Interaction of Retinal Pigmented Epithelial Cells and CD4 T Cells Leads to T-Cell Anergy

Dale S. Gregerson, Neal D. Heuss, Kathleen L. Lew, Scott W. McPherson, and Deborah A. Ferrington

PURPOSE. Retinal pigmented epithelial (RPE) cells may contribute to retinal immune privilege. Daily phagocytosis and degradation of photoreceptor cell outer segment tips by RPE provide substantial amounts of retinal autoantigens for potential MHC occupancy. RPE are well placed to modulate antigen (Ag)-specific activation of T cells in the outer retina under conditions in which inflammatory mediators may upregulate major histocompatibility complex (MHC) on RPE cells. The Ag-presenting ability of RPE cells was examined to determine whether they induce Ag-dependent modulation of CD4 T-cell activity.

METHODS. The effects of RPE on Ag-specific activation of naive, Ag-specific CD4 T cells were tested in cultures with immortalized, syngeneic murine RPE cells. Flow cytometry, proliferation, and cytokine production were used to assess T-cell activation and phenotype.

RESULTS. Naive CD4 T cells exposed to peptide-pulsed RPE upregulated expression of CD25, CD69, and CD44, showing receptor occupancy. However, T-cell proliferation and production of IL-2, IL-17, and IFN-γ were severely depressed. Provision of whole β-gal, as opposed to β-gal peptide, gave no evidence of T-cell activation. T cells recovered from RPE cocultures were hyporesponsive to restimulation with splenic APC and Ag, but did not exhibit significant regulatory activity. Although CD25 was upregulated on RPE-activated T cells, expression of FoxP3 was similar to that found after activation of regulatory T cells. 17

CONCLUSIONS. RPE cells directly presented extracellular peptides through MHC class II to naive CD4 T cells, leading to an anergic state in the T cells. The anergic T cells survived, but were not immunoregulatory. The ability to modulate T-cell responsiveness in this manner may underlie the contribution of the RPE to immune privilege. (Invest Ophthalmol Vis Sci. 2007;48:4654–4663) DOI:10.1167/iovs.07-0286

The tight junctions of the retinal vascular endothelium and the retinal pigment epithelium (RPE) form the blood–retinal barrier (BRB). The RPE is asymmetric; the apical surface of the RPE is inside the BRB and interacts with retinal photoreceptors. The basal surface of the RPE is outside the BRB and separates the retina from the highly fenestrated blood vessels of the choriocapillaris and choroid. The RPE, as part of its normal function, removes the tips of the photoreceptor cells by phagocytosis on a diurnal cycle and digests them. 1

Retinal photoreceptor cells express, or are surrounded by, several retina-specific autoantigens (auto-Ags) that are targets of immunopathogenic autoimmunity. 2 Although central, thymic mechanisms of tolerance, including the thymic expression of tissue-specific Ags as a result of the autoimmune regulator (AIRE) gene, contribute to tolerance to retinal Ags, 3,4 there is evidence of peripheral mechanisms of tolerance to retinal Ags. 5–8 These mechanisms require the interaction of T cells with Ag-presenting cells (APCs) bearing Ag derived from retina. Naive T cells, and even rested, quiescent T cells from a pathogenic CD4 T-cell line have not been found to enter immunologically quiescent retina. 9 Further, there appear to be very few Ag-presenting cells in the quiet retina. 10–12 Together, these results suggest that initial T-cell interactions with retinal Ag may occur outside the BRB. Given the large quantity of photoreceptor cell outer segment tips processed by the RPE, these retinal Ags may be made available to T cells via presentation in MHC expressed on the basal surface of the RPE. The presentation of Ag in the absence of danger signals 13–14 or by “non-professional” APCs 15,16 may produce T-cell anergy or induce regulatory T cells. 17

RPE cells are known to possess properties that affect innate and adaptive immune responses. Their expression of major histocompatibility complex (MHC) class II has been reported to be upregulated by proinflammatory cytokines. 18–20 RPE cells are also known to produce several cytokines, including IL-6, IL-8, granulocyte-macrophage–colony-stimulating factor (GM-CSF), and TGF-β in response to stimulation with IL-1α, IFN-γ, and TNF-α or combinations thereof. 21–25 Ag presentation by nonprofessional APCs, perhaps including RPE cells induced to express MHC and various cytokines by local inflammation, may further promote induction of retinal Ag-specific peripheral tolerance through interaction with circulating, autoreactive naive T cells not deleted by central tolerance. RPE cells induced to express MHC class II have been reported to be capable of presenting Ag to Ag-experienced CD4 T cells. 26–28 Conversely, others have examined the effects of mitogen-activation of T cells in the presence of RPE cells and found suppression of their responses. 27–29 Inhibition is due in part to production of PGE2 and NO by the RPE. 30–31 Fas L-dependent induction of apoptosis in activated T cells by RPE has also been observed and proposed to form a barrier against T-cell infiltration. 32

In most cases, the experimental systems used to study the effects of RPE on T-cell responses have not used purified or well-characterized cell populations or defined Ags, complicating interpretation of the results. To bring greater definition to the study of this important interaction, we initiated experiments with well-defined components, including purified, Ag-specific naive CD4 T cells from TCR transgenic mice, providing conditions in which bone marrow-derived APCs were ex-
cluded. This strategy allowed examination of Ag presentation by the RPE without the confounding effects of contamination by conventional APC. In this study, we asked whether cultured RPE expressed MHC class II in a functionally significant manner when assayed with naive T cells specific for a defined Ag. If so, we wanted to determine whether RPE cells were able to process and present exogenous or endogenous Ags and whether the resultant T-cell response differed from that associated with Ag presentation by professional APC. We found that the RPE interact with naive CD4 T cells in a manner that dissociates the Ag-stimulated responses of the T cells and yields T cells with suppressed effector functions but that did not possess significant regulatory properties. T-cell unresponsiveness to Ag was maintained in T cells recovered from RPE cocultures and rechallenged with splenic APC and Ag, indicating the induction of anergy.

**Materials and Methods**

**Mice**

Wild-type and transgenic mice on the B10.A background were used. The 3E9 TCR-transgenic mice were used as a source of naive 3E9 CD4 T cells specific for a class II–restricted epitope of βgalactosidase (β-gal). Mice were housed and handled in accordance with the Institutional Animal Care and Use Committee at the University of Minnesota, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Cell Culture Conditions**

Immortalized murine B10.A RPE cells were made as described. All cells were grown at 37°C in a humidified chamber containing 8% CO2. Cells were grown to near confluence before characterization or use in experiments. To assess interactions with lymphocytes, the RPE cells were cultured in multiwell plates or flasks, as indicated, in a medium containing RPMI 1640 and supplemented with nonessential amino acids, glucose, pyruvate, 2-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum. IFN-γ and/or TNF-α were added as described in the Results section. An mAb specific for lα/β and known to block Ag recognition (clone 10-3.6), was used to determine the role of TCR recognition of peptide in MHC class II in blocking experiments. An isotype control (clone G155-178; IgG2a,k) was also used. Both antibodies were applied to the RPE cultures at 10 μg/mL 1 hour before addition of the β-gal peptide and 3E9 T cells. The Abs were left in the wells for the duration of the experiment.

**Antigens**

Purified β-gal was purchased from Prozyme (San Leandro, CA). A 14-residue synthetic peptide of β-gal recognized by class II restricted CD4 T cells (peptide 70-71, YVVDEANIELTHGMV) was made in the University of Minnesota Microchemical Facility. Ovalbumin (OVA) was purchased from Sigma-Aldrich (St. Louis, MO).

**Preparation of Ag-Coated Latex Beads**

Carboxylyl YG 1-μm microspheres (Fluoresbrite; Polysciences, Inc. Warrington, PA) were coated with Escherichia coli β-gal or OVA per manufacturer’s instructions. Briefly, 0.5 mL beads were washed three times in 0.1 M borate buffer (pH 8.5) and resuspended in 100 μL of borate buffer containing 300 μg of protein. After overnight incubation at room temperature on a rocker, the beads were washed with calcium and magnesium-free PBS, resuspended in 50 μL of PBS, and irradiated (50,000 R) to eliminate contamination. Ag-coated and control beads were added to RPE (grown to confluency in 24- or 6-well plates), splenic APC or purified DC at concentrations from 2.8 × 105 to 1.4 × 106 beads/well. The uptake and content of beads per RPE cell were determined by flow cytometry, as previously described.

**RPE and CD4 T-Cell Interaction in T-Cell Anergy**

**Cell Purification**

Fresh 3E9 CD4 T cells were prepared from pooled spleens and lymph nodes (LNs) of 3E9 TCR Tg mice. The cells were first depleted with Abs to CD19, CD11b, and CD11c using the magnetic cell sorting system and two passes over LD columns (MACS; Miltenyi Biotec, Inc., Auburn, CA). The flow-through cells were then positively selected for CD4+ or CD90+ cells using LS columns. Dendritic cells (DCs) were isolated by positive selection for CD11c cells by magnetic cell sorting.

**Preparation, Recovery, and Purification of 3E9 T Cells from RPE Cocultures**

Spleen and lymph node cells from 3E9 mice were pooled and depleted of CD8+, CD11c+, and CD25+ cells using antibody-conjugated beads and LD columns (Miltenyi Biotec, Inc.). Unbound cells were positively selected with anti-CD90 using LS columns. Purified cells (CD90+; 6.5 × 106) were cultured either with or without β-gal peptide 70-71 in 7.5 mL flasks already confluent with RPE cultures that had been pretreated for 5 days with IFN-γ (100 U/mL) and TNF-α (1 ng/mL). These flasks were cultured for a total of 7 days, exchanging 50% of the culture supernatant with fresh media on days 2 and 4, also adding 5 μL of magnetic particles (BioMag; Bangs Laboratories, Fishers, IN) on day 4 to each flask. On day 7, RPE and T cells were harvested from the flask using trypsin/EDTA, washed, and separated in a 15-mL conical tube based on RPE phagocytosis of the particles with a magnet (Magnet; BD Biosciences, San Jose, CA). All cells not retained by this magnetic separation were cultured overnight in fresh flasks and were added at 5 μL of fresh magnetic particles, then harvested and separated with the magnet again. Since approximately 2% of the original number of RPE cells were still present and would contaminate the next cultures, the cells were further separated based on forward and side scatter (FACSaria sorter; University of Minnesota Stem Cell Institute Flow Core Facility). Complete separation was confirmed by culturing 2 × 107 flow cytometry-purified T cells into a well of a 96-well plate. No RPE cells were observed after 4 days.

**CFSE-Stained 3E9 CD4 T Cells**

T cells purified from 3E9 spleens and LNs were resuspended to 50 × 106 cells/mL in PBS. CFSE (5 (6-carboxyfluorescein diacetate, N-succinimidyl ester) was added to a final concentration of 4 μM, and the cells were incubated for 10 minutes at 37°C with gentle mixing. Ten to 15 mL of RPMI with 10% fetal calf serum (FCS) was added to stop the reaction. The cells were washed three times and used in cultures.

**Flow Cytometry**

Since many T cells adhere to the plastic and the RPE monolayer, recovery of the T cells was enhanced by incubation in trypsin/EDTA for 5 minutes at 37°C. The contents of the wells were quantitatively transferred to tubes, washed, incubated in culture medium for 1 hour at 37°C to replenish surface expression, washed once, and stained in 100 μL of staining buffer (PBS with 2% FCS, 0.05% Na azide) containing labeled antibodies. After 20 minutes on ice, cells that were incubated with biotin-labeled Abs were washed once and resuspended in streptavid conjugates. After 15 minutes, all cells were washed once and resuspended for flow cytometry in a flow cytometer (FACS Calibur, with CellQuest software; BD Biosciences). The entire contents of each well were acquired. T cells were distinguished from RPE based on staining for Thy 1. Also, RPE cells are much larger than T cells, and the populations were easily distinguished on the scatterplots.

**Cytokine Assays**

The cytokine content of culture supernatants taken from lymphocyte cultures as described earlier was determined by ELISA according to the manufacturer’s suggestions, as previously reported. The antibody pairs and the murine cytokine standards for IL-2, IL-17, and IFN-γ were
RESULTS

Expression of Class II MHC and Costimulatory Molecules on Murine RPE

Ag recognition by CD4 T cells requires expression of class II MHC molecules by APCs, and the processing pathways to load them with peptides. Class II MHC was undetectable on untreated RPE cells in culture, and IFN-γ treatment alone had little effect (Fig. 1). TNF-α alone also had little effect on class II expression (data not shown), but the combination of IFN-γ and TNF-α induced an increase in expression detectable by 4 days after treatment. Analysis of cell surface molecules capable of providing the costimulation required for Ag-specific activation of naive CD4 T cells showed that CD80 was expressed constitutively and that B7-H1 was upregulated by IFN-γ treatment (Fig. 2). Cell surface expression of CD86, CD40, and CD134L was not found under these cytokine treatment conditions (data not shown).

Ag Presentation by Cultured, Cytokine-Treated RPE

RPE cultures induced to express MHC class II were tested for the ability to present Ag to naive CD4 T cells using the 3E9 T cells from TCR Tg mice in which the TCR is specific for an immunodominant, class II–restricted epitope of β-gal (Ref. 33 and manuscript in preparation). Ag was provided as a 14-residue synthetic peptide containing the cognate epitope in β-gal. Irradiated splenic APCs were used as positive controls for Ag presentation. Evidence of TCR ligation by peptide-treated RPE was found by several measures including the upregulation of T-cell surface expression of CD25 and a slight increase in CD44 (Fig. 3). CD69 was also elevated (data not shown). RPE cells that were not treated with both cytokines were much less able to induce evidence of TCR occupancy in the 3E9 T cells. OVA control cultures and no-Ag negative control cultures showed no evidence of 3E9 T-cell activation or Ag recognition on splenic APCs or RPE cells. Purified T cells alone had no response to Ag, demonstrating that they were not functionally contaminated with APCs (data not shown). As a further control for the Ag dependence of the unresponsiveness, a blocking Ab

![Figure 1](image1.png)

![Figure 2](image2.png)
specific for I-A^k was used to show that Ag presentation by class II MHC was required for upregulation of CD69 and CD25 on the 3E9 T cells (Fig. 4). Presence of the blocking Ab reduced the Ag-dependent expression of CD69 and CD25 to near control levels.

**TCR Occupancy but Nonproductive Activation**

Although RPE cells expressing both MHC class II and CD80 presented peptide to β-gal-specific CD4 T cells so that TCR ligation was detected, the activation was not productive, in that T-cell proliferation and cytokine production (IL-2 and IFN-γ) were minimal (Fig. 5). Instead, the RPE promoted the survival of the 3E9 T cells far beyond that found in cocultures with splenic APCs. Note that the number of 3E9 T cells recovered after 4 days in the presence of RPE cells was greater than that recovered from cultures with splenic APCs. The survival-promoting activity was not dependent on the addition of Ag to the cultures. Several attempts to provide costimulatory activity...
or to inhibit APC regulatory activity associated with B7-like molecules or CD40 by adding antibodies with agonist or antagonist activity for costimulatory molecules had little effect on the responses of the 3E9 T cells to Ag presented by RPE (data not shown).

**RPE Fail to Present Ag that Requires Uptake and Processing**

Unlike results obtained with the β-gal peptide, the ability to process and present whole β-gal was lacking in the RPE, including the IFN-γ/TNF-α-treated RPE described above in Figure 3. Although the RPE failed to take up soluble Ag and process it into class II, it was possible that directing Ag to their highly active phagocytic pathway may reveal Ag processing. To this end, β-gal was adsorbed to 1-μm latex beads and added to the cultures. Uncoated beads and beads coated with an irrelevant protein (OVA) were used as control samples. The extensive uptake of the latex beads into RPE has been described in detail.35 DCs were used as the positive control cultures for Ag presentation and were found to present the bead-bound Ag and other forms of the β-gal Ag efficiently to naive 3E9 cells (Fig. 6). Conversely, there was no evidence of T-cell activation by the RPE cells, including cytokine-activated RPE, despite their extensive uptake of the beads. A wide range of bead concentrations was examined in several experiments, but no evidence of recognition of bead-bound β-gal by the 3E9 T cells was found.

**Inhibition of T-Cell Activation in RPE/Splenocyte Cocultures**

The inability of RPE to activate cytokine production or induce proliferation in naive 3E9 T cells was not affected by addition of antibodies specific for CD28, CD80, CD86, CD274 (PD-L1; B7-H1), or CD40 (data not shown) that might either provide

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**Figure 5.** 3E9 T cells stimulated with peptide on cytokine-treated RPE upregulated activation markers, but failed to produce cytokines. After coculture with or without peptide Ag for 2 to 3 days, supernatants were collected for cytokine (IL-2 and IFN-γ) assays, and the cellular contents of the wells were suspended and assessed for the indicated markers of activation and recovery. (■) Irradiated splenic APCs (positive control); (□), RPE cells.

**Figure 6.** RPE failed to present Ag in class II MHC when the Ag required uptake and processing. Top: As positive controls, DCs (CD11c⁺) presented peptide Ag, whole β-gal, and β-gal-coated beads to naive 3E9 T cells. Uncoated beads and beads coated with an irrelevant protein Ag (OVA) were used as controls. Bottom: Irradiated spleen cells (SPL) were used as the positive control. The RPE cells were pretreated with IFN-γ and TNF-α. The naive 3E9 T cells failed to respond to whole β-gal, whether as soluble Ag or bead-bound Ag, if RPE cells were used as the APCs. Results are representative of several assays. Unstimulated, resting T cells are in the bottom left area, whereas activated T cells are found in the top right area.
second signal, or block an inhibitory activity. Studies from other laboratories have demonstrated the presence of several RPE-derived inhibitory factors, including TGF-β, PGE₂, and NO, that contribute to the inhibition of lymphocyte activation.³⁰,³¹ To examine the effect of RPE cells on lymphocyte activation by conventional APCs, we dispensed suspensions of irradiated splenocytes and naive 3E9 T cells onto monolayers of RPE cells, to determine whether the presence of RPE would inhibit Ag-dependent T-cell activation by professional APCs. Compared with cultures containing only RPE cells as APCs, the Ag-stimulated increases in CD25, CD44, and CD69 expression on 3E9 T cells approached near-positive control levels by addition of splenocytes to the T-cell–RPE cultures (Fig. 7). Similar results were found with DCs, and GM-CSF-treated DCs (data not shown). Clearly, TCR occupancy was not compromised in these cocultures, since CD25, CD69, and CD44 were substantially upregulated. Conversely, IL-2 and IFN-γ production was inhibited in the cocultures, and forward scatter (FSC) was reduced. A greater portion of the cells remained at a small, “resting” size in cocultures (P < 0.05, paired t-test). As described in the next section, T-cell proliferation was also inhibited by the RPE cells.

**Inhibition of T-Cell Proliferation**

Ag-dependent proliferation of the 3E9 T cells in response to Ag and splenic APC was inhibited when stimulated on a monolayer of RPE cells (Fig. 8). Although approximately 80% of the 3E9 T cells in control cultures of splenic APCs had diluted their CFSE content by day 2, less than 10% of the T cells in the RPE/spleen cell cocultures had divided. By day 7, significant division was found in the RPE/spleen cell cultures, but it was substantially less than that with splenic APCs alone. Although there was evidence of TCR occupancy in cultures of 3E9 cells and RPE in the presence of peptide Ag as shown earlier, there was little dilution of CFSE up to 7 days later.

**Properties and Responsiveness of 3E9 T Cells Recovered from Cocultures with RPE Cells**

A defining feature of T-cell anergy is functional inactivation that is manifested even when the T cells are later given Ag and fully competent APCs.⁴⁰ This distinguishes anergy from immunoregulation, which is dependent on the ongoing inhibitory activity of other cells in the population, such as regulatory cells.⁴¹ To test for evidence of anergy, 3E9 T cells were recovered and purified after 7 days in coculture with cytokine-pretreated RPE cells, with or without the β-gal peptide. The recovered cells were then tested for responsiveness on irradiated splenic APCs and Ag. Control cultures included fresh, naive 3E9 T cells (condition 1). The responsiveness of 3E9 cells recovered after a cycle of stimulation with splenic APCs and Ag was also tested for comparison (condition 4A).

From a starting number of 65 × 10⁶ T cells, approximately two times more T cells were recovered from RPE monolayers if the cognate peptide for the 3E9 T cells was included during the incubation (22.7 × 10⁶ vs. 10.1 × 10⁶). The activity of the recovered 3E9 T cells was compared to fresh control 3E9 cells, by assessing the production of cytokines (Fig. 9, conditions 1 versus 2A) when given fresh splenic APCs and peptide. IL-2, IL-17, and IFN-γ production remained strongly reduced in the recovered 3E9 cells relative to cytokine production by an equal number of fresh 3E9 control cultures. These results are consistent with induction of anergy in the T cells.
which RPE cells affect T-cell responsiveness. 3E9 T cells recovered from parallel cultures with splenic APCs and peptide were also included as control cultures (condition 4A versus 1), and gave strong responses, as expected.

**RPE-Induced FoxP3 Expression without Regulatory Activity**

Flow cytometry was performed to detect markers of regulatory T cells, including coexpression of CD25 and FoxP3 on 3E9 cells recovered from cocultures with RPE. Approximately 3% of the 3E9 T cells recovered from cultures containing RPE and Ag were double positive for CD25/FoxP3 (Fig. 10). FoxP3 was upregulated in cultures containing peptide and limited to cells that also expressed CD25 and CD69 (data not shown). FoxP3 expression in cocultures with RPE in the absence of conventional APC was dependent on the inclusion of the β-gal peptide in the cultures, showing that peptide-pulsed RPE provided the signals required to induce FoxP3 expression (paired t-test; \( P < 0.001 \)). The average results of five separate experiments are given in Table 1. FoxP3 was induced to near normal expression levels, with respect to the frequency of positive cells and their GMFI (geometric mean fluorescence intensity), in cultures with RPE cells and Ag, relative to that expressed on 3E9 T cells from cultures with spleen cells and Ag. A somewhat higher frequency was found if 3E9 T cells were stimulated with peptide on the combination of RPE and splenic APC relative to RPE alone (\( t \)-test; \( P = 0.03 \)).

Since the recovered T cells survived well, exhibited a significant degree of unresponsiveness with respect to cytokine production, and contained a CD4+CD25+FoxP3+ population, the possibility that they had been induced to become regulatory was examined. Recovered 3E9 T cells from RPE cocultures with and without Ag were incubated with an equal number of freshly harvested 3E9 T cells and stimulated with peptide and irradiated splenic APCs (Fig. 9, conditions 3A and 3B, respectively). IL-2, IL-17, and IFN-γ production by fresh naive 3E9 T cells was not significantly affected by the 3E9 T cells recovered after 7 days on RPE cells, providing no evidence of regulatory activity in either population.

**Discussion**

Since RPE cells phagocyte copious quantities of photoreceptor cell rod outer segments (ROS), they are in a position to process and present large amounts of known, immunopathogenic retinal self-Ags, including interphotoreceptor retinoid-binding protein (IRBP), arrestin, rhodopsin, and recoverin. If these molecules were presented under conditions that promoted T-cell unresponsiveness, the RPE could play an important role in maintaining peripheral tolerance. The results show that RPE could participate in the generation of peripheral tolerance to retinal Ags by the induction of anergy in naive T cells specific for a retina-derived protein. Presentation of exogenous peptide in class II MHC of RPE led to TCR occupancy and assays to detect regulatory activity found little evidence of Treg activity, and FoxP3 expression was induced, but not elevated above levels induced by activation with splenic APCs and Ag. Taken together, the evidence showed that the functional status of the recovered T cells was most consistent with
an anergic state. Our results differ in several respects from those reported by Liversidge et al.\textsuperscript{30,31,42} We found little evidence of TCR-independent RPE-mediated T-cell activation activities described in those papers. In particular, there was no T-cell proliferation resulting from cocultures of RPE and T cells, and IL-2 secretion was not induced in our naïve 3E9 T cells incubated with RPE alone. Further, T-cell proliferation was not restored, in our studies, by inclusion of exogenous IL-2. Most of our experiments were performed with highly purified, naïve CD4 T cells in the absence of classic APCs from LNs or spleen, and this difference may contribute to the disparity in our results.

Alternative pathways of T-cell activation that result in loss of effector functions have been described. Several members of the B7-like family have been found to downregulate T-cell activation, including responses in the nervous system, contributing to the maintenance of peripheral tolerance. B7-H1 expression on microglia was found to limit T-cell responses.\textsuperscript{43} Treatment of mice with anti-B7-1 in vivo reduced the severity of experimental autoimmune encephalomyelitis, whereas treatment with anti-B7-2 exacerbated disease progression.\textsuperscript{44} As a result, evidence of expression of several B7-like family members on the RPE cells was sought. Cultured RPE was found to express CD80 (B7-1), well-known to promote T-cell activation by Ag-primed APCs. Levels were increased by addition of IFN-γ/H\textsubscript{9253} and the combination of IFN-γ/H\textsubscript{9253} and TNF-α/H\textsubscript{9251}. Conversely, CD86 (B7-2) and OX-40L(CD134L), which are also known to promote T-cell activation, were not found on untreated or treated RPE cultures. Uregulation of B7-H1 (PD-L1), often considered to be a regulatory member of the B7 family,\textsuperscript{45} was found on IFN-γ-treated RPE cells. However, experiments to attribute the inhibitory effect of RPE on 3E9 T-cell activation to B7-H1 expression found no evidence of its participation (data not shown). Given the complex interrelationships between the B7 family of molecules and their ligands and the evidence that these interactions can have unpredictable outcomes apparently based on undefined local conditions, further detailed in vivo and in vitro analyses are needed to elucidate the roles of these molecules.\textsuperscript{46} Although CD40 has been detected on human RPE cell cultures,\textsuperscript{47} we found no staining on our murine cultures.

Tolerance-promoting conditions may be found in inflamed retina in which damage to retinal cells leads to release of antigens into the extracellular space. These antigens could be loaded into MHC class II on RPE by several pathways. In the classic pathway, newly synthesized class II may be loaded with peptides derived from material taken up by the RPE via phagocytosis/endocytosis. The peptide-class II complex is chaperoned by invariant chain (Ii, CD74) to the cell surface for presentation to T cells. The alternative pathway loads peptide into recycled class II from the cell surface in early endosomes; in this case, antigen may be taken up at the time of class II endocytosis.\textsuperscript{48} A somewhat different set of peptides may be loaded compared with the classic route.\textsuperscript{49} Some studies show that class II recycling is most efficient, perhaps dependent on, class II molecules that are associated with cell surface CD74.\textsuperscript{50,51} Since we found that RPE cells are relatively deficient in CD74 (preliminary results), the alternative pathway may be much less active than in conventional APCs. In the mouse model of EAE induced with the myelin oligodendrocyte glycoprotein, it has been shown that li-deficient mice cannot present the encephalitogenic peptide, but are susceptible to

### Table 1. Coexpression of CD25 and FoxP3 on 3E9 T Cells after Culture on RPE Cells and/or Irradiated SPLs\textsuperscript{*}

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<thead>
<tr>
<th>Cultures</th>
<th>(−) Ag</th>
<th>(+) Ag</th>
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<tbody>
<tr>
<td>SPL</td>
<td>0.3 ± 0.4</td>
<td>3.6 ± 2.4</td>
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<tr>
<td>RPE</td>
<td>0.6 ± 0.5</td>
<td>2.5 ± 0.8</td>
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<tr>
<td>RPE and SPL</td>
<td>1.7 ± 1.1</td>
<td>4.5 ± 1.9</td>
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\textsuperscript{*} Percentage of \textsuperscript{V}β\textsubscript{10}\textsuperscript{+} lymphocytes expressing CD25 and FoxP3.
challenge with the pathogenic peptide of MOG. In any case, the functional outcome in our studies is that the alternative pathway still does not provide for degradation of intact β-gal that would lead to loading of the specific β-gal peptide.

Finally, the occasional peptide will dissociate from surface class II, providing a brief opportunity for exogenous, extracellular peptide to load. Given the lack of evidence for the classic and alternative pathways in RPE, this pathway may contribute significantly. Proteases in the extracellular space in inflamed areas could degrade proteins to fragments able to load into RPE cell surface class II MHC whose expression was induced by the proinflammatory cytokine milieu. In any case, the route to class II occupancy does not alter our conclusions regarding induction of an anergic state. Regardless of the route taken by the peptide onto class II, the class II–peptide complex contributes to the unresponsiveness of T cells that recognize Ag on RPE cells.

Recognition of retinal Ags in the MHC class II of RPE was also limited by other properties of the RPE. First, relatively little class II was expressed and only under specific conditions. Second, loading peptides into the class II molecules of the RPE required providing the peptides exogenously. Despite the enormous phagocytic ability of RPE cells, we found that they had no detectable ability to process and present Ag when supplied as the whole molecule. The loading of class II molecules with peptides, whether self or foreign, may be compromised by the low levels of CD74 expression. When care was taken to eliminate contaminating APCs from the T-cell preparations, no evidence for Ag processing was found. This is a factor that further distinguishes our studies from those of other laboratories where the T cells were supplied as unpurified and uncharacterized populations of spleen or LN cells. Elsewhere, we showed that the intracellular processing pathways that lead to expression of endogenous peptides in MHC class I are active, making a β-gal-expressing RPE cell line susceptible to β-gal-specific cytotoxic T lymphocytes (CTLs). The inability of the RPE to load Ag requiring processing into MHC class II was clearly unrelated to limits on phagocytosis. RPE cells have a prodigious ability to phagocytose and degrade photoreceptor cell outer segments and ingest a wide variety of particulates in vitro, including the Ag-coated latex beads that we found to be potent sources of Ag for DCs. Occupancy of the peptide-binding groove of class II molecules by invariant chain (Ii, CD74), while in transit from the endoplasmic reticulum (ER), preserves the ability of those class II molecules to be loaded with exogenous peptides in the endosomal pathway, and chaperones the class II molecules through delivery to the cell surface. In preliminary studies, we found that CD74 (invariant chain) was expressed at low levels in RPE. These low levels could permit loading of class II with self-peptides, limiting the occupancy of MHC class II by peptides from exogenous Ags. It could also compromise the maturation and transfer of peptide-loaded class II to the cell surface, as well as recycling pathways.

The ability of these immortalized RPE cells to inhibit T-cell activation was not limited to Ag-specific naïve 3E9 T cells. The activation of LN cells from wild-type B10.A mice by concanavalin A (ConA), as measured by proliferative responses, was also inhibited in cocultures of LN cells that contained RPE supernatant alone, or in direct T cell-RPE monolayer contact, or in a split-well configuration (data not shown). Significant inhibition was found in all cases, consistent with other reports of the effects of soluble factors and contact-mediated effects of various RPE cell lines and cultures on mitogen-activated LN cells. Assays of cytokine production showed that IL-2 and IFN-γ secretion production were significantly inhibited. Control adherent cell cultures, including VA-13 and Cos-7 cells (data not shown), had little effect on cytokine synthesis by the ConA-activated LN cells.

In summary, RPE cells were found to present an extracellular peptide on MHC class II to naïve CD4 T cells in a manner that led to the induction of anergy in the T cells. The anergic T cells did not display immunoregulatory activity when tested with naïve T cells of the same specificity. The ability of the RPE to induce T-cell unresponsiveness in this manner may contribute to retinal immune privilege.

Acknowledgments

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