0, 1,
7, and 14 of in vitro culture. Cells were fixed with 5% buffered
formalin for 30 minutes at room temperature, permeabilized
with 0.1% Triton X100 and 0.1% sodium citrate for 2 minutes
at 4°C, and washed twice. Thereafter, the cells were blocked
with PBS containing 2% FBS for 20 minutes on ice, incubated
with anti-pan keratin antibody for 30 minutes on ice, and
washed twice, after which they were analyzed by flow cytom-
etry.

**Immunohistochemistry**

Frozen sections of BALB/c eyes were fixed and permeabilized
with acetone, blocked with blocking solution (PBS containing
2% FBS) for 1 hour and then incubated with anti-pan-keratin
antibody at 37°C for 2 hours. Sections were washed with
blocking solution and examined under the fluorescence micro-
scope.

**MLR**

Cultured I/CB cells (after 14 days’ culture) were seeded in
flat-bottomed 96-well culture plates and cultured overnight.
Spleens were removed from BALB/c or C57BL/6 mice and
pressed through nylon mesh to produce a single-cell suspen-
sion. Red blood cells were lysed with Tris-NH\(_4\)Cl. Responder
BALB/c spleen cells (2.5 \(\times\) 10\(^5\) cells/well) were cultured with
x-irradiated (2000R) C57BL/6 stimulator spleen cells (2.5
\(\times\) 10\(^5\) cells/well) in the presence or absence of I/CB cells. Cultures
were incubated with 200 \(\mu\)l culture medium at 37°C in a 5%
CO\(_2\) atmosphere. Supernatants of the cultures were collected
on day 3 for cytokine enzyme-linked immunosorbent assay
(ELISA), and the cells were pulsed with [\(^3\)H] thymidine for
the terminal 8 hours and then harvested by automated cell har-
cer (Tomtec, Orange, CT). Incorporated radioactivity was
measured by liquid scintillation counter, and the amount was
expressed in counts per minute (cpm).

**Activation of DO11.10 Tcr Transgenic T Cells**

DO11.10 T-cell receptor (Tcr) transgenic T cells were prepared from
DO11.10 mouse spleens and lymph nodes, as previously
described,\(^{30}\) and purified with T-cell enrichment columns
(R&D, Minneapolis, MN). Purified DO11.10 T cells (2.5 \(\times\) 10\(^6\))
were stimulated with peritoneal exudate cells and 50 to 200
\(\mu\)g/ml OVA in 96-well plates. Supernatants were collected at 48
hours, and the cells were harvested at 72 hours after they were
pulsed for 8 hours with [\(^3\)H] thymidine.

**Activation of Purified Naive T Cells by Anti-CD3 Antibody**

T cells were prepared from naive BALB/c or C3H/HeJ mouse
spleens, then purified through a T-cell enrichment column
(R&D). Purified T cells (1 \(\times\) 10\(^7\)) were stimulated with 0 to 10
\(\mu\)g/ml anti-CD3 antibody (Clone 2C11; Pharmingen, San Diego,
CA). T cells were harvested on day 3 after the exposure of
[\(^3\)H] thymidine for the terminal 8 hours.
Cytokine Assays
To measure the cytokine (interleukin [IL]-2, interferon [IFN]-γ, IL-4, or IL-10) content in supernatants of MLR and DO11.10 T-cell cultures, a quantitative capture ELISA was used. Culture supernatants were collected at 72 hours from MLR, and at 48 hours from DO11.10 T-cell cultures, immediately frozen, and stored at −20°C until used. ELISA was performed according to the manufacturer’s instructions (PharMingen). Rat monoclonal antibodies to mouse cytokine IFN-γ (Clone:R4-6A2), IL-2 (JES6-1A12), IL-4 (11B11), or IL-10 (JES-2A5; all from PharMingen) were used as coating antibodies. Biotinylated rat monoclonal antibodies to mouse IFN-γ (XMG1.2), IL-2 (JES6-5H4), IL-4 (BVD6-24G2), or IL-10 (SXC-1; all from PharMingen) were used as detecting antibodies. All recombinant cytokines used to standardize the assays were purchased from PharMingen.

TUNEL Assay
To perform the Tdt-dUTP terminal nick-end labeling (TUNEL) assay, we cultured naïve C3H/HeJ T cells for 24 and 48 hours with anti-CD3 antibodies in the presence of PE cells cultured from C3H/HeJ (wild-type) or C3H/HeJ.gld/gld (CD95L-deficient) donors. T cells were then collected by pipetting, washed twice with 2% FBS-PBS and fixed with 5% formalin-PBS for 30 minutes at room temperature. The cells were washed twice again and incubated for 1 hour at 37°C in a humidified chamber with 50 μl of a reaction mixture containing 5 μl of 0.1 mM rhodamine-6-UTP, 3 μl of 1 mM dATP, 2 μl of 25 mM CoCl₂, 0.5 μl of 25 U TdT, or water and 10 μl 1× TdT buffer. At completion of the induction, the reaction was stopped with 2 μl of 0.5 M EDTA, and the cells were washed twice with 2% FCS-PBS. Stained cells were analyzed on a flow cytometer (Epics XL analyzer; Coulter Inc., Hialeah, FL). Each experiment was repeated at least three times with similar results. The figure presented (Fig. 5) displays results of a representative experiment.

Reverse Transcription–Polymerase Chain Reaction
I/CB PE cells were cultured for 14 days as described. Thereafter, the culture medium was exchanged for serum-free medium. After overnight culture, I/CB PE cells were washed twice with PBS, then total RNA was extracted immediately by using an RNA extraction kit (RNaseasy; Qiagen, Chatworth, CA). Extracted total RNA was treated with RNase free DNase (Promega, Madison, WI) for 15 minutes at 37°C, followed by phenol-chloroform extraction twice. RNA was precipitated by ethanol precipitation and dried. The RNA was then dissolved in water, re-precipitated with 50% ethanol, and stored at −20°C until used. The RNA was then dissolved in water and 20 μl of a reaction mixture containing 1 U RNase-free DNase, 1 μl of 25 mM CoCl₂, 2 μl of 1 mM dATP, 2 μl of 25 mM CoCl₂, 0.5 μl of 25 U TdT, or water and 10 μl 1× TdT buffer. At completion of the induction, the reaction was stopped with 2 μl of 0.5 M EDTA, and the cells were washed twice with 2% FCS-PBS. Stained cells were analyzed on a flow cytometer (Epics XL analyzer; Coulter Inc., Hialeah, FL). Each experiment was repeated at least three times with similar results. The figure presented (Fig. 5) displays results of a representative experiment.

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RESULTS
Preparation of Pure PE Cell Lines from I/CB
I/CB tissues were removed from eyes of normal BALB/c mice. The tissues were rendered enzymatically and with trituration into a single-cell suspension and placed in tissue culture. In mouse eyes, pigment epithelium and corneal epithelium contain cytokeratin as an intermediate filament, whereas nonpigmented epithelium and nonepithelial cells of I/CB were keratin negative (data not shown). Single-cell suspensions prepared immediately from I/CB tissues contained approximately 20% of the cells that were keratin positive. After overnight culture, the rate of keratin-positive cells increased to 50% to 55%, because nonadherent cells had been washed out. Because these cultures were maintained in vitro, the proportion of keratin-positive cells increased. By day 14, more than 95% of the cultured cells were keratin positive (data not shown). At this time, when examined by contrast microscopy, the cells displayed an epithelial configuration, being predominantly hexagonal in shape, and they formed a monolayer (data not shown). In all subsequent experiments, I/CB PE cells that had been in culture for 14 days were used.

Effect of I/CB PE Cells on T-Cell Proliferation
To assess the influence of I/CB PE cells on T-cell proliferation, three different types of T-cell assays were performed: 1) traditional
MLR in which BALB/c spleen cells (responders) were stimulated with x-irradiated (2000R) C57BL/6 spleen cells (stimulators); 2) OVA peptide-specific, Tcr transgenic T cells from DO11.10 mice were stimulated with OVA-pulsed BALB/c spleen cells; and 3) anti-CD3 monoclonal antibody was added in mitogenic concentrations to naïve purified T cells from BALB/c spleen cells. Each of these cellular reactant sets was added to wells containing 14-day cultured I/CB PE cells, and, at appropriate intervals thereafter, [3H]thymidine was added. The results of a representative set of experiments are displayed in Figures 1a, 1b, and 1c and indicate the effects of I/CB PE cells on MLRs. As few as 3000 cultured I/CB PE cells inhibited proliferation of alloreactive T cells in these cultures, although even more profound inhibition was observed when 24,000 I/CB PE cells were present (Fig. 1a). The extent of inhibition was not dependent on the amount of allogeneic stimulators added to the culture, because I/CB PE cells inhibited proliferation in cultures containing 50 × 10^3 C57BL/6 stimulators as well as cultures containing only 1.6 × 10^4 stimulators (Fig. 1b). Moreover, inhibition of T-cell proliferation was complete, whether the cultures were stopped at 72, 96, or even 120 hours (Fig. 1c). When DO11.10 T cells were stimulated in vitro with OVA-pulsed BALB/c spleen cells in the presence of I/CB PE cells, dose-dependent inhibition of T-cell proliferation was observed (Fig. 1d). Suppression was partial when 2,000 I/CB PE cells were present but complete when 20,000 I/CB PE cells were present. Finally, T-cell proliferation resulting from ligation of the Tcr for antigen with anti-CD3 antibodies was also inhibited by I/CB PE cells (Fig. 1e). Even at the highest dose of anti-CD3 added (10 μg/ml), 20,000 I/CB PE cells completely prevented [3H]thymidine incorporation. Thus, irrespective of the mechanism of T-cell activation studied, I/CB PE cells inhibited T-cell proliferation in vitro.

Effect of I/CB PE cells on Cytokine Production by T Cells

To determine whether inhibition of T-cell proliferation correlated with suppression of cytokine production, supernatants were collected from cultures in which cultured I/CB PE cells were added to MLRs and to OVA-stimulated DO11.10 T cells. Supernatants were harvested and assayed by ELISA for IL-2, IFN-γ, IL-4, and IL-10. Results of cytokine measurements in supernatants of mixed lymphocyte cultures are presented in Figures 2a through 2d. In a dose-dependent manner, I/CB PE cells suppressed secretion of IFN-γ, IL-2, IL-4, and IL-10 in these cultures. The effect on IFN-γ production was particularly strong. In the presence of 20,000 I/CB PE cells, very small amounts of IFN-γ and IL-4 were detected. Similarly, I/CB PE cells inhibited IFN-γ and IL-2 production by OVA-stimulated DO11.10 T cells (Figs. 2e, 2f). Because DO11.10 T cells failed to secrete either IL-4 or IL-10 in response to OVA stimulation, no effect on I/CB PE cells could be discerned. These results indicate that the inhibition of T-cell activation achieved by exposure to I/CB PE cells includes both proliferation and cytokine production.

Effect of Cell-to-Cell Contact on Inhibition of T-Cell Activation

Ocular parenchymal cells have been reported to inhibit T-cell function by secreting soluble immunomodulatory factors and by direct cell-to-cell contact. To examine these possibilities for I/CB PE cells, supernatants were harvested from I/CB PE cells cultured alone for 3 days. The supernatants, either harvested from the usual culture conditions (5 × 10^5 I/CB PE cells in 1 ml medium) or in high cell-to-medium ratio (2.5 × 10^7 I/CB cells in 1 ml medium), were added to lymphocyte cultures. Results of representative experiments are presented in Figures 3a and 3b. Supernatants of cultured I/CB PE cells in the usual cell-to-medium ratio had only trivial inhibitory effects on T-cell proliferation (Fig. 3a). When I/CB PE supernatants were in a high cell-to-medium ratio, a modest degree of inhibition was observed. MLRs conducted in medium containing 50% concentrated supernatant from I/CB PE cultures displayed T-cell proliferation that was significantly reduced (data not shown). In companion experiments, MLRs were conducted in culture wells in which lymphocytes were separated from I/CB PE cells by a transwell membrane. These membranes prevent T cells from physically encountering I/CB PE cells but permit soluble factors secreted by the latter cells to pass. As revealed in Figure 3b, T cells cultured across a transwell membrane from I/CB PE cells proliferated vigorously, to a level comparable to T cells cultured without I/CB PE cells. Similarly, I/CB PE cells separated by a transwell membrane were unable to inhibit proliferation of T cells exposed to anti-CD3 antibodies (data not shown). Based on these results, we conclude that the ability of I/CB PE cells to inhibit T-cell activation was primarily dependent on cell-to-cell contact between T cells and PE cells, rather than through soluble factors secreted by the PE cells.

Role of CD95/CD95 Ligand Interactions and Apoptosis in Effector I/CB PE Cells

Many ocular cells constitutively express CD95 ligand (CD95L) on their surface membrane,15,16 this expression has been linked to the ability of these cells to suppress T-cell functions and to confer immune privilege on the anterior chamber.18 We next examined the possibilities that inhibition of T-cell activation by PE cells was dependent on CD95L expression and secondary to apoptosis induced among the responding T cells. In the first experiments, PE cells were removed from eyes of wild-type C3H/HeJ mice and from the mutant strain C3H/HeJ gld/gld, which is deficient in expression of CD95L. After 2 weeks in culture, I/CB PE cells were added in graded numbers to cultures containing C3H/HeJ spleen cells (responders) plus anti-CD3 antibodies. After 48 hours, these cultures were terminated, and the amount of [3H]thymidine incorporated was assessed. Results of a representative experiment are presented in Figure 4. Increasing numbers of PE cells from both wild type and CD95L-deficient donors suppressed the proliferation of responding T cells, and the amount of suppression was comparable for both sources of I/CB PE. This indicates that the suppression of T-cell proliferation that is mediated by PE cells is not dependent on CD95L expression. In the second experiments, BALB/c spleen cells were stimulated with anti-CD3 antibodies in the presence or absence of cultured BALB/c I/CB PE cells, as described. After 24 and 48 hours the responding T cells were harvested and subjected to TUNEL assay to determine the proportion of cells undergoing apoptosis. The results of a representative experiment are presented in Figure 5. In the absence of I/CB PE cells, approximately 9% (24 hours) and 17% (48 hours) of responding T cells were found to be TUNEL positive by flow cytometry. By contrast, in the presence of I/CB PE cells, less than 2% of the responding T cells were TUNEL positive.
FIGURE 1. Effect of cultured I/CB PE cells on T-lymphocyte activation in vitro. (a, b, and c) Mixed lymphocyte cultures. Responder BALB/c spleen cells were mixed with irradiated (2000R) stimulator C57BL/6 spleen cells and cultured in the presence or absence of cultured BALB/c I/CB PE cells. Proliferation was measured by assessing the amount of [\(^{3}\)H]thymidine incorporated during the terminal 8 hours of cultures. (a) Responder cells (\(2.5 \times 10^5\)) were mixed with irradiated stimulator cells (\(2.5 \times 10^5\)) and added to graded numbers of BALB/c I/CB PE cells. After 3 days' culture, [\(^{3}\)H]thymidine was added. (b) Responder cells (\(2.5 \times 10^5\)) were stimulated with graded numbers of irradiated stimulator cells and cultured in the presence or absence of a constant number of I/CB PE cells (\(2 \times 10^5\)). (c) Responder cells were mixed with stimulator cells in the presence or absence of BALB/c I/CB PE cells and cultured for 72, 96, and 120 hours. (d) Responder OVA-specific Tcr transgenic D011.10 T cells (\(2.5 \times 10^4\)) were stimulated with BALB/c peritoneal exudate cells (\(1 \times 10^5\)) pulsed with OVA (100 \(\mu\)g/ml) in the presence of graded numbers of I/CB PE cells. [\(^{3}\)H]thymidine incorporation was assessed after 3 days of culture. (e) T cells purified from spleens of naive BALB/c mice were stimulated with graded amounts of anti-CD3 antibodies in the presence of graded numbers of I/CB PE cells. [\(^{3}\)H]thymidine incorporation was assessed after 3 days of culture. Bars, mean ± SE incorporation of radioisotope in triplicate cultures. Asterisks and daggers indicate mean values significantly lower than positive controls: (a), \(* P < 0.0001\); (b), \(* P < 0.0001, ** P < 0.01\); (c), \(* P < 0.0001\); (d), \(* P < 0.05, ** P < 0.005, *** P < 0.0001\); (e), \(* P < 0.0005, ** P < 0.005, *** P < 0.001, ††† P < 0.0001\).
positive at both 24 and 48 hours. These results indicate that I/CB PE cells do not promote apoptosis among T cells responding to anti-CD3 stimulation. On the contrary, I/CB PE cells appear to protect T cells from undergoing apoptosis after stimulation by anti-CD3 antibodies.

### Survey of I/CB PE Cells for Pertinent Immunomodulatory Molecules

In an effort to understand the molecular basis by which I/CB PE cells suppress T-cell activation, we prepared cellular extracts from the cultured cells and analyzed them by RT-PCR for the following molecules: TGF-β1, TGF-β2, IL-4, IL-6, IL-10, IFN-γ, TNF-α, POMC, and CD95L (Fas L). The PCR products were electrophoresed in 2% agarose gel and visualized by staining with ethidium bromide. The results are presented in Figure 6.

mRNA species from cultured I/CB PE cells were identified for TGF-β1, TGF-β2, IL-6, IL-10, and TNF-α. No message was detected for IFN-γ, IL-4, proopiomelanocortin (POMC), or CD95L.

Based on this information, we attempted to reverse I/CB PE-dependent inhibition of T-cell proliferation by adding pertinent neutralizing antibodies to the cultures. The following antibodies were used: a pan anti-TGF-β antibody, anti-IL-10, and anti-TNF-α. Each of these antibodies was added to mixed lymphocyte cultures grown in the presence or absence of I/CB PE cells. The results of representative experiments are presented in Figures 7a, and 7b. As can be seen, anti-TGF-β antibodies failed to relieve the inhibition of T-cell proliferation imposed by I/CB PE cells. Similarly, anti-IL-10 antibodies displayed no ability to permit T cells to proliferate in these
cultures (data not shown). Anti-TNF-α antibodies tended to promote T-cell proliferation in mixed lymphocyte cultures not containing I/CB PE cells (a result reported previously by others; see Fig. 7b). When this effect was taken into account, anti-TNF-α antibodies were ineffective at restoring T-cell proliferation in the presence of I/CB PE cells. Thus, none of the immunomodulatory factors that were detected by RT-PCR in cultured I/CB PE cells appeared to account for the ability of the cells to inhibit T-cell activation.

Effects of IL-12 and IL-2 on Suppression Achieved by I/CB PE Cells

The failure of T cells to proliferate when a signal is delivered through the Tcr may be due either to immunosuppressive factors in their microenvironment or to the absence of costimulatory molecules required for activation. IL-12 is a potent costimulatory molecule that is required during the activation of T cells in the MLR. IL-2 is the prototypic growth factor for T cells and is required for proliferation after Tcr ligation. In the following experiments IL-12 or IL-2 was added to mixed lymphocyte cultures in the presence or absence of cultured I/CB

For Figures 3, 4, and 5, the experiments were performed as described in the Methods section. Briefly, responder BALB/c spleen cells were stimulated with irradiated stimulator C57BL/6 spleen cells in medium containing graded amounts of supernatants obtained from cultured I/CB PE cells. Responder cells were mixed with stimulator cells in wells to which I/CB PE cells were added directly or were separated from the lymphocytes by a semipermeable (transwell) membrane. [3H]thymidine was added to the cultures during the terminal 8 hours. Bars, mean ± SE incorporation of radioisotope in triplicate cultures. Asterisks, mean values not significantly different from positive controls: *P < 0.0001, **P < 0.05.
When T-cell proliferation was assessed (data now shown), it was observed that both IL-12 and IL-2 enhanced \[^{3}H\]thymidine incorporation in cultures without I/CB PE cells. However, neither IL-12 nor IL-2 was very effective at promoting T-cell proliferation when I/CB PE cells were present. At the highest density of I/CB PE cells (20,000/culture), T-cell proliferation in the presence of IL-12 and IL-2 was no greater than that of the syngeneic (negative) control. These findings indicate that the inhibitory effect of I/CB PE cells on T cells is sufficiently powerful to overcome the potent costimulatory and proliferation-inducing properties of IL-12 and IL-2, respectively.

**DISCUSSION**

Cultured PE Cells from the I/CB of normal mouse eyes possess immunomodulatory properties, particularly with regard to the functional activation of T lymphocytes. When naive T cells are stimulated through the Tcr for antigen in the presence of appropriate costimulation, the cells undergo a functional transformation that leads to proliferation and secretion of cytokines. This singular event is pivotal to induction and elicitation of immune responses to antigens. To examine the inhibitory effect of I/CB PE cells on naive T-cell activation, three different T-cell assays were used: 1) MLRs, in which the responding T cells directly recognize and respond to alloantigens encoded by the major histocompatibility complex; 2) the response of naive Tcr transgenic T cells when stimulated with their cognate antigen; and 3) the global T-cell response to anti-CD3 antibody. When I/CB PE cells were present in cultures of each of these types of assays, the proliferative responses of T cells were profoundly curtailed. Moreover, the responding T cells were impaired in their capacity to secrete IFN-\(\gamma\), IL-1, IL-4, and IL-10. A similar pattern of failed proliferation and cytokine secretion was reported previously when T-cell stimulation assays were conducted in the presence of explants of I/CB. The similar outcome, however, does not necessarily reflect a similarity of mechanism. Whereas cultured I/CB PE cells effected their inhibition of T-cell activation largely through a contact-dependent mechanism, explants of whole I/CB tissue secrete immunosuppressive factors into the supernatant, and these soluble factors act in an inhibitory manner on responding T cells. Thus, the normal I/CB contains cells that inhibit T-cell activation by two different mechanisms: soluble immunosuppressive factors and direct cell-to-cell contact.

At present, several different parenchymal cell types within the eye have been demonstrated to possess immunomodulatory properties that have a direct or indirect effect on T-cell function. Corneal epithelium and keratocytes secrete soluble factors, including TGF-\(\beta\), that inhibit lymphocyte activation and suppress inflammation. In addition to secreting soluble,
but still undefined, immunosuppressive factors,²⁰ corneal endothelial cells also express CD95L, which inhibits effector T cells from destroying orthotopic corneal allografts,¹³,¹⁶ presumably by initiating programmed cell death in the T cells. Müller cells, especially when treated with IFN-γ, acquire immunosuppressive properties that are mediated in part by direct cell contact.²⁵

Retinal pigment epithelial (RPE) cells bear ontogenetic similarity to the pigment epithelium of I/CB. During the past few years, several groups have investigated the capacity of RPE to function as antigen-presenting cells and also as modulators of T-cell activation. Regarding the latter, Liversedge and Forrester³⁷ reported that RPE cells suppress lymphocyte proliferation even in the presence of exogenous antigen-presenting cells, implying an immunomodulatory role. These researchers have gone on to show that RPE cells suppress T-cell activation through soluble factors (prostaglandin E₂ [PGE₂]) and through membrane-bound mechanisms. Several soluble factors in addition to PGE₂ have been found to be secreted by RPE, including TGF-β.³⁸,³⁹ More recently, Jorgenson et al.⁴¹ have reported that human RPE cells induce apoptosis among activated T cells through a CD95-CD95L process. However, the precise mechanism of T-cell death was not defined by this study, and the veracity of CD95L expression on human RPE cells was even called into question. In fact, in a very recent publication, Farrokh–Siar et al.⁴⁰ reached the conclusion that human fetal RPE cells suppress proliferation of an autonomously proliferating T-cell line through apoptosis but that the apoptosis was not mediated through the CD95-CD95L pathway or through secretion of apoptosis-inducing cytokines.

Our analysis of the contact-dependent mechanism by which I/CB PE cells inhibit T-cell activation is relevant to this issue. We found that cultured I/CB PE cells from CD95L-deficient mice were comparable to wild-type PE cells in inhibiting T-cell activation. Moreover, we found no evidence that the CD95L gene was even transcribed in cultured I/CB PE cells from normal mice. Finally, I/CB PE cells appear to protect responding T cells from apoptosis, rather than to promote programmed cell death. We conclude that the capacity of I/CB PE cells to inhibit T-cell activation is independent of CD95L interactions and does not depend on triggering of apoptosis among responding T cells.

We are unable as yet to identify the molecular mechanism that accounts for the ability of cultured I/CB PE cells to inhibit T cells by a contact-dependent mechanism. Although we found mRNA evidence for the activity of genes encoding TGF-β, IL-6, TNF-α, and IL-10, we were unable to neutralize the inhibitory effects of I/CP PE cells with neutralizing antibodies to these cytokines. Taylor et al.⁴¹ have recently reported that T cells that have been activated in the presence of aqueous humor acquire novel properties, including the capacity to suppress the activities of bystander T cells. Experiments similar in design are now under way to determine whether T cells that have come in contact with I/CB PE cells also acquire regulatory functions.

The capacity of I/CB PE cells to suppress T-cell activation by a direct cell-to-cell contact mechanism resembles some of the properties ascribed to RPE cells. However, the I/CB PE cells do not form a physical barrier (through extensive tight junctions) similar to that created by RPE. Thus, they do not participate in the so-called blood–ocular barrier. In fact, unlike RPE, the PE cells of the ciliary body are effectively outside the blood–ocular barrier, because tight junctions between and among secretory ciliary epithelial cells create a barrier at the level of the ciliary processes between intraocular compartments and the extraocular environment. This unique anatomic location has caused us to wonder why ciliary body PE cells possess immunomodulatory properties. It is relevant that the PE cell layer of the ciliary body actually separates the secretory epithelium from the microvessels of the ciliary processes in the stroma. Thus, migratory T cells that escape from local vessels into the

**Figure 8.** Traffic of T cells through ciliary body epithelium during which T cells are functionally disarmed. AC, anterior chamber; NPE, nonpigmented epithelium; PC, posterior chamber.
stroma must pass through the PE layer if they intend to penetrate into the posterior chamber. With this microanatomic arrangement, the opportunity exists for PE cells to influence the functional properties of T cells destined to enter the eye. Our data suggest that, given this opportunity, PE cells disarm the T cells by preventing them from inducing a differentiation program that could lead to immunogenic inflammation. A diagram describing this process is presented in Figure 8. There are other examples of T-cell migration in which the cells escape the microvasculature and penetrate through an epithelial layer into another compartment. Populations of T cells regularly leave the microvessels of the intestinal villi and migrate through the absorptive epithelial layer (which is also linked by tight junctions) into the intestinal lumen.42

Whether a similar process takes place in the iris is debatable. The anatomic relationships between the PE of the iris and the iris vessels differ from that of the ciliary body PE in that there is no superficial layer of secretory epithelium maintaining a barrier. Indeed, the iris microvessels themselves are believed to create a type of barrier that limits ingress of blood-borne leukocytes.

Our demonstration that cultured I/CB PE cells inhibit T-cell activation by a contact-dependent mechanism should be interpreted cautiously for several reasons. First, cells that have been explanted in culture often adopt functional phenotypes that are not representative of their native properties when present in intact tissues. Thus, the possibility exists that the properties we have discovered among cultured I/CB PE cells do not reflect in vivo properties of PE cells. Second, for technical reasons, we have worked with PE cells from both the iris and ciliary body. Although PE cells from these two tissues are closely linked ontogenetically, they may adopt different functions when they differentiate respectively into the PE layer of the iris and the PE layer of the ciliary body. Thus, the ability to inhibit T-cell activation through direct contact may be a property of only one or the other type of PE.

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