Suppression of Bystander T Helper 1 Cells by Iris Pigment Epithelium-Inducing Regulatory T Cells via Negative Costimulatory Signals

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Purpose. To determine whether iris pigment epithelium (IPE)-induced T regulatory (Treg) cells can suppress the activation of bystander T cells with cell contact via costimulatory interactions.

Methods. $CD8^+$ T cells were cocultured with IPE, x-irradiated, and then used as regulators (IPE-induced Treg cells). The target $CD4^+$ T cells from wild-type control or knockout donors were used for the assay. T-cell activation was assessed for proliferation by examining both [^{3}H]-thymidine incorporation and cytokine production. Expression of costimulatory molecules on IPE-induced Treg cells was evaluated using RT-PCR, immunostaining, and flow cytometry. Expression of costimulatory receptors on target T cells or Treg cells was evaluated by flow cytometry. Neutralizing antibodies were then used to abolish regulatory function.

Results. $CD8^+$ IPE-induced Treg cells significantly suppressed the activation of effector target T cells, e.g., T-cell proliferation and cytokine production such as Th1, Th2, and Th17 cytokines. Although IPE-induced Treg cells expressed various costimulatory molecules, including programmed cell death 1 ligand 1 (PD-L1), only PD-L1 on the Treg cells was actually delivered to target Th1 cells using cell-to-cell interaction (T-T interaction). If neutralizing antibodies for PD-L1 were cocultured with Treg cells, Th1 suppression was impaired. Moreover, Treg cells failed to suppress IFNγ production by target $CD4^+$ T cells from programmed cell death 1 (PD-1) knockout donors. Th1-specific inhibition was exclusively achieved with direct cell contact.

Conclusions. T cells exposed to IPE in the eye that acquires full regulatory capacity express negative costimulators and suppress bystander Th1-type effector cells. (*Invest Ophthalmol Vis Sci.* 2010;51:2529–2536) DOI:10.1167/iovs.09-4460

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A monolayer of pigment epithelium (PE) lines the iris, ciliary body, and retina of the inner eye, all of which contribute to the immunoprivileged site. The neural crest-derived epithelial cells participate in ocular immunoprivilege.[1,2] To avoid the blinding consequences of intraocular inflammation, the PE monolayer expresses an extensive array of mechanisms by which innate and adaptive immune effectors can be regulated. For instance, cultured iris PE cells from the anterior segment in the eye have been demonstrated to induce $CD8^+$ regulatory T cells in vitro.[2] Thus, ocular PE cells can create and maintain peripheral immune tolerance.

T regulatory (Treg) cells act at multiple levels of an immune response to suppress self-reactive T cells.[3–5] Treg cells are potent modulators of T cell-mediated immune responses in vitro and in vivo and can be subdivided into two main groups: $CD4^+CD25^+$ naturally occurring Treg cells, which acquire their suppressive functions in thymus, and induced Treg cells, which acquire their functions in the periphery.[3–5] Forkhead-winged helix transcription factor gene/Forkhead box p3 (Foxp3), which is constitutively expressed on naturally occurring Treg cells and on peripherally induced Treg cells, are essential for Treg cell development and function. In several ocular studies, investigators reported that murine T cells are converted to Treg cells by in vitro exposure to ocular PE cells.[6–10] Ocular PE-induced Treg cells suppress the activation of bystander target T cells by anti–CD3 antibodies (first signal) when added to secondary cultures containing target T cells plus Treg cells. The iris PE (IPE)-induced Treg cells that acquire regulatory function expressed CD8, CD25, CD80, CD86, CD152 (CTLA-4), glucocorticoid-induced TNF-R family-related gene, and Foxp3.[7–9] The phenotype of the induced Treg cells was similar to naturally occurring $CD4^+CD25^+$ Treg cells. The ability of naturally occurring Treg cells to suppress bystander T cells depends solely on cell-to-cell contact.[3] Similarly, IPE-induced Treg cells were suppressed by direct cell contact when added to secondary cultures.[8] The Treg cells were induced to express Foxp3 transcripts through the transforming growth factor-beta (TGFβ) signal, while another population in IPE-induced Treg cells also exhibited the Th3 phenotype and secreted soluble active TGFβ.[7,9] TGFβ-secreting Treg cells do not express Foxp3 but do suppress effector T cells independently of cell contact, emphasizing that suppression mechanisms by the eye-specific Treg cells are still unclear.

In the present study, we examined whether IPE-induced Treg cells suppress the activation of effector target T cells in vitro. $CD8^+$ IPE-induced Treg cells greatly expressed programmed cell death 1 ligand 1 (PD-L1) costimulatory molecules and suppressed the activation of bystander IFNγ-producing T helper 1 (Th1)-type cells that express the programmed cell death 1 (PD-1) costimulatory receptor in vitro.

METHODS

Mice

Adult C57BL/6 mice (CLEA Japan, Inc., Tokyo, Japan) were used as donors of the lymphoid cells and ocular pigment epithelium. PD-1 knockout (KO) donor (PD-1$^{-/-}$) mice were kindly provided by Taku Okazaki and Tasuku Honjo. KO and wild-type mice were used as target T-cell donors.[11–13] All experiments were approved by the Institutional Animal Research Committee of Tokyo Medical and Dental University and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Cultured Iris Pigment Epithelium

IPE cells were cultured as previously described.[14,15] Iris tissues were separated and incubated in PBS containing 1 mg/mL dispase and 0.05 mg/mL DNAse I (both from Boehringer-Mannheim, Mannheim, Germany) for 1 hour Single-cell suspensions were then incubated for 14 days. IPE cells were stained with FITC-labeled anti–pan cytokeratin antibody (clone PCK-26; Sigma-Aldrich, St. Louis, MO) at the completion of the 14-day primary culture greater than 99%. The cultured IPE contained neither CD45$^+$ nor major histocompatibility complex class II$^+$ cells.[14]

Preparation of Treg and Target T Cells and Assay for Determining T-Cell Activation

Suspensions of responder cells were pressed through nylon mesh (Immulan mouse T-cell kit; Biotex Laboratories, Houston, TX) to produce a single-cell suspension of cells that were >95% CD3$^+$. Regulatory T cells were prepared from purified CD8$^+$ T cells exposed to IPE and were referred to as CD8$^+$ IPE-induced Treg cells. T cells were enriched with CD8$^+$ cells using superparamagnetic beads (MACS cell isolation kits; Miltenyi Biotec, Auburn, CA; >95% of cells expressed the relevant surface marker), cultured for 24 or 48 hours in the presence of IPE cells with anti–CD3 (0.1 μg/mL, clone 2C11; BD PharMingen, San Diego, CA) and were harvested, x-irradiated (20 Gy), and used as Treg cells. To avoid Treg cell proliferation, irradiated IPE-induced Treg cells were used in secondary cultures. Flow cytometry with anti-cytokeratin antibodies showed that the amount of IPE cells contaminating the harvested T cells was <0.97%.

For anti–CD3-driven T-cell activation of target T cells, purified CD4$^+$ T cells were added (2×10^5 cells/well) to culture wells with x-irradiated IPE-induced Treg cells. Anti–CD3 antibody (1.0 μg/mL) was added to the wells, and the cultures were maintained for 48 hours (for evaluation of cytokine production) or 72 hours (for evaluation of cell proliferation). The cultures were then assayed for [^{3}H]-thymidine (1 μCi/mL for the last 8 hours of culture) uptake as a measure of cell proliferation. Serum-free medium was used in cultures and assays involving T cells stimulated by anti–CD3 antibodies to mimic, as closely as possible, the intraocular microenvironment outside the blood-ocular barrier. Serum-free medium was composed of RPMI 1640 medium without the addition of FBS and was supplemented with 0.1% bovine serum albumin (BSA; Sigma-Aldrich) and 0.2% insulin, transferrin, selenium culture supplement (ITS$^+$; Collaborative Biochemical Products, Bedford, MA).

RT-PCR

Total RNA was extracted from CD8$^+$ IPE-induced Treg cells and control T cells (not exposed to IPE). For PCR amplification, cDNA was amplified using primers for PD-L1 (B7-H1) and GAPDH, as previously described.[16] After 35-cycle amplification, PCR products were separated by 1.0% agarose gel containing ethidium bromide.

Flow Cytometry

Flow cytometric analysis of CD8$^+$ IPE-induced Treg cells was performed using phycoerythrin-labeled anti–mouse PD-L1 monoclonal antibodies (B7-H1, clone MIH5; eBioscience, San Diego, CA). CD8$^+$ IPE-induced Treg cells were harvested and stained with anti–PD-L1 monoclonal antibodies. CD8$^+$ IPE-induced Treg cells were also stained with anti–PD-L2 (B7-DC, clone 122), CD80 (B7–1, clone 16–10A1), CD86 (B7–2, clone GL1), ICOS-ligand (ICOS-L/B7-H2, clone HK5.3), CD276 (B7-H3, clone M3.2D7), CD252 (OX40L, clone RMI134L), CD152 (CTLA-4, clone UC10–4B9), and PD-1 (clone RMP1–30) (all eBioscience). Before staining, cocultured T cells were incubated with anti–CD16/CD32 antibodies (Fcγ III/II receptor; BD PharMingen) for 15 minutes at 4°C. Phycoerythrin-conjugated rat IgG isotype (eBioscience) was used as the control.

Flow cytometry was also used to analyze the expression on target T cells of the costimulatory receptors. Phycoerythrin-conjugated anti–mouse PD-1 mAb was used to stain the purified T cells. At 24, 48, or 72 hours after activation with anti–CD3 antibodies, the target CD4$^+$ T cells were harvested, washed twice, and stained with anti–PD-1 mAb. Before staining, the cocultured cells were incubated with mouse Fc block for 15 minutes. As an isotype control for the molecules, we used the phycoerythrin-conjugated rat IgG isotype.

Immunohistochemistry

Cultured CD8$^+$ IPE-induced Treg cells were prepared for staining with anti–PD-L1 antibodies. After washing with PBS, these T 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 antibodies (10 μg/mL, clone MIH6), or control rat IgG (1:20) as the isotype control. Subsequently, the cells were washed with PBS; this was followed by 1-hour incubation with fluorescence-labeled secondary antibody. The secondary antibody used was Alexa Fluor 488 (anti-rat antibodies; Invitrogen). Fluorescence signals were detected using confocal microscopy (Radiance 2000; Bio-Rad Laboratories, Tokyo, Japan).

Blocking Antibodies

In some in vitro experiments, purified anti–mouse PD-L1/B7-H1 mAb (10 μg/mL), anti–mouse PD-L2/B7-DC mAb (10 μg/mL), or rat IgG (isotype control, 10 μg/mL) was added to cultures with CD8$^+$ IPE-induced Treg cells plus target CD4$^+$ T cells. The supernatants of IPE-induced Treg cells were also pretreated with anti–mouse PD-L1 mAb.

Cytokine Concentrations

The concentration of cytokines in supernatants of target CD4$^+$ T cells was measured by a cytometric beads array (CBA) assay kit including IL-2, IL-4, IL-5, TNF-α, or IFNγ according to manufacturer's instructions (mouse Th1/Th2 cytokine kit; BD PharMingen). The concentration of IL-17 (R&D Systems, Minneapolis, MN) in the supernatants of the T-cell cultures was also measured by ELISA.

Preparation of Pure OT-I T Cells

OT-I TCR transgenic mice (C57BL/6 background, obtained originally from The Jackson Laboratory, Bar Harbor, ME) were maintained in our colony. Normal C57BL/6 mice were used as a source of peritoneal exudate cells (PECs). T cells from OT-I mice recognize a peptide (residues 257–264) derived from ovalbumin (OVA) presented in the context of K^b. OVA-specific TCR transgenic T cells in OT-I mice were identified by surface flow cytometry for the expression of CD8 and Vβ5.[16] OVA, purchased from Sigma-Aldrich, was dissolved in Hanks balanced salt solution at a concentration of 25 mg/mL. Complete Freund's adjuvant containing heat-killed *Mycobacterium tuberculosis* strain H37 Ra was purchased from Difco Laboratories (Detroit, MI).

Spleens were removed from OT-I mice and strained through nylon mesh to produce a single-cell suspension. Red blood cells were lysed with Tris-NH_4Cl. The remaining cells were then washed three times with RPMI 1640 and passed through T-cell columns. After enrichment, the percentages of T cells were monitored by CD4 and CD8 staining and flow cytometric analyses. Together, CD4$^+$ and CD8$^+$ cells ac-

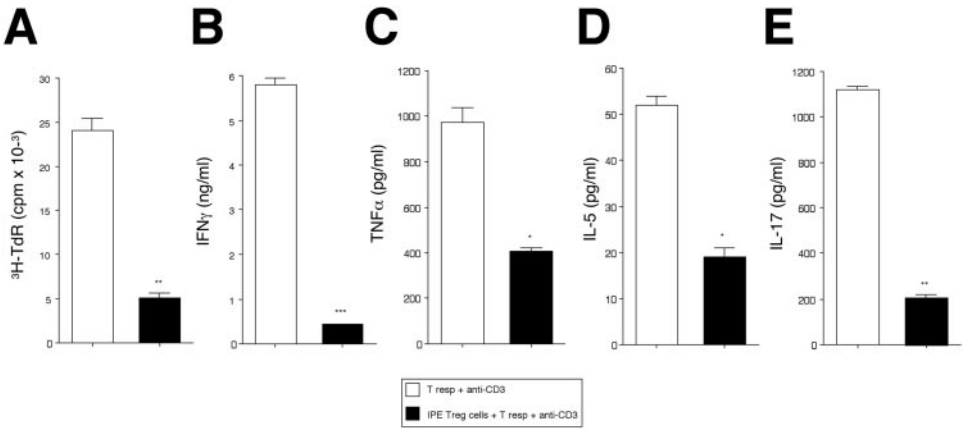

FIGURE 1. Capacity of IPE-induced Treg cells to suppress the activation of bystander $CD4^+$ T cells. Purified naive $CD4^+$ T cells in the presence of anti–CD3 antibodies were cocultured with $CD8^+$ IPE-induced Treg cells (2.5×10^5 cells/well, respectively). (**A**) One set of cultures was terminated at 72 hours and was followed by the addition of [^{3}H]-thymidine to assay the amount of proliferation. Mean ± SEM cpm for triplicate cultures. From another set of similar cultures, supernatants were harvested after 48 hours and assayed for IFNγ (**B**), TNF-α (**C**), IL-5 (**D**), and IL-17 (**E**) by cytokine CBA or ELISA. Data are mean ± SEM of three assay determinations. *White bars*: responder $CD4^+$ T cells (T resp) + anti–CD3. *Black bars*: IPE-induced Treg cells + anti–CD3 stimulated responder T cells. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$, compared with positive control cultures.

counted for >90% of the cells in enriched suspensions. PECs were harvested from normal C57BL/6 mice that received 2.5 mL thioglycolate (Sigma-Aldrich) intraperitoneally 3 days earlier, as described in a previous report.[16]

Statistical Analysis

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

RESULTS

Capacity of IPE-Induced Treg Cells to Suppress Activation of Bystander T Cells

We first examined whether $CD8^+$ T cells exposed to IPE cells can suppress the activation of bystander effector T cells in vitro. Because IPE-induced Treg cells are exclusively CD8,[7–9] we used $CD8^+$ T cells in the following assays. To assess the influence of $CD8^+$ IPE-induced Treg cells on T-cell activation, different types of T-cell assays were performed. The initial results showed that IPE-induced Treg cells significantly suppressed T-cell proliferation induced by anti–mouse CD3 after 72-hour culture (Fig. 1A). IPE-induced Treg cells also profoundly suppressed various types of cytokine production by anti–CD3-stimulated responder T cells (Figs. 1B-E). For instance, IPE-induced Treg cells significantly suppressed Th1-type cytokines IFNγ (Fig. 1B), TNF-α (Fig. 1C), and IL-2 (data not shown). Similarly, IPE-induced Treg cells significantly suppressed Th2-type cytokines IL-5 (Fig. 1D) and IL-4 (data not shown). In addition, IPE-induced Treg cells significantly suppressed Th17-type cytokine IL-17 (Fig. 1E). These results indicated that $CD8^+$ T cells exposed to IPE exhibit global suppression against activated responder T cells in vitro.

Capacity of IPE-Induced Treg Cells to Suppress T-Cell Activation in an Antigen-Specific Manner

Next, we examined whether cultured IPE could convert preactivated T cells to regulators. Purified OT-1 T cells were cultured with PECs in the presence of OVA. After 24-hour culture, harvested OT-1 T cells were cocultured with IPE cells for 48 hours and were used as T regulators. The IPE-induced Treg cells significantly suppressed T-cell activation (T-cell proliferation and IFNγ production by responder T cells), whereas control T cells did not (Fig. 2A). These results indicated that IPE cells were able to convert preactivated T cells to regulators.

We also examined whether IPE-induced Treg cells could suppress T-cell activation in an antigen (Ag)-specific manner.

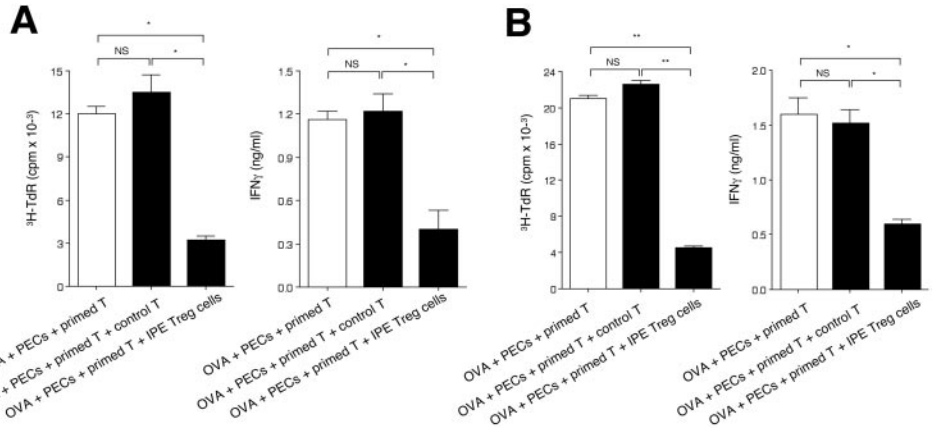

FIGURE 2. Capacity of IPE-induced Treg cells to suppress T-cell activation in an Ag-specific manner. (**A**) Capacity of cultured IPE to convert preactivated T cells into regulators. Purified naive OT-1 T cells were cultured with PECs in the presence of OVA. After a 24-hour culture period, harvested OT-1 T cells were cocultured with IPE cells for 48 hours and were used as T regulators (IPE Treg cells). For control, OT-1 T cells were cultured in the absence of IPE cells (control T). X-irradiated IPE Treg cells (1×10^5/well) or control T cells (1×10^5/well) were then added to cultures containing primed T cells (3×10^5/well) plus PECs + OVA. To prepare OVA-primed T cells, C57BL/6 mice were immunized with OVA 7 days before and OVA-pulsed PECs were prepared 1 day before experimentation. (**B**) First, the primed T cells were cultured with PECs in the presence of OVA. After 24 hours of culture, the T cells were used as target-activated T cells. IPE-induced Treg cells from OT-1 mice were added with the target-activated T-cell cultures. *White bars*: positive control cultures containing primed T cells plus PECs + OVA. After 48 hours, the supernatants of cultures were assayed for IFNγ production by ELISA. After 72 hours, the cultures were assayed for [^{3}H]-thymidine uptake. Mean ± SEM cpm for triplicate cultures are presented. $^*P < 0.05$ and $^{**}P < 0.005$ compared with positive control cultures. NS, not significant.

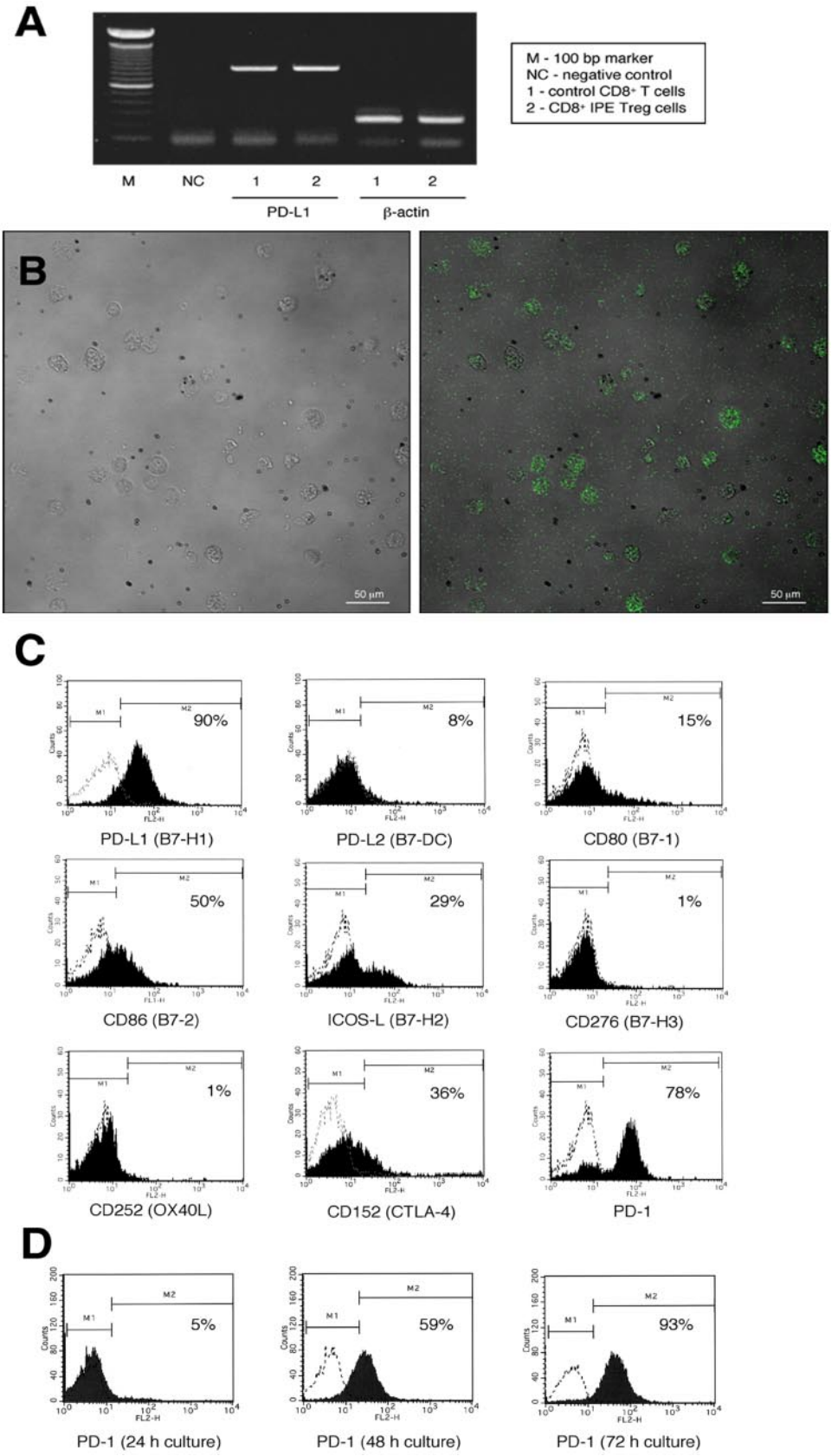

FIGURE 3. Detection of PD-L1 costimulatory molecules by IPE-induced Treg cells. (**A**) Detection of mRNA expression of PD-L1 in IPE-induced Treg cells. mRNA was extracted from $CD8^+$ IPE-induced Treg cells or control $CD8^+$ T cells, then reverse transcribed and amplified by PCR using PD-L1 and GAPDH primers. PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide. M, 100-bp marker. (**B**) $CD8^+$ IPE-induced Treg cells were stained with anti–mouse PD-L1 antibodies and then examined by fluorescence confocal microscopy. In the bright-field image (*left*), T cells are round and can be seen along with numerous pigment particles from IPE. *Right*: it is clear that PD-L1 (*green*) is expressed by IPE-induced Treg cells. PD-L1 is highly expressed on the surfaces of the T cells. (**C**) By examination of flow cytometric analysis, IPE-exposed $CD8^+$ T cells were stained with specific mouse antibodies as follows: PD-L1 (B7-H1), PD-L2 (B7-DC), CD80 (B7–1), CD86 (B7–2), ICOS-L (B7-H2), CD276 (B7-H3), CD252 (OX40L), CD152 (CTLA-4), and PD-1. *Dotted histogram*: T cells that stained with isotype control antibodies. Percentages in the upper right corners indicate the positive cells. (**D**) Detection of PD-1 on target responder $CD4^+$ T cell. $CD4^+$ T cells in the presence of anti–CD3 antibodies (1 μg/mL) were cocultured for 24 to 72 hours. After 24, 48, or 72 hours, these T cells were harvested and stained with phycoerythrin-labeled anti–mouse PD-1 antibodies, followed by flow cytometry examination. *Dotted histogram*: T cells that stained with isotype control antibodies. Percentages in the upper right corners indicate PD-1–positive cells.

To assay, primed T cells were cultured with PECs in the presence of OVA for 24 hours as the target cell. The activated T cells were then cocultured with IPE-induced Treg cells from OT-1 mice. As shown in Figure 2B, IPE-induced Treg cells, but not control T cells, significantly suppressed bystander T-cell activation in an Ag-specific manner, indicating that IPE-induced

Treg cells are able to suppress the activated T-cell response (i.e., when added after effector-target T-cell activation).

Detection of PD-L1 Costimulatory Molecules by IPE-Induced Treg Cells

We previously showed that $CD8^+$ IPE-induced Treg cells greatly express B7 and CTLA-4 costimulatory molecules.[8] If $CD8^+$ IPE-induced Treg cells were depleted of $B7\text{–}2^+$ and $CTLA\text{-}4^+$ T cells, the suppressive activity to bystander T cells was reduced. Therefore, we next examined whether IPE-exposed $CD8^+$ T cells can express costimulatory molecules. First we checked the expression of PD-L1 and PD-1 costimulatory molecules on the Treg cells because PD-L1 costimulatory molecules bind to PD-1 on responder T cells and suppress the activation of T cells through PD-1–negative signals.[17,18] $CD8^+$ T cells were cultured with or without IPE and then harvested and assayed by RT-PCR for content of mRNA for PD-L1 and GAPDH. Control T cells without IPE expressed significant amounts of PD-L1 mRNA, and IPE-induced Treg cells also expressed significantly greater levels of PD-L1 mRNA (Fig. 3A). Immunohistochemical analysis showed that PD-L1 was highly expressed on the surfaces of almost all IPE-exposed $CD8^+$ T cells (Fig. 3B). Positive staining was not obtained when we used an isotype control antibody (data not shown).

Next, we examined whether IPE-exposed $CD8^+$ T cells can express various costimulatory molecules and receptors such as PD-L1 (B7-H1), PD-L2 (B7-DC), CD80 (B7–1), CD86 (B7–2), ICOS-ligand (B7-H2), CD276 (B7-H3), CD252 (OX40L), CD152 (CTLA-4), and PD-1. We used flow cytometry to confirm the expression of these costimulatory molecules by $CD8^+$ IPE-induced Treg cells. As shown in Figure 3C, IPE-induced Treg cells expressed costimulatory molecules, including PD-L1, PD-L2, CD80, CD86, and ICOS-L, but not CD276 and CD252. IPE-induced Treg cells expressed costimulatory receptors, including CD152 and PD-1. Among the positive expression, IPE-induced Treg cells exhibited particularly high expression of PD-L1 (90% positive) and PD-1 (78% positive). Similarly, x-irradiated IPE-induced Treg cells also expressed these costimulatory molecules (data not shown).

We next examined whether target responder T cells expressed the PD-1 costimulatory receptor. $CD4^+$ T cells were stimulated with anti–CD3 antibodies. T cells were removed at 24, 48, or 72 hours and then were examined by flow cytometry for PD-1 expression. As displayed in Figure 3D, though anti–CD3 stimulated $CD4^+$ T cells poorly expressed PD-1 in 24-hour cultures (only 5% positive), these T cells expressed PD-1 in the 48-hour cultures (59% positive). Moreover, these T cells greatly expressed PD-1 in the 72-hour cultures (93% positive) (Fig. 3D). Thus, anti–mouse CD3 stimulation acts synergistically to significantly enhance PD-1 expression by target $CD4^+$ T cells.

Capacity of Neutralizing Antibody to Interfere with the Suppression of T-Cell Activation by PD-L1–Expressing Treg Cells

We examined the effect of anti–PD-L1 and PD-L2 neutralizing antibodies on the activation of $CD4^+$ T cells exposed to IPE-induced Treg cells. Purified $CD4^+$ T cells that stimulated with anti–CD3 antibodies were placed in culture wells containing IPE-induced Treg cells in the presence of either anti–PD-L1 or anti–PD-L2 antibodies. When anti–mouse PD-L1 antibodies were used in the cultures in vitro, IPE-induced Treg cells failed to suppress T-cell activation of responder T cells (Fig. 4A). In contrast, IPE-induced Treg cells in the presence of isotype control antibody significantly suppressed T-cell activation (Fig. 4A). Similarly, anti–PD-L1 blocking antibody impaired IFNγ production by target responder T cells in the presence of IPE-induced Treg cells (data not shown). On the other hand,

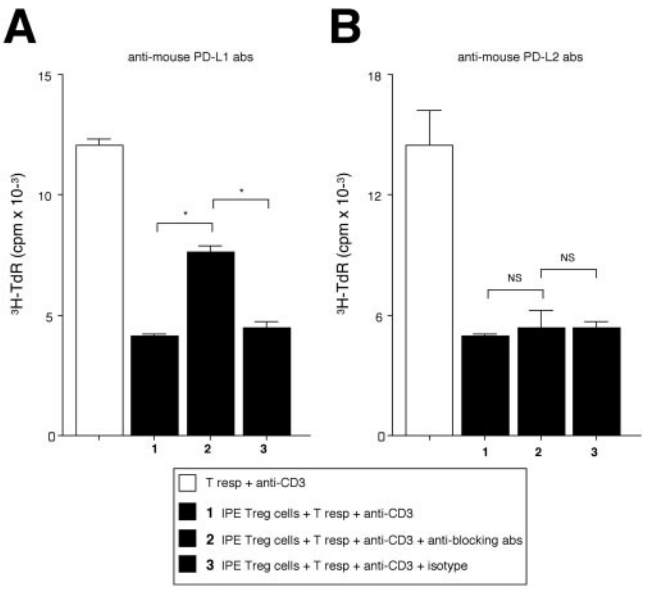

FIGURE 4. Capacity of neutralizing antibodies to PD-L1 to prevent the suppression of T-cell activation by IPE-induced Treg cells. Purified splenic $CD4^+$ T cells were stimulated with anti–CD3 antibody and cocultured with IPE-induced Treg cells for 72 hours. Anti–mouse PD-L1–neutralizing antibody (**A**, 10 μg/mL), anti–mouse PD-L2–neutralizing antibody (**B**, 10 μg/mL), or isotype rat IgG (10 μg/mL) was added in some wells. T-cell activation was evaluated with cell proliferation assay, as described. $^*P < 0.05$, comparing two groups. NS, not significant.

IPE-induced Treg cells in the presence of anti–PD-L2 antibodies and isotype IgG significantly suppressed T-cell activation (Fig. 4B). It is assumed that the expression of PD-L2 by IPE-induced Treg cells is not necessary for T-cell suppression.

Capacity of PD-L1$^+$ IPE-Induced Treg Cells to Suppress Activation of Responder T Cells from PD-1–Deficient Donors

We examined whether IPE-induced Treg cells can suppress the activation of bystander T cells from PD-1 KO donors. Target $CD4^+$ T cells from wild-type mice were used as controls. $CD8^+$ IPE-induced Treg cells failed to suppress the cell proliferation of $CD4^+$ T cells from PD-1 KO donors, whereas IPE-induced Treg cells significantly suppressed the T-cell proliferation from wild-type donors (Fig. 5A). Treg cells significantly suppressed IFNγ production by activated T cells from wild-type donors (Fig. 5B). By contrast, Treg cells failed to suppress the activation of T cells from PD-1 KO donors. Suppression by Treg cells completely disappeared. When target T cells from PD-1 KO donors were used in similar cultures in vitro, IPE-induced Treg cells significantly suppressed IL-17 production by these target T cells (Fig. 5C). Although these Treg cells greatly suppressed IL-17 production, as has been shown in a previous experiment (see Fig. 1E), the expression of PD-L1 by IPE-induced Treg cells was not necessary for Th17 suppression. Otherwise, PD-L1 costimulatory negative signal was required to achieve Th1-specific inhibition.

Use of Cell Contact by IPE-Induced Treg Cells to Suppress Bystander T Cells

We previously reported that $B7^+$ IPE-induced Treg cells bind to $CTLA\text{-}4^+$ responder T cells to suppress the T-cell activation of responder T cells through cell contact.[8] We also showed that some populations of IPE-induced Treg cells secrete inhibitory factors such as TGF-β1 and IL-10 and suppress the T-cell acti-

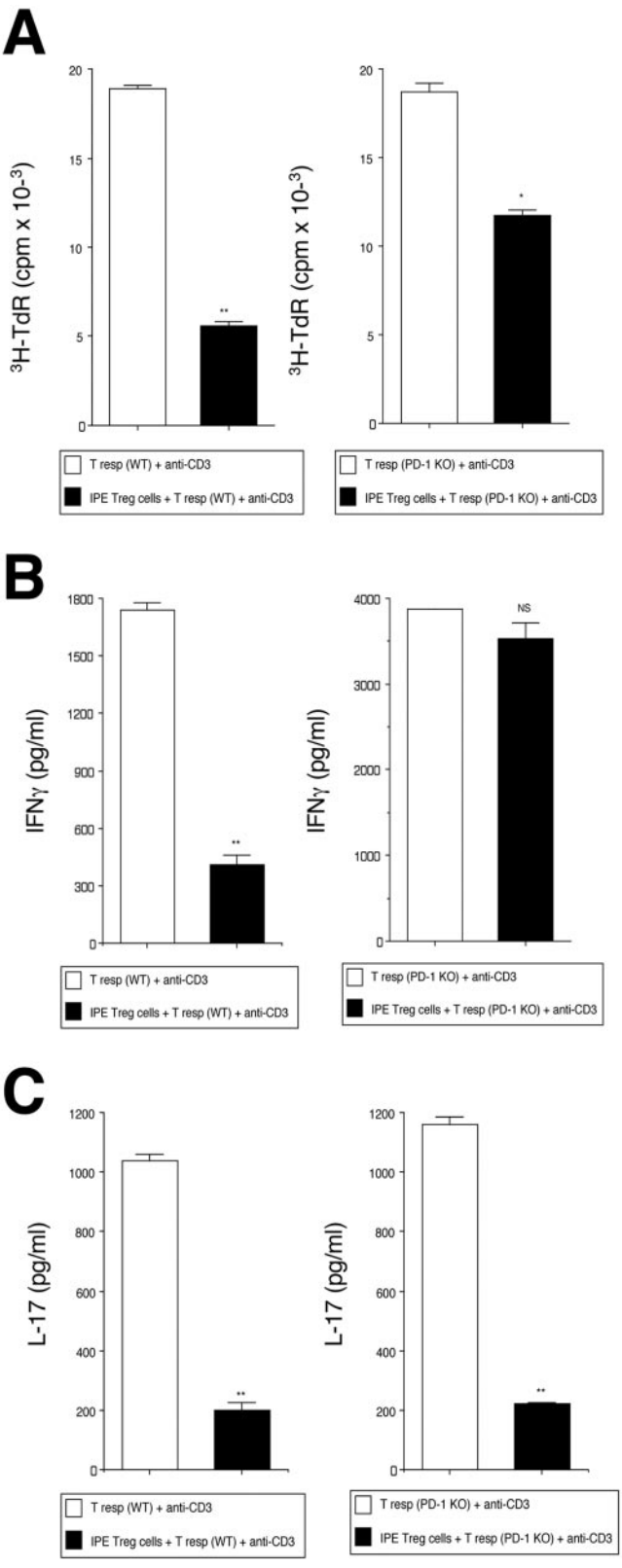

FIGURE 5. Capacity of IPE-induced Treg cells to T-cell activation from PD-1 KO donors. (**A**) Target $CD4^+$ T cells were obtained from C57BL/6 wild-type controls or from PD-1 KO mice. Purified $CD4^+$ T cells (wild-type or KO T cells) in the presence of anti–CD3 antibodies were cocultured with $CD8^+$ IPE-induced Treg cells. Culture was terminated at 72 hours, followed by the addition of [^{3}H]-thymidine to assay for the amount of proliferation. Mean ± SEM cpm for triplicate cultures. After 48 hours, supernatants were harvested and then assayed for IFNγ (**B**) or IL-17 (**C**) by cytokine CBA or ELISA. $^*P < 0.05$ and $^{**}P < 0.005$ compared with positive control cultures. *White bars*: responder T cells + anti–CD3. NS, not significant.

vation in a contact-independent manner.[9] We, therefore, examined whether PD-L1 cell surface molecules by IPE-induced Treg cells can suppress PD-1^+ IFNγ-producing Th1 type cells in a cell contact–dependent manner. As expected, supernatants of IPE-induced Treg cells partially, and significantly, suppressed IFNγ production by $CD4^+$ activated T cells (Fig. 6A). By contrast, no suppression was observed by supernatants of control T cells. If the supernatants of IPE-induced Treg cells were pretreated with anti–PD-L1 blocking antibodies, supernatants of IPE-induced Treg cells significantly suppressed IFNγ production by activated T cells (Fig. 6B). Similarly, supernatants of the Treg cells in the presence of anti–PD-L1 antibodies significantly suppressed cell proliferation of activated T cells (data not shown). Taken together, these results suggest that PD-$L1^+$ IPE-induced Treg cells are able to suppress PD-1^+ Th1 cells through cell-cell interaction.

DISCUSSION

Some studies recently documented that PD-L1 plays a crucial role in downregulating immune responses and maintaining or promoting peripheral immune tolerance.[17–21] A noteworthy feature of PD-L1 is its broad expression on thymus, spleen, heart, placenta, pancreas, endothelium, epithelium, tumors, immunocytes, and even ocular tissues.[22–24] The peripheral tissue-specific expression indicates that it may have a key role in regulating immune responses in inflamed tissues. Although most studies for nonlymphoid tissues or lymphoid tissues to date show that PD-L1 upregulation in these tissues can suppress T-cell activity as assessed by T-cell proliferation and cytokine production by activated T cells,[17–21] the function of lymphoid cell PD-L1 expression in the inflamed eye is still unfolding. Therefore, we examined whether T cells exposed to IPE can suppress target bystander T cells in vitro. The $CD8^+$ IPE-induced Treg cells constitutively expressed the PD-L1 costimulatory molecules and suppressed the activation of bystander IFNγ-producing Th1 cells that express the PD-1 receptor in vitro. A large body of in vitro evidence demonstrated that ligation of PD-L1 with the receptor PD-1 on activated T cells downregulates TCR-medicated T-cell proliferation and cytokine production.[17–19] In ocular studies, cultured ocular PE cells expressing PD-L1 suppressed PE-mediated T-cell activation by PD-L1–PD-1 interaction.[17,25] In a recent report by Hattori et al.,[26] primary cultured human iris PE cells established from fresh iris tissues inhibited T-cell proliferation through the costimulatory interaction. In the present study, PD-L1 molecules on IPE-induced Treg cells were delivered to target Th1 cells that secrete IFNγ cytokines and express PD-1 in direct cell contact–dependent mechanisms. These results suggest that the T cell–T cell interaction may also regulate T-cell immunity in the presence of iris PE cells that have powerful immune inhibitory activities.

Latchman et al.[27] previously showed that PD-$L1^{-/-}$ mice indicated PD-L1 on T cells and antigen-presenting cells and that host tissue negatively regulate T cells. Moreover, Nishimura et al.[28] reported that aged PD-1 (PD-L1 receptor)$^{-/-}$ mice with C57BL/6 backgrounds spontaneously developed autoimmune diseases such as characteristic lupus-like arthritis and glomerulonephritis. In addition, the costimulatory molecules are upregulated by IFNγ Th1 cytokines, and the PD-L1/PD-1 interactions are able to suppress T-cell activation.[22,29] We also demonstrated that retinal pigment epithelium (RPE) exposed

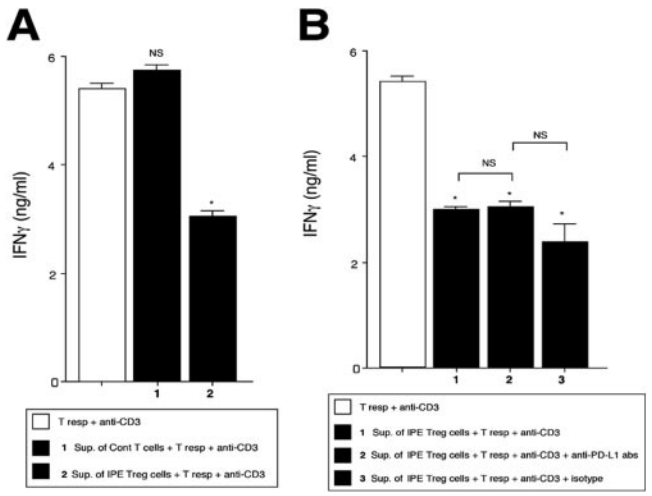

FIGURE 6. Capacity of IPE-induced Treg cell supernatants to T-cell activation. (**A**) Supernatants from IPE-induced Treg cells were harvested and used for in vitro assay. Supernatants from control $CD8^+$ T cells were also harvested as a control. Target responder $CD4^+$ T cells were stimulated with anti–CD3 and cocultured with these T-cell supernatants. $^*P < 0.05$, compared with positive control cultures. *White bars*: responder $CD4^+$ T cells + anti–CD3. NS, not significant. (**B**) Anti–mouse PD-L1 neutralizing antibodies or isotype rat IgG was added to supernatants from IPE-induced Treg cells. After 48 hours, supernatants from anti–CD3-stimulated responder T cells in the presence of Treg cell supernatants were harvested and then assayed for IFNγ by ELISA. $^*P < 0.05$, compared with positive control cultures. *White bars*: responder $CD4^+$ T cells + anti–CD3). NS, not significant.

to Th1 cytokine IFNγ, through the inducible expression of PD-L1, suppresses T-cell activation by engaging PD-1 on the IFNγ-secreting T cells.[17] IFNγ-pretreated RPE cells that express PD-L1 significantly suppress bystander Th1 cells that are induced by recombinant mouse IFNγ and IL-12 plus anti–CD3 antibody. Moreover, Treg cells failed to suppress IFNγ production by $CD4^+$ T cells from PD-1 null donors.[17] This resulted in changes to the T-cell functional program and suppressed T-cell susceptibility to activation through the first signal (anti–CD3 stimulation) plus the second costimulatory signal (PD-1–PD-L1 interactions). Unlike ocular PE cells, T cells constitutively express PD-L1, but not PD-L2, molecules without stimulation.[21,23] PD-L2 expression is restricted on macrophages and dendritic cells.[19,23]

On the other hand, naive resting T cells do not express PD-1. After activation, its surface expression is inducible on T cells in the presence of anti–CD3 antibodies (Fig. 3D). It is assumed that the PD-L1/PD-1 pathway between Treg cells and bystander T cells may occur during inflammatory conditions. The T cell-specific inhibition by IPE-induced Treg cells that expressed PD-L1 costimulatory molecules on the surface was achieved exclusively with direct cell contact. Furthermore, the PD-L2/PD-1 pathway was not relevant to Treg inhibition because IPE-induced Treg cells poorly expressed PD-L2 (Fig. 3C) and because Treg cells in the presence of anti–mouse PD-L2 blocking antibodies significantly suppressed the activation of effector T cells (Fig. 4B).

As shown in this study, $CD8^+$ T cells exposed to IPE cells can acquire regulatory functions and exhibit global suppression against bystander T cells in vitro. For instance, IPE-induced Treg cells significantly suppressed T-cell proliferation and cytokine production by activated responder T cells in the presence of anti–CD3. Under appropriate conditions, activated $CD4^+$ T cells are able to produce various cytokines such as Th1 (IFNγ, TNF-α, and IL-2), Th2 (IL-4, IL-5, and IL-13), and Th17 (IL-17). Our established Treg cells significantly suppressed these cytokines through activated T cells. Among them, Th1 cytokine IFNγ and Th17 cytokine IL-17 as inflammatory cytokines have been shown to be critical mediators for ocular inflammatory disease in animal models and in human inflammatory disorders.[30–32] We, therefore, evaluated with the cytokine production by activated responder T cells. If neutralizing antibodies for PD-L1 were cocultured with IPE-induced Treg cells and if target $CD4^+$ T cells from PD-1 KO donors were used, the Th1-specific suppression by IPE-induced Treg cells was impaired. Importantly, the PD-1/PD-L1 interaction played a critical role in the Th1-mediated, but not Th17-mediated, inflammation. We have had similar results and recently reported that cultured RPE cells in the IFNγ-treated cell cultures greatly expressed PD-L1 costimulatory molecules and suppressed activation of the bystander IFNγ-producing Th1-type cells that express the PD-1 costimulatory receptor in vitro.[17] Thus, Th1 cytokine-exposed ocular PE cells can express the negative costimulatory molecule, resulting in suppression of the bystander Th1-type cells. During inflammatory conditions, a subpopulation of $PD\text{-}1^+$ T cells is the first to encounter $PD\text{-}L1^+$ ocular resident cells, and another subpopulation of $PD\text{-}1^+$ T cells is also able to access the $PD\text{-}L1^+$ T cells, which means T-T interactions. This can account for the findings that in culture, these T cells eventually are able to cross-regulate bystander $CD4^+$ T cells.

$CD8^+$ IPE-induced Treg cells exhibit a regulatory phenotype (e.g., IPE-induced Treg cells express Foxp3 molecules), and the expression of Foxp3 was necessary for the cells' regulatory function.[8,9] There is no evidence, however, that the expression of Foxp3 is required for PD-L1 expression by our induced Treg cells. Some reported that naturally occurring Treg cells constitutively express Foxp3 and express both PD-1 and PD-L1 molecules. Their influence on Treg function is, however, unknown.[33–35] As revealed in the present study, IPE-induced Treg cells greatly expressed these costimulatory molecules. If the Treg cells express both PD-L1 and PD-1, the interaction between $CD8^+$ Treg cells has a deleterious effect on their survival. It is assumed that a PD-1 signal (negative costimulatory signal) might be required for acquiring Treg function such as a CTLA-4, which is an immunoglobulin superfamily. PD-1 is most closely related to CTLA-4 because it shares approximately 24% amino acid sequence identity. This molecule is also a transmembrane protein. In fact, the $CD8^+$ IPE-induced Treg cells greatly express CTLA-4 and CTLA-4 ligand (B7–2).[8] On the other hand, IPE-induced Treg cells also secrete soluble inhibitory factors. The supernatants of IPE-induced Treg cells still inhibit IFN-γ production in the presence of anti–PD-L1 neutralizing antibody (see Fig. 6B), strongly suggesting that other mechanisms are also involved. We have previously shown a role for other immunosuppressive molecules such as CTLA-4, TGF-β, and IL-10 in the inhibitory effect of IPE cells and IPE-induced Treg cells.[8,9] The inhibition of these molecules in the supernatants should be included.

In conclusion, IPE-induced Treg cells greatly expressed the PD-L1 cell surface molecules and suppressed the activation of IFNγ-producing Th1 cells that express PD-1 in vitro. Although the Treg cells exhibited global suppression (e.g., the suppression of T-cell proliferation and Th1, Th2, and Th17 cytokines by target T cells), the PD-L1 costimulatory negative signal was required to achieve Th1-specific inhibition. Using T-T interactions, IPE-induced Treg cells are able to achieve the suppression of IFNγ Th1 cytokine, because inflammatory cytokines have been shown to be critical mediators of ocular inflammatory disease.[30–32] Thus, the IPE-derived regulatory T cells acquire functions that play a role in establishing immune regulation in the eye.

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