T-Cell Suppression by Programmed Cell Death 1 Ligand 1 on Retinal Pigment Epithelium during Inflammatory Conditions

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Purpose. To determine whether retinal pigment epithelial (RPE) cells can inhibit in vitro T-cell activation during inflammatory conditions.

Methods. Primary cultured RPE cells were established from normal C57BL/6 mice. Target bystander T cells were established from normal splenic T cells with anti-CD3 antibodies. T-cell activation was assessed for proliferation by both examining [^{3}H]-thymidine incorporation and the production of interferon (IFN)γ or IL-17, as determined by ELISA. Expression of programed cell death 1 ligand 1 (PD-L1) on RPE or recombinant mouse IFNγ-pretreated RPE cells was evaluated using oligonucleotide microarray, RT-PCR, immune staining, and flow cytometry. Expression of programed cell death 1 (PD-1)$^+$ on target T cells was evaluated by flow cytometry. Anti-mouse PD-L1 or PD-L2 neutralizing antibodies or target T cells from PD-1 knockout donors were used for the assay.

Results. IFNγ-pretreated RPE greatly suppressed activation of bystander T cells, especially the IFNγ production by the target T cells (Th1 cells, but not Th17 cells) via direct cell contact. By examining cell surface candidate molecules, IFNγ-pretreated RPE expressed much higher levels of PD-L1 compared with the control nontreated RPE. Although primary RPE did not express the costimulatory molecule, expression of the molecule was induced on the surface of IFNγ-pretreated RPE. PD-L1$^+$ RPE in the presence of IFNγ selectively suppressed PD-1$^+$ T-cell activation. IFNγ-pretreated RPE in the presence of anti-PD-L1 neutralizing antibodies, but not anti-PD-L2, failed to suppress T-cell production of IFNγ. In addition, these RPE cells failed to suppress the production of IFNγ by CD4$^+$ T cells from PD-1 null donors.

Conclusions. Suppression of T-cell activation was obtained in cultures only when RPE expressed negative costimulators. Therefore, the authors propose that in vitro, Th1 cytokine-exposed ocular resident cells can express this molecule and it is this expression that causes the suppression of the bystander Th1-type cells. (*Invest Ophthalmol Vis Sci.* 2009;50:2862–2870) DOI: 10.1167/iovs.08-2846

During severe inflammatory conditions, immune tolerance mechanisms in the eye become bankrupt; that is, there is infiltration of inflammatory cells in the eye, with the subsequent intraocular inflammation often leading to blindness. To avoid the consequences of this inflammation, the eye employs an extensive array of mechanisms through which innate and adaptive immune effectors can be regulated, and even silenced. These mechanisms include an intraocular microenvironment (aqueous humor and vitreous fluids) that is rich in soluble immunoregulatory factors[1–3]; retina barriers that limit the infiltration of inflammatory cells and other molecules from the blood that are capable of mediating immunogenic inflammation[4]; and constitutive expression on the ocular parenchymal cells of the CD95 ligand can trigger apoptosis of the effector T cells.[5] These mechanisms help to explain why experimentally the eye has been shown to be an immune privileged site.

Ocular pigment epithelia (PE) of the retina have been identified as important participants in helping to create and maintain this immune tolerance.[6] The retinal PE (RPE) layer has a primary vision-related function that serves as a "light sink," quenching any unfocused light that might otherwise enter the eye and compete with the focused images that pass through the visual axis to the retina.[7] RPE also has secondary functions that are related in part to the region of the eye in which they are located. For example, RPE provides nutritive and biochemical support for the proper functioning of the rod and cone photoreceptor cells of the retina.[8] In addition, primary cultured RPE cells have been demonstrated to suppress T-cell activation in vitro, and it has been proposed that this immunoregulatory property of RPE is related to the intraocular suppression of immunogenic inflammation.[9–13]

We have previously reported that cultured iris PE cells (IPE) from the anterior segment in the eye suppress anti–CD3-driven T-cell activation in vitro by direct cell contact.[9–11] Primary cultured IPE cells uniquely express B7-2 (CD86) costimulatory molecules, and these IPE cells significantly suppressed CTLA-4$^+$CD4$^+$ effector T cells.[9] On the other hand, cultured RPE from the posterior segment suppressed the T-cell activation by both cell contact and by the secretion of immunosuppressive factor(s).[12–14] We recently reported that cultured RPE cells established from normal mice and humans can greatly suppress

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the activation of T cells by secreting TGFβ.[15] In addition, the human RPE cell lines constitutively express the programed cell death 1 ligand 1 (PD-L1/B7-H1) costimulatory molecules, and RPE cells can fully suppress bystander programed cell death 1 (PD-1)$^+$ human T cells.[16] PD-L1 costimulatory molecules are expressed on various organs, tissues, and cells.[17] These molecules are greatly up-regulated by interferon (IFN)γ, and the PD-L1/PD-1 interactions can suppress T-cell proliferation and cytokine production by activated T cells.[17,18] Thus, cultured RPE cells are able to produce immunoregulatory molecules on their surface and secrete immunoregulatory soluble factors into the supernatants of the culture medium.

In present study, we examined whether murine RPE cells can suppress bystander T cells during inflammatory conditions. To achieve this, we used Th1 cytokine IFNγ-pretreated RPE cells, as inflammatory cytokines have been shown to be critical mediators for ocular inflammatory disease in animal models,[19,20] as well as in human inflammatory disorders.[21–23] In the IFNγ-treated cell cultures, the RPE cells greatly expressed the PD-L1 costimulatory molecules, and suppressed the activation of the bystander IFNγ-producing Th1-type cells that express the PD-1 costimulatory receptor in vitro.

METHODS

Mice

Adult C57BL/6 mice purchased from CLEA Japan Inc. (Tokyo, Japan) were used as donors of the lymphoid cells and ocular pigment epithelium. PD-1 knockout donors (PD-1$^{-/-}$), as well as wild-type mice, were used as donors of target T cells.[24] All experiments were approved by the Institutional Animal Research Committee of Tokyo Medical and Dental University, and conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Culture Media

Dulbecco's modified Eagle medium (DMEM) complete medium that contained 20% fetal bovine serum (FBS) was used for the primary cultures of RPE. To mimic as closely as possible the intraocular microenvironment outside the blood-ocular barrier, serum-free medium was used in the cultures and in the assays involving the T cells stimulated by anti-CD3 antibodies. Serum-free medium was composed of RPMI 1640 medium without the addition of FBS, and supplemented with 0.1% bovine serum albumin (0.1% BSA; Sigma-Aldrich, St. Louis, MO) and 0.2% insulin, transferrin, selenium (ITS$^+$) culture supplement (Collaborative Biochemical Products, Bedford, MA).

Preparation of Cultured Retina Pigment Epithelium

RPE cells were cultivated as has been previously described.[9] Eyes were enucleated, and cut into two halves along a circumferential line posterior to the ciliary process, creating a ciliary body–free posterior eyecup. The eyecup was incubated in 0.2% trypsin (Biowhitaker, Walkersville, MD) for 1 hour. The RPE tissues were triturated to make a single cell suspension, and then resuspended in DMEM complete medium. Samples were then placed into 6-well plates and incubated for 2 weeks. As determined by flow cytometry, the primary RPE cultures were found to be greater than 98% cytokeratin positive (Clone PCK-26; Sigma).

Preparation of Purified T Cells and the Assay for Determining T-Cell Activation

Separately cultured, cytokeratin-positive RPE cells (1.0 or 2.0 $\times$ 10^4 cells/well) were seeded in flat-bottomed 96-well culture plates and incubated overnight. For stimulation with anti-CD3 antibodies, CD4$^+$ T cells were prepared separately from donor spleens, using isolation kits (MACS Cell Isolation Kits; Miltenyi Biotec, Auburn, CA). These cells, which were purified by a single immunomagnetic depletion step that used MACS magnetic beads, proved to be more than 94% CD4 positive. Purified T cells (2.5 $\times$ 10^5 cells/well) were stimulated with anti-CD3 antibody (Clone 2C11; BD PharMingen, San Diego, CA) with incubation for 48 hours (for cytokine production) or 72 hours (for T-cell proliferation). Depending on the individual experiment, the concentrations of the soluble anti-CD3 abs in these cultures ranged from between 0.5 to 1 μg/mL. The amounts of IFN-γ or IL-17 in the supernatant of T cells exposed to RPE cells were measured by ELISA (R&D Systems, Minneapolis, MN). As a measure of T-cell proliferation, the cultures were assayed for the uptake of [^{3}H]–thymidine after the incubation. Incorporated radioactivity was measured by a liquid scintillation counter, with the amount expressed in counts per minute (cpm).

Microarray Analysis

Pigment epithelial cells were cultured from the retina of normal eyes of C57BL/6 mice. After 14 days, when the cultures contained a virtually pure population of cytokeratin$^+$ RPE cells (1 $\times$ 10^6 cells), the culture medium was discarded and replaced with fresh serum-free medium. Primary cultured RPE cells were treated (or not) with recombinant mouse IFNγ (100 U/mL) for 24 hours. Total RNA was isolated with reagent (Trizol; Invitrogen-Life Technologies, Carlsbad, CA). RNA was purified from total cellular RNA using a purification kit (Nucleospin RNA II; Macherey-Nagel, Inc., Düren, Germany) and the quality of total RNA was assessed by electrophoresis using a 1% agarose gel.

Experimental procedures for microarray analysis were performed according to the manufacturer's instructions (Affymetrix GeneChip Expression Analysis Technical Manual; Affymetrix; Santa Clara, CA) as has been previously reported.[25,26] Briefly, double-strand standard cDNA with a T7 promotor was synthesized from 5 μg total RNA with a synthesis kit (Super Script Choice System; Invitrogen-Life Technologies). Approximately 50 μg of biotin-labeled cRNA was synthesized by in vitro transcription with T7 polymerase. After purification and fragmentation, cRNA was hybridized to an oligonucleotide microarray (Mouse Genome 430 2.0). After washing and staining, the scanned images were interpreted using commercial software (Microarray Suite 5.0 [MAS 5]; Affymetrix). For global normalization, the average signal in an array was set equal to 100. The experiment was repeated and the reproducibility of the results for the same cell type was found to be approximately the same. The microarray data were deposited in the GEO public database (accession number: GSE9428).

Reverse Transcription–Polymerase Chain Reaction

Cellular extracts were prepared from cultured RPE cells or fresh RPE tissues (n = 6) and analyzed by RT-PCR. Other RPE cells that were exposed to recombinant mouse IFNγ were also prepared. The RPE cells were cultured with serum free media for 24 hours in the presence of recombinant IFNγ (100 U/mL). These RPE cells were washed twice with PBS, and then the total RNA was isolated with reagent (Trizol; Invitrogen-Life Technologies). After cDNA synthesis, PCR was carried out using the standard PCR method. The PCR conditions and primer sequences for the mouse PD-L1 and for the mouse IFNγ and GAPDH have been previously reported.[9,11] The PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide. To standardize the mRNA expression level, GAPDH expression was used as an internal control.

Flow Cytometry

Flow cytometry was used to analyze the expression on T cells of the costimulatory molecule, PD-1. Phycoerythrin-conjugated anti–PD-1 mAb (Clone RMP 1-14), and FITC-conjugated anti-CD4 mAbs were used to stain the purified T cells. At 24 or 48 hours after activation with anti-CD3 abs, the CD4$^+$ T cells (RPE-exposed T cells or control T cells) were harvested, washed twice, and then stained with anti–PD-1 mAb.

Before staining, the co-cultured cells were incubated with mouse Fc block (Fcγ III/II Receptor, Clone 2.4G2; BD PharMingen) for 15 minutes. As an isotype control for the molecules, we used the phycoerythrin-conjugated rat IgG isotype (BD PharMingen).

Flow cytometric analysis of the cultured RPE cells was also performed using phycoerythrin-labeled anti-mouse PD-L1 mAbs (Clone MIH6). IFNγ-pretreated RPE cells or nontreated RPE cells were harvested and then stained with anti-PD-L1 mAbs. Phycoerythrin-conjugated rat IgG isotype (BD PharMingen) was used as the control.

Immunohistochemistry

Cultured RPE cells were grown on a 4-well chamber (cell culture slide; BD Biosciences, Bedford, MA). RPE cells in the presence of recombinant mouse IFNγ were also prepared. After washing with PBS, these RPE cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, followed by permeabilization with 0.1% Triton X. The cells were incubated for 3 hours with the monoclonal antibodies anti-mouse PD-L1/B7-H1 abs (10 μg/mL) or the control rat IgG (1:50) as the isotype control. Subsequently, the cells were washed with PBS followed by an additional 1-hour incubation with fluorescence-labeled secondary antibody. The secondary antibody used was Alexa Fluor 488 (anti-rat abs; Invitrogen). Fluorescence signals were detected using confocal microscopy (Radiance 2000; Bio-Rad Laboratories, Tokyo, Japan).

EAU Induction

The high-pressure liquid chromatography–purified mouse IRBP peptide (mIRBP; sequence, GPTHLFQPSLVLDMAKILLD) was purchased from Qiagen K.K. (Tokyo, Japan). Complete Freund's adjuvant (CFA) and *Mycobacterium tuberculosis* H37Ra were purchased from Difco (Detroit, MI), while purified *Bordetella* pertussis toxin (PTX) was purchased from Sigma-Aldrich.

Normal mice were immunized using 150 μg of mIRBP peptide 1-20. This peptide, which was administered SC in the neck, has been shown to induce EAU.[26] The mice were also immunized SC in the neck using the peptide in a 0.2-mL emulsion of CFA containing 2.5 mg/mL *M. tuberculosis* H37Ra that was supplemented by an intraperitoneal injection of 0.5 μg PTX. Clinical scores were graded from 0 to 4 using half-point increments, as has been previously described.[27,28]

Statistical Evaluation of Results

Each experiment was repeated at least twice with similar results. All statistical analyses were conducted with a Student's *t*-test. Values were considered statistically significant when $P < 0.05$.

Results

Capacity of IFNγ-Pretreated Murine RPE Cells to Suppress Activation of Bystander T Cells

Cultured RPE cells can fully suppress the activation of bystander T cells in vitro, which includes the T cell–activated T-cell proliferation and cytokine production.[9,11] We first examined whether primary cultured RPE cells could suppress activation of bystander T cells during inflammatory conditions. To determine this, we used IFNγ-pretreated RPE cells. Naïve $CD4^+$ T cells were stimulated with anti-CD3 antibodies in the presence (or absence) of cultured RPE. After 72 hours, T-cell proliferation was evaluated by thymidine-uptake. Both RPE cells and IFNγ-treated RPE cells significantly suppressed the T-cell proliferation, although there was no statistically significant difference between these RPE cells (Fig. 1A).

Proliferation is only one manifestation of the anti–CD3-driven T-cell activation. Another manifestation is the production of cytokines. Since cytokines do have the ability to cross-regulate bystander T cells, we next examined the extent to which T cells could be stimulated with anti-CD3 in the presence of RPE produced by the effector cell–associated cytokines IFNγ or IL-17. Recently, some investigators have reported that IL-17 is also important in animal inflammatory models.[29–31] $CD4^+$ T cells were stimulated with anti-CD3 in the presence (or absence) of the two RPEs listed above. After 48 hours, supernatants were removed and analyzed by ELISA for the IFNγ and IL-17 contents. The results, which are displayed in Figure 1B, show that the production of IFNγ was significantly reduced (compared to positive controls) in T cell cultures that were stimulated with anti-CD3 in the presence of RPE. Moreover, production of IFNγ was profoundly reduced in T cell cultures stimulated with anti-CD3 in the presence of IFNγ-treated RPE cells, and the differences between the nontreated RPE and the IFNγ-treated RPE were statistically significant (Fig. 1B, left panel). On the other hand, both IFNγ-pretreated RPE cells and nontreated cells significantly suppressed IL-17 production by the target T cells (Fig. 1B, right panel). These results suggest that RPE cells exposed to the inflammatory Th1 cytokine IFNγ inducibly express immunoregulatory molecule(s).

To confirm the capacity of the RPE cells, we next used difference concentrations of anti-CD3 antibody. $CD4^+$ T cells were stimulated with 0.5 μg/mL or 1.0 μg/mL anti-CD3 in the presence (or absence) of RPE. As revealed in Figure 1C, IFNγ-treated RPE cells fully and significantly suppressed IFNγ production by bystander $CD4^+$ T cells compared with the nontreated RPE cells. Next, we used difference concentrations of recombinant mouse IFNγ to treat the RPE cells. RPE cells in the presence of IFNγ ranging from 100 U-500 U/mL significantly suppressed IFNγ production by the bystander $CD4^+$ T cells (Fig. 1D).

Previously, our group along with other investigators have demonstrated that cultured RPE cells can suppress T-cell activation via soluble inhibitory factors.[12,15] In addition, other investigators have also shown that RPE cells are able to suppress T-cell activation via cell contact.[10,14] Accordingly, to avoid T cell–RPE cell contact, we examined the influence of IFNγ-treated RPE plus anti–CD3-treated T cells on the IFNγ content in culture supernatants when only the T cells were cultured in a transwell membrane. As revealed in Figure 1E, supernatants from cell insert cultures in which the T cells were stimulated with anti-CD3 in the presence of IFNγ-treated RPE contained much larger amounts of IFNγ compared with IFNγ-treated RPE plus T cells (no transwell membrane). When taken together, these results indicate that RPE cells exposed to IFNγ inducibly express immunoregulatory molecule(s) on their surfaces and are able to suppress the activation of bystander T cells by cell-contact dependent mechanisms. Although RPE cells secrete inhibitory factors such as TGFβ and thrombospondin,[12,15,26] the culture cells also share cell surface molecules that are able to suppress bystander T cells similar to other ocular cells, e.g., the iris pigment epithelium. Thus, we hypothesized that inactivated effector T cells might be induced by cell surface molecule(s) that are produced by the Th1 cytokine IFNγ-exposed RPE cells.

Identification of Highly Expressed Genes in IFNγ-Treated RPE Cells by GeneChip Analysis

As our next step, we examined the gene expression profiles for IFNγ-treated mouse RPE cells (GeneChip analysis; Affymetrix). The microarray used here contained 45,102 genes. We found that the number of genes expressed at a signal level more than 50 (i.e., a significant signal) was 14,580 in the IFNγ-treated RPE cells and 13,993 in the nontreated control RPE cells.

We first compared the highly and significantly expressed genes in two RPE cells (Table 1). We found that the transcripts for IL-18 binding proteins, suppression of cytokine signaling (SOCS)-1, interferon regulatory factor (IFR)1, and some IFNγ induced chemokines, which included chemokine (C-X-C motif) ligand 9, a monokine induced by IFN-γ (MIG), chemokine

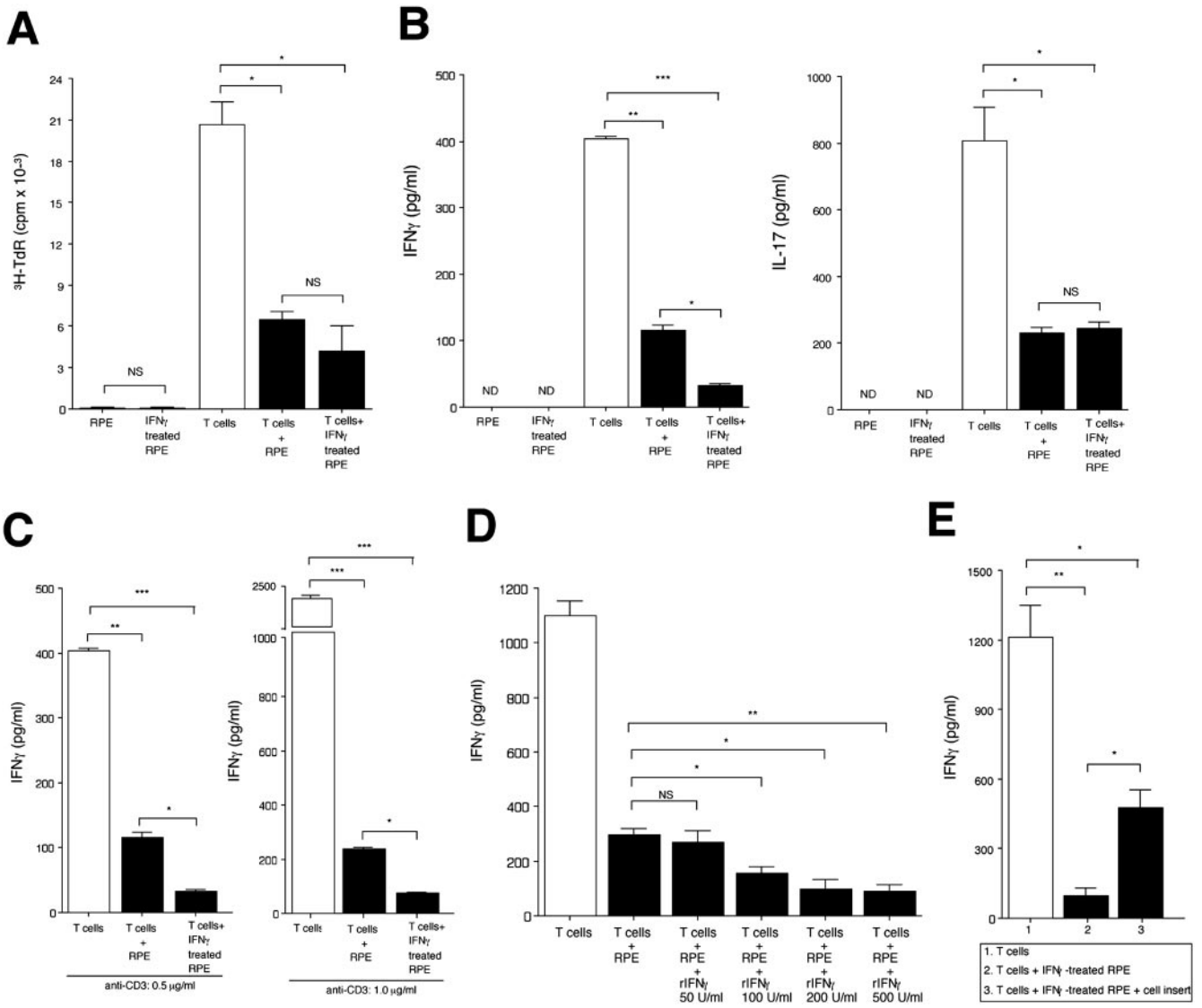

FIGURE 1. Capacity of IFNγ-pretreated RPE to suppress activation of bystander CD4$^+$ T cells. Purified naïve CD4$^+$ T cells (2.5×10^5 cells/well) in the presence of anti-CD3 antibodies were co-cultured with two RPE cells, primary cultured RPE cells and IFNγ-pretreated RPE cells (1.0×10^4 cells/well, respectively). (**A**) One set of cultures was terminated at 72 hours followed by the addition of [^{3}H]-thymidine to assay for the amount of proliferation. Mean cpm for triplicate cultures are presented ± SE. (**B**) From another set of similar cultures, supernatants were harvested after 48 hours and assayed for IFNγ or IL-17 content by ELISA. Data are the mean ± SE of three ELISA determinations. (**C**) Purified CD4$^+$ T cells were co-cultured with primary cultured RPE cells or IFNγ-pretreated RPE cells in the presence of anti-CD3 antibodies that was administered in a dose-dependent manner (0.5 or 1.0 μg/mL). (**D**) CD4$^+$ T cells were co-cultured with IFNγ (0, 50, 100, 200, 500 U/mL)-pretreated RPE cells in the presence of 1.0 μg/mL anti-CD3 antibodies. The supernatants were harvested after 48 hours and assayed for IFNγ content by ELISA. (**E**) Cultured RPE cells in the presence of IFNγ were grown in 24-well culture plates (2×10^5 cells/well). Transwell cell culture inserts were placed in these wells, followed by the addition of CD4$^+$ T cells plus anti-CD3 antibodies. After 48 hours, the supernatants were assayed for IFNγ content by ELISA. *Asterisks* indicate significance levels: $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$, compared with the indicated groups. NS, not significant. ND, not detected.

(C-X-C motif) ligand 10, an IFN-γ induced protein (IP)10, and chemokine (C-X-C motif) ligand 11, an IFN inducible T cell alpha chemoattractant (I-TAC), were all expressed at a much higher level in the IFNγ-treated RPE than in the control RPE (Table 1). In the signal transducer and activator of transcription (Stat) family, IFNγ-treated RPE expressed much higher levels of Stat1 transcripts compared with the control RPE (signal log ratio, 3.0). We also found that the IFNγ-treated RPE expressed high levels of toll-like receptor type (TLR)2 and -3, and MHC class II (Table 1). In the costimulatory molecules, IFNγ-treated RPE expressed much higher levels of programed cell death 1 ligand 1 (PD-L1/B7-H1) transcripts compared with the control (signal log ratio, 4.8). PD-L2 (B7-DC) costimulatory molecules that also bind to PD-1 on T cells were not expressed. The expression of other costimulatory molecules, e.g., CD40, -80, -86, and ICOS ligand, was found to be very poor on these RPE cells (Table 1). Among the many genes that were highly expressed in the IFNγ-treated RPE cells, only the PD-L1 costimulatory molecules were able to provide a negative signal to the T cells via a cell-to-cell contact. Because of this, we decided to focus on the role of PD-L1 in RPE in our further experiments.

Detection of PD-L1 Costimulatory Molecules by IFNγ-Treated RPE

We used RT-PCR, flow cytometry, and immunohistochemistry to confirm the expression of PD-L1 by IFNγ-treated RPE cells. As shown in Figure 2A, IFNγ-pretreated RPE cells expressed

TABLE 1. Representative Genes Expressed at Higher Levels in IFNγ-Treated RPE Cells as Compared to the Nontreated RPE Cells

Probe Set	Accession Number*	Gene Description	Abbreviations	Signal in RPE	Signal in IFNγ-treated RPE	SLR†
		Cytokines and Cytokine Signal				
1450424_a_at	AF110803	Interleukin 18 binding proteins	(IL-18BP)	18	366	4.5
1450446_a_at	AB000710	Suppressor of cytokine signaling 1	(SOCS-1)	42	244	2.5
1448436_a_at	NM_008390	Interferon regulatory factor 1	(IFR1)	319	3391	3.5
1450033_a_at	AW214029	Signal transducer and activator of transcription 1	(Stat1)	418	3801	3.0
1450403_at	AF088862	Signal transducer and activator of transcription 2	(Stat2)	134	386	1.7
1426587_a_at	AI325183	Signal transducer and activator of transcription 3	(Stat3)	1744	1837	0.3
1448713_at	NM_011487	Signal transducer and activator of transcription 4	(Stat4)	<5	<5	—
		Chemokines				
1418652_at	NM_008599	Chemokine (C-X-C motif) ligand 9	(MIG)	32	9127	8.4
1418930_at	NM_021274	Chemokine (C-X-C motif) ligand 10	(IP-10, CRG-2)	144	5461	5.0
1419697_at	NM_019494	Chemokine (C-X-C motif) ligand 11	(I-TAC)	17	408	4.9
		Toll-like Receptors				
1419132_at	NM_011905	Toll-like receptor 2	(TLR2)	158	796	2.2
1422781_at	NM_126166	Toll-like receptor 3	(TLR3)	40	272	2.4
		MHC Class I and Class II				
1452431_s_at	AF119253	Histocompatibility 2, class II antigen A, alpha	(H-2 class IIAa)	19	485	4.8
1451721_a_at	NM_010379	Histocompatibility 2, class II antigen A, beta 1	(H-2 class IIAb1)	11	802	4.8
1422527_at	NM_010386	Histocompatibility 2, class II, locus DMa	(H-2 class IIDMa)	11	802	4.8
1449580_s_at	NM_010388	Histocompatibility 2, class II, locus DMb1	(H-2 class IIDMb1)	100	456	2.7
1417025_at	NM_010382	Histocompatibility 2, class II antigen E beta	(H-2 class IIEb)	12	757	5.7
1425519_a_at	BC003476	Ia-associated invariant chain		34	2322	6.4
		Costimulatory Molecules				
1439221_s_at	BB220422	CD40	(CD154 ligand)	14	28	1.1
1432826_a_at	AK019867	CD80	(B7-1)	11	54	2.1
1420404_at	NM_019388	CD86	(B7-2)	18	5	1.0
1419714_at	NM_021893	Programmed cell death 1 ligand 1	(PD-L1/B7-H1)	14	420	4.8
1450290_at	NM_021396	Programmed cell death 1 ligand 2	(PD-L2/B7-DC)	<5	<5	—
1419212_at	NM_015790	Icos ligand	(ICOSL)	33	20	0.2

SLR, signal log ratio.

* Accession number from GenBank or TIGR database. Genes shown are expressed at higher levels in cultured RPE cells than in IFNγ-pretreated RPE cells.

† Comparative analysis with RPE and IFNγ-treated RPE.

the mRNA for PD-L1 to a much greater degree than was seen in the nontreated RPE cells. Immunohistochemical analysis showed that while PD-L1 is highly expressed on the surface of IFNγ-treated RPE cells, it was not expressed on the surface of primary RPE (Fig. 2B). Also, positive staining was not obtained when we used an isotype control antibody (data not shown). Flow cytometric analysis confirmed that PD-L1 was expressed by IFNγ-treated RPE, whereas the expression of the molecules was poor on the primary control RPE cells (Fig. 2C). In a separate experiment, we also examined whether fresh RPE tissues include PD-L1 mRNA in an ocular inflammation model. For this assay, we extracted the RNA from EAU donors. As revealed in Figure 2D, mRNA for PD-L1, as well as mRNA for IFNγ inflammatory cytokines were highly observed in fresh RPE tissues in EAU. However, they were absent from normal donor tissues (Fig. 2D). Together these results imply that PD-L1 costimulatory molecules on RPE are greatly up-regulated during inflammatory conditions.

We next examined whether RPE-exposed target $CD4^+$ T cells are able to express the PD-1 costimulatory receptor. $CD4^+$ T cells were stimulated with anti-CD3 in the presence or absence of RPE. T cells were removed at 24 or 48 hours and then examined by flow cytometry for PD-1 expression. As displayed in Figure 2E, while anti-CD3 stimulated $CD4^+$ T cells poorly expressed PD-1 in 24-hours cultures, these T cells greatly expressed PD-1 in the 48-hours cultures. Similarly, T cells exposed to RPE in the presence of anti-CD3 stimulation showed surface expression of PD-1, especially in the 48-hours cultures (Fig. 2E). Thus, RPE and anti-CD3 stimulation appear to act synergistically to significantly enhance PD-1 expression by $CD4^+$ T cells.

Capacity of Anti-mouse PD-L1 Antibody to Interfere with the Suppression of the T-Cell Activation by PD-$L1^+$ RPE

In the following experiments, we examined the effect of anti-mouse PD-L1 blocking antibody on the activation of $CD4^+$ T cells exposed in vitro to RPE, which was then followed by treatment with anti-CD3 and recombinant IFNγ. We also tested the effect of the anti-mouse PD-L2 antibody. As before, purified

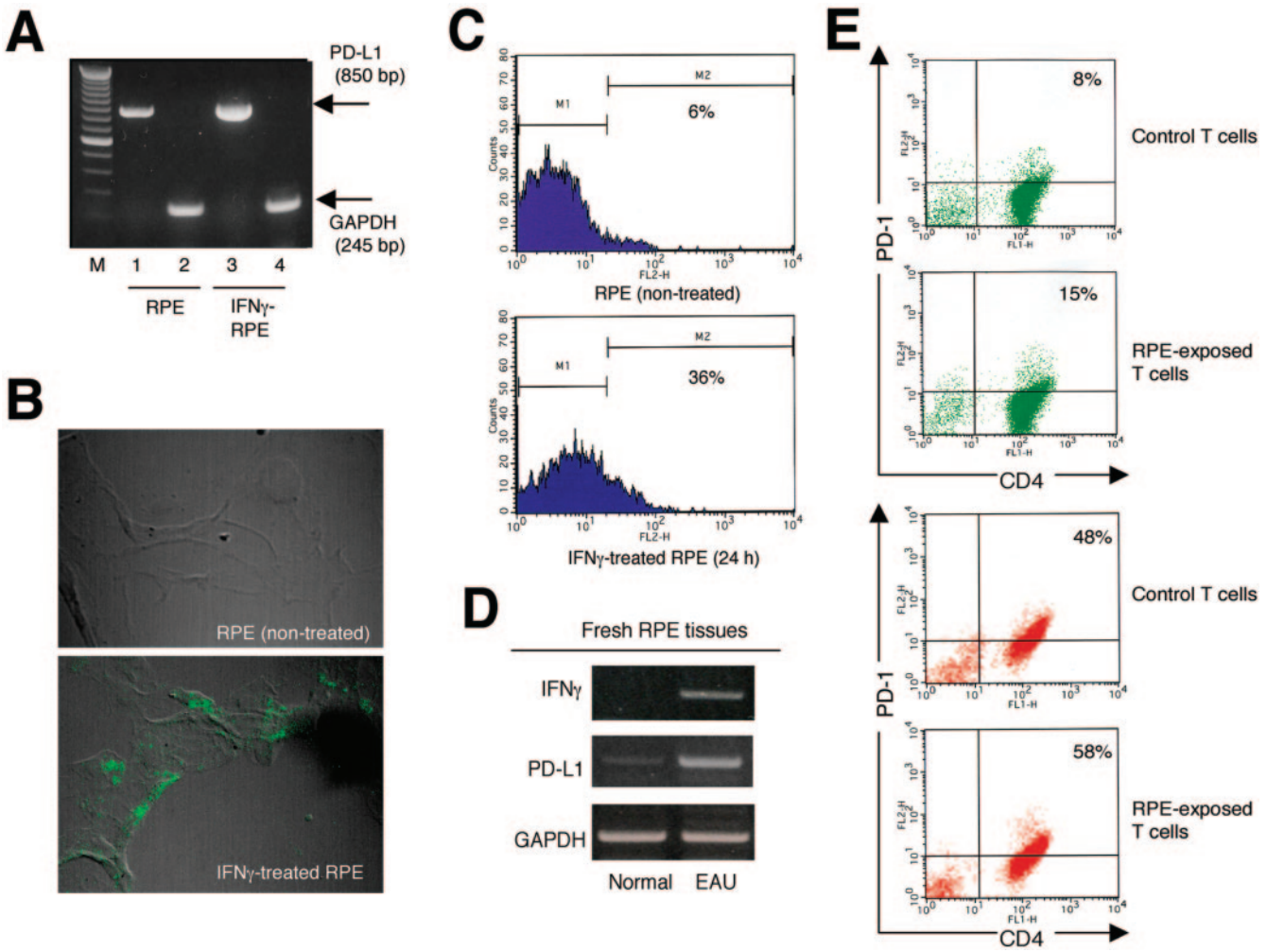

FIGURE 2. Detection of PD-L1 costimulatory molecules by IFNγ-pretreated RPE cells. (**A**) Detection of mRNA expression of PD-L1 in IFNγ-pretreated RPE cells. mRNA, extracted from cultured RPE cells in the presence (3,4) or absence (1,2) of IFNγ, was reverse transcribed and amplified by PCR using primers for PD-L1 and GAPDH. PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide. M, 100-bp marker. (**B**) IFNγ-pretreated RPE cells were stained with anti-mouse PD-L1 antibodies, and then examined by fluorescence confocal microscopy. In the *lower* figure it is clearly seen that PD-L1 (*green*) is expressed by IFNγ-pretreated RPE cells. PD-L1 is partially expressed on the surfaces of the RPE cells. The primary cultured RPE cells (not exposed to IFNγ) are seen in the *upper* figure displays. In this image, the expression of PD-L1 was not detected on the cells. (**C**) Cultured RPE cells or IFNγ-pretreated RPE cells (24 hours culture) were stained with phycoerythrin-labeled anti-mouse PD-L1 antibodies, and examined by flow cytometry. Percentages in the *upper right corners* indicate positive cells. (**D**) Detection of mRNA expression of PD-L1 in fresh RPE cells. mRNA, extracted from fresh RPE tissues from EAU ($n = 6$) or normal mice ($n = 6$) was reverse transcribed and amplified by PCR using primers for mouse IFNγ, PD-L1, and GAPDH. PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide. (**E**) Detection of PD-1 on RPE-exposed T cell. CD4$^+$ T cells in the presence of anti-CD3 abs (1 μg/mL) were co-cultured with or without IFNγ-pretreated RPE cells. After 24 hours (*upper green histograms*) or 48 hours (*lower red histograms*), the T cells were harvested and stained with phycoerythrin-labeled anti-mouse PD-1 and FITC-labeled anti-mouse CD4 antibodies, followed by examination by flow cytometry with double staining methods. Percentages in the *upper right corners* indicate CD4/PD-1 double positive cells.

splenic CD4$^+$ T cells were placed in culture wells containing cultured IFNγ-pretreated RPE and then stimulated with anti-CD3 antibodies in the presence of 1.0 or 10 μg/mL of either anti–PD-L1 or anti–PD-L2 antibodies. ELISA was used to measure the production of cytokines (IFNγ and IL-17) by CD4$^+$ T cells at 48 hours. As the results displayed in Figure 3A reveal, with a concentration above 10 μg/mL of anti-PD-L1 blocking antibody, the T cells exposed to RPE underwent significant levels of IFNγ production compared with that seen for the T cells plus IFNγ-treated RPE without blocking antibody. In contrast, IFNγ-treated RPE in the presence of isotype control antibody greatly suppressed IFNγ production by the CD4$^+$ T cells (Fig. 3A). IFNγ-treated RPE cells in the presence of both abs (anti–PD-L1 and isotype control) significantly suppressed IL-17 production by the target T cells (data not shown). When anti-mouse PD-L2 antibodies were used in similar cultures in vitro, IFNγ-pretreated RPE was observed to significantly suppress the IFNγ production (Fig. 3B) and the IL-17 production (data not shown) by target T cells. Although these RPE cells greatly suppressed IL-17 production, as has been documented in a previous experiment (see Fig. 1B), it is assumed that the expression of PD-L1 by RPE cells is not necessary in order for Th17 suppression to occur.

We next examined whether primary cultured IFNγ-treated RPE cells can suppress activation of bystander T cells from PD-1 knockout (KO) donors. As a control, CD4$^+$ T cells from wild-type mice were also used. IFNγ pretreated RPE cells significantly suppressed IFNγ production by activated T cells from wild-type donors (Fig. 3C). By contrast, these IFNγ-pretreated RPE cells failed to suppress the activation of T cells from PD-1 KO donors.

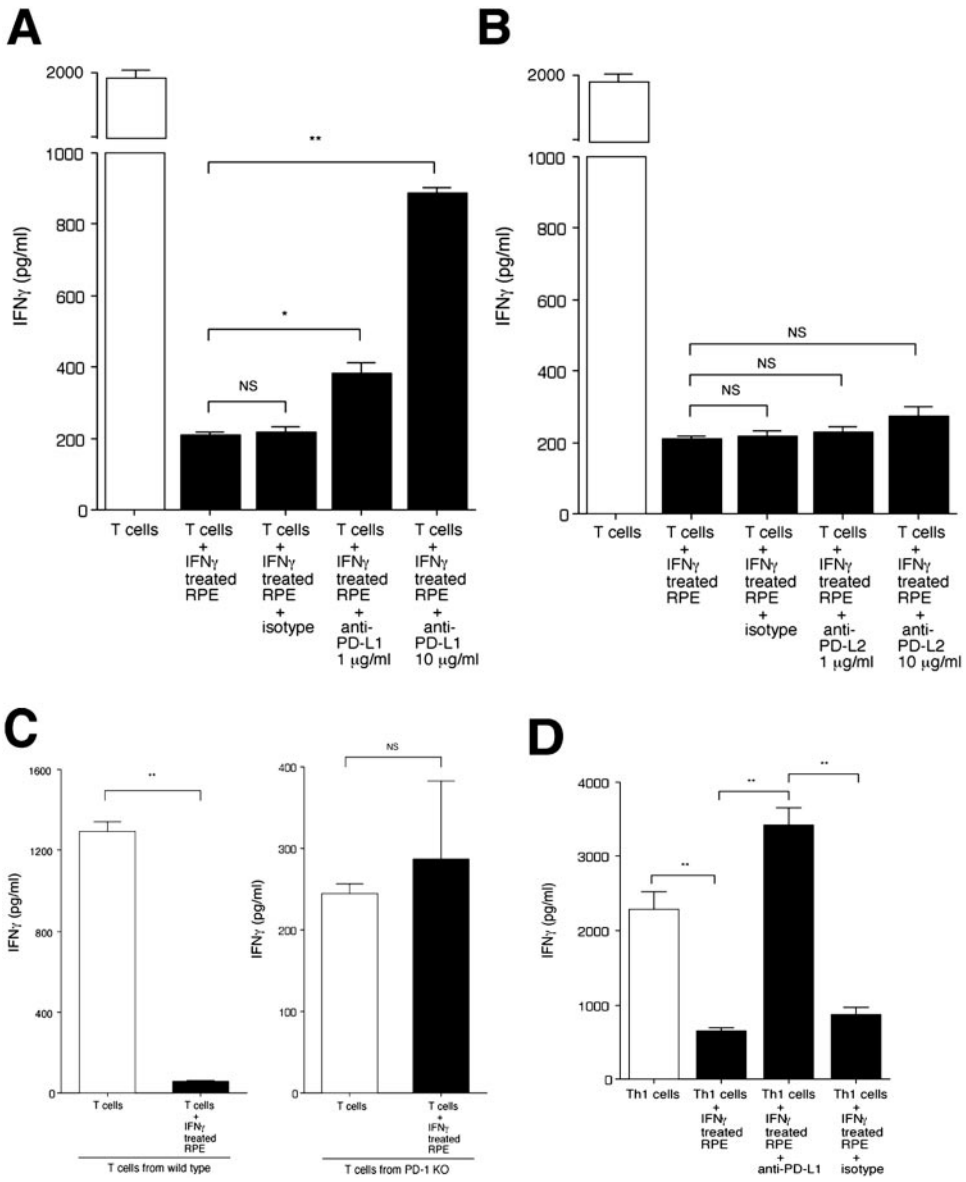

FIGURE 3. Capacity of neutralizing antibodies to PD-L1 and PD-L2 to prevent suppression of T-cell activation by IFNγ-treated RPE. (**A**) $CD4^+$ T cells were stimulated with anti-CD3 antibody and co-cultured with IFNγ-treated RPE cells for 48 hours. Anti-mouse PD-L1 neutralizing abs (clone MIH6, 1 or 10 μg/mL) or isotype rat IgG was added in some wells. (**B**) Purified splenic $CD4^+$ T cells were also co-cultured with IFNγ-pretreated RPE cells in the presence of anti-mouse PD-L2 neutralizing abs (clone TY25, 1 or 10 μg/mL) or isotype rat IgG. (**C**) $CD4^+$ T cells were obtained from C57BL/6 wild-type controls or from PD-1 knockout (KO) mice. (**D**) Using recombinant mouse IL-12 (1 ng/mL) and IFNγ (500 U/mL) plus anti-CD3 antibodies (1 μg/mL), purified $CD4^+$ T cells were cultured for 3 days. These T cells greatly produced Th1 type cytokines IFNγ. T cells in the presence of anti-PD-L1 abs (clone MIH6, 1 μg/mL) or isotype controls (rat IgG, 1 μg/mL) were co-cultured with IFNγ-treated RPE cells for 48 hours. After 48 hours, supernatants were harvested and then assayed for IFNγ content by ELISA. Data are the mean ± SE of three ELISA determinations. *Asterisks* indicate significance levels: $^*P < 0.05$, $^{**}P < 0.005$, compared with the indicated groups.

We finally confirmed whether these RPE cells were able to suppress polarized murine Th1 cells. Using recombinant mouse IL-12 and IFNγ plus anti-CD3 antibodies, purified $CD4^+$ T cells were cultured for 3 days. These Th1 cells greatly express PD-1 (data not shown) and produce IFNγ. IFNγ-treated RPE cells significantly suppressed activation of the Th1 cells whereas these RPE cells in the presence of anti–PD-L1 neutralizing abs failed to suppress these target T cells (Fig. 3D). Together, these results are suggesting that RPE exposed to inflammatory cytokines IFNγ inducibly express ligand for PD-1 (PD-L1) to suppress $PD\text{-}1^+$ effector Th1 type cells in vitro.

DISCUSSION

PD-L1 costimulatory molecules are widely expressed on thymus, spleen, heart, placenta, pancreas, endothelium, epithelium, tumors, and immunocytes such as T cells, B cells, dendritic cells, and monocytes.[17,32] In ocular studies, the molecules are constitutively expressed on corneal endothelium[33] and inducibly expressed on retinal epithelium that has been exposed to IFNγ.[16] Recently, Hori et al.[33] demonstrated that PD-L1 costimulatory molecules expressed on corneal endothelial cells provide a negative costimulation for the effector T cells helping to inhibit corneal allograft rejection. We have previously reported that RPE expressing PD-L1 suppressed the RPE-mediated T-cell activation.[16] Moreover, aged PD-1 knockout mice with C57BL/6 backgrounds spontaneously developed autoimmune diseases such as characteristic lupus-like arthritis and glomerulonephritis.[34] In addition, the costimulatory molecules are greatly upregulated by the Th1 cytokine IFNγ, and the PD-L1/PD-1 interactions are able to suppress T-cell activation.[17,18] Cultured RPE has the capacity to suppress activation of $CD4^+$ T cells, and certain T cells are able to respond to anti-CD3 stimulation in the presence of RPE by secreting IFNγ. Therefore, we hypothesize that RPE exposed to IFNγ, via the inducible expression of PD-L1, suppresses T-cell activation by engaging the PD-1 (PD-L1 receptor) on the IFNγ-secreting T cells. In the present study, we found that RPE exposed to the inflammatory cytokines inducibly expressed PD-L1, and that this molecule bound directly to PD-1 on the bystander T cells. Although primary RPE did not express the costimulatory molecule, expression of the molecule was induced on the surface of IFNγ-pretreated RPE. In addition, IFNγ-pretreated RPE greatly expressed signal transducer and activator of transcrip-

tion 1 (Stat1) that is a critical transcription factor in IFN-dependent responses.[35] Loke et al.[36] previously reported that PD-L1 expression is regulated by Stat1 signal. Therefore, IFNγ-induced Stat1 activation may contribute to the expression of PD-L1 in RPE cells. This results in changes to the T cells' functional program and suppresses their susceptibility to activation through the first signal (anti-CD3 stimulation) plus the second costimulatory signal (PD-1-PD-L1 interactions).

Because experimental autoimmune uveitis is a Th1- and Th17-mediated inflammatory disease,[19,20,29–31] we investigated whether the enhanced inflammatory response in the absence of the PD-1/PD-L1 pathway was associated with T-cell activation and an elevated type 1 and 17 T cell response. To document this, we used anti-mouse PD-L1 blocking antibodies to interfere with the RPE-T interaction. IFNγ-pretreated RPE cells in the presence of the neutralizing abs fail to suppress the production of IFNγ by activated CD4$^+$ T cells. However, the RPE cells were able to significantly suppress the production of IL-17. Similarly, IFNγ-pretreated RPE cells failed to suppress the production of IFNγ by CD4$^+$ T cells from PD-1 null donors, but these RPE cells significantly suppressed the production of IL-17. These results indicate that the PD-1/PD-L1 interaction plays a critical role in the Th1-mediated inflammation. Although cultured RPE cells greatly suppress the Th17 cells, the PD-1/PD-L1 interaction between the local ocular resident cells and infiltrating T cells may be irrelevant with regard to the suppression. Thus, PD-L1 via the ocular tissues (e.g., RPE cells) and cells (e.g., T cells) can directly suppress the PD-1$^+$ cells that secrete Th1-cytokines under inflammatory conditions.

We previously reported that T cells exposed to ocular pigment epithelial cells acquire a T-regulator phenotype that can express Foxp3 (a regulatory T-cell marker) and produce TGFβ (inhibitory cytokine).[37,38] T cells that encounter ocular pigment epithelium in vitro are inhibited from undergoing T-cell receptor–triggered activation and instead acquire the capacity to suppress the activation of the bystander T cells. It is assumed that a subpopulation of PD-1$^+$ T cells is the first to encounter the PD-L1$^+$ RPE (RPE-T interaction), and that another subpopulation of PD-1$^+$ T cells is also able to access the PD-L1$^+$ T cells (T–T interaction). This can account for the findings that in cultures, these T cells eventually are able to cross-regulate the bystander CD4$^+$ T cells. In fact, both naïve and activated T cells that have been found to constitutively express PD-L1 and regulatory T cells can also express the molecule.[32] At the present time, we are conducting further experiments using pigment epithelial cell–induced regulatory T cells to explore these possibilities.

Previous studies have shown that cultured RPE cells have the capacity to present antigens to the T cells.[39,40] The antigen-specific T-cell activation generally requires antigen presentation by the antigen-presenting cells and the second signal via the B7-CD28 costimulatory pathway. As shown in the present study and in our previous report, when compared to the murine iris pigment epithelial (IPE) cells, the RPE cells poorly express the B7 costimulatory molecules.[9] Cultured IPE cells established from the anterior segment in the eye uniquely express B7 costimulatory molecules and greatly suppress CD152$^+$ bystander T cells.[9,11] In the present study, we found that PD-L1, which is thought to be an important negative regulator in the immune system, is inducibly expressed on the murine RPE cells in response to IFNγ in vitro. In addition, the PD-L1/PD-1 pathway is speculated to contribute to the immune system in the eye. Our data also show that highly purified mouse RPE cells can inducibly express PD-L1 but not PD-L2 (B7-DC) in response to IFNγ. These results suggest that PD-L2 costimulatory molecules, which are also ligands for PD-1, do not play a critical role in the T cell–RPE cell interaction.

In summary, we have shown that the PD-L1 molecules, but not PD-L2, are expressed on the isolated RPE cells in the presence of the inflammatory mediator, IFNγ. Furthermore, we were able to show that the interaction between PD-L1–expressing RPE and IFNγ-producing Th1 cells have a negative effect on Th1 cytokine production. These results suggest that Th1 cytokine–exposed RPE cells can express the negative costimulatory molecule, which results in the suppression of the bystander Th1-type cells.

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