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Acquisition of T Regulatory Function in Cathepsin L-Inhibited T Cells by Eye-Derived CTLA-2α during Inflammatory Conditions

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Pigment epithelium isolated from the eye possesses immunosuppressive properties such as regulatory T (Treg) cell induction; e.g., cultured retinal pigment epithelium (RPE) converts CD4+ T cells into Treg cells in vitro. RPE constitutively expresses a novel immunosuppressive factor, CTLA-2α, which is a cathepsin L (CathL) inhibitor, and this molecule acts via RPE to induce Treg cells. To clarify CTLA-2α’s role in the T cell response to RPE in ocular inflammation, we used the experimental autoimmune uveitis (EAU) animal model to examine this new immunosuppressive property of RPE. In EAU models, TGF-β, but not IFN-γ inflammatory cytokines, promotes the up-regulation of CTLA-2α in RPE. Similarly, CTLA-2α via RPE was able to promote TGF-β production by the CD4+ T cells. The RPE-exposed T cells (RPE-induced Treg cells) greatly produced TGF-β and suppressed bystander effector T cells. There was less expression of CathL by the RPE-exposed T cells, and CathL-inhibited T cells were able to acquire the Treg phenotype. Moreover, CathL-deficient mice spontaneously produced Treg cells, with the increase in T cells potentially providing protection against ocular inflammation. More importantly, CD4+ T cells from EAU in CathL knockout mice or rCTLA-2α from EAU animals were found to contain a high population of forkhead box p3+ T cells. In both EAU models, there was significant suppression of the ocular inflammation. These results indicate that RPE secretes CTLA-2α, thereby enabling the bystander T cells to be converted into Treg cells via TGF-β promotion. The Journal of Immunology, 2009, 183: 5013–5022.

Immune tolerance in the eye becomes bankrupt during severe inflammatory conditions; i.e., inflammatory cell infiltration in the eye leads to intraocular inflammation that can ultimately cause 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. To study severe uveitis disorders in humans, the experimental autoimmune uveitis (EAU) animal model has been developed. EAU is a T cell-mediated autoimmune disease that leads to the destruction of the neural retina and related tissues, including serous retinal detachment, retinal folding, photoreceptor damages, vasculitis, retinitis and vitreitis, and, ultimately, blindness (1–3). Initiation of the major pathogenic events in EAU occurs by activating effector T cells that then react with retinal Ags such as interphotoreceptor retinoid-binding proteins (IRBP) (3). The activated effector T cells have an increased ability to enter target organs. Subsequently, they produce inflammatory cytokines and chemokines that recruit other inflammatory cells that can then cause retinal tissue damage. In contrast, there are also regulatory T (Treg) cells associated with ocular autoimmune disease that have suppressive activity (4–6). There are a number of mechanisms that can contribute to the spontaneous recovery from this autoimmune disease, including uveitis.

Cysteine proteinases are widely distributed in a variety of organisms that are involved in the process of intra- and extracellular protein degradation and turnover. Likewise, protein inhibitors of cysteine proteinases have also been found in a variety of organisms. Recently, a novel class of cysteine proteinase inhibitors has been reported. These inhibitors include mouse-activated T cells and mast cells that express mRNAs encoding the proteins

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Author contributions: S.S., who was the principal investigator, designed and performed the experiments, and wrote the manuscript. S.H. and H.T. established the Treg cells and carried out the EAU induction and flow cytometry. O.N. performed the qPCR, Western blotting, and CathL activity analysis. K.M. performed genotyping for KO mice. Y.U. (Dr. Usui) and M.T. carried out the EAU induction in KO mice. K.I. produced anti-CathL Ab. M.K. performed immunohistochemical experiments. Y.U. (Dr. Uchiyama) and C.P. supervised the CathL KO mice. Y.Y. produced recombinants and Ab for CTLA-2α. M.M. designed and conceptualized the study, and drafted and edited the manuscript.

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3 Abbreviations used in this paper: EAU, experimental autoimmune uveitis; CathL, cathepsin L; EGFP, enhanced GFP; Foxp3, forkhead box p3; IRBP, interphotoreceptor retinoid-binding protein peptide; iTreg, induced T regulatory; KO, knockout; nTreg, naturally occurring T regulatory; PBS-T, PBS that contained 0.01% Tween 20; qRT-PCR, quantitative RT-PCR; Rhe, retinal pigment epithelium; Treg, T regulatory; T resp, responder target T cells.

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CTLA-2α and CTLA-2β, both of which are highly similar to the proregios of mouse cathepsin L (CatHL) (7–9). The cysteine peptidase CatHL broadly affects the immune system (10–12). For instance, CatHL influences the levels of extracellular matrix components in lymphoid organs, the thymic output, and the number of T cells in the periphery (11). Moreover, CatHL-deficient NOD mice have an increased proportion of Treg cells within the T cell compartment (12). In previous eye studies, retinal pigment epithelium (RPE) has been found to constitutively express CTLA-2α. When the RPE encounters T cells, these secreted proteins can selectively inhibit the CatHL on the effector T cells (13). The RPE-CTLA-2α-controlled CatHL activity and CatHL-inhibited T cells were able to acquire the Treg phenotype in vitro (13). Moreover, the CTLA-2α, which affects Treg cell induction, occurs via the TGF-β signaling pathway, because the CTLA-2α can promote activation of TGF-β in the eye. The CD4+ RPE-induced Treg cells express forkhead box p3 (Foxp3). It is well known that CD4+ Treg cells expressing high levels of the transcription factor Foxp3 are required for the maintenance of peripheral tolerance.

The present study was designed to clarify the role(s) that the RPE immunosuppressive factors, CTLA-2α and TGF-β, play when intraocular inflammation occurs in an immune privilege site such as the eyes.

Materials and Methods

Mice

RPE cells were obtained from adult C57BL/6 and ICR mice (Taconic Farms or CLEA Japan). Cathepsin L (Cat HL) homozygous and control littermate mice, backcrossed onto a C57BL/6D background for at least 6 generations, were obtained from the Institute of Experimental Animal Sciences, Osaka University Graduate School of Medicine (14, 15).

Preparation of primary cultures of RPE and RPE-induced Treg cells

Primary RPE were cultivated, as has been previously described (16–20). The RPE were cultured in complete DMEM, placed into culture plates, and incubated for 2 wk.

T cells exposed to RPE (RPE-induced Treg cells) in the presence of anti-mouse CD3ε Ab (0.1 µg/ml, clone 2C11; BD Pharmingen) were harvested, y irradiated (2000 rad), and added (10⁶ cells/well) to 96-well plates containing fresh T cells (responder target T cells (T resp); 10⁶ cells/well) and anti-CD3 Ab. The harvested T cells contained ≤0.99% cytotkeratin+ RPE, whereas the cultured RPE did not contain any CD45+, F4/80-, or MHC class II+ cells, as has been previously reported (13).

Preparation of purified T cells and the cell proliferation assay

Cytokeratin-positive RPE were cultured separately in 96-well plates (1–2 × 10⁴ cells/well) or 24-well plates (5–10 × 10⁴ cells/well). Suspensions of responder cells were pressed through a nylon mesh (Immumal mouse T cell kit; Biotex Laboratories) to produce a single-cell suspension. CD4+ T cells were prepared separately using MACS cell isolation kits (Miltenyi Biotec). These cells were found to be more than 95% CD4+. After stimulating the target T cells (2.5 × 10⁴ cells/well) with anti-CD3 Ab, cells were incubated for 72 h. After incubation, the cultures were assayed for cell proliferation by measuring the uptake of [³H]thymidine.

Flow cytometry

Flow cytometry was used to analyze the expression of Foxp3 on T cells, such as 1) CTLA-2α-overexpressing T cells; 2) rTGF-β2-treated T cells; 3) rTGF-β2-treated CTLA-2α-overexpressing T cells; and 4) control T cells. Before staining, the cells were incubated with a mouse Fc block (FcγRIII/II; BD Pharmingen) at 4°C for 15 min. After permeabilization, the cells were stained with FITC-labeled anti-mouse Foxp3 (eBioscience) or with isotype rat control Ab at 4°C for 30 min. In separate experiments, the fresh CD4+ T cells that were harvested from the spleen of EAU mice (day 21) in the wild-type or CatHL knockout (KO) donors were permeabilized and then stained with FITC-conjugated anti-mouse Foxp3 or isotype control Ab at 4°C for 30 min.

GeneChip

As per a previous report (13, 21), total RNA for the RPE was isolated using TRIzol reagent (Invitrogen Life Technologies) in accordance with the manufacturer’s instructions. RNA was purified from total cellular RNA using a Nucleosip RNA II (Macherey-Nagel). Experimental procedures for the GeneChip were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual, which has been described previously (21). The cRNA was hybridized to an oligonucleotide microarray (Mouse Genome 430 2.0). The microarray data are deposited in the Gene Expression Omnibus (GEO) public database http://www.ncbi.nlm.nih.gov/geo/ under accession number GSM5134.

RT-PCR and quantitative RT-PCR (qRT-PCR)

Cellular extracts were prepared from cultured RPE. Total RNA for the RPE was isolated using TRIzol reagent. The forward and reverse primers for CTLA-2α, CatHL, and GAPDH, and the PCR conditions have been previously described (13). The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide. The level of CTLA-2α mRNA was normalized to that of GAPDH. The qRT-PCR analysis was conducted using primers for CTLA-2α or CatHL (13).

Immunohistochemistry

The harvested T cells, RPE-induced Treg cells or control CD4+ T cells, were fixed with 4% paraformaldehyde for 10 min at room temperature and then permeabilized with 0.1% Triton X-100. Subsequently, the cells were incubated with 1:500 anti-mouse CatHL or isotype control Ab (rabbit) for 1 h, followed by Alexa Fluor 488-conjugated anti-rabbit Ab (Invitrogen). rCTLA-2α-treated or rTGF-β2-treated T cells were also stained with anti-CatHL. Fluorescence signals were detected by confocal microscopy using a Radiance 2000 microscope (Bio-Rad). Established rCTLA-2α proteins and anti-CTLA-2α Ab were used, as previously described (13).

CTLA-2α overexpression in RPE

For CTLA-2α overexpression in RPE, mouse CTLA-2α-pIRES-enhanced GFP (EGFP) vector was synthesized and used with transfection reagent (Effectene Transfection Reagent; Qiagen). As a control, the assay was conducted using only the pIRES vector. Fluorescence microscopy and qRT-PCR or semiqualitative RT-PCR were used to confirm the expression of CTLA-2α.

Induction of EAU and rCTLA-2α transfer

Mice from wild-type or CatHL KO donors were immunized s.c. in the neck region with 200 µg of IRBPp29 (Biosynthesis) emulsified in CFA (Difco) containing the Mycobacterium tuberculosis strain H37Ra (Difco) and injected i.p. with 100 µg of pertussis toxin (Sigma-Aldrich) as an additional adjuvant, as has been previously described (13, 22). Funduscopy examination was conducted at 14, 17, and 21 days with the histological examination conducted on day 21 after the immunization. The clinical scores were calculated on days 14, 17, or 21 in the EAU mice, in accordance with the previous method (23). Inflammation was evaluated based on the fundus and histological findings. In addition, cells were harvested from spleens on day 21 in the EAU mice to perform Foxp3 staining for flow cytometry.

rCTLA-2α from normal C57BL/6 mice was administered to the EAU animals. After immunization, rCTLA-2α administration (1 µg/body, i.p. injection) was performed nine times during the evaluation periods. Inflammation was evaluated on days 14, 17, or 21 using fundus scores (clinical score, grade 0–4) and histological findings (day 21). For the controls, EAU donors that were not treated with rCTLA-2α (i.e., treated with PBS only) were used.

Purified splenic CD4+ T cells were collected from the EAU mice in the CatHL KO or wild-type donors and then evaluated using the IRBP Ag-specific proliferation assay. After incubation for 72 h, the cultures were assayed for cell proliferation by measuring the uptake of [³H]thymidine.

Western blotting

Cultured RPE were washed in PBS twice, homogenized by sonication for 5 min at 4°C, and then centrifuged at 15,000 rpm for 20 min. Protein concentration was detected using a bicinchoninic acid protein assay reagent kit (Pierce). We used 15% SDS-PAGE to separate the total protein (10 µg), and the samples were transferred onto polyvinylidine difluoride membranes. Immunodetection was conducted using anti-mouse CatHL (R&D Systems) diluted 1/2000 in PBS that contained 0.01% Tween 20 (PBS-T) with 0.1% BSA. For the detection assay, first we blocked incubation using

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FIGURE 1. Role of CTLA-2α in T cell response to RPE cells. A, Fresh RPE tissues from EAU (n = 6) or normal donors (n = 6) were harvested, and the expression of mRNA for TGF-β1, IFN-γ, Foxp3, and CTLA-2α was assessed. B, Ocular fluids were collected from EAU (n = 10) or normal donors (n = 10), and the production of the cytokines, TGF-β1 and IFN-γ, was measured. Error bars represent the SEM. **, p < 0.005 between two groups. C, Cultured RPE cells were treated with mouse rIFN-γ (100 U/ml) for 24 h. For the examination of the GeneChip microarray (left panel) and RT-PCR (right panel), RNA of the IFN-γ-treated or untreated (control RPE) was extracted. IFN-γ expression was normalized by GAPDH expression. D, RNA was harvested from cultured RPE and analyzed for the presence of TGF-β1 or β2 transcripts by GeneChip (left panel) and RT-PCR (right panel). TGF-β expression was normalized by GAPDH expression. E, Supernatants of primary cultured RPE and RPE cell lines were collected to evaluate the production of active TGF-β1. F, Cultured RPE or CD4+ T cells were treated with rTGF-β2 (5 or 20 ng/ml) for 24 h. The expression of CTLA-2α by RPE (left panel) or T cells (right panel) exposed to rTGF-β was evaluated by qRT-PCR.

5% skim milk in PBS for 1 h at room temperature, followed by washing of the membrane in PBS-T and then incubation with the primary Ab at 4°C overnight. After incubation, the membrane was washed three times for 10 min each with PBS-T, followed by incubation in peroxidase-conjugated anti-goat secondary Ab (DakoCytomation) diluted 1/2500 in PBS-T for 1 h at room temperature. The membrane was washed three times for 10 min each in PBS-T, and then processed for detection using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer).

CathL activity assay

Activities of CathL were determined using previously published methods (8). Briefly, 10 μl of RPE lysate (2.5 μg of total protein) was mixed with 400 μl of reaction buffer (50 mM sodium acetate buffer at pH 5.5) containing 4 mM DTT. To be able to assay only the CathL activity, a selective cathepsin B inhibitor, CA-074 (Merck-Calbiochem), was added to the incubation mixture. Then, 500 μl of 10 μM Z-Phe-Arg-MCA was added to start the enzymatic reaction at 37°C. CathL activity was measured using a spectrofluorometer with excitation and emission wavelengths of 380 and 440 nm, respectively.

TGF-β ELISA

The concentrations of TGF-β in the supernatants of primary cultured RPE cells, rCTLA-2α-treated T cells, CTLA-2α-overexpressing T cells, and ocular fluids in EAU donors were measured as the active forms using TGF-β ELISA (R&D Systems). The ocular fluids (n = 10) were collected from EAU using microcapillaries.

Statistical evaluation

All experiments were repeated at least twice with all experimental results found to be similar. All statistical analyses were conducted using Student’s t test. Results of the EAU experiments were analyzed using the Mann-Whitney U test. Values were considered statistically significant if p was less than 0.05.

Results

Relationship between CTLA-2α and TGF-β produced by RPE in inflamed eyes

RPE cells obtained from the posterior segment of the eye mainly use soluble inhibitory factors to suppress T cell activation, especially the soluble forms of TGF-β. TGF-β plays a critical role in down-regulating bystander T cells (24, 25) and in the acquisition of the Treg phenotype (13, 26, 27). In addition, RPE also produce CTLA-2α, which is a promoter for TGF-β (13). To clarify CTLA-2α’s role in the T cell response to RPE, we examined fresh tissues and fluids in normal eyes and in eyes with inflammation to determine whether they contained CTLA-2α and TGF-β. Using the experimental uveitis model, we harvested fresh RPE tissues from EAU or normal donors and then assessed the expression of mRNA for CTLA-2α, IFN-γ (inflammatory cytokine), TGF-β1 (immunosuppressive cytokine), and Foxp3 (Treg marker). We also collected ocular fluids from EAU or normal donors, and measured the production of the TGF-β1 and IFN-γ cytokines. On day 21 after immunization for EAU, high levels of the IFN-γ and TGF-β1 mRNA were expressed in the fresh RPE tissues (Fig. 1A). In normal donors, the RPE tissues expressed TGF-β1 mRNA, but not IFN-γ mRNA. EAU RPE tissues also clearly expressed mRNA for...
Foxp3 and CTLA-2α. This suggests that the ocular infiltrating cells contain Foxp3+ Treg cells because ocular tissues constitutively express immunoregulatory molecules such as TGF-β and CTLA-2α. Although the expression of CTLA-2α mRNA was clearly seen in normal RPE tissue, this expression was much higher in the RPE from EAU. If the RPE tissues were collected from EAU-recovered mice on day 30, the expression of CTLA-2α mRNA was still highly expressed in EAU RPE tissues when compared with normal RPE (data not shown). Similarly, ocular fluids from EAU (on day 21) expressed high levels of both IFN-γ and TGF-β (Fig. 1B). These results indicate that ocular infiltrating cells and tissues produce high levels of these cytokines during inflammatory conditions.

Because large amounts of IFN-γ inflammatory cytokines and TGF-β immunosuppressive cytokines were found in the eyes of our experimental inflammatory models, we examined whether these cytokines were effective against the expression of CTLA-2α by RPE. In the first set of experiments, we treated the cultured RPE with mouse rIFN-γ for 24 h. When compared with untreated cells, the RPE exposed to rIFN-γ expressed similar levels of the CTLA-2α transcripts in both the GeneChip and RT-PCR analyses (Fig. 1C).

Next, we examined whether cultured RPE produces active forms of TGF-β. Results indicated that cultured RPE expressed mRNAs for TGF-β1 and β2 (Fig. 1D), and that cultured RPE (primary cells and cell lines) produced high levels of the active forms of TGF-β1 (Fig. 1E). We also treated cultured RPE with rTGF-β. Results from our qRT-PCR analysis indicated there was an up-regulation of the expression of CTLA-2α as compared with the untreated cells (Fig. 1F, left panel). Interestingly, after the cells were pretreated with anti-CD3 plus rTGF-β, the CD4+ T cells greatly expressed CTLA-2α mRNA (Fig. 1F, right panel). This suggests that TGF-β promotes the up-regulation of the CTLA-2α expression on responding T cells. Similar to CTLA-4, which is a negative costimulatory receptor for the activated T cells (25, 26), TGF-β stimulation of T cells might be the key to the initiation of CTLA-2α synthesis.

**Capacity of rCTLA-2α to induce Treg cells in experimental uveitis models**

We next examined whether rCTLA-2α could induce Treg cells in EAU donors. Compared with untreated mice (control), administration of rCTLA-2α in EAU mice significantly suppressed the ocular inflammation (Fig. 2A). Inflammatory cell infiltration was noted throughout all of the retinal layers in these EAU mice. In addition, we also observed that there was a partial destruction of the RPE layer (Fig. 2A, lower picture). When rCTLA2α was administered in EAU mice, only a few cells exhibited inflammatory cell infiltration in the retinal layer, with the retinal layers remaining intact. These histological scores also exhibited significant suppression when there was administration of rCTLA-2α in EAU mice (data not shown). These results indicate that CTLA-2α promotes suppression of ocular inflammation in this model.

We also examined whether Foxp3+ Treg cells were found within fresh splenic CD4+ T cells from EAU donors that had been previously treated with rCTLA-2α. The CD4+ T cells from the EAU animals that were administered the CTLA-2α proteins had a high population of Foxp3+ T cells (13% positive) as compared with the CD4+ T cells from the nontreated EAU donors (PBS only, 6% positive) and nonimmunized normal mice (1% positive) (Fig. 2B). We also collected the serum from these mice to examine the production of TGF-β. As compared with the nontreated EAU donors (PBS only) and the nonimmunized normal mice, the EAU donors had significantly high levels of TGF-β as compared with the untreated EAU donors (Fig. 2C). This suggests that TGF-β was produced by rCTLA-2α to induce Treg cells in EAU donors. The EAU donors administration of rCTLA-2α resulted in high levels of TGF-β production.
animals given CTLA-2α had higher levels of active TGF-β1 (Fig. 2C).

When CD4+ T cells are exposed to rCTLA-2α, they acquire a regulatory phenotype in vitro (13). Therefore, to test whether the T cells could produce active TGF-β-like RPE-induced Treg cells, supernatants from the rCTLA-2α-pretreated T cells were harvested and then examined for the production of active TGF-β1. Data collected indicated that the rCTLA-2α-treated CD4+ T cells secreted significant levels of active TGF-β1 in a dose-dependent manner (Fig. 2D). When taken together, these results suggest that some of the CD4+ T cell populations that were exposed to CTLA-2α by RPE were actually of the Th3 type.

Expression of CathL by RPE and RPE-induced Treg cells

In the next step, because CTLA-2α is a cytokine protease inhibitor known to inhibit lysosomal cysteine peptidase CathL, we examined whether CTLA-2α release by RPE could selectively inhibit CathL. Western blotting was used to confirm the expression of CathL in RPE. Results indicated that primary RPE cultures expressed CathL proteins, both pro-CathL and mature CathL proteins (Fig. 3A). When cultured RPE was treated with anti-CTLA-2α-neutralizing Abs, an increase was noted in the expression of CathL proteins, especially for the mature CathL proteins (Fig. 3A). We also examined whether CTLA-2α produced by RPE was able to inhibit CathL activity. When RPE was treated with anti-CTLA-2α-neutralizing Abs, there was increased CathL activity as compared with that seen for the RPE cells with isotype control Abs (Fig. 3B). This suggests that CTLA-2α controls CathL activity in the eye.

RPE-induced Treg cells were stained with anti-CathL and examined by fluorescence microscopy. We also stained rCTLA-2α- and rTGF-β2-treated T cells with anti-CathL Abs. As seen in Fig. 3C, immunohistochemical analysis indicated there was less expression of CathL, with a discontinuous pattern noted on the surface (upper left panel) for the RPE-induced Treg cells. In contrast, there was clear expression of the proteins by the control CD4+ T cells (upper right panel). In addition, both the rCTLA-2α-treated and rTGF-β2-treated T cells expressed the molecule to a much lesser extent as compared with the control T cells (Fig. 3C, lower panels). Similarly, these
T cells along with the RPE-exposed, rCTLA-2α-treated, and rTGF-β2-treated T cells all expressed transcripts for CathL to a much lesser degree than the control T cells in our RT-PCR analysis (Fig. 3D). These results imply that the soluble factors produced by RPE can inhibit CathL activities in the T cells when these T cells encounter RPE.

Because we previously reported that the loss of CathL activities due to an inhibitor such as CTLA-2α by RPE results in differentiation to Treg cells (13), we decided to examine whether RPE could convert CathL-overexpressing T cells into Treg cells in vitro. For control cells, we used T cells that were treated by plasmids only. These cells were then harvested and examined by qRT-PCR. Results showed that CathL-overexpressing T cells contained greater levels of mRNA for CathL as compared with the control T cells (Fig. 3E). As expected, RPE failed to convert the CathL-overexpressing T cells into Treg cells, whereas RPE was able to convert control T cells into Treg cells (Fig. 3F). Thus, our results indicate that when CathL-overexpressing T cells are exposed to RPE, they fail to acquire a regulatory function.

Expression of Foxp3 by CathL null CD4⁺ T cells
We further examined whether CathL-lacking T cells exhibit the Treg phenotype. For this in vitro Treg assay, we used CD4⁺ T cells from CathL KO or wild-type donors. When using T cells from CathL KO donors, we found that fresh splenic CD4⁺ T cells from wild-type donors only had a small population of Foxp3⁺ (3%), whereas there was a spontaneous increase in the population of CD4⁺ T cells from the KO donors (10% positive; Fig. 4A). After CathL KO or wild-type T cells were added to secondary cultures, there was a significant suppression of the responder T cells observed for the KO CD4⁺ T cells when compared with the wild-type cells in vitro (Fig. 4B). Experiments also showed that there was a significant production of active forms of TGF-β1 by the KO T cells (Fig. 4C).

We also examined whether EAU in the CathL KO donors could suppress inflammation in the eye. Fresh CD4⁺ T cells (CathL KO or wild type) in EAU or nonimmunized mice were stained with anti-Foxp3. As seen in Fig. 5A, there was a high population of Foxp3⁺ T cells (17% positive) noted for the CD4⁺ T cells from EAU in the CathL KO donors. In contrast, there was only a small Foxp3⁺ T cell population found for the CD4⁺ T cells from the nonimmunized wild-type (2% positive) and EAU wild-type donors (4% positive). More importantly, EAU in CathL KO mice fully and significantly suppressed ocular inflammation as compared with that seen for the wild-type mice (Fig. 5B, left panel). With regard to the histological scores, EAU in CathL KO mice significantly suppressed ocular inflammation as compared with that seen for the wild type (Fig. 5B, right panel). For the wild-type controls, infiltration of the inflammatory cells was found throughout all of the retinal layers, including the RPE layer (Fig. 5B, lower picture). In contrast, there was less retinal layer infiltration by the inflammatory cells for EAU in the CathL KO donors. When taken together, these results imply that there may be an increase in the circulation of the Treg cells, and it is this increase that helps to protect the CathL-deficient mice from intraocular inflammation.

Subsequently, after we collected purified splenic CD4⁺ T cells from the EAU in the CathL KO or wild-type donors, we evaluated these cells using an IRBP Ag-specific proliferation assay. There was a significant response by the purified CD4⁺ T cells from the wild-type EAU donors to the IRBP Ags. This response was not noted for the CD4⁺ T cells from EAU in the CathL KO donors (Fig. 5C). Moreover, there was a significant reduction of the proliferation by the wild-type CD4⁺ T cells in the presence of Ags when CathL KO CD4⁺ T cells were added to the cultures (Fig. 5D). These results suggest that Treg cells may be found in some of the CD4⁺ T cell populations in the CathL-deficient EAU donors.

Capacity of CTLA-2α-overexpressing RPE to induce Treg cells
We examined whether CTLA-2α-overexpressing RPE was more efficient in promoting the induction of Treg cells in vitro. For control cells, we harvested and examined RPE cells that had been pretreated only by plasmid. Fluorescence microscopy detected the presence of EGFP-labeled CTLA-2α in RPE after a 48-h culture. This expression was more clearly visualized in many of the cells after a 72-h culture (Fig. 6A). Both semiquantitative RT-PCR and qRT-PCR indicated there were relatively greater amounts of mRNA in the CTLA-2α-overexpressing RPE as compared with the control RPE (Fig. 6B).

We also examined whether CTLA-2α-overexpressing T cells could produce TGF-β. For control cells, we used CD4⁺ T cells
that were pretreated with only plasmid. As shown in Fig. 6C, the overexpressing CD4+ T cells contained significant amounts of the CTLA-2α mRNA as compared with the control cells. Moreover, the CTLA-2α-overexpressing T cells secreted significantly more active TGF-β1 as compared with the control T cells (Fig. 6D). In addition, the CD4+ T cells exposed to CTLA-2α-overexpressing RPE greatly and significantly suppressed the activation of the bystander T cells as compared with the T cells that were exposed to control RPE (Fig. 6E). Thus, CTLA-2α-overexpressing RPE can more efficiently promote the induction of Treg cells.

**Foxp3 expression by CTLA-2α-overexpressing T cells**

As a final step, we examined the expression of Foxp3 by the CTLA-2α-overexpressing T cells. Purified CD4+ T cells or CTLA-2α-overexpressing CD4+ T cells in the presence of anti-CD3 were cultured for 48 h. These T cells were also cocultured with or without rTGF-β2. As seen in Fig. 7A, there was a larger expression of Foxp3 (4%) by the CTLA-2α-overexpressing T cells as compared with the control T cells (1%). As expected, the rTGF-β2-treated T cells greatly expressed Foxp3 (15%). Moreover, rTGF-β2-treated CTLA-2α-overexpressing T cells also expressed much higher levels of Foxp3 (35%).

For the in vitro assay, similar to the control T cells, there was no significant suppression of the bystander T cells by the CTLA-2α-overexpressing T cells. In contrast, significant suppression was seen for the rTGF-β2-treated T cells (Fig. 7B). Moreover, the rTGF-β2-pretreated CTLA-2α-overexpressing T cells were able to fully and significantly suppress T cell activation (Fig. 7B). These results suggest that these in vitro manipulated T cells were able to acquire the regulatory phenotype and carry out the regulatory function.

**Discussion**

Today, it is widely accepted that Treg cells have immunoregulatory abilities (28). However, it is still unknown how these Treg cells become functionally active and what factor(s) specifically regulates their functions. Based on our previous reports (13), the goal of the present study was to clarify the suppressive mechanisms of RPE’s novel inhibitory factor, CTLA-2α. The primary goal of the experiment was to determine how the T cell populations are able to generate and interact with the autoimmune uveitis pathogenesis. RPE-induced Treg cells are able to express Foxp3 via TGF-β and CTLA-2α, and they can produce soluble active TGF-β. It is assumed that a part of the RPE-exposed T cell population contains Foxp3+-inducible Treg cells, whereas the other part contains the TGF-β-producing Th3-type T cells (29). It is also assumed that there are at least two types of CD4+ T cells that have suppressive abilities that provide protection from inflammatory infiltrating T cell attacks.

Treg cells act at multiple levels of the immune response to suppress self-reactive T cells that have escaped from central tolerance (30). Treg cells are potent modulators of T cell-mediated immune responses both in vitro and in vivo and can be subdivided into two main groups. The CD4+CD25+ are naturally occurring Treg (iTreg) cells that acquire their suppressive functions in the thymus. The induced Treg (iTreg) cells are cells that acquire their functions in the periphery (31). Functional abrogation of the iTreg cells provokes multirorgan autoimmunity, which leads to increases in immunity to tumors, grafts, allergens, and microbial pathogens (30). Foxp3 is essential for nTreg cell development and function, and its importance can be best illustrated in Foxp3-deficient mice.
and in patients suffering from immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome, in which mutations or deletions in the Foxp3 gene halt the development of the nTreg cells and lead to a fatal autoimmune and inflammatory disease (32–35). Although most studies support the notion that the thymus represents the major site of nTreg cell development, some

![FIGURE 6](http://www.jimmunol.org/)  
Capacity of CTLA-2α-overexpressing RPE to promote the induction of Treg cells. A, Primary cultured RPE were transfected with plasmid of mouse CTLA-2α/pIRES-EGFP for 48 or 72 h. For the control cells, the RPE were pretreated by plasmid only (pIRES-EGFP), with these cells then harvested and examined. Fluorescence microscopy was used to confirm the expression. Vector-pIRES-EGFP. B, Semiquantitative RT-PCR (left panel) and qRT-PCR (right panel) were performed to confirm the expression of CTLA-2α by CTLA-2α-overexpressing RPE. C, qRT-PCR was also performed to confirm the expression of CTLA-2α mRNA by CTLA-2α-overexpressing T cells. D, Supernatants of CTLA-2α-overexpressing T cells were collected for measurement of active TGF-β production. Supernatants of untreated CD4⁺ T cells and control plasmid-pretreated T cells were also collected. E, Purified CD4⁺ T cells (1 x 10⁶/well) were cultured with CTLA-2α-overexpressing RPE or control RPE for 24 h in the presence of anti-CD3 (0.1 μg/ml), harvested, x-irradiated, and then used as Treg cells (RPE-induced Treg cells). RPE-induced Treg cells were added (1 x 10⁵/well in a 96-well plate) to cultures containing T resp (1 x 10⁵/well) plus anti-CD3. Positive control cultures containing T cells (T resp) and anti-CD3 are represented by □. Error bars represent the SEM. *p < 0.05, and **p < 0.005 between two groups.

![FIGURE 7](http://www.jimmunol.org/)  
Capacity of CTLA-2α-overexpressing T cells plus rTGF-β2 to inhibit bystander target T cells in vitro. A, CTLA-2α-overexpressing T cells or control CD4⁺ T cells in the presence of anti-CD3 were cultured for 48 h. These T cells were also cocultured with or without rTGF-β2. After 48 h, harvested T cells were stained with FITC-conjugated Foxp3 Abs, and then examined by flow cytometry. The numbers in the histograms indicate the percentages of cells that were positive for Foxp3. B, CTLA-2α-overexpressing CD4⁺ T cells were cultured for 24 h in the presence of anti-CD3 plus rTGF-β2, harvested, x-irradiated, and then used as Treg cells. As a control, T cells were cultured in the absence of CTLA-2α transfection and additional rTGF-β2. Positive control cultures containing T cells (T resp) and anti-CD3 are represented by □. Error bars represent the SEM. *p < 0.05 between positive control cultures and Treg cells.
reports have recently suggested that the Foxp3+ T cell populations may also be induced in the periphery (36–38). As shown in the current study, iTreg cells can be generated during inflammatory conditions in peripheral organs such as the eye.

TGF-β1 is a potent, suppressive cytokine that is critically involved in the induction of tolerance and the regulation of immune responses (39). This can be best illustrated by the onset of a severe autoimmune-like syndrome in TGF-β1−/− mice, and which is characterized by a spontaneous and progressive, multiorgan infiltration of mononuclear cells and pathogenic autoantibodies (40). Furthermore, disruption of the TGF-β signaling in T cells by deletion of the TGF-β receptor type II or inactivation of the receptor-activated Smad3 gene results in dysregulated T cell responses (41–43). The mechanism through which TGF-β1 mediates its tolerogenic functions has yet to be totally understood. The potential role of TGF-β1 in the development, differentiation, expansion, or suppression mechanism of the iTreg cells, as well as the nTreg cells, is believed to be of great importance. TGF-β1 has been recently shown to promote nTreg cell expansion as well as the generation of Foxp3+ iTreg cells from CD4+CD25− T cells. However, the underlying molecular mechanisms have yet to be clearly and conclusively defined. In the current study, we evaluated whether a novel inhibitory factor that is secreted by the RPE cells, CTLA-2α, could promote TGF-β, and whether the CTLA-2α-TGF-β pathway plays an important role in the induction of the Treg cells (eye-derived iTreg cells). Interestingly, similar to the previous reports for TGF-β1 (36), TGF-β2, which is the dominant isoform in the eye, can convert CD4+CD25−T cells in the presence of anti-CD3 into Foxp3+ T cells in vitro. Moreover, CTLA-2α via RPE can promote the effect of TGF-β both in vivo and ex vivo. For example, rCTLA-2α-pretreated CD4+ T cells can significantly produce active TGF-β1, and the serum that is collected from experimental uveitis models that have been administered rCTLA-2α has been found to contain significant amounts of active TGF-β. It has been speculated that at least two types of Treg cells may exist in inflamed eyes, as follows: 1) Foxp3+ iTreg cells and 2) Foxp3−Th3 cells, which produce TGF-β. In fact, it has been reported that there are some CD4+ T cell populations that, when exposed to ocular pigment epithelium, can express Foxp3 and suppress the bystander effector T cells by cell contact. In addition, other T cell populations can produce soluble TGF-β (13, 21, 44). These latter cells are able to suppress the effector T cells in a cell contact-independent manner.

At the present time, many kinds of lysosomal cysteine proteases, including CathL, have been identified. During cell differentiation, the actual cathepsin that is induced changes depending upon the type of differentiation that is occurring. For instance, cathepsin O/K is induced during differentiation from monocytes to macrophages, whereas cathepsin O/K is induced during differentiation from monocytes to osteoclasts (45). Recently, Urbich et al. (46) reported that CathL is required for endothelial progenitor cell-induced neovascularization. CathL has been reported to be essential for the onset of autoimmune diseases such as type 1 diabetes (12). In the diabetes models in the CathL null mice, an increase in the proportion of Foxp3+ Treg cells has been observed. When the previous reports are taken together with our present results, the findings suggest that the increased Foxp3+ Treg cells may be homing in on the peripheral tissues to control the inflammatory conditions. In fact, EAU in CathL null or EAU treated with rCTLA-2α has been found to greatly suppress the ocular inflammation. In addition to the findings for CTLA-2α, TGF-β has also been shown to inhibit the expression of CathL in T cells and can control the CathL activities in RPE. This suggests that significant amounts of the immunosuppressive factors (e.g., CTLA-2α and TGF-β) are actually losing CathL activities due to the RPE that is contained within the subretinal space and infiltrating cells. Ultimately, however, the CathL-inhibited T cells that can express Foxp3 are able to acquire Treg function.

In conclusion, we have examined the process by which CTLA-2α via RPE mediates the conversion of CD4+ T cells into Treg cells. We hypothesize that intraocular migratory T cells that escape from the choroid vessels into the stroma need to pass through the RPE layer to be able to penetrate into the retinal space. Once in the subretinal space, which includes the RPE layer, secretion of CTLA-2α by RPE converts the intraocular effector T cells into Foxp3+ Treg cells that then acquire regulatory functions. This theory is supported by the fact that CTLA-2α via the T cells and RPE is able to inhibit CathL activities and promote TGF-β activation. Therefore, RPE-derived CD4+ Treg cells play an important role in the immune tolerance found in the posterior segment of the eye.

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Disclosures

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