# RPE Cells Resist Bystander Killing by CTLs, but Are Highly Susceptible to Antigen-Dependent CTL Killing

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**Purpose.** Retinal pigmented epithelial (RPE) cells maintain the blood–retinal barrier, sustain retinal photoreceptor cell health and function, and may play a role in ocular immune privilege. If RPE immunomodulatory activities were antigen specific, their expression would require antigen presentation. In a study of antigen processing and major histocompatibility complex (MHC) class I–restricted presentation by RPE cells, the cells' sensitivity to the activity of cytotoxic T lymphocytes (CTLs) was determined.

**Methods.** RPE was cultured, with and without proinflammatory cytokines and antigen, followed by the addition of β-galactosidase (β-gal)–specific CTLs. Cytotoxic activity was measured by the CTL-dependent activation of caspase-3 in the RPE. Sensitivity to the CTLs was used to evaluate the activity of pathways of antigen processing and presentation using an antigen (β-gal) that was either applied to or expressed in RPE.

**Results.** RPE cells were sensitive targets for activated CTL-mediated killing in vitro only if prepulsed with cognate peptide, or if β-gal-expressing RPE was pretreated to induce upregulation of immunoproteasome. Activated CTLs induced apoptosis in RPE within 3 hours of coculture with antigen-positive RPE monolayers. Application of CTLs in a resting state to antigen-positive RPE led to their activation in the absence of exogenous antigen-presenting cells (APCs). This antigen-dependent activation and killing required 24 hours of co-incubation of RPE with resting CD8 T cells specific for β-gal. Although RPE cells are highly phagocytic, functional evidence for processing that allowed phagocytosed antigens to load into class I MHC was not detected. RPE was minimally sensitive to bystander killing by activated CTLs.

**Conclusions.** Although there are many reports of T-cell inhibition by RPE, we found that CTLs efficiently killed RPE cells by induction of apoptosis in an antigen-dependent manner. The survival of RPE in the face of extensive CTL destruction of adjacent photoreceptor cells in vivo appears to be based on their insensitivity to injury via bystander mechanisms. (*Invest Ophthalmol Vis Sci.* 2006;47:5385–5394) DOI:10.1167/iovs.06-0636

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Supported by National Eye Institute Grants EY014176 and EY013623 (DAF) and EY011542 (DSG), an unrestricted grant to the Department of Ophthalmology from Research to Prevent Blindness, and the Minnesota Lions Clubs.

Submitted for publication June 12, 2006; revised July 21, 2006; accepted September 14, 2006.

Disclosure: **D.S. Gregerson**, None; **K.L. Lew**, None; **S.W. McPherson**, None; **N.D. Heuss**, None; **D.A. Ferrington**, None

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Two critical interfaces form the blood–retinal barrier (BRB): the tight junctions of the retinal vascular endothelium and the retinal pigment epithelium (RPE). The apical surface of the RPE lies inside the BRB and interacts with retinal photoreceptor cells (PCs). The RPE basal surface is outside the BRB and separates the retina from the choriocapillaris, a heavily vascularized network of highly fenestrated choroidal blood vessels. As part of its normal function, the RPE engulfs and digests the tips of the PCs in a diurnal cycle.

Several autoantigens that are targets of immunopathogenic autoimmune responses are found in or around retinal PCs, including arrestin and interphotoreceptor retinoid binding protein.[1] Expression of a foreign protein in PCs as a transgene with the retinal arrestin or rhodopsin promoters also serves as a target for retinal autoimmune disease.[2,3] Evidence of immunologic tolerance to retinal antigens (Ags) has been reported by several laboratories.[4,5] Undoubtedly, central, thymic mechanisms contribute to tolerance of some of these Ags,[6] including several PC Ags.[5,7] However, evidence of peripheral mechanisms has also been found, consistent with interactions between T cells and antigen-presenting cells (APCs) bearing Ag derived from retina.[8] Because naive T cells have not been found in immunologically quiescent retina, the interaction that leads to recruitment of regulatory T cells may occur outside the BRB.

Given the prodigious quantity of outer segment tips processed daily by the RPE, these Ags may be made available via presentation in major histocompatibility complexes (MHCs) expressed on the basal surface of the RPE, outside the BRB. Consequences of such expression could include the sensitization of the RPE to attack by T cells with specificity for outer segment Ags and generation of a tolerance-inducing signal resulting from the presentation of Ag by a "nonprofessional" APC. One of the well-established paradigms of peripheral tolerance is the presentation of Ag in the absence of danger signals, which may lead to unresponsiveness, as well as induction of regulatory T cells.[9,10] Presentation by nonprofessional APCs, such as RPE cells, induced to express MHC by local inflammation may promote the local induction of peripheral tolerance. In this study, the Ag-presenting activities of RPE for MHC class I–restricted CD8 T cells were examined in vitro. We asked whether the RPE can present intracellular Ag or cross-present exogenous Ag to CD8 T cells and whether the RPE becomes sensitive to attack by CD8 T cells.

## Materials and Methods

### Mice

RPE cells, T cells, and APCs were from B10.A mice. 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.

### RPE Cultures

Murine RPE cells were harvested, immortalized, and cultured as previously described.[11] Culture conditions for RPE characterization and

assays included seeding cells in 12-, 24-, or 96-well plates ($5 \times 10^5$–$1 \times 10^4$ cells/well, respectively), in a T75 flask ($1 \times 10^6$ cells), or on glass chamber slides (BD Falcon, Bedford, MA) with RPMI growth and assay medium containing RPMI 1640, 0.1 mM nonessential amino acids, 0.3% glucose, 2 mM glutamine, 100 μg/mL pyruvate, 5 μM 2-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum. Cytokines were added as will be described in the Results section. Cells were grown to near confluence before characterization or use.

## Transduction of RPE Cell Lines with β-Gal

After immortalization, RPE cells were transduced with MFGnlsLacZ, a MoMLV-based retrovirus vector produced by the ψ CRIP packaging cell line (generously provided by Richard Mulligan, Harvard University, Boston, MA). The vector encodes a nuclear-localized form of *E. coli* β-gal. Details of retroviral transduction of cultured cells were described previously.[12] β-Gal activity in the transduced cells was confirmed by X-gal staining of RPE cultures. Quantitation of expression was assayed using the β-gal substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) substrate, measured spectrophotometrically, as previously reported.[8] Purified *E. coli* β-gal (Prozyme, San Leandro, CA) was used as the standard.

## Ags and Immunizations

A synthetic peptide of β-gal recognized by H-2L$^d$-restricted T cells (peptide TPHPARIGL) was made in the University of Minnesota Microchemical Facility. Purified β-gal was purchased from Prozyme. Bovine rod outer segments (ROS) were prepared as previously described.[13] For the conjugation of bovine ROS with β-gal, 3.2 mg of ROS, and 3.2 mg of β-gal were mixed with 5 mM disuccinimidyl suberate (DSS; Pierce Biotechnology, Rockford, IL) in 1 mL of PBS (pH 7.5) for 30 minutes at RT. The reaction was quenched with 50 μL of 1 M glycine. Conjugated ROS were separated from reactants by centrifugation and two washes. β-Gal was quantitated in a slot-blot immunoassay, as we have recently described,[14] with purified β-gal used for the standard curve, and a monoclonal Ab to β-gal (Sigma-Aldrich, St. Louis MO). Total protein was determined by the BCA protein assay kit (Pierce Biotechnology), with bovine serum albumin used as the standard. The conjugate was found to contain 72 ng β-gal/μg total ROS protein. This estimate may be low, as some β-gal may have lost Ab-binding activity due to cross-linking.

## Generation of the β-Gal-Specific CD8 T-Cell Line (β4)

CD8 T cells specific for an immunodominant, L$^d$-restricted epitope of β-gal (TPHPARIGL)[15,16] were prepared from splenocytes harvested from vaccinia virus VSC 56-vaccinated female B10.A mice, as previously described.[8] Use of the virus, and generation and maintenance of the T cells were as previously described.[17] Ag-responsive T cells were restimulated on a 10- to 14-day cycle with 10 to 20 U/mL IL-2 added 24 hours after each restimulation with β-gal peptide-pulsed, irradiated splenic APCs. This oligoclonal CD8 T-cell line was designated β4. When activated, it has CTL activity.

## Cell Purification

Resting β4 CD8 T cells were isolated from cultures by positive selection for Thy 1 (MACS and LS columns; Miltenyi Biotec, Inc., Auburn, CA) as previously described.[2,8] Dendritic cells (DCs) were isolated by positive selection of pooled bone marrow, spleen, and lymph nodes for CD11c$^+$ cells (MACS; Miltenyi Biotec, Inc.).

## Western Immunoblot Analysis

Western immunoblot analysis was performed as previously described.[18] Proteasomal subunits were detected with primary antibodies to α6/C2, β1, β5, PA28α, LMP2, or LMP7 (Affinity Bioreagents, Golden, CO). Antibodies were used at 1:1000 dilution. Goat anti-mouse or anti-rabbit alkaline phosphatase-conjugated secondary antibody (1:5000) was used in conjunction with the substrate 5-bromo-4-chlor-3′-iodolyl phosphate p-toluidine/nitro blue tetrazolium chloride (BCIP-NBT) to visualize the immune reaction. Images were captured (Fluor-S MultiImaging System; BioRad, Hercules, CA), and relative content of specific proteins was determined by densitometric analysis of immune reactions (Sigma Scan; SPSS, Chicago, IL).

## Measurement of CTL-Induced Apoptosis of RPE Cells

Select groups of RPE cells grown in six-well plates were incubated with no cytokines, IFN-γ (25–100 U/mL), TNF-α (0.5–4 ng/mL), or a combination of both cytokines for 2 to 5 days before the experiments. To load cell surface class I with cognate peptide, RPE cells were pulsed for 1 hour with 1 μM β-gal peptide or medium only. RPE cells were washed and then cultured with CTLs (from 0 to $8 \times 10^5$/well) for 2 to 3 hours. At the indicated times, the cells were harvested by trypsinization, washed, resuspended in polypropylene tubes, and returned to the incubator for 30 minutes. Apoptosis was determined by staining with an antibody specific for the activated form of caspase-3, or by analyzing for annexin V binding (BD-PharMingen, San Diego, CA). Activation of caspase-3 and -6 was also measured by hydrolysis of specific fluorogenic peptide substrates. These included the GDEVDGI (caspase-3) and LVEIDNG (caspase-6) substrates (PhiPhiLuxG2D2 and CyToxiLux Kits; OncoImmunin, Gaithersburg, MD).

## Flow Cytometry

Staining of MHC class I (H-2K$^k$), CD25, and Thy 1 was done according to the manufacturer's recommendations (BD-Pharmingen). Staining for the activated form of caspase 3 and IFN-γ was performed by an intracellular staining protocol according to the manufacturer's recommendations. Annexin V binding was assessed as recommended by the manufacturer. After 20 minutes on ice, cells that were incubated with biotin-labeled Abs were washed once and resuspended in streptavidin conjugates. After 15 minutes, all cells were washed once and resuspended for flow cytometry (FACSCalibur with CellQuest software; BD Biosciences, San Jose, CA). CTLs were distinguished from RPE based on staining for Thy 1. Also, RPE cells are much larger than CTLs and were easily distinguished on scatterplots.

# Results

## Effect of IFN-γ and TNF-α on MHC Class I Expression on RPE Cells

CTL recognition of target cells requires surface expression of MHC class I containing cognate Ag on the target cell. We previously reported that MHC class I was upregulated on murine RPE cells after incubation in IFN-γ.[11] Flow cytometry confirmed the presence of a low level of class I on the surface of untreated, cultured RPE from B10.A mice (Fig. 1). On incubation with IFN-γ, class I expression was substantially elevated. The addition of TNF-α alone to cultured RPE cells did not appreciably increase class I expression. Incubation of RPE with IFN-γ plus TNF-α produced little or no increase over that induced by IFN-γ alone.

## Effect of CTLs on RPE Preincubated with the Target Peptide

To demonstrate that Ag-specific CTLs recognize a class I–restricted Ag in an Ag-dependent and T-cell-dose–dependent manner, RPE cells were incubated for 1 hour with or without a β-gal peptide (TPHPARIGL) before addition of graded numbers of activated, β-gal-specific CTLs (β4 T-cell line). These H-2L$^d$-restricted CTLs have been shown to be cytotoxic for

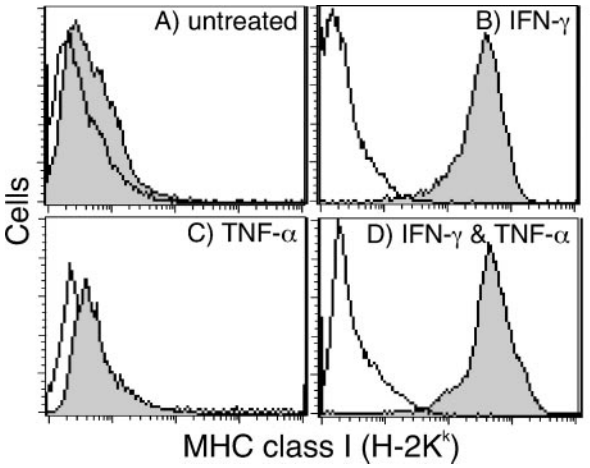


**FIGURE 1.** Expression of class I MHC on RPE was upregulated by incubation with the proinflammatory cytokine IFN-γ, but not by TNF-α. RPE monolayers were incubated with the indicated cytokines for 4 days and assayed for class I expression by flow cytometry. (**A**) Untreated RPE; (**B**) IFN-γ, 100 U/mL; (**C**) TNF-α, 1 ng/mL; and (**D**) TNF-α, 1 ng/mL + IFN-γ, 100 U/mL. RPE cells were grown to near confluence before the cytokines were added. Isotype control (*unfilled plot*); anti-H-2K$^k$ (*shaded plot*).

peptide-loaded P815 cells in vitro[11] and to be immunopathogenic for β-gal$^+$ tissues in transgenic mice in vivo.[2,8] Addition of CTLs to peptide-pulsed RPE led to increased intracellular levels of activated caspase-3 in the RPE, consistent with induction of apoptosis (Fig. 2). A dose response that correlated with the number of CTLs was found in the percentage of caspase-3–positive cells that resulted (Fig. 2). The low, endogenous

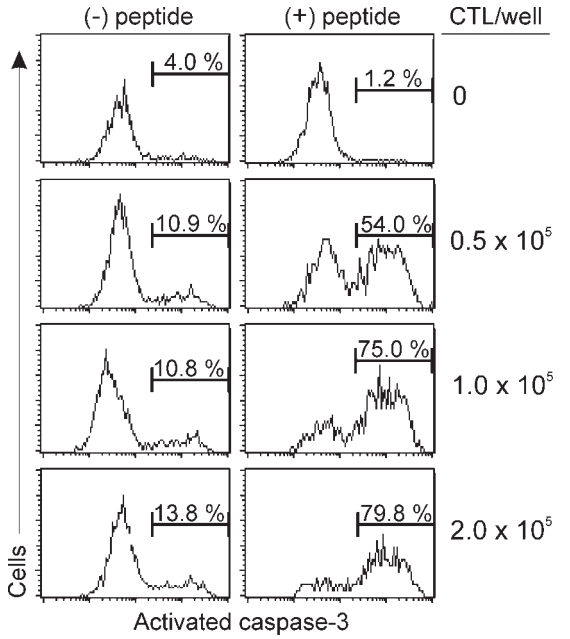


**FIGURE 2.** Activated CTLs recognize peptide-pulsed RPE in vitro. Graded numbers of activated CTLs were added to monolayers of RPE cells that were not pretreated with exogenous cytokines. RPE cells in the indicated wells were pulsed with 1 μM peptide and washed immediately before addition of CTLs. Cytotoxicity was measured by determining the activation of caspase-3 as detected by intracellular staining with an antibody specific for the activated form. The analysis was gated on the RPE, and Thy1$^+$ cells were excluded.

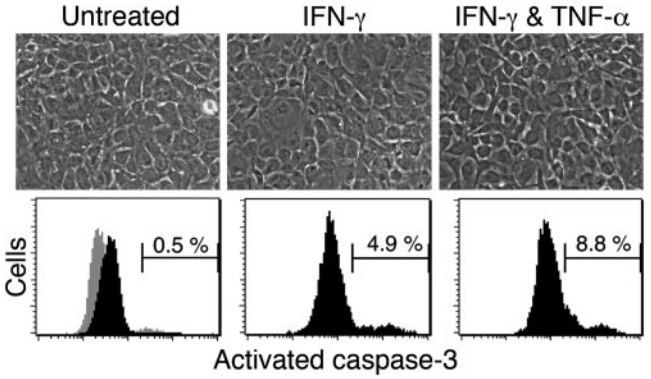


**FIGURE 3.** RPE cells express low levels of caspase-3, and modestly upregulate caspase-3 on treatment with proinflammatory cytokines. RPE cell confluent monolayers were untreated, treated with IFN-γ (100 U/mL), or treated with both cytokines (100 U/mL IFN-γ and 1 ng/mL TNF-α) as indicated for 4 days. No CTLs were added. After microscopy, the cells were collected and stained for the activated form of caspase-3 by flow cytometry. *Top*: phase-contrast microscopy of the cells revealed similar morphology under all culture conditions. *Bottom*: representative histograms of caspase-3 activation. The shaded profile indicates the isotype control antibody. $P = 0.057$ (IFN-γ-treated) and 0.003 (IFN-γ–and TNF-α–treated) relative to untreated controls.

level of class I is sufficient for CTL recognition when loaded with the exogenous β-gal peptide. The validity of the activated caspase-3 immunoassay as a measure of CTL killing of RPE cells is demonstrated later in this section.

Because several of the following experiments use RPE cells treated with cytokines to alter expression of molecules associated with Ag processing and presentation, it was necessary to determine whether these treatments alone alter the levels of activated caspase 3. RPE cells incubated without cytokines expressed a low level of endogenous caspase 3 activation (Fig. 3). RPE cells cultured with IFN-γ or a combination of IFN-γ and TNF-α were found to contain slightly elevated levels of the activated form of caspase-3 relative to untreated RPE (5.3% ± 3.2%, 8.5% ± 2.2%, and 0.4% ± 0.2%, respectively; mean ± SD of three trials; Fig. 3). These low levels of caspase 3 activity were well tolerated by the RPE based on their normal appearance and absence of appreciable cell death, even after 10 to 14 days in the presence of these cytokines.

## Involvement of IFN-γ–Induced Upregulation of MHC Class I in CTL Killing of RPE Cells

Incubation of RPE with IFN-γ or the combination of IFN-γ and TNF-α, for four days before peptide pulsing and addition of activated CTLs significantly elevated the levels of activated caspase-3 in a CTL- and peptide-dependent manner and led to destruction of the RPE monolayer (Fig. 4). Because the peptide is a known immunodominant epitope that can load directly into cell-surface MHC (H-2L$^d$), this experiment eliminates the upstream intracellular Ag processing and presentation pathways normally required for generation of cell surface MHC–peptide complexes. There was also substantial killing (49.4%) of peptide-loaded RPE that was not cytokine treated. Increasing class I expression by approximately 100-fold with cytokine pretreatment (Fig. 1) increased killing of the RPE by less than 2-fold. Although this could be a biologically significant increase, it appears that the low cell-surface level of class I on untreated cells was not the rate-limiting step for cytotoxicity.

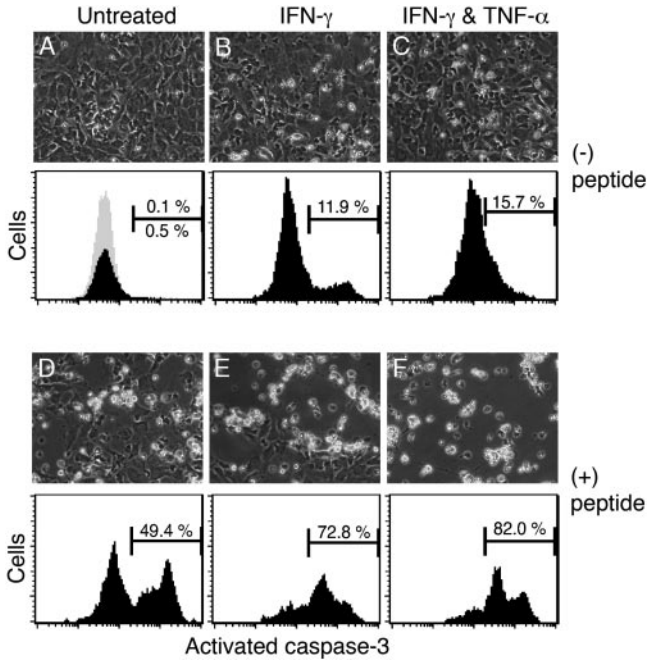


**FIGURE 4.** CTL killing of RPE targets is promoted by pretreatment of the RPE with proinflammatory cytokines. RPE cells ($3 \times 10^5$/well) were untreated (**A**, **D**), treated with 100 U/mL IFN-γ (**B**, **E**), or treated with both cytokines (**C**, **F**) for 4 days before addition of activated CTLs. Samples (**A–C**) were not pulsed with peptide. Samples (**D–F**) were pulsed with 1 μM peptide. All samples received CTLs ($1 \times 10^5$/well) and were harvested 4 hours later. (Number on *top* of the bracket indicates the percentage of cells with activated caspase-3; the number *below* the bracket is the isotype control.) $P < 0.05$, for experiment (**D**) versus (**E**). $P < 0.01$, for experiment (**D**) versus (**F**).

## Use of Caspase-3 Activation to Assess CTL Killing of RPE

Transfer of lytic granules from CTLs into target cells after recognition of cognate Ag on the target is an important mechanism by which CTLs kill cells. Proteases in the lytic granules lead to activation of caspases-3 and -6, and subsequent apoptosis of the target cell.[19] We have shown that increased intracellular levels of the activated form of caspase-3 correlates with TUNEL staining in P815 targets.[11] To validate further the applicability of this assay for RPE cells, CTL-induced increases in levels of activated caspase-3, annexin-V binding, and hydrolysis of caspase-3 – and -6–specific substrates in IFN-γ-treated RPE were examined. All these additional measures of apoptosis were elevated specifically in response to peptide-dependent CTL attack on the RPE (Fig. 5). The following studies were performed with flow cytometry used to detect activation of caspase-3 as the principal measure of CTL recognition of Ag on RPE.

## Ag Dependency of CTL Killing of RPE Cells

We previously reported that RPE expressed Fas, especially after cytokine pretreatment, and was sensitive to killing after ligation by anti-Fas Ab.[11] Furthermore, CTLs are widely found to express FasL. Regardless, killing by CTL in our assays was substantially Ag dependent (Table 1), especially in the absence of exogenous IFN-γ or TNF-α. Evidence for bystander killing was not appreciable until TNF-α in the RPE pretreatment was raised to 4 ng/mL or higher.

## CTL Recognition of Endogenous β-Gal

To determine whether RPE can process β-gal and generate the β-gal peptide required to activate the CTLs, a β-gal-expressing RPE clone was prepared and tested as CTL targets. Expression of β-gal in the transduced RPE cells was detected by cleavage of the X-gal substrate, leading to deposition of blue product in the nuclei, consistent with the nuclear localizing signal on the recombinant β-gal (Fig. 6). Spectrophotometric assays based on *o*-nitrophenyl β-D-galactopyranoside cleavage, a soluble col-

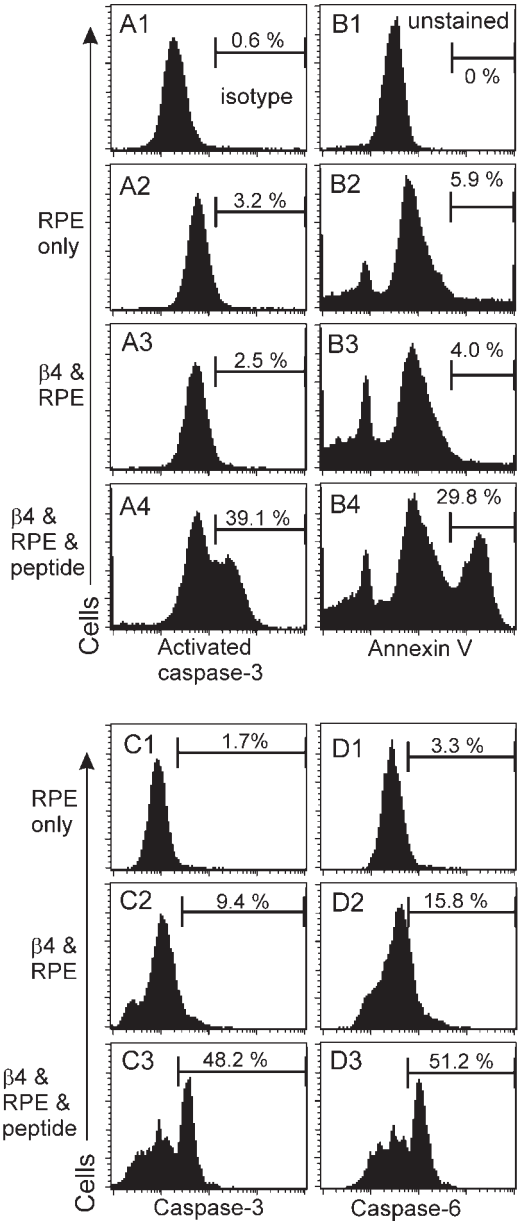


**FIGURE 5.** Intracellular staining for the activated form of caspase-3 correlates with other measures of the induction of apoptosis. RPE cell monolayers were treated with IFN-γ (100 U/mL) for 7 days. Selected wells were pulsed with cognate peptide as indicated. β4 CTLs ($3 \times 10^5$/well) were added as indicated. (**A**) Immunostaining with antibody specific for the activated form of caspase-3 using an intracellular staining protocol. (**B**) Binding of annexin V. (**C**) Hydrolysis of the caspase-3–specific substrate. (**D**) Hydrolysis of the caspase-6–specific substrate. The wells were harvested for analysis 3 hours after the CTLs were added.

**TABLE 1.** Peptide Dependency of Killing of RPE by CTLs

| RPE Treatment | RPE | RPE + CTL* | RPE + CTL + Pep† |
|---|---|---|---|
| Untreated | | | |
| Exp. 1 | 4.1 ± 1.3‡ | 6.9 ± 4.8 | 37.4 ± 3.5 |
| | | $P > 0.1$‖ | $P < 0.001$¶ |
| Exp. 2 | 2.0 ± 1.6 | 7.7 ± 1.0 | 44.0 ± 4.2 |
| | | $P > 0.05$ | $P < 0.0001$ |
| IFN-γ§ | | | |
| Exp. 1 | 5.3 ± 3.2 | 9.7 ± 0.4 | 58.3 ± 13.4 |
| | | $P > 0.1$ | $P < 0.001$ |
| Exp. 2 | 5.6 ± 0.6 | 9.8 ± 1.8 | 71.2 ± 19.3 |
| | | $P < 0.05$ | $P < 0.01$ |
| IFN-γ & TNF-α | | | |
| Exp. 1 (0.5 ng/mL)§ | 8.5 ± 2.2 | 13.8 ± 2.7 | 82.5 ± 6.2 |
| | | $P > 0.05$ | $P < 0.0001$ |
| Exp. 1 (4 ng/mL)§ | 14.0 ± 2.2 | 38.1 ± 1.1 | 88.9 ± 9.7 |
| | | $P < 0.01$ | $P < 0.001$ |

* CTLs were used at $2 \times 10^5$/well.

† Peptide TPHPARIGL was incubated with RPE for 1 hour. The RPE was then washed before addition of CTLs.

‡ Percentage of cells positive for the activated form of caspase-3; mean ± SD. All from groups of two to four samples/experiment.

§ IFN-γ was applied at 100 U/mL; TNF-α was applied at the indicated concentrations. Cytokines were applied to near-confluent RPE monolayers for 4 days before assay with the CTLs.

‖ *P* (*t*-test) comparing RPE only with RPE + CTL.

¶ *P* (*t*-test) comparing RPE + CTL with RPE + CTL + peptide.

orimetric β-gal substrate, showed an expression level of 78 ng/$10^6$ RPE cells.

Caspase 3 activation by CTLs was measured in untreated β-gal$^+$ RPE in either the absence (control) or presence of CTLs (Fig. 7). In untreated RPE, the histograms of activated caspase-3 were minimally elevated, indicating that the CTLs were largely ignorant of Ag expressed in untreated β-gal$^+$ RPE. However, RPE pretreatment with IFN-γ, TNF-α, or a combination of IFN-γ and TNF-α before exposure to CTLs produced a substantial increase in caspase 3 activation (Fig. 7). Thus, IFN-γ or TNF-α pretreatment substantially promoted CTL recognition of peptide generated from endogenous β-gal and susceptibility to cytotoxicity.

## Role of Upregulation of Immunoproteasome in CTL Killing of β-Gal$^+$ RPE

Results shown in Figure 2 indicate that endogenous levels of class I expression were sufficient for recognition of exogenously loaded β-gal peptide, indicating that the level of class I on the surface was not the limiting step in providing adequate signal for CTL-induced killing of β-gal–transduced RPE. Instead, an event upstream of class I presentation is implicated. One critical upstream event is the proteasomal degradation of intracellular proteins to produce antigenic peptides.[20] Exposure to selected cytokines upregulates a specialized form of proteasome, the immunoproteasome,[21] which is more efficient than constitutive proteasome at generating immunodominant peptides.

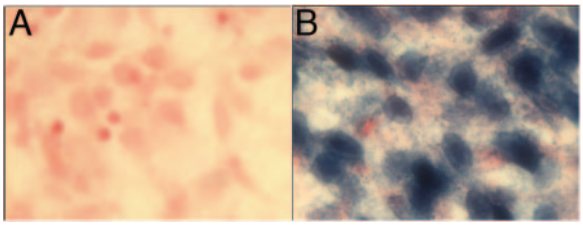


**FIGURE 6.** Expression of β-gal in retrovirally transduced RPE cells. Control RPE cells (**A**) and transduced cells (**B**) were incubated with a colorimetric β-gal substrate (Bluo-gal; Invitrogen, Carlsbad, CA), to visualize the blue staining resulting from hydrolysis of the substrate by the β-gal enzyme.

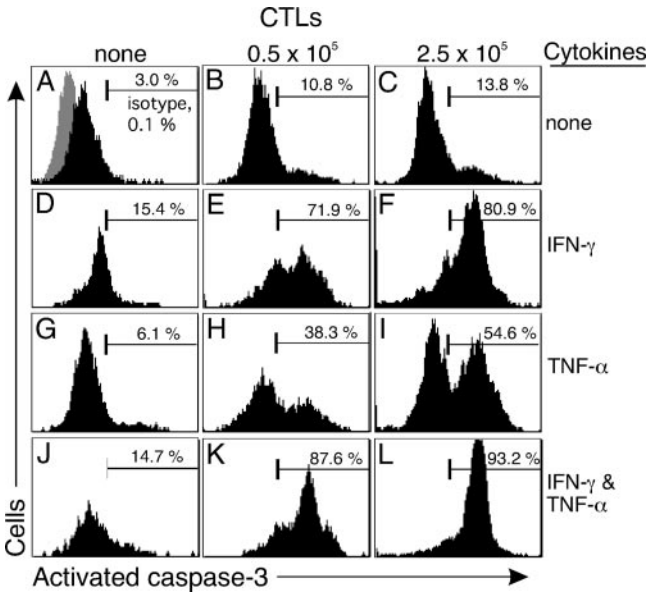


**FIGURE 7.** CTL-induced apoptosis in RPE transduced with β-gal requires cytokine pretreatment of the RPE. Monolayers of β-gal$^+$-RPE cells were untreated (**A–C**), treated with 100 U/mL IFN-γ () (**D–F**); treated with TNF-α (1 ng/mL) (**G–I**); or treated with both cytokines (**J–L**) for 4 days before addition of the indicated number of preactivated CTLs. Caspase 3 activation detected by flow cytometry was used to assess induction of apoptosis in the RPE resulting from CTL recognition. The isotype control is shown by the shaded plot in (**A**). $P > 0.1$: comparing (**A**) and (**B**); $P > 0.05$: (**A**) versus (**C**); $P < 0.05$: (**D**) versus (**E**), (**G**) versus (**H**), and (**J**) versus (**K**); $P < 0.01$: (**D**) versus (**F**), (**G**) versus (**I**), and (**J**) versus (**L**).

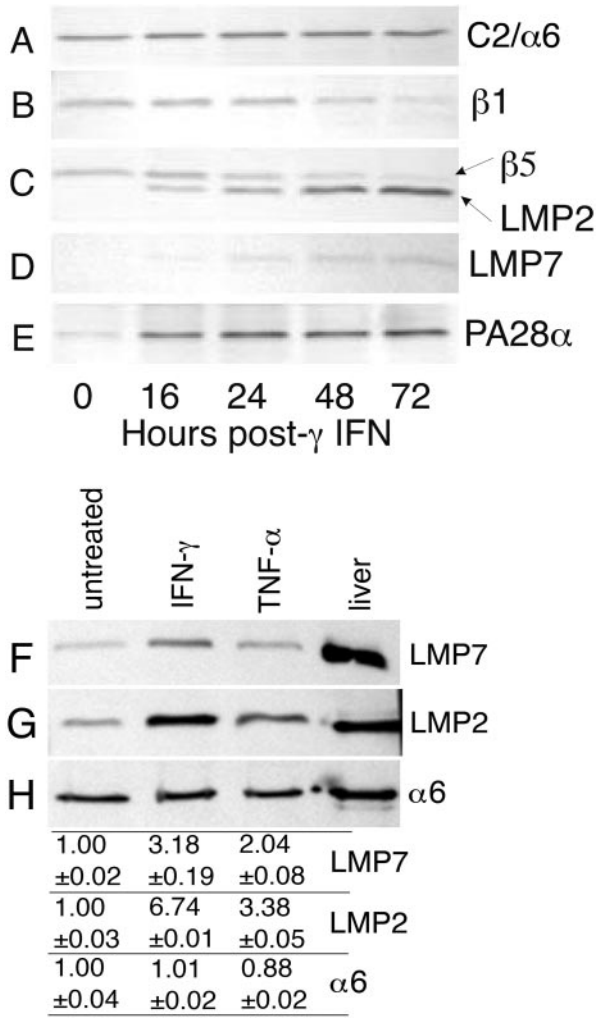


**FIGURE 8.** Induction of immunoproteasome in RPE by IFN-γ and TNF-α. *Top*: Western immunoblots of RPE cell lysates showing INF-γ–induced alteration of proteasomal subunits including α6 (**A**), β1 (**B**), β5 and LMP2 (**C**), LMP7 (**D**), and PA28 (**E**). Lanes correspond to protein (20 μg) from untreated RPE (0 hours), and RPE treated with IFN-γ for 16, 24, 48, and 72 hours. IFN-γ (100 U/mL) was applied at time 0. *Bottom*: immunoblots showing upregulation of immunoproteasome subunits after incubation in TNF-α (1 ng/mL) relative to IFN-γ (100 U/mL). Subunits shown include LMP7 (**F**), LMP2 (**G**), and α6 (**H**). 20S proteasome purified from liver was used as a control (0.05 μg). Densitometry readings of immunoblots for (**F**), (**G**), and (**H**) were normalized to levels in untreated cells and shown directly *below* the blot.

The relative concentration of proteasomal subunits associated with either the immunoproteasome (LMP2, LMP7) or constitutive proteasome (β1, β5) was measured in RPE cell homogenates by Western immunoblot with subunit-specific antibodies (Fig. 8). A two- to sixfold increase in immune reaction for LMP2 and LMP7, two subunits associated with the immunoproteasome, was observed in RPE treated with IFN-γ (Fig. 8, top). Furthermore, there was a corresponding decrease in content of the β1 and β5 subunits that are associated with the constitutive proteasome. The immune reaction of the C2/α6 subunit, which is present in both the immunoproteasome and constitutive proteasome, was also measured. The composition of the α-subunits is not altered by changes in cell conditions and accurately reflects the total proteasome content. There was little change in staining for the α6 subunit, consistent with the replacement of constitutive proteasome with immunoproteasome in IFN-γ–treated RPE. In addition to proteasomal subunits, we also monitored the cellular content of the α subunit of a regulatory complex (PA28) that increases the efficiency of the immunoproteasome in generating immunogenic peptides. Treatment with IFN-γ caused upregulation of the PA28 regulatory complex. No difference in upregulation of the immunoproteasome for IFN-γ doses between 25 and 150 U/mL was found (data not shown).

The critical role of the immunoproteasome, compared with class I expression, was revealed by studies in which RPE cells were incubated with TNF-α. To test for the significance of immunoproteasome degradation of endogenous β-gal in the sensitivity of RPE to the CTLs, we took advantage of our observation that treatment of RPE with TNF-α induced immunoproteasome synthesis (Fig. 8, bottom) without increasing MHC class I on the cell surface (Fig. 1). CTL killing of β-gal$^+$ RPE pretreated with TNF-α suggests upregulation of immunoproteasome and not the concentration of class I MHC, as the rate-limiting step in CTL recognition of RPE-generated Ag. These results demonstrate that endogenous β-gal is degraded via immunoproteasomes, which was detected by CTL killing, and that generation of the immunoproteasome-dependent peptide of β-gal is a limiting step.

## Effect of Incubation with RPE on Cytotoxicity of Resting CD8 T Cells

The experiments above were performed with activated CTLs. Numerous studies have shown that RPE cells inhibit T-cell activation in vitro.[22,23] Most prior studies were performed with CD4 T cells, or with unseparated cells from lymph nodes or spleen. To examine the effects of RPE on activation of resting CD8 T cells, β-gal$^+$ RPE or control RPE were incubated for 4 days with IFN-γ or IFN-γ+TNF-α. The indicated RPE cultures were pulsed with β-gal peptide for 1 hour and washed immediately before addition of resting β-gal-specific β4 T cells. No APCs were added. Resting T cells were recovered from cultures that had been stimulated 2 weeks earlier with Ag-pulsed splenic APCs, and maintained in medium containing IL-7 and -2. RPE/CTL cocultures harvested after 4 and 6 hours showed no evidence of killing in an analysis for caspase-3 activation, nor by microscopy (data not shown). At 24 hours, substantial killing was found in cultures of normal RPE that were treated with cytokine and peptide-pulsed, and also in cultures that contained cytokine-treated β-gal$^+$ RPE (Fig. 9). RPE cells were sensitive to attack by resting CTLs if they expressed endogenous β-gal or acquired Ag via peptide loading of cell surface class I. In the absence of cognate peptide, the resting T cells did not induce caspase-3 activation.

## Cross-Presentation of Exogenous Ag

Because RPE cells phagocytose large amounts of PC ROS, they are in a position to process and present known, immunopathogenic self-Ags. If peptide from these molecules were presented in class I under conditions that promoted unresponsiveness, RPE could play an important role in maintaining peripheral tolerance. To do so would require that RPE be capable of cross-presentation—the loading of peptides derived from exogenous Ag into class I MHC. Functional assays show that DCs are capable of cross-presentation.[24] If this pathway were active in RPE, cross-presentation of exogenous Ag could be detected by the targeting of these RPE by β-gal-specific CTLs. This possibility was explored by providing β-gal in several forms, particulate and soluble, to RPE. Purified DCs served as the positive control.

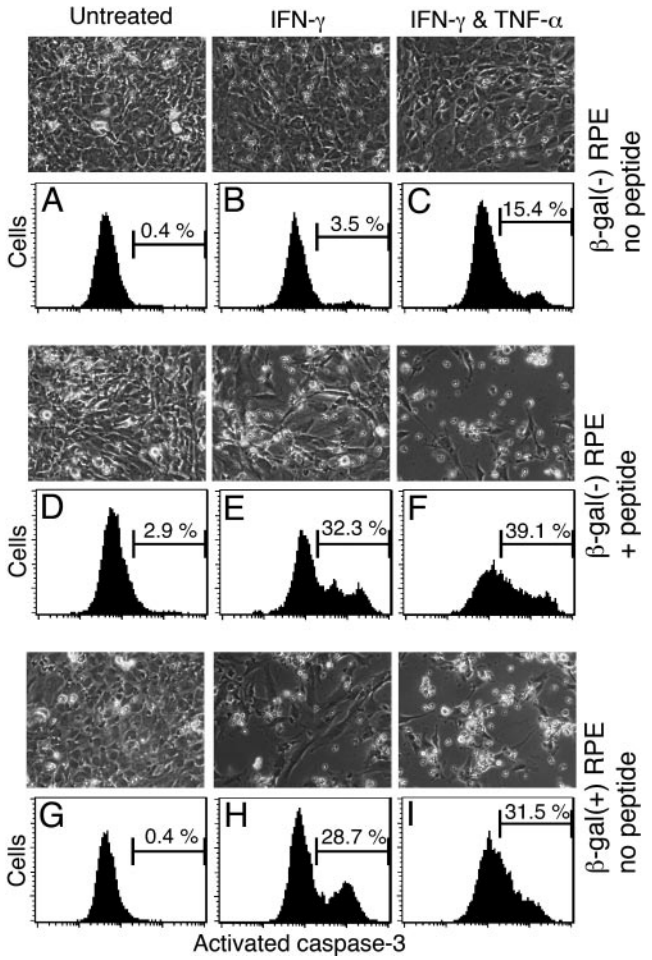


**FIGURE 9.** Application of resting CTLs to antigen-expressing or peptide-coated RPE monolayers leads to activation of the CTLs and attack on the RPE after 24 hours of coculture. RPE cells were untreated (**A**, **D**, **G**), treated with 100 U/mL IFN-γ (**B**, **E**, **H**), or treated with both cytokines (**C**, **F**, **I**) for 4 days before addition of resting β4 CTLs. Samples (**A–C**) were normal RPE cells without exogenous peptide; samples (**D–F**) were normal RPE cells with peptide pulsing, and samples (**G–I**) were β-gal$^+$ RPE cells. All samples received resting CTLs; no exogenous APCs were added. Photomicrographs above the histograms show the corresponding cultures 24 hours after addition of β4 T cells. (**A**), (**D**), and (**G**) were not different ($P > 0.1$) from controls without T cells. $P < 0.05$ (**D**) versus (**E**) or (**F**), and (**G**) versus (**H**) or (**I**), were significant.

Because the background level of activated caspase-3 in cultured DCs is relatively high, it was not possible to use caspase-3 activation to measure antigen recognition by CTLs. Instead, H-2L$^d$–restricted peptide presentation by DC to CD8 T cells was based on detecting the activation of resting CTLs, with flow cytometry used to monitor increases in intracellular IFN-γ levels and cell surface CD25, markers of T-cell activation. β-Gal was applied to the DCs in several forms; as the cognate peptide, soluble full-length β-gal protein, and β-gal$^+$ ROS. We observed upregulation of both IFN-γ and CD25 after addition of any form of β-gal to the T cell/DC cultures, confirming degradation of exogenous β-gal by proteasomes in these professional APCs, and its cross-presentation (Fig. 10). Conversely, no consistent CTL killing of RPE after incubation with soluble β-gal protein or β-gal$^+$ ROS was found. A wide range of doses of β-gal$^+$ ROS was tested (data not shown). Similar results were found in other experiments using β-gal–coated carboxylate beads or β-gal$^+$ ROS isolated from transgenic mice expressing β-gal in the photoreceptor cells (data not shown). In another control experiment, the β-gal–conjugated bovine ROS and β-gal coated beads were also able to stimulate naive, β-gal–specific CD4 T cells when applied to DCs, further confirming their antigenic activity (data not shown).

## DISCUSSION

The immunomodulatory activities of RPE have been reported to include the ability to (1) induce apoptosis in lymphoid cells by FasL-dependent and independent mechanisms,[25–29] (2) phagocytose T cells,[30] (3) inhibit or alter[31] T-cell activation by soluble molecules, including $PGE_2$ and TGF-β[23,32] or cell-associated molecules,[22,23] and (4) evade immune recognition by low expression of MHC.[33] RPE has been reported to be relatively resistant to immune attack, including the application of activated, alloreactive T cells.[33] Results to date have largely explored the effects of several RPE populations, some uncharacterized, in conjunction with unpurified T cells, usually without phenotypic characterization, and of unknown specificity or function. There has been little attempt to rigorously exclude or systematically include BM-derived APCs. Most experiments have used polyclonal T-cell activators, with or without exogenous costimulation.

To bring greater definition to studies of RPE immunology, we used a defined system in which a specific T-cell population with a well-defined Ag[2] and a defined RPE line,[11] were used to examine T cell–RPE interactions. The initial studies explored the contributions of Ag-specific activities by asking whether RPE could process and present Ag, from intracellular or extracellular sources, to CTLs. We found very efficient CTL-induced killing of RPE cells after exogenous loading of an immunodominant peptide (TPHPARIGL) into cell surface class I. Further, class I presentation of the immunodominant peptide produced by cleavage of endogenously produced β-gal made RPE excellent CTL targets. Apoptotic cell death was induced via a caspase-dependent pathway based on the increased content of the cleaved (activated) forms of caspase-3 and -6, and other measures of apoptosis. No evidence of RPE cross-presentation of exogenous Ags was found. Resting CD8 T cells were activated by Ag-expressing or Ag-incubated RPE, and became cytotoxic for the RPE on which they were cultured; no evidence of T-cell inhibition was observed.

Comparing the extent of CTL killing after exogenous and endogenous loading of class I, in combination with cytokine pretreatment, allowed us to examine different components of the Ag-presentation pathway. Our results show that while class I levels are low in cultured RPE, they were sufficient to elicit targeted killing by CTLs when the peptide was loaded from an exogenous source. Incubation with cytokines upregulated class I, and the extent of killing was greater. In contrast with these results, RPE cells endogenously producing β-gal were targeted by CTLs only after conditioning with either TNF-α or IFN-γ.

Class I–restricted Ag presentation by a target cell encompasses a number of steps, including proteasome-mediated generation of antigenic peptides, transport of the peptides into the endoplasmic reticulum through the transporter associated with antigen processing (TAP), loading of peptides into class I, and translocation of class I to the cell surface.[34] Exposure of cells to inflammatory cytokines is known to enhance Ag presentation. Further, cytokines upregulate production of immunoproteasome, a specialized form of the proteasome that is more efficient at generating immunogenic peptides than the constitutively expressed proteasome. We probed for subunits

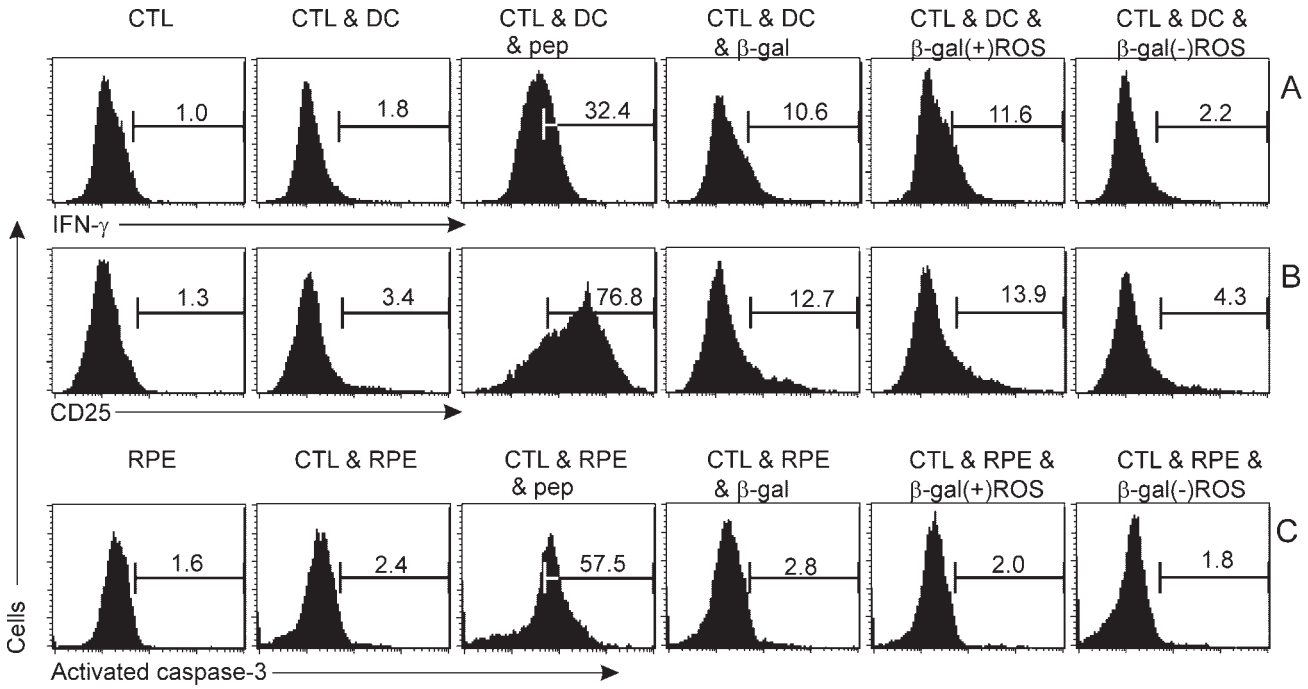


**FIGURE 10.** DCs cross-presented β-gal with resting β4 CTLs, but RPE did not. DCs were isolated and treated with 1 ng/mL GM-CSF (granulocyte/monocyte colony stimulating factor) for 2 days ($5 \times 10^4$/well). β-Gal protein (100 μg/mL) and ROS (62 μg/mL total protein) were applied at the time of DC plating. The β-gal$^+$ ROS contained 4.5 μg/mL β-gal. Immediately before addition of the T cells, the indicated DCs were pulsed for 1 hour with 1 μM β-gal peptide and washed. Resting CTLs ($1 \times 10^5$/well) were added and harvested 2 days later. Flow cytometry was used to detect upregulation of (**A**) IFN-γ production and (**B**) CD25 expression by the β4 CTLs. (**C**) The same Ags were similarly applied to IFN-γ–pretreated RPE cells, followed by activated β4 T cells. RPE cells were tested 2 days later for upregulation of activated caspase-3. The results are representative of three assays. The peptide-pulsed RPE ($P < 0.05$) was the only population in (**C**) in which there was significant activation of caspase-3. Increases in CD25 and IFN-γ (**A**, **B**) were significant ($P < 0.05$) in all experiments where β-gal was supplied to the DCs compared with controls without Ag.

of the immunoproteasome and found that it was expressed at a low level in untreated cells. With cytokine treatment, expression of immunoproteasome was significantly upregulated, as was cytotoxicity, suggesting that immunoproteasome cleavage of endogenous β-gal was a critical step in presentation of the immunodominant peptide from β-gal. The requirement for immunoproteasome can be explained by its greater efficiency for generating antigenic peptides. In addition, the constitutive form and the immunoproteasome exhibit discrete cleavage motifs and as a consequence, can generate different antigenic peptides.[35,36]

Cross-presentation of exogenous Ags is a well-known function of DCs, and was recently proposed to involve fusion between the ER and plasma membrane at sites of ingestion.[37,38] Although the details of this mechanism have been challenged,[39] the process does not exclude cross-presentation by other cells. Any pathway that delivers exogenous Ag to the cytosol provides for proteasomal cleavage that yields the desired sequence for class I occupancy. Cross-presentation of PC Ags acquired by RPE during outer-segment recycling could have two opposing consequences. First, it could deliver PC Ags into MHC on the basal surface of the RPE, where they would be accessible to circulating lymphocytes. This expression could make RPE susceptible to attack by T cells specific for those Ags. Second, it could allow RPE to provide on-going Ag-specific induction of peripheral tolerance to retina-specific Ags. Because RPE cells are postmitotic, susceptibility to immune attack based on MHC-restricted presentation of Ag could be very detrimental. The limitations on RPE Ag presentation and their susceptibility to killing by CTLs suggest that preservation of the RPE may be more important than their potential to induce peripheral tolerance. Their role in maintaining PC health may outweigh their potential function as APC.

We have found that intravenous adoptive transfer of activated β4 CTLs to mice expressing β-gal in retinal PCs is capable of destroying the entire PC layer.[2] The underlying RPE frequently exhibits vacuoles, but survives in the convalescent mice, yielding a confluent, apparently normal monolayer a few weeks later. Despite the vigorous attack on the neighboring cells, RPE in vivo are quite resistant to CTL bystander killing. For example, with CTL attack evoked by viral retinitis, the RPE remained intact, even though the PCs undergo CTL-mediated apoptosis.[40] In another study, direct subretinal injection of activated, alloreactive CTLs primed for class I Ag expressed in recipient eyes showed only focal defects in the RPE monolayer, limited to the injection site.[33] When added directly to the eye cups, the CTLs caused only minor RPE killing, only if eyes were pretreated with IFN-γ, consistent with our observation in cultured RPE that IFN-γ treatment causes upregulation of class I molecules and immunoproteasome.

The resistance of the RPE to CTL-mediated killing in vivo has been proposed to result, in part, from the ability of the RPE to induce apoptosis in CTLs via the Fas-mediated pathway[28] and a non-FasL pathway.[25,33] In contrast to RPE in vivo, we and others have reported that cultured human[41] or murine RPE cells do not express FasL.[42] Even with cytokine treatment, we observed no upregulation of FasL on RPE. However, Fas was constitutively expressed on cultured RPE, was upregulated by cytokines,[43,44] and sensitized the RPE to apoptosis via ligation with an anti-Fas antibody.[11] Untreated RPE cells were resistant to anti-Fas antibody–induced cell killing. These results are consistent with previous reports demonstrating that cultured RPE

cells are resistant to cell death via stimulation by anti-Fas antibody.[44] Using a variety of cytokines and TLR ligands, we have been unable to induce detectable levels of FasL on murine RPE (Gregerson DS, unpublished results, 2004–2006), consistent with a report that the resistance of RPE to alloreactive T cells applied to murine eye cups was not FasL-dependent.[33] Further, we found no evidence that T cell:RPE coculture induces apoptosis in T cells, even in prolonged cocultures (Gregerson DS, unpublished data, 2001–2006). Instead, survival of the T cells was greatly enhanced in those cocultures.

The critical role of RPE cells in maintaining PC health and function depends on the ability of RPE to survive in a challenging environment. To meet this challenge, RPE cells are particularly able to upregulate molecules associated with the inhibition of apoptosis.[45–47] Several of these molecules may contribute to the resistance of RPE to bystander CTL killing.

## *Acknowledgments*

The authors thank Tina Tran and Thien Sam for technical assistance.

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