Immunosuppressive Properties of Regulatory T Cells Generated by Incubation of Peripheral Blood Mononuclear Cells with Supernatants of Human RPE Cells

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Purpose. To determine whether supernatants of human retinal pigment epithelium (RPE) cells can convert $CD4^+$ T cells into regulatory T cells (Tregs) under Treg-induction conditions in vitro and in vivo.

Methods. Peripheral blood mononuclear cells were cocultured with supernatants from TGFβ2-pretreated human RPE lines on anti-CD3–coated plates. Cells were then separated with a $CD4^+CD25^+$ Treg isolation kit and cultured with supernatants from RPE, anti-CD3/CD28 antibodies, high-dose IL-2, and TGFβ2. By flow cytometry sorting, $CD25^+CD45RA^-$ Tregs were separated. Expressions of $CD25^{high}$, Foxp3, CD152, and TNFRSF 18 on Tregs were analyzed by flow cytometry. Cytokine production by Tregs was measured by ELISA. Proliferation of target T cells was assessed by [^{3}H]thymidine incorporation or CFSE incorporation. In addition, mouse RPE–induced Tregs were used for the in vitro assay and in vivo experimental autoimmune uveitis (EAU) models.

Results. Human RPE–induced Tregs expressed higher levels of the Treg markers $CD25^{high}$, Foxp3, CD152, and TNFRSF 18. In addition, RPE-induced Tregs included significant numbers of $CD4^+CD25^{high}Foxp3^{high}CD45RA^-$ active effector Tregs that significantly suppressed the activation of Th1/Th17 cell lines, indicating that they have immunosuppressive properties. Furthermore, $CD4^+CD25^{low}Foxp3^{low}CD45RA^-$ nonsuppressing cytokine-secreting T cells were removed from the in vitro–manipulated Treg population. Administration of mouse RPE–induced Tregs significantly suppressed ocular inflammation in mice with EAU. In addition, the Tregs suppressed retinal antigen-specific T cells in vitro.

Conclusions. It is hoped that through the data provided in this study that Tregs might become useful as individualized therapeutic agents for ocular autoimmune diseases. (*Invest Ophthalmol Vis Sci.* 2012;53:7299–7309) DOI:10.1167/iovs.12-10182

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Ocular pigment epithelium (PE) cells of the retina are important for the creation and maintenance of ocular immune privilege.[1,2] The immunoregulatory property of retinal PE (RPE) is related to intraocular suppression of immunogenic inflammation. Murine T cells have been converted into regulatory T cells (Tregs) by in vitro exposure to PE cells harvested from the retinas of uninflamed, healthy eyes.[3–9] In addition, RPE-induced Tregs suppress activation of bystander target T cells in vitro. In animal uveitis models, inflamed eyes contain $CD4^+CD25^+Foxp3^+$ Tregs.[7,8] Ocular immune privilege is created by numerous mechanisms, including anterior chamber–associated immune deviation and inhibitory molecules, whereas RPE is assumed to play a relatively minor role. Recently, we showed that RPE-dependent human regulatory T cells that were expanded in vitro acquired a suppressive function.[10] These human RPE–induced Tregs greatly suppressed intraocular T cells from active uveitis patients. Moreover, eye-specific Treg induction depends on transforming growth factor beta (TGFβ) signaling.[6–8] Therefore, in vitro–manipulated RPE-induced Tregs have potential therapeutic applications for patients with refractory ocular inflammatory disorders, such as uveitis.

The clinical use of Tregs as personalized therapeutic agents for the treatment of autoimmune diseases, graft-versus-host disease (GVHD), and transplant rejection is under investigation. However, numerous issues must be addressed before introducing Treg therapy to the clinic. For example, Foxp3 expression can be induced in human T cells without necessarily conferring a suppressive function.[11] Human $Foxp3^+$ Tregs are unstable in vivo, which complicates their usefulness for adoptive transfer therapies.[12] Therefore, recent studies have focused on deciphering mechanisms that regulate the stability of the Treg cell lineage. More recently, human $Foxp3^+$ Tregs were divided into three groups based on the expression of CD45RA, a transmembrane protein tyrosine phosphatase expressed by naïve T cells: $CD25^{low}CD45RA^+$ resting Tregs, $CD25^{high}CD45RA^-$ active Tregs, and $CD25^{low}CD45RA^-$ nonsuppressive Tregs.[13] These three subsets of human $Foxp3^+$ cells display distinct phenotypes and functions. Both resting and active Tregs have potent immunosuppressive properties in vitro. On the other hand, $CD25^{low}CD45RA^-$ nonsuppressive Tregs produce proinflammatory cytokines.[13,14]

$Foxp3^+$ Tregs can also be generated from naïve $CD4^+$ T cells in vitro in the presence of TGFβ.[15–17] Naïve T cells are isolated from a patient's blood, converted into Tregs in vitro, and then expanded in vitro to produce large numbers for therapy. This de novo approach for Treg generation is particularly attractive because some patients with autoimmune diseases have functional Treg defects, and so their cells cannot be used therapeutically.[14,18]

The present study was designed to determine whether RPE-induced $CD4^+$ Tregs can be expanded under Treg-induction conditions and if these RPE-induced Tregs can suppress the

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activation of effector T cells. To improve the procedure for induction of Tregs, we used high-dose recombinant IL-2, anti-human CD3/CD28 antibodies, and supernatants from recombinant TGFβ-pretreated RPE cells. Among the in vitro–manipulated RPE-induced Tregs studied, $CD4^+CD25^{high}Foxp3^{high}CD152^{high}CD45RA^-IFN\text{-}\gamma^-IL\text{-}17^-$ active RPE-induced Tregs greatly suppressed the activation of effector T cells in vitro. We were able to remove $CD4^+CD25^+Foxp3^+CD152^+CD45RA^-IFN\text{-}\gamma^+IL\text{-}17^+$ nonsuppressive T cells from the remainder of the Treg population. In addition, we examined whether mouse RPE–induced Tregs can suppress intraocular inflammation in mice with experimental autoimmune uveitis (EAU). The administration of RPE-induced Tregs that greatly expressed Foxp3 significantly suppressed ocular inflammation in EAU. In vitro, the retinal antigen–specific cytokine response was significantly reduced when intraocular T cells were cocultured with RPE-induced Tregs.

Materials and Methods

RPE-Induced Tregs

This research was performed according to the Declaration of Helsinki standards and was approved by the Institutional Review Board of Tokyo Medical and Dental University. Human RPE cell lines (ARPE-19 cells) were kindly provided by Nobuyuki Ebihara (Juntendo University School of Medicine, Tokyo, Japan). As described previously,[10] we used $CD4^+$ T cells exposed to RPE cell–free supernatants as Tregs, to avoid contamination with allogeneic RPE cells. The supernatants of human RPE cell lines that were cultured in the presence or absence of recombinant human TGFβ2 (rTGFβ2, 5 ng/mL; R&D Systems, Minneapolis, MN) and high-dose recombinant human IL-2 (rIL-2, 200 U/mL; PeproTech, Inc., Rocky Hill, NJ) were harvested for 72 hours. Peripheral blood mononuclear cells (PBMCs) were cocultured with the RPE supernatants on anti-human CD3-coated plates (BD Biosciences, Bedford, MA) for 24 hours and then harvested. Cells were separated with a human $CD4^+CD25^+$ Treg isolation kit (MACS Systems; Miltenyi Biotec, Auburn, CA). Flow cytometry revealed 95% $CD4^+CD25^+$ purity. In secondary cultures for Treg induction, prepared $CD4^+CD25^+$ T cells were recultured with high-dose rIL-2, anti-human CD3 antibody (2 μg/mL; Ancell Corp., Bayport, MN), anti-human CD28 antibody (2 μg/mL; BD PharMingen, San Diego, CA), or rTGFβ2 (5 ng/mL) for 3 days. The harvested Tregs contained no $cytokeratin^+$ RPE cells ($\leq$0.3%), as shown by staining with anti-pan-cytokeratin antibody (Clone PCK-26; Sigma Chemical Co., St. Louis, MO).[10]

The concentrations of TGFβ1, IL-10, IFN-γ, IL-17, and TNF-α in the supernatants of RPE-induced Tregs or control T cells (not exposed to RPE supernatants) were measured by ELISA (R&D Systems). The concentration of IFN-γ or IL-17 in the supernatants of target T cells was also measured to evaluate suppression by RPE-induced Tregs. The proliferation of Tregs was assessed by [^{3}H]thymidine incorporation.

Preparation of Murine RPE Cells and Supernatants

Primary cultured murine RPE cells were prepared as described previously.[19–21] RPE cells were harvested from adult C57BL/6 mice (CLEA Japan, Inc., Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) was used for culturing human and murine RPE.[19–21] All animal protocols were approved by Tokyo Medical and Dental University. Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of Murine RPE Supernatant-Induced Tregs

The supernatants of mouse RPE cells cultured in the presence of porcine TGFβ2 (5 ng/mL; R&D Systems) were harvested for the generation of murine Tregs. The procedure was similar to the methods used for human RPE–induced Tregs, as described above. Adult C57BL/6 mice were used as spleen cell donors. To avoid contamination with RPE cells, $CD4^+$ T cells exposed to RPE supernatants (no RPE cells) were used as Treg cells. T cells were separated with mouse $CD4^+$ T-cell isolation kits (MACS Systems). For the induction of Treg cells, $CD4^+$ T cells in the presence of anti-mouse CD3 antibody (0.5 μg/mL; BD PharMingen) and anti-mouse CD28 antibody (0.5 μg/mL; BD PharMingen) were cultured in RPMI 1640 with 10% FBS, recombinant mouse IL-2 (rIL-2, 200 U/mL), and TGFβ2-pretreated RPE supernatants for 72 hours. The $CD4^+$ T cells exposed to the RPE supernatants were harvested, x-irradiated (2000 rad), and added (2×10^5 cells/well) to target cells. The harvested Treg cells contained no $cytokeratin^+$ mouse RPE cells ($\leq$1%) when the T cells were stained with anti-pan-cytokeratin antibody.

Establishment of Target T Cells

Target-activated T cells, pan-T cells, or $CD4^+$ T cells (Th1 cells and Th17 cells) were established from the PBMCs of healthy volunteers. The cells were cultured in RPMI 1640 medium with 10% FBS and rIL-2 (100 U/mL), anti-human CD3 (2 μg/mL), and anti-human CD28 (2 μg/mL).

Human Th1 cell lines were established by culture with human rIL-12 (20 ng/mL; Wako, Osaka, Japan), human rIL-2, anti-human CD3 antibody (2 μg/mL), anti-human CD28 antibody (2 μg/mL), and anti-human IL-4 antibody (5 μg/mL; R&D Systems). T cells that produced a high level of IFN-γ were used as Th1 cells. For the induction of human Th17 cell lines, purified $CD4^+$ T cells from healthy donors were cultured with anti-human CD3 (2 μg/mL), anti-human CD28 (2 μg/mL), anti-human IFN-γ (5 μg/mL; R&D Systems), anti-human IL-4 (5 μg/mL), and the recombinant human proteins IL-1β (20 ng/mL; Pepro Tech, Inc.), IL-6 (20 ng/mL; R&D Systems), IL-23 (20 ng/mL; R&D Systems), and TNF-α (20 ng/mL; R&D Systems). After 5 to 7 days of culture, the harvested T cells that produced large amounts of IL-17 were used for the Treg assay.

For T-cell activation assays, the established target T cells were added (1.0×10^5 cells/well) to 96-well plates with and without RPE-induced Tregs. The RPE-induced Tregs were seeded directly onto 96-well plates ($1–5 \times 10^4$ cells/well). T-cell activation was assessed by [^{3}H]thymidine incorporation, 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) incorporation by flow cytometry, or cytokine production (IFN-γ and IL-17), as in previous reports.[3,19–21]

Flow Cytometry

The cultured RPE-induced Tregs were prepared for Treg phenotype sorting by flow cytometry. Control T cells from the same donor (fresh $CD4^+CD25^+$ natural Tregs) were isolated by using a human $CD4^+CD25^+$ Treg isolation kit. These T cells were stained with fluorescein isothiocyanate (FITC)–conjugated anti-human CD25 antibody (eBioscience, San Diego, CA), anti-human Foxp3 antibody (eBioscience), anti-human CD152 antibody (BD PharMingen), anti-human TNFRSF 18 antibody (GITR; BD PharMingen), and phycoerythrin (PE)-conjugated antibody to anti-human CD4 antibody (BD PharMingen). Before staining, cocultured cells were incubated with an Fc-receptor blocker (Miltenyi Biotec) for 15 minutes. After permeabilization, the cells were stained with mouse IgG (R&D Systems) or rat IgG (eBioscience) as isotype controls at 4°C for 30 minutes.

To detect Foxp3 on murine RPE–induced Tregs, Tregs and control T cells were also prepared. After Fc-receptor blockade and permeabilization, cells were stained with FITC-conjugated anti-mouse Foxp3 antibody (eBioscience) and PE-conjugated anti-mouse CD4 antibody (eBioscience) at 4°C for 30 minutes and then analyzed by flow cytometry.

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CD45RA and CD25 Staining

Human RPE–induced Tregs, cultured control $CD4^+$ T cells, and fresh $CD4^+$ T cells from healthy donors were stained with FITC-labeled anti-human CD25 antibody and PE-labeled anti-human CD45RA antibody (Beckman Coulter, Marseille, France) at 4°C for 30 minutes. The T cells were also stained with FITC-labeled rat IgG (eBioscience) and PE-labeled mouse IgG (MBL, Nagoya, Japan) as isotype controls.

For purification of $CD4^+CD25^{high}CD45RA^-$ T cells from RPE-induced Tregs, cells were stained with anti-CD45RA-PE and anti-CD25-FITC and sorted on a fluorescence-activated cell sorter (BD FACS Vantage; BD, Franklin Lakes, NJ). As controls, $CD4^+CD25^+CD45RA^-$ T cells were also sorted by using a fluorescence-activated cell sorter. Both populations were stained with the following antibodies: anti-human Foxp3, anti-human CD152, anti-human IFN-γ (R&D Systems), and anti-human IL-17 (R&D Systems). These T cells were also used in the in vitro suppression assays (T-cell proliferation or cytokine production by CFSE-labeled pan-T cells, Th1-type T cells, and Th17-type T cells).

Treg Transfer into Mice with EAU

Normal mice were subcutaneously immunized in the neck region with 200 μg of interphotoreceptor retinoid-binding protein peptide ($IRBP_{1-20}$) emulsified in complete Freund's adjuvant (Difco, Detroit, MI) and containing *Mycobacterium tuberculosis* strain H37Ra (Difco).[22–24] Funduscopic examination was performed on days 14, 17, and 21 after immunization, and histologic examination was performed on day 21 after immunization. Clinical scores (grades 0–4) were assigned in accordance with the previous method. Inflammation was evaluated based on fundus and histologic findings. In addition, cells were harvested from the EAU eyes ($n = 12$) on day 21 for use in in vitro assays.

T cells from green fluorescent protein (GFP) transgenic mice were used for the assay. For adoptive transfer experiments, GFP-labeled murine RPE-induced Tregs (1×10^6 cells) and control $CD4^+$ T cells (1×10^6 cells) were prepared. These T cells were injected intravenously (IV) into mice 14 days after immunization with $IRBP_{1-20}$. After injection into mice with EAU, GFP-positive T cells were observed. For visualization, whole-mount retinas were viewed under inverted and upright confocal laser-scanning microscopes (Leica TSC-SP2; Leica Microsystems, Wetzlar, Germany). Digital images of the flat mounts were taken by using a spot image analysis system, and an area (450×450 μm) at the center of the optic nerve was analyzed by using ImageJ image-analysis software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsbweb.nih.gov/ij/index.html). GFP-positive cells were counted. Intraocular cells were harvested to detect Foxp3 by flow cytometry and RT-PCR.

RNA Extraction and RT-PCR Analysis

Cellular extracts were prepared from mouse RPE–induced Tregs that were cultured as described above. The RPE-induced Tregs and control T cells (mouse $CD4^+$ T cells without RPE) were washed twice with PBS, and then total RNA was isolated with a commercial ready-to-use reagent (TRIzol; Invitrogen/Life Technologies, Carlsbad, CA). After cDNA synthesis, RT-PCR was performed by a standard PCR method. PCR amplification of cDNA was performed by using primers for murine Foxp3, as described previously.[6] The PCR products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide. The Foxp3 mRNA concentrations were normalized to the amount of GAPDH mRNA present.

Statistical Evaluation

Each experiment was repeated at least twice with similar results. Parametric data were analyzed with the Student's *t*-test. Nonparametric data were analyzed with the Mann-Whitney *U* test. Statistical significance was defined as $P < 0.05$.

Results

Phenotype of Human RPE–Induced Tregs

We first assessed the expression of CD25, Foxp3, CD152 (CTLA-4), and TNFRSF 18 (GITR) in RPE-induced Tregs that were generated with methods similar to those described in our previous report.[10] Previously, we conducted experiments to measure the Foxp3 expression on Treg cells that were cultured with recombinant TGFβ2 (rTGFβ2) only, RPE cell line supernatant only, or rTGFβ2-pretreated RPE cell line supernatant.[10] The T cells that were exposed to supernatants of rTGFβ2-treated RPE cells expressed a much greater level of Foxp3 than that of the T cells that were treated with rTGFβ2 only, supernatant of RPE cells only, or a control chemical. Therefore, in the current study, we used T cells exposed to supernatants of rTGFβ2-pretreated RPE cells as RPE-induced Tregs.

Representative results showed that RPE-induced Tregs expressed a higher level of CD25 (94% $CD4^+CD25^+$) than did fresh Tregs obtained from the same healthy donor (naturally occurring Tregs; 80%+) (Fig. 1A). In addition, RPE-induced $CD4^+CD25^+$ Tregs greatly expressed Foxp3, CD152, and TNFRSF 18 molecules, as compared with control Tregs from the same donor (Fig. 1A).

In secondary cultures for Treg induction, prepared $CD4^+CD25^+$ T cells already exposed to RPE supernatants were recultured with a high-dose of rIL-2, anti-human CD3 antibody, and anti-human CD28 antibody in the presence or the absence of recombinant TGFβ2 for 3 days. RPE-induced Tregs cultured with rTGFβ2 greatly expressed Foxp3 (85%+) as compared with those cultured without rTGFβ2 (59% CD4/Foxp3 double positive) (Fig. 1B).

We next evaluated whether RPE-induced Tregs can be expanded in vitro. Because of the limited number of regulatory T cells in vivo and the inherent loss of cells during the isolation procedures, it became necessary to expand regulatory T cells in vitro. As revealed in Figure 1C, RPE-induced Tregs expanded approximately 50-fold over 10 days in culture with stimulation by a high-dose of rIL-2, anti-CD3 antibody, and anti-CD28 antibody in the presence of rTGFβ2; control $CD4^+$ T cells expanded to an even greater extent.

Capacity of RPE-Induced Tregs to Produce Immunoregulatory Cytokines

We next evaluated whether RPE-induced Tregs can secrete inhibitory cytokines. Compared with control T cells, RPE-induced Tregs produced high levels of the immunoregulatory cytokines TGFβ1 and IL-10 (Fig. 2). In contrast, the effector cell–associated cytokines IFN-γ, IL-17, and TNF-α were produced at lower levels compared with controls (Fig. 2). Thus, our RPE-induced Tregs secreted high levels of inhibitory cytokines and low levels of inflammatory cytokines.

Effect of RPE-Induced Tregs on Proliferation of Activated Target T Cells

We next examined whether the expanded RPE-induced Tregs could suppress the activation of bystander effector T cells in vitro. For the assay, we used human RPE-induced Tregs after 3 or 7 days of secondary culture. The 3-day cultured cells significantly suppressed proliferation of target effector T cells (Fig. 3A). However, the 7-day cultured Tregs had less

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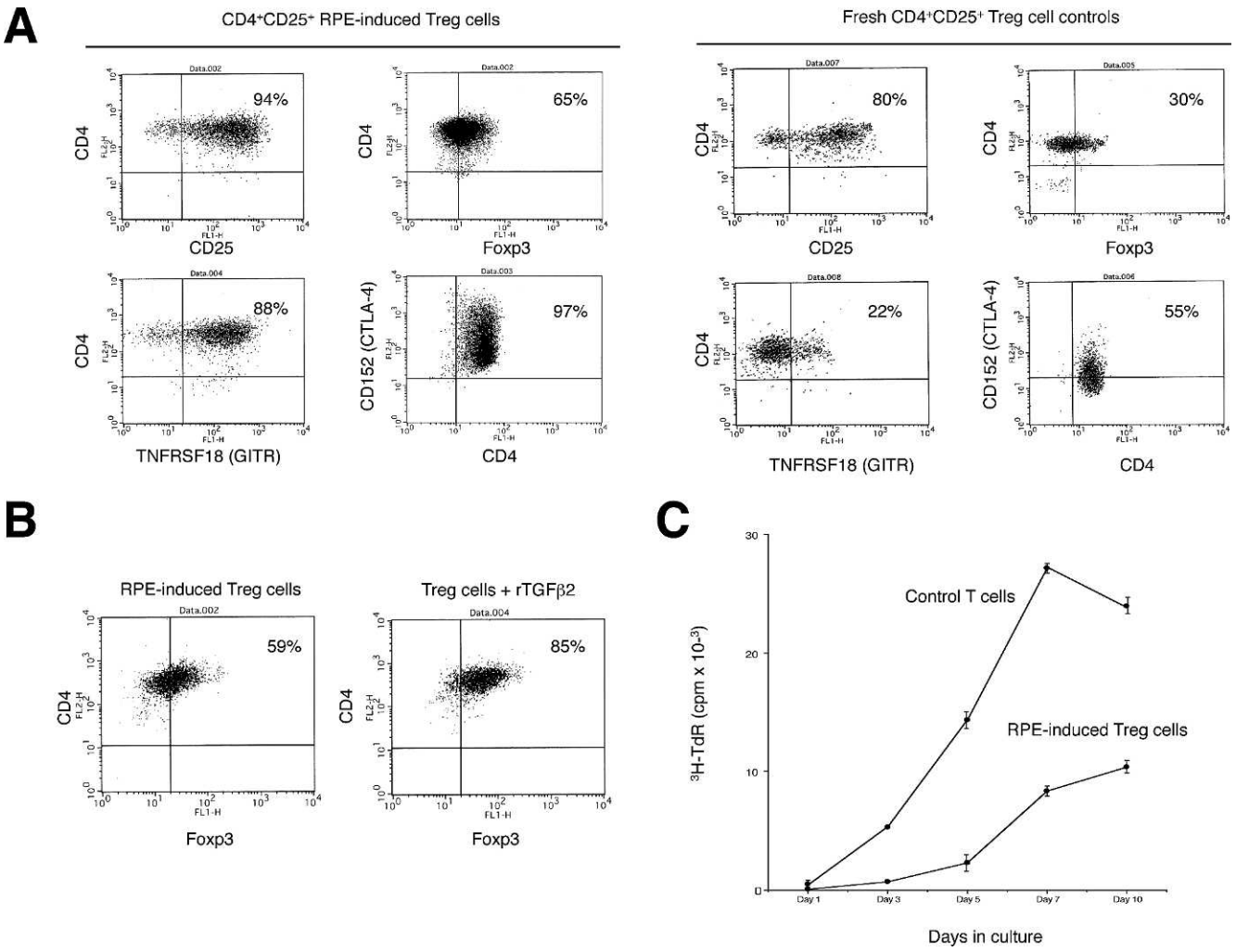

Figure 1. Phenotype of $CD4^+$ $CD25^+$ human RPE-induced Tregs. (**A**) Isolated $CD4^+CD25^+$ cells from RPE supernatant–induced Tregs stained with anti-human antibodies and analyzed by flow cytometry. Control: fresh $CD4^+CD25^+$ Tregs (naturally occurring Tregs) from the same healthy donor. (**B**) Isolated RPE supernatant–exposed $CD4^+CD25^+$ T cells recultured with a high-dose of rIL-2 and anti-human CD3/CD28 antibodies with or without rTGFβ2 for 3 days, then stained with anti-human Foxp3/CD4 antibodies. The percentages of double-positive cells are shown. (**C**) RPE-induced Tregs and uninduced control $CD4^+$ T cells (1×10^5/well) stimulated with a high-dose of rIL-2, anti-CD3 antibody, and anti-CD28 antibody in the presence of rTGFβ2; [^{3}H]thymidine incorporation. Mean ± SEM of triplicate samples.

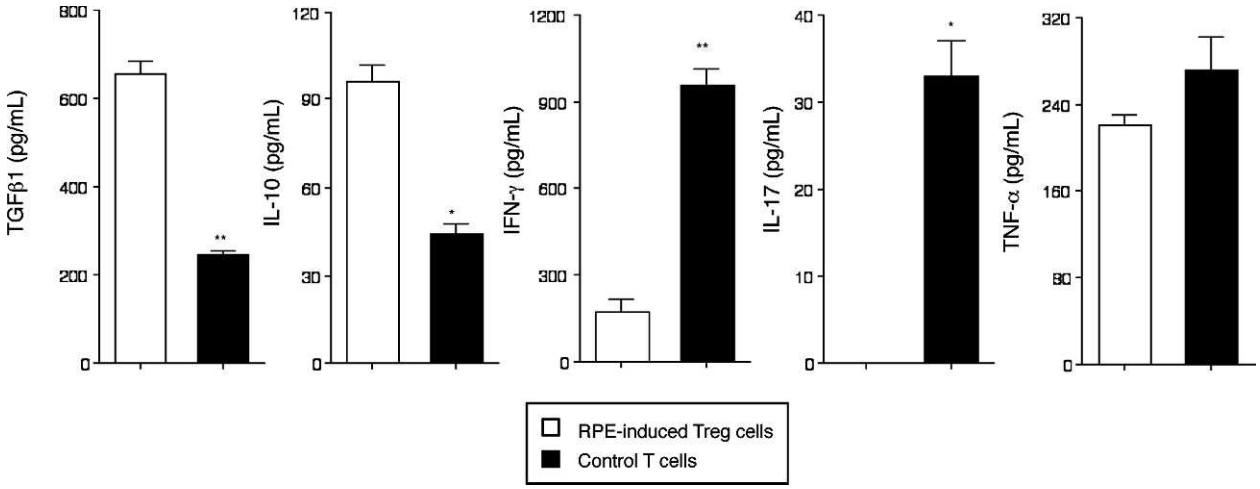

Figure 2. Production of cytokines by RPE-induced Tregs. Supernatants from RPE-induced Tregs (*open bars*) and uninduced control $CD4^+$ T cells (*black bars*) were assayed for TGFβ1, IL-10, IFN-γ, IL-17, and TNF-α by ELISA. $^*P < 0.05$ and $^{**}P < 0.005$.

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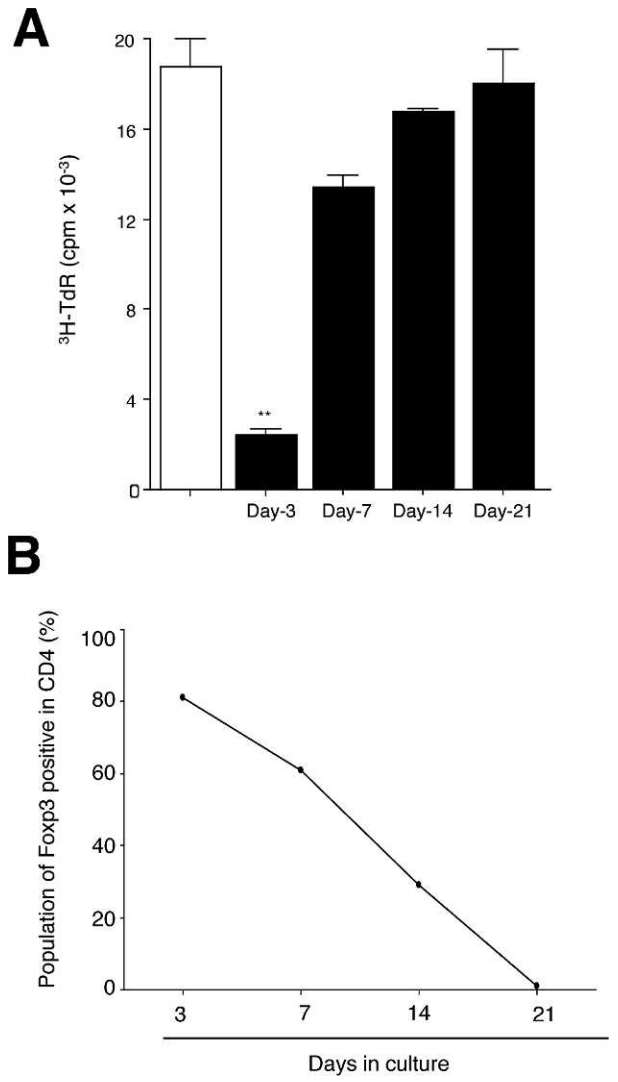

Figure 3. Effect of RPE-induced Tregs on proliferation of activated target T-cell lines. (**A**) Purified target pan-T cells stimulated by anti-CD3/CD28 antibody cultured for 72 hours in the presence (*black bars*) or absence (*open bars*) of RPE-induced Tregs. Exposed cells (3-, 7-, 14-, or 21-day cultures) were assayed for [^{3}H]thymidine uptake. $^{**}P < 0.005$, compared with *open bar*. (**B**) Expression of Foxp3 in long-term RPE-induced Treg culture harvested on days 3, 7, 14, or 21 and stained with anti-human Foxp3 and CD4 antibodies.

suppressive activity. Unexpectedly, the expression of Foxp3 was dramatically reduced during the secondary culture (Fig. 3B). On day 21, the expression was less than 5%. These results indicate that RPE-induced Tregs may lose their regulatory phenotype during long-term culture. Therefore, we changed our procedure to obtain only active Tregs, excluding less active T cells.

Isolation of Human RPE–Induced CD45RA$^-$CD25^{high} Active Tregs

Human CD4$^+$Foxp3$^+$ Treg cells are composed of three phenotypically and functionally distinct subpopulations: CD25^{high}CD45RA$^-$ active Tregs, CD25^{low}CD45RA$^+$ resting Tregs, and CD25^{low}CD45RA$^-$ nonsuppressive T cells.[13] The combination of CD25 and CD45RA staining of CD4$^+$ RPE-induced Tregs revealed five subpopulations, two of which, Gate 1 (G1) and Gate 2 (G2), are shown in Figure 4A. Gate 1 includes CD25^{high}CD45RA$^-$ active Tregs. RPE-induced Tregs included significant numbers of these active Tregs, compared with control T cells without RPE supernatants (middle histogram) and naïve T cells (left histogram). On the other hand, both RPE-induced Tregs and control T cells included CD25^{low}CD45RA$^-$ nonsuppressive T cells (Gate 2 in Fig. 4A). Next, we assessed whether CD25^{high}CD45RA$^-$ active RPE-induced Tregs express Foxp3 and CD152 (CTLA-4) and produce inflammatory cytokines (IFN-γ and IL-17). By using the sorting system, CD25^{high}CD45RA$^-$ active Tregs (Gate 1) and CD25^{low}CD45RA$^-$ nonsuppressive T cells (control: Gate 2) were separated from human RPE–induced Tregs. RPE-induced active Tregs (CD25^{high}CD45RA$^-$) greatly expressed Foxp3 and CD152 as compared with RPE-induced CD25^{low}CD45RA$^-$ nonsuppressive T cells (Fig. 4B). As expected, RPE-induced CD25^{low}CD45RA$^-$ nonsuppressive T cells also expressed these molecules, but in lower amounts. Importantly, RPE-induced active Tregs produced less IFN-γ and IL-17, whereas CD25^{low}CD45RA$^-$ nonsuppressive T cells produced significantly higher levels of these cytokines (Fig. 4B). Based on the mean fluorescence intensity determined by flow cytometric analysis, active Tregs (CD25^{high}CD45RA$^-$) expressed high levels of Foxp3 and CD152 as compared with CD25^{low}CD45RA$^-$ nonsuppressive T cells (Fig. 4C). These results suggest that separated active Tregs do not include inflammatory cytokine–secreting nonsuppressive T cells and significantly express Treg cell markers.

Capacity of RPE-Induced Active Tregs to Suppress Bystander Effector T Cells

Finally, we determined whether CD25^{high}CD45RA$^-$ RPE-induced active Tregs are able to suppress bystander effector T cells in vitro. We prepared as targets Th1 cell lines induced with anti-human CD3 antibody, anti-human CD28 antibody, human rIL-12, human rIL-2, and anti-human IL-4 antibody. For the induction of target Th17 cells, purified CD4$^+$ T cells were cultured with anti-human CD3 antibody, anti-human CD28 antibody, anti-human IFN-γ antibody, anti-human IL-4 antibody, human rIL-1β, human rIL-6, human rIL-23, and human rTNF-α. The active Tregs fully suppressed the proliferation of target Th1 cells (Fig. 5A) and Th17 cells (Fig. 5A). In contrast, the CD25^{low}CD45RA$^-$ T cells separated from RPE-exposed T cells did not suppress proliferation (Fig. 5A). Similarly, active Tregs significantly suppressed cytokine production by target Th1 or Th17 cells (Fig. 5B). These results suggest that in vitro–expanded CD4$^+$CD25$^+$ RPE-induced Tregs, particularly isolated active Tregs, may have immunosuppressive activities. If CD4$^+$CD25$^+$ RPE-induced Tregs are used to treat human inflammatory ocular disease, we must first remove CD25^{low}CD45RA$^-$ cytokine-secreting nonsuppressive T cells from the total RPE-induced Treg cell population.

Capacity of RPE-Induced Tregs to Suppress Experimental Uveitis Models

We assessed the ability of RPE-induced Tregs to suppress EAU in mice by injecting cells IV into EAU mice 14 days after immunization with IRBP$_{1-20}$. First, we assessed the expression of Foxp3 in mouse RPE-induced Tregs under the same procedure for humans. As shown in Figure 6A, the mouse RPE–induced Tregs greatly expressed Foxp3 (CD4/Foxp3 double positive = 62%) as compared with controls (4%+).

Ocular inflammation was significantly suppressed in EAU mice that received RPE-induced Tregs, as compared with control EAU mice that received CD4$^+$ T cells (Fig. 6B).

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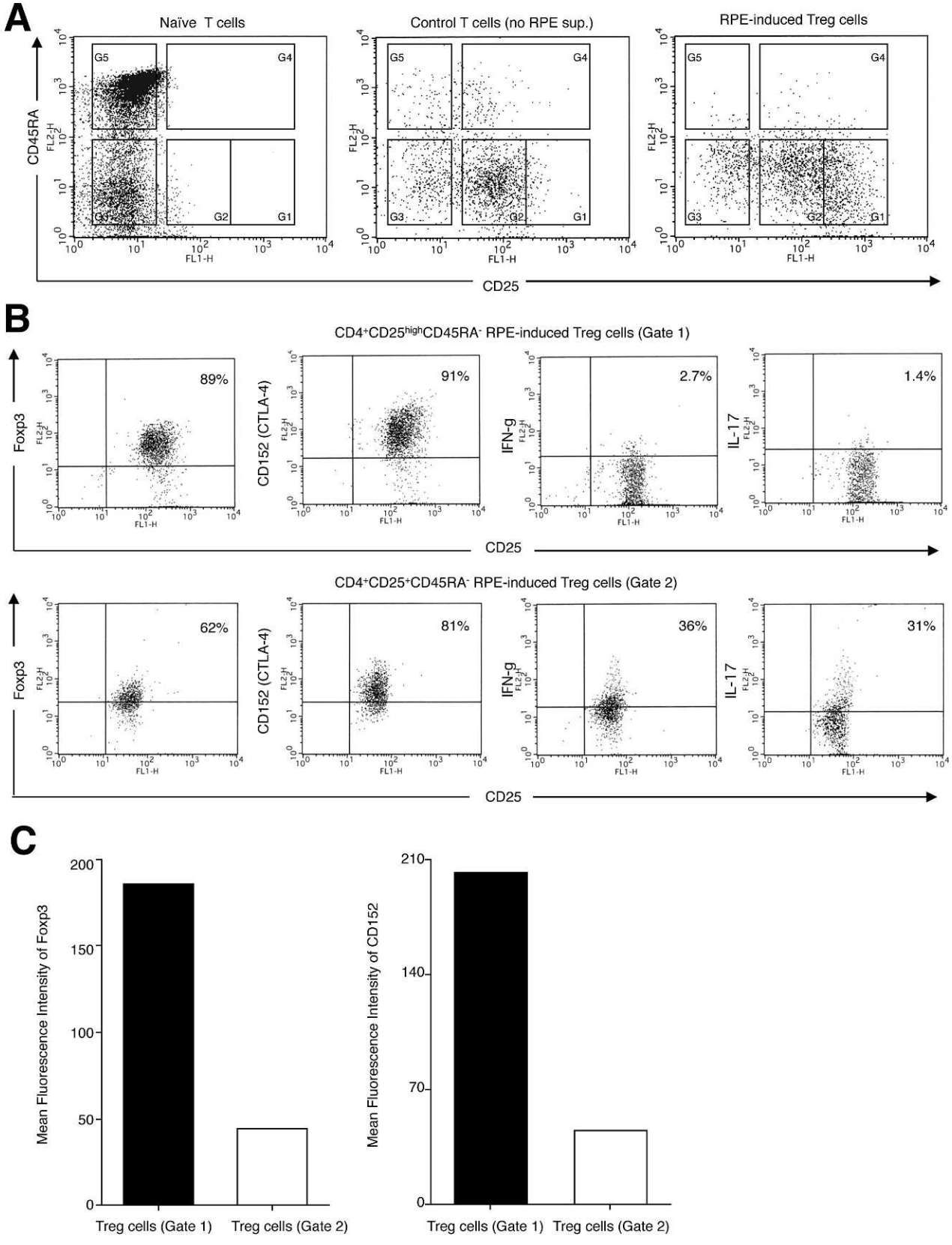

Figure 4. Separation of $CD4^{+}CD25^{high}CD45RA^{-}$ RPE-induced active Tregs. (**A**) Five T-cell subpopulations identified by flow cytometry. G1: $CD25^{high}CD45RA^{-}$ active Tregs. G2: $CD25^{+}CD45RA^{-}$ nonsuppressive T cells. (**B**) After permeabilization, G1 and G2 cells were stained with anti-human antibodies as indicated. The percentages of double-positive cells are shown. (**C**) The graph on the *left* indicates mean fluorescence intensity of Foxp3, and the graph on the *right* indicates mean fluorescence intensity of CD152 (CTLA-4) in $CD25^{high}CD45RA^{-}$ active Tregs (G1, *black bars*) and $CD25^{+}CD45RA^{-}$ nonsuppressive T cells (G2, *open bars*).

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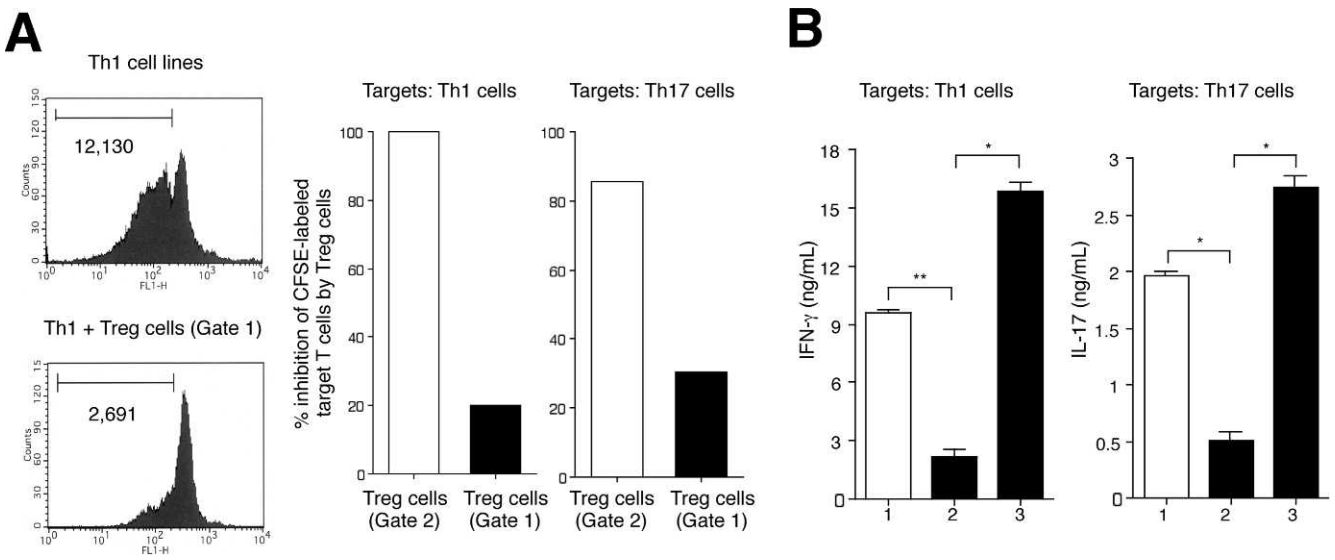

Figure 5. Suppression of RPE-induced active Tregs. (**A**) Capacity of active RPE-induced Tregs to suppress proliferation of bystander target T cells. Isolated active RPE-induced Tregs ($CD4^+CD25^{high}CD45RA^-$; *black bars*) added to secondary cultures containing target $CD4^+$ T cells, as indicated. Control: G1 cells (*open bars*). Histograms (*left*) show the active RPE-induced Tregs plus CFSE-labeled target Th1 cells or target Th1 cells only (positive control: *upper histogram*). The number of CFSE-positive cells is shown. Bar graphs (*right*) show the percentage inhibition of CFSE-labeled target Th1/Th17 cells. (**B**) Capacity of active RPE-induced Tregs to suppress cytokine production by bystander target T cells. 1: target cells only (target Th1 or Th17 cells). 2: target cells + G1 cells. 3: target cells + G2 cells. $^*P < 0.05$ and $^{**}P < 0.005$.

Inflammatory cells had infiltrated all retinal layers in the control mice, and the RPE layer was partially destroyed. In EAU mice that received RPE-induced Tregs, only a few inflammatory cells infiltrated the retinal layers, which remained intact (Fig. 6B, lower pictures). These results suggest that transferred RPE-induced Tregs can inhibit inflammatory cells in ocular inflammation models.

We next used confocal laser-scanning microscopy to determine whether IV-injected RPE-induced Tregs from GFP transgenic mice could infiltrate the eyes of EAU mice. As shown in Figure 6C, we found GFP-positive T cells in whole-mount retinas obtained after the transfer of RPE Tregs and control T cells. GFP-positive control T cells were observed at much higher levels than RPE-induced Tregs. In addition, intraocular GFP-positive cells expressed high levels of Foxp3 after Treg transfer, but had poor expression after control cell transfer (Fig. 6D). Likewise, the Foxp3 mRNA levels were greater in the RPE-induced Tregs than in the control T cells (Fig. 6E). These results suggest that RPE-induced Tregs, which inducibly express Foxp3, migrated into the EAU-affected eyes to suppress the inflammation.

We next assessed whether RPE-induced Tregs suppress retinal antigen–specific T cells in vitro. Eye-derived T cells were harvested from mice with EAU and evaluated by using an IRBP retinal antigen–specific assay. We found a significant response of increased IFN-γ and IL-17 inflammatory cytokines in the purified T cells from EAU mice immunized with IRBP antigens (Fig. 6F). This Ag-specific cytokine response was significantly reduced when intraocular T cells were cocultured with RPE-induced Tregs. In contrast, control T cells did not suppress T-cell activation. These results indicate that RPE-induced Tregs suppress in vitro retinal antigen–specific activation of intraocular T cells.

Discussion

We demonstrated that RPE-induced Tregs expressed high levels of Treg cell markers. In addition, the RPE-induced Treg population included significant numbers of $CD4^+CD25^{high}CD45RA^-$ active cells that significantly suppressed the activation of Th1/Th17 cell lines. Our results indicate that in vitro–manipulated RPE-induced Tregs suppress T-cell activities, such as proliferation and cytokine production by activated inflammatory T-helper cells. We were able to remove the nonsuppressing cytokine-secreting T cells ($CD4^+CD25^{low}CD45RA^-$) from the Treg cell population. The administration of murine RPE–induced Tregs that greatly expressed Foxp3 significantly suppressed ocular inflammation in mice with EAU. In vitro, the retinal antigen–specific cytokine response (IFN-γ and IL-17) was reduced when intraocular T cells were cocultured with RPE-induced Tregs. Thus, RPE-induced Tregs suppressed ocular inflammation in vivo and in vitro.

Our results support the development of RPE-induced Treg immunotherapy for ocular inflammatory diseases such as refractory uveitis (Fig. 7). For immunotherapy, $CD4^+CD25^{high}CD45RA^-$ active Tregs would be isolated and transferred into the inflamed eye in a single vitreous injection. Our strategy for Treg induction is advantageous because we first ascertained Treg mechanisms in the eye, which is part of the peripheral immune system and an immune-privileged site. As in our previous report, the soluble inhibitory factor TGFβ secreted by RPE cells upregulates the expression of CD25 and CD152 on $CD4^+$ T cells, and $CD25^{high}Foxp3^+$ T cells acquire the regulatory phenotype.[10] Like mouse $CD4^+CD25^+$ Tregs, the suppressive effects of human $CD4^+$ Tregs are not antigen specific. Once activated, therefore, these Tregs have broad suppressive effects. As revealed in this study, $CD4^+$ RPE-induced Tregs can produce large amounts of soluble inhibitory factors, but not inflammatory cytokines. The antigens responsible for noninfectious uveitis are still unknown. Therefore, antigen nonspecific Tregs are appropriate for immunotherapy.

Other advantages to our proposed immunotherapy include the use of only autogeneic, never allogeneic, T cells for introduction into the eye. Systemic side effects would be unlikely because the Tregs can be transferred by local injection (perhaps vitreous injection). Approximately 80% of uveitis patients have associated autoimmune pathogenic mechanisms and are treated with corticosteroids, immunosuppressive

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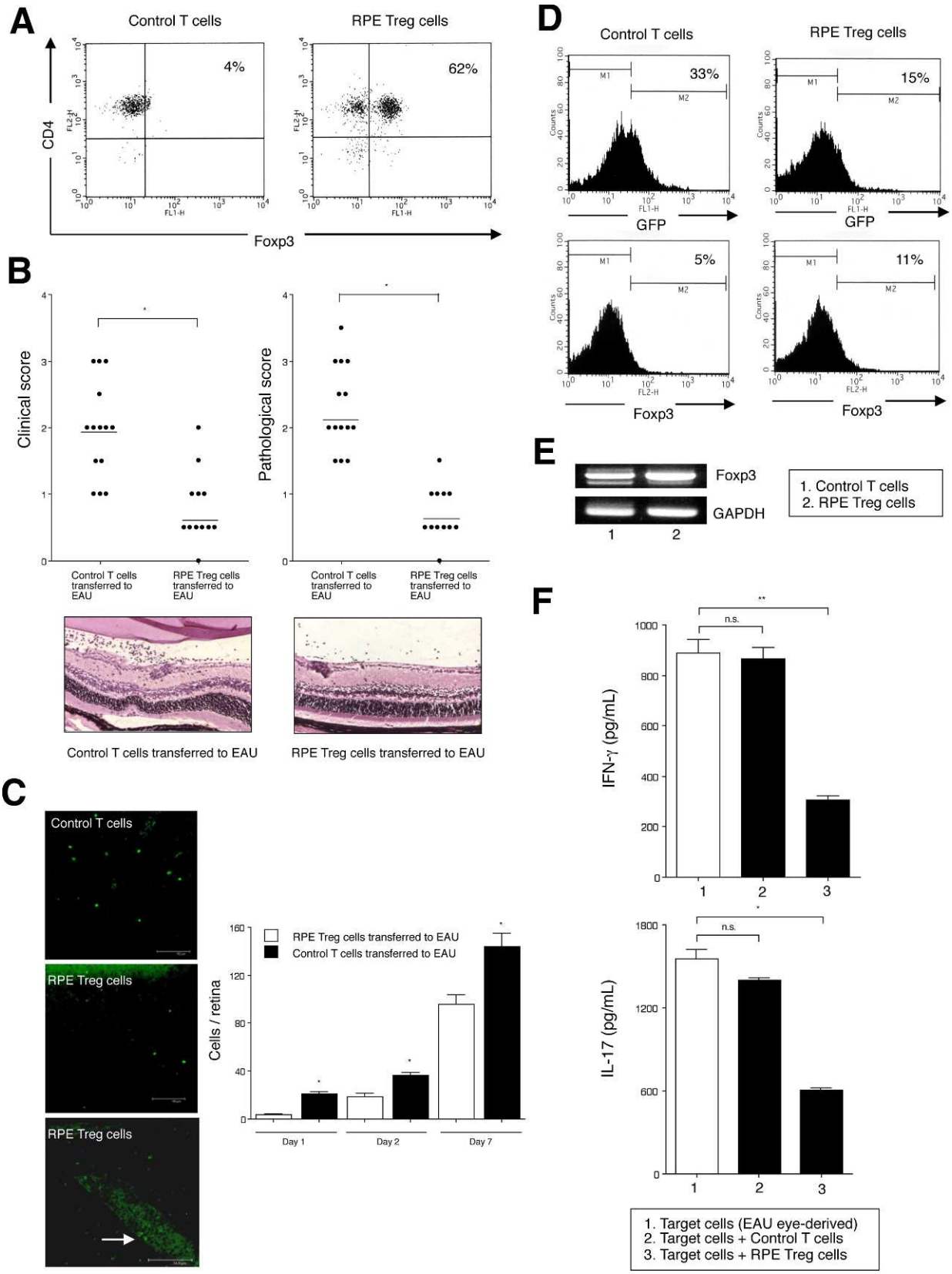

Figure 6. Capacity of murine RPE-induced Tregs to suppress ocular inflammation in EAU models. (**A**) Detection of Foxp3 in murine RPE-induced Tregs. rTGFβ-pretreated RPE-supernatants were cocultured with $CD4^+$ T cells in the presence of anti-mouse CD3/CD28 antibodies. Control $CD4^+$ T cells were cultured without RPE supernatants. Cells were stained with anti-CD4 and anti-Foxp3 antibodies and analyzed by flow cytometry. The percentages of double-positive cells are shown. (**B**) Capacity of RPE-induced Tregs to suppress inflammation in EAU models. RPE-induced Treg cells (1×10^6) were administered to EAU donor mice by IV injection 14 days after immunization with $IRBP_{1-20}$. Inflammation was evaluated on day 21 by fundus score (grade 0–4; *left panel*) and pathologic score (*right panel*). $CD4^+$ T cells without RPE supernatant induction (1×10^6) were used as controls. $^*P <$

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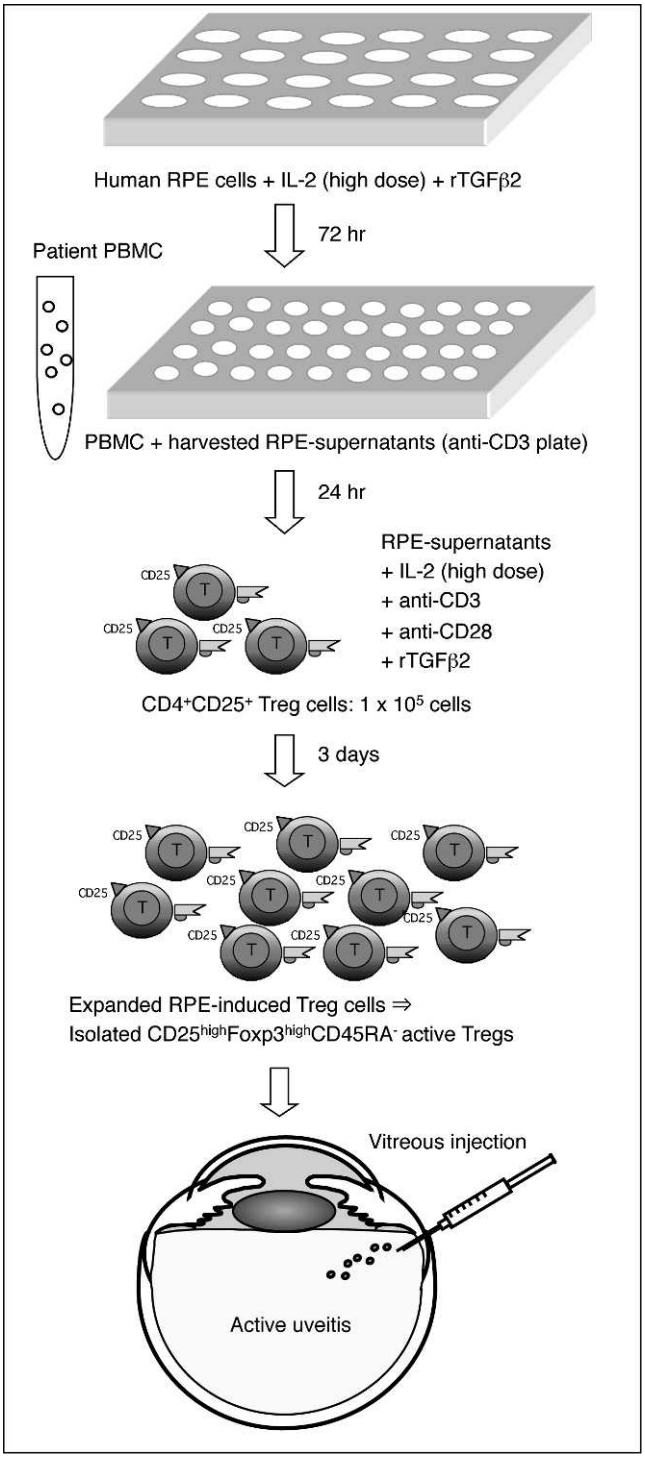
0.05. (**C**) GFP-labeled RPE-induced Tregs and control T cells were injected into mice with EAU on days 1, 2, and 7. GFP-positive T cells were quantified by upright confocal laser-scanning microscopy. After the day 7 injection, intraocular cells were harvested to detect Foxp3 by flow cytometry ($^*P < 0.05$) (**D**) and RT-PCR (**E**). (**F**) Capacity of RPE-induced Tregs to suppress cytokine production in activated bystander T cells. Intraocular T cells from EAU eyes (day 21 EAU, $n = 12$) were evaluated by IRBP retinal Ag–specific assays. T cells (1×10^5/well) were cocultured with RPE-induced Tregs and antigen-presenting cells (x-irradiated spleen cells: 1×10^4/well) + 10 μg/mL mouse IRBP peptide for 48 hours. $CD4^+$ T cells were prepared as controls. Supernatant cytokine (IFN-γ or IL-17) concentrations were measured by ELISA. $^*P < 0.05$ and $^{**}P < 0.005$. n.s., not significant.

agents, and anticytokine antibodies (e.g., infliximab), which have adverse side effects. By using our approach, the patient could be treated with autologous Tregs a few days after providing a blood sample. Moreover, the number of injections and the number of Tregs introduced can be individually titrated by using fundoscopic examinations. After the injection, we can directly and quickly determine whether the treatment was effective.



Over the past decades, researchers have made substantial strides in the understanding of clinical applications for Tregs in experimental models for transplantation and autoimmune diseases. In animal models, the adoptive transfer of Tregs provides protection from GVHD,[25] colitis,[26] arthritis,[27] and diabetes.[28] In murine eye models, $CD4^+CD25^+$ T cells inhibit severe ocular inflammation in EAU.[29] Recently, in human patients, immune-suppressing regulatory T cells have appeared in the clinical setting for inflammatory disorders. Immune-suppressive cellular immunotherapy requires the use of a large number of antigen-specific Tregs that suppresses certain immune responses. Adoptive transfer is a mode of treatment in which a patient's immune cells are modified in vitro and then reintroduced into the patient. The adoptive transfer of Tregs is advantageous because Tregs have the potential to mediate antigen-specific immunosuppression, thus avoiding the toxicity of general immunosuppressive drugs. Additionally, Tregs are generated from the patient's peripheral blood and thus should not be rejected by his or her immune system.

In the adoptive transfer of Tregs for therapy, naturally occurring Tregs are isolated from a patient's blood sample, expanded in vitro, and infused back into the patient.[30] Alternatively, naïve T cells are isolated from the patient's blood, converted into Tregs in vitro, and then expanded in vitro.[30] In vivo targeting of Tregs for therapy involves the use of monoclonal antibodies (e.g., antibodies to CD3/CD28) or pharmacologic agents (e.g., rapamycin) that activate and expand Tregs in the patient.[30] In the adoptive transfer of Tregs for human therapy, Tregs are divided into three groups based on the expression of CD45RA: $CD25^{high}CD45RA^-$ active Tregs, $CD25^{low}CD45RA^+$ resting Tregs, and $CD25^{low}CD45RA^-$ cells, classified as non-Tregs.[13] These three subsets of human $Foxp3^+$ cells display distinct phenotypes and functions. Although both active and resting Tregs have potent immunosuppressive properties, our protocol for uveitis treatment does not include the use of resting Tregs, because the number of cells used is small. Importantly, we were able to remove non-Treg cells ($CD25^{low}CD45RA^-$), which produce the proinflammatory cytokines IL-2, IL-17, and IFN-γ and do not have an immunosuppressive function, from our Treg population.

Human $Foxp3^+$ Tregs are unstable in vivo, which complicates their use in adoptive transfer therapies.[12,13] $CD25^{low}CD45RA^-$ nonsuppressive Tregs express the retinoic acid receptor–related orphan nuclear receptor γt (RORγt) and,

Figure 7. RPE-induced Treg immunotherapy for uveitis. PBMCs are isolated from a 20-mL blood sample from a patient with active uveitis. Supernatants of human RPE cell lines cultured with rTGFβ2 and high-dose rIL-2 are cocultured with PBMCs for 24 hours in anti-human CD3-coated plates. Isolated $CD4^+CD25^+$ T cells are recultured with a high-dose of rIL-2, anti-human CD3/CD28 antibodies, and rTGFβ2 for 3 days. Active $CD4^+CD25^{high}CD45RA^-$ Tregs are isolated from the expanded population and transferred into the uveitic eye by vitreous injection. Total preparation time: 4 days.

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in an inflammatory milieu, can become Th17 cells, which play a central role in autoimmune disease.[13] Moreover, studies from mice suggest that some Foxp3$^+$ Tregs may lose Foxp3 expression in vivo as a result of epigenetic modifications of the *Foxp3* gene, causing the cells to differentiate into inflammatory memory T cells, which produce inflammatory cytokines.[12] The topic of Treg stability was recently summarized in an article by Bailey-Bucktrout and Bluestone.[31] Another possible drawback of Treg therapies is the possibility of unanticipated detrimental effects; in fact, a recent clinical study of Tregs in patients was discontinued due to detrimental effects.

Several approaches have been used for the generation and expansion of antigen-specific human Tregs.[32] Foxp3$^+$ Tregs represent less than 10% of CD4$^+$ T cells in human peripheral blood, and large numbers may be required for effective Treg-based cellular immunotherapy.[32] Hippen et al.[33] recently reported a protocol that allows human peripheral blood CD4$^+$CD25$^+$ Tregs that can to be expanded 3000-fold. After isolation from blood, the Tregs were expanded by culturing in the presence of IL-2, rapamycin, and artificial human antigen-presenting cells. Several clinical trials have tested the ability of in vitro–expanded CD4$^+$CD25$^+$ Tregs to prevent GVHD after allogeneic bone marrow transplantation, and many other trials are under way. A clinical trial led by Di Ianni et al.[34] used freshly isolated CD4$^+$CD25$^+$ Tregs from donor blood to treat GVHD in 28 cancer patients, and none of the patients developed chronic GVHD. In addition to the clinical trials, other ongoing studies indicate that adoptive Treg cellular therapy is proving to be safe and effective for the treatment of many diseases.[33–35]

In our present study, we used murine RPE–induced Tregs that were induced by supernatants of primary cultures of mouse RPE cells and cultured with TGFβ, rIL-2, and anti-CD3/CD28 antibodies. These RPE-induced Tregs effectively suppressed ocular inflammation by impairing the activation of in vivo–sensitized T cells and may have therapeutic potential for the treatment of uveitis. Although several practical difficulties remain to be overcome prior to clinical applications, technical improvements to expand CD4$^+$Foxp3$^+$ T cells would allow multiple transfers of regulatory T cells. To improve the procedure for possible human use, the in vitro–manipulated RPE-induced Tregs gained immunosuppressive properties by culture with high-dose recombinant IL-2, anti-human CD3/CD28 antibodies, and supernatants from recombinant TGFβ-pretreated human RPE cells. For the treatment of refractory uveitis patients, we will prepare only active effector Treg cells by removing CD4$^+$Foxp3$^+$ nonsuppressive T cells that produce IFN-γ and IL-17.

In conclusion, we successfully isolated active Tregs induced by supernatants of RPE cells under Treg conditions, including TGFβ, rIL-2, and antibody to CD3/CD28. These active Tregs exhibit immunosuppressive effects. One potential disadvantage of our Treg therapy method is the possible instability of Tregs in the vitreous gel after injection. We are now investigating whether RPE-induced active Tregs can suppress various inflammatory cells, including T cells established from active uveitis, under our conditions. In addition, we plan to test whether active Tregs obtained from systemic inflammatory disorders that include active ocular inflammation (such as Behçet's disease) can be induced to have immunosuppressive properties.

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