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Role of Thrombospondin-1 in T Cell Response to Ocular Pigment Epithelial Cells¹,²

Yuri Futagami,* Sunao Sugita,³* Jose Vega,† Kazuhiro Ishida,‡ Hiroshi Takase,* Kazuichi Maruyama,† Hiroyuki Aburatani,§ and Manabu Mochizuki*

Ocular pigment epithelium (PE) cells promote the generation of T regulators (PE-induced Treg cells). Moreover, T cells exposed to PE acquire the capacity to suppress the activation of bystander T cells via TGFβ. Membrane-bound TGFβ on iris PE cells interacts with TGFβ receptors on T cells, leading to the conversion of T cells to CD8⁺ Treg cells via a cell contact-dependent mechanism. Conversely, soluble forms of TGFβ produced by retinal PE cells can convert CD4⁺ T cells into Treg cells in a manner that is independent of cell contact. In this study, we looked at the expression of immunoregulatory factors (TGFβ, thrombospondins, CD59, IL-1 receptor antagonist, etc.) in PE cells as identified via an oligonucleotide microarray. Several thrombospondin-binding molecules were detected, and thus we focused subsequent analyses on thrombospondins. Via the conversion of latent TGFβ to an active form that appears to be mediated by thrombospondin 1 (TSP-1), cultured iris PE and retinal PE cells induce a PE-induced Treg cell fate. After conversion, both ocular PE and PE-induced Treg cells express TSP-1. Regulatory T cell generation was amplified when the T cells also expressed TSP-1. In addition, PE-induced Treg cells significantly suppressed activation of bystander T cells via TSP-1. These results strongly suggest that the ability of ocular PE and PE-induced Treg cells to suppress bystander T cells depends on their capacity to produce TSP-1. Thus, intraocular TSP-1 produced by both ocular parenchymal cells and regulatory T cells is essential for immune regulation in the eye. The Journal of Immunology, 2007, 178: 6994–7005.

immune privilege in the eye is created by a pigment-containing epithelial layer that lines the posterior surface of the iris, ciliary body, and neural retina. Pigment epithelium (PE)² cells in sites of immune privilege protect the delicate internal structures of the visual axis from the blinding consequences of innate and adaptive immune inflammation (1–4). Specifically, the retinal PE (RPE) monolayer functions as the immune privileged tissue (5) and RPE cells create an immune-specific microenvironment in the subretinal space via the release of soluble immunosuppressive factors (6–8). Similarly, the iris PE (IPE) in the anterior segment of the eye creates an immunosuppressive microenvironment in the anterior chamber. For example, cultured IPE cells inhibits activated effector T cells by using cell surface immunomodulatory molecules in vitro (9–12). Both RPE and IPE cells contribute to the integrity of blood-ocular barriers, thereby securing immune privilege within the eye.

T cells exposed to the ocular PE can acquire the capacity to suppress the activation of bystander T cells. Currently, it is thought that there are at least two possible pathways leading to regulatory T cell (Treg) induction, both of which involve cross-talk between the ocular PE and responding T cells. The first involves the interaction between B7 on the IPE and CTLA-4 on T cells (9, 10). Cultured IPE cells constitutively express B7 costimulatory molecules, particularly B7-2 (CD86), on their surface. The second pathway involves interaction between TGFβ on PE cells and TGFβ receptors on T cells (12, 13). IPE and IPE-exposed T cells constitutively express membrane-bound TGFβ (12). In dominant negative TGFβ receptor II (DN TGFβ RI) mice, membrane-bound TGFβ-expressing IPE cells fail to suppress the activation of bystander T cells. IPE cells also fail to convert T cells from similarly affected transgenic mice into Treg cells. Two types of cell surface molecules, B7 costimulatory molecules and membrane-bound TGFβ, are important for conversion to a PE-induced Treg cell state and thus, global T cell suppression.

Several groups have demonstrated a relationship between TGFβ and thrombospondin 1 (TSP-1); for example, there is clear evidence that TSP-1 binds and activates TGFβ (14–17). In this study we provide evidence that TSP-1 is essential for the induction of Treg fate and the subsequent suppression of bystander T cells in vitro and also provide evidence that the effect is mediated via TSP-1 produced by PE cells.

Materials and Methods

Mice

Adult C57BL/6 or BALB/c mice purchased fromCLEA Japan were used as donors of lymphoid cells and ocular PE. The generation of C57BL/6
mice with TSP-1 mutations has been described previously (18, 19). TSP-1 null mice were a generous gift from Dr. J. Lawler of Beth Israel-Deaconess Medical Center (Boston, MA). DN TGFβRII background (C57BL/6) transgenic mice were generated and provided by Drs. R. E. Gress and P. J. Lucas of the National Cancer Institute (National Institutes of Health, Bethesda, MD) using a human CD2 promoter/enhancer (20).

Cell culture
Primary cultures of IPE cells and ciliary body PE (CBPE) cells were cultured in RPMI 1640 complete medium supplemented with 10% FBS as described previously (9, 10). A primary culture of RPE cells was done in DMEM complete supplemented with 20% FBS (9, 10). T cells stimulated with anti-CD3 Abs were grown in serum-free medium composed of complete medium without FBS supplemented with 0.1% BSA (Sigma-Aldrich) and 0.2% insulin, transferrin, and selenium culture supplement (Collaborative Biochemical Products).

Preparation of cultured cells
IPE CBPE, and RPE cells were isolated and cultured as described previously (9, 10). Briefly, eyes were enucleated from 6- to 8-week-old C57BL/6 or BALB/c mice and bisected to isolate the anterior and posterior halves. For IPE and CBPE cells, iris and ciliary body tissues were dissected and then incubated for 1 h in 1 mg/ml Dispase and 0.05 mg/ml DNAse I (Boehringer Mannheim). For RPE cells, eyes were enucleated from 6- to 8-week-old male C57BL/6 mice and bisected along a circumferential line posterior to the ciliary process, creating a ciliary body-deficient posterior eyecup. The eyecup was then incubated for 1 h in 0.2% trypsin (BioWhittaker). These tissues were triturated to form a single cell suspension and then resuspended in culture medium. Primary IPE cultures were >99% cytokeratin positive (clone PCK-26; Sigma-Aldrich) as determined by flow cytometry. The CBPE and RPE cell isolates were 95–97% cytokeratin positive after 14 days of incubation. Human RPE cells (ARPE-19) were kindly provided by Dr. N. Eihara (Juntendo University, Tokyo, Japan).

GeneChip assay
IPE, CBPE, and RPE cells were isolated from normal eyes of C57BL/6 mice as described above and cultured for 14 days, at which point the cultures contained near pure populations of cytokeratin-positive cells (IPE, 99%; CBPE, 95%; RPE, 97%), and the culture medium was replaced with serum-free medium. Next, total RNA was isolated from 1 × 106 cells of each type using TRIzol (Invitrogen Life Technologies) as described by the manufacturer. RNA was further purified using a NucleoSpin RNA II column (Macherey-Nagel) and RNA quality was assessed after electrophoresis by spectrophotometry. cDNAs were synthesized by the manufacturer (Affymetrix) (21). Briefly, double-strand standard cDNA with a T7 promoter was synthesized from 5 μg of total RNA using the SuperScript choice system (Invitrogen-Life Technologies). Approximately 50 μg of biotin-labeled cRNA was synthesized by in vitro transcription with T7 polymerase. After purification and fragmentation, cRNA was hybridized to the oligonucleotide microarray (mouse genome 430 2.0 array; Affymetrix). The scanned images were interpreted by using the microarray suite 5.0 (Affymetrix). The CBPE and RPE cell isolates were >99% cytokeratin positive after 14 days of incubation. Human RPE cells (ARPE-19) were kindly provided by Dr. N. Eihara (Juntendo University, Tokyo, Japan).

Detection of TSP-1 transcripts in PE cells and T cells exposed to ocular PE
Cellular extracts were prepared from cultured PE cells, fresh ocular tissues, or purified T cells exposed to PE cells as described above. Fresh tissues from the iris or ciliary body or whole retina from normal C57BL/6 mice were also prepared as described previously (9). In separate experiments, recombinant TGFβ (5 ng/ml; R&D Systems), TSP-1 peptide (10 ng/ml; Hematologic Technologies), and a TSP-1 type 1 repeat (3×) recombinant protein (3Tsk) (final concentration of 10 μg/ml; from Dr. J. Lawler, Beth Israel-Deaconess Medical Center) were used to treat PE cells. Purified T cells from wild-type C57BL/6 DN TGFβRII donors were added to the ocular PE cell culture. Using a previously reported method (12), membrane-bound TGFβ. IPE Treg cells were used in the detection of TSP-1 transcripts. Anti-TGFβAb followed by biotin-conjugated anti-mouse IgG was used to detect the CD8+ IPE-induced Treg cell population as described previously (12). Next, cultured PE and T cells were washed twice and treated with RNA STAT-60. PCR was conducted using the hot-start PCR method with AmpliTaq and AmpliWax. The following conditions were used: 40 cycles of denaturation at 94°C for 60 s, annealing at 54°C (TSP-1) or 60°C (TSP-2) for 60 s, and extension at 72°C for 60 s. Oligonucleotide primers were as follows. For TSP-1 (733-bp product), 5′-GTTCCTGGTGAAAGATTGTAA-3′ and 5′-CTATTTCAAGGCAACAGG-3′. For TSP-2 (649-bp product), 5′-CAGATCTCGGGCGCTGGCTA-3′ and 5′-ATAAGACGGCGACACACATACAG-3′. PCR products were separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining. The levels of expression of mRNA were standardized using GAPDH expression as an internal control.

Bioassay for TGFβ
To measure the concentration of mature (active) and total (active plus latent) TGFβ, the supernatants of IPE cell cultures from wild-type C57BL/6 or TSP-1-null donors were collected and added to cultured Mv1Lu cells (American Type Culture Collection cell line CCL-64) as described previously (10, 12). Purified TSP-1 protein from platelets at a final concentration of 20 μg/ml were obtained from Dr. J. Lawler and used in the assay. To measure the total amount of TGFβ in the cell culture supernatants, supernatants were first collected and treated with 1 N HCl to lower the pH to 2.0. The concentration of TGFβ was then calculated by comparing the suppression of Mv1Lu cell proliferation to the suppression of proliferation observed when known amounts of TGFβ2 (R&D Systems) were used. For some assays, neutralizing anti-TGFβAb (R&D Systems) was added. The results of these assays are presented in picograms per milliliter TGFβ.

Detection of TSP-1 on cultured PE and T cells by flow cytometry
Flow cytometry was used to analyze the expression of surface or intracellular TSP-1 on cultured PE or T cells. In brief, harvested PE cells were blocked with anti-CD16/CD32 Abs (Fcy III/II receptor, clone 2.4G2; BD Pharmingen) at 4°C for 15 min, washed, and stained with anti-TSP-1 Abs (clone 20; BD Biosciences) or control mouse IgG (BD Pharmingen) at 4°C for 30 min. Afterward, cells were stained with FITC-conjugated streptavidin (BD Pharmingen) at 4°C for 15 min. The cells were washed and analyzed by flow cytometry. T cells exposed to PE were treated similarly. To detect intracellular TSP-1, cultured PE and T cells were incubated in culture medium. Primary IPE cultures were performed in 1% agarose gel. GeneChip assays were as described by the manufacturer. RNA was further purified using a NucleoSpin RNA II column (Macherey-Nagel) and RNA quality was assessed after electrophoresis by spectrophotometry. cDNAs were synthesized by the manufacturer (Affymetrix) (21). Briefly, double-strand standard cDNA with a T7 promoter was synthesized from 5 μg of total RNA using the SuperScript choice system (Invitrogen-Life Technologies). Approximately 50 μg of biotin-labeled cRNA was synthesized by in vitro transcription with T7 polymerase. After purification and fragmentation, cRNA was hybridized to the oligonucleotide microarray (mouse genome 430 2.0 array; Affymetrix). The scanned images were interpreted by using the microarray suite 5.0 (Affymetrix). The CBPE and RPE cell isolates were 95–97% cytokeratin positive after 14 days of incubation. Human RPE cells (ARPE-19) were kindly provided by Dr. N. Eihara (Juntendo University, Tokyo, Japan).

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Western blots for TSP-1 in supernatants of cultured PE cells

After 14 days in culture in standard medium, PE cells were cultured for an additional day in serum-free medium. Next, the supernatants were collected and assayed for TSP-1. The total protein content of the supernatants was determined using a bicinchoninic acid protein assay kit (Pierce). Recombinant TSP-1 was used as a positive control (Hematologic Technologies). A HRP-conjugated secondary Ab (Santa Cruz Biotechnology) was added at a concentration of 1 g/ml and sections were incubated for 2 h at room temperature in the dark. Isotype controls include biotin-conjugated mouse IgG at a concentration of 1 g/ml.

TSP-1 ELISA in the supernatants of human RPE cells

The concentration of TSP-1 in the supernatants of the human RPE cell (ARPE-19) culture was measured by ELISA (human TSP-1 ELISA kit; American Research Products). The supernatants from the RPE cell cultures were also collected and assayed after the addition of anti-TSP-1 Abs or an isotype control (0.25 µg/ml).

Immunohistochemical detection on whole eye sections

Frozen sections of a fresh ocular sample were prepared to confirm the expression of TSP-1 at the cell level. Normal mouse eyes were removed and quick frozen in OCT embedding compound with dry ice. Ten-micrometer sections were cut and mounted on glass slides and dried for 60 min. After fixation in acetone for 10 min, sections were rinsed three times in 1X PBS and blocked with 1% BSA in 1X PBS for 10 min at room temperature. Biotin-conjugated Abs that recognize TSP-1 were added at a concentration of 1 µg/ml and sections were incubated for 2 h at room temperature. Sections were washed five times for 3 min in 1X PBS following by the addition of FITC-streptavidin (1:50) and incubation for 30 min at room temperature in the dark. Isotype controls include biotin-conjugated mouse IgG at a concentration of 1 µg/ml.

Statistical evaluation of results

Each experiment was repeated at least twice with similar results. All statistical analyses were conducted using a Student’s t test. Values were considered statistically significant if p < 0.05.

Results

Eye-related immunoregulatory genes are expressed in cultured ocular PE cells

To identify genes that are up-regulated in response to immunological changes in the eye, we used a GeneChip microarray assay in which ~45,102 genes were tested. IPE, CBPE, and RPE cells were tested. The number of genes that showed significant signals (above a cutoff value of 50) was 15,073 genes for IPE cells, 13,993 genes for CBPE cells, and 12,384 genes for RPE cells. Expression profiles for representative genes are summarized in Tables I and II.

Table I summarizes the results of a GeneChip assay to look at the expression of eye-related immunoregulatory molecules in ocular PE cells and presents a comparison of our results with those other expression studies on ocular PE from other groups. We found that TGFβ1–3 and TGFβ receptor 1–3 exhibited high levels of expression in PE cells (Table I). Among members of the thrombospondin superfamily of genes, only TSP-1 and TSP-2 exhibited a high level of expression. Among the neuropeptides known to be enriched in the aqueous humor (34–36), only the calcitonin gene-related peptide was detected in all PE cells. Macrophage migration inhibitory factor, which inhibits NK function in the aqueous humor (37), showed a high level of expression in our

### Table I. Summary for expression of eye-related immunoregulatory molecules in GeneChip

<table>
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<tr>
<th>Probe Set Signal in GeneChip</th>
<th>GenBank Accession</th>
<th>Gene Name</th>
<th>Gene Designator</th>
<th>IPE</th>
<th>CBPE</th>
<th>RPE</th>
<th>Referenceb</th>
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<td>7, 12, 22, 23</td>
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<td>637</td>
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<td>7153</td>
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<td>2750</td>
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<td>2027</td>
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</table>

*Low indicate a signal of 5–50. ND, Not detectable because of a signal <5.

bOthers who have reported the expression of the molecule in ocular PE.
assay, and among the eye-related complement regulators only CD59a exhibited a high level of expression. Costimulatory molecules such as CD80 and CD86 that are uniquely expressed on cultured IPE cells (9) did not exhibit a significant level of expression.

In regard to members of the TNF receptor superfamily, including CD95, TNF receptor I, and TNF receptor II, all of the PE cell types tested expressed these genes; however, the CD95 ligand was not detected. One of the anti-inflammatory cytokines, the IL-1 receptor antagonist, also showed significant levels of expression in the ocular PE. From among the molecules that showed high levels of expression in the assay, we focused on TSP-1 because the roles of these others in ocular immune privilege are not known.

Expression of thrombospondin in freshly procured ocular pigment epithelium and tissues

We next confirmed that, as expected, TSP-1 and TSP-2 are expressed on cultured ocular PE cells and fresh tissues from the iris, ciliary body, and retina. From among the molecules that showed high levels of expression in the assay, we focused on TSP-1 because the roles of these others in ocular immune privilege are not known.

Expression of thrombospondin in freshly procured ocular pigment epithelium and tissues

We next confirmed that, as expected, TSP-1 and TSP-2 are expressed on cultured ocular PE cells and fresh tissues from the iris, ciliary body, and retinal pigment epithelium. PE cells cultured from all three ocular tissues for 14 days were prepared for RT-PCR and flow cytometry. As seen in Fig. 1A, cultured IPE, CBPE, and RPE cells expressed TSP-1 mRNA. In addition, a high level of TSP-2 mRNA was detected in CBPE and RPE cells, whereas in IPE cells TSP-2 mRNA was expressed at significant but weak levels (Fig. 1B). Fresh iris, ciliary body, and retinal pigment epithelium tissues express TSP-1 mRNA (Fig. 1C), and all ocular tissues expressed TSP-2 mRNA at least at low levels (data not shown). These results indicate that ocular tissues, including the pigment epithelium, express thrombospondins, and in particular the thrombospondin gene TSP-1. Consistent with the results of our flow cytometry analysis, cultured IPE cells express TSP-1 on the cell surface whereas surface TSP-1 was detected only poorly on CBPE and RPE cells (Fig. 1C). In contrast, intracellular TSP-1 was clearly expressed in all cultured PE cells.

We next asked whether ocular PE cells secrete TSP-1. PE cells were cultured in serum free medium for 24 h, after which time the TSP-1 protein in the cell culture supernatant was assayed by Western blotting. The TSP-1 protein was clearly detected in the supernatants of CBPE and RPE cells (Fig. 1D). In contrast, the protein was poorly detected in IPE cell supernatants. And as expected, the TSP-1 protein was not detected in the supernatants of CBPE cells from TSP-1-null donors (Fig. 1D).

FIGURE 1. Detection of thrombospondins in freshly obtained ocular pigment epithelium and tissues. A and B, The presence of TSP-1 and/or TSP-2 transcripts was assayed by PCR and gel electrophoresis in a 14-day culture of ocular PE cells (IPE, CBPE, and RPE) (A) or fresh ocular tissues (iris, ciliary body (CB), and whole retina) (B) from normal C57BL/6 mice. The levels of expression of mRNAs were standardized to GAPDH. C, Flow cytometry of intracellular TSP-1 on ocular PE cells using anti-TSP-1 or a mouse IgG isotype control (dotted lines) with CBPE cells from TSP-1 knockout (KO) donors used as a negative control. TSP-1 in cells was visualized using a biotin-conjugated, anti-mouse IgG secondary Ab followed by treatment with FITC-conjugated streptavidin. The percentages (%) of positive cells for FITC-labeled TSP-1 are indicated, with the mean fluorescence intensity values in parentheses. D, Western blotting of cultured PE cells from wild-type (WT) or TSP-1-null donor cells using the indicated Abs. M, Molecular marker. E, Section of a fresh ocular sample that was frozen and prepared for immunohistochemistry to detect TSP-1. Biotin-conjugated mouse IgG. CB, Ciliary body; CP, corneal epithelium; IR, iris; RE, retina. F, Cultured human RPE (ARPE-19) cell supernatant was analyzed by TSP-1 ELISA, without treatment or with treatment with anti-TSP-1/-2 Abs (filled bar, 0.25 μg/ml) or a goat IgG isotype control (crosshatched bar). The mean ± SEM of two ELISA is shown. * p < 0.05, comparing two groups.
FIGURE 2. Relationship between TGFβ and TSP-1 produced by ocular PE. A, Cultured PE cells were prepared for detection of TGFβ2 and TSP-1 transcripts in the presence or absence of recombinant mouse TGFβ2. PE cells treated with recombinant TGFβ2 (5 ng/ml; filled bars) and nontreated cells (open bars) were harvested by RT-PCR, separation by gel electrophoresis, and visualization by ethidium bromide staining. Negative images were analyzed using NIH Image software. The TGFβ2 or TSP-1/GAPDH ratios based on semiquantitative RT-PCR are indicated. B, Cultured PE cells from wild-type donors (open bar) or TSP-1-null donors (filled bar) were harvested for detection of TGFβ2 via RT-PCR. Left, The TGFβ2/GAPDH ratio based on semiquantitative RT-PCR. Right, IPE cell supernatant that was collected and added to Mv1Lu cells to perform a TGFβ bioassay to detect active TGFβ2. Counts per minute of triplicate cultures incubated for 24 h are presented as mean ± SEM. *p < 0.05, comparing two groups. C, RPE cell supernatants were collected and added to Mv1Lu cells to perform a TGFβ bioassay (filled bars, total TGFβ2; open bars, active TGFβ2). Additional RPE cell supernatant samples were treated with recombinant TSP-1 or anti-TGFβ2 neutralizing Abs. Mean counts per minute for triplicate cultures incubated for 24 h are presented as mean ± SEM. *p < 0.05; **p < 0.0005, compared with RPE supernatants only, group A. ND, Not detected. D, Detection TGFβ2 transcripts in the presence of TSP-1 peptide in PE cells. TSP-1 peptide-treated PE cells (10 ng/ml; crosshatched bars), 3TSR-treated PE cells (10 μg/ml; filled bars), and nontreated cells (open bars) were harvested, followed by RT-PCR. The negative image was analyzed using NIH Image software. TGFβ1 or TGFβ2/GAPDH ratio based on the results of semiquantitative RT-PCR are indicated.

Next, we attempted to confirm that TSP-1 is expressed in fresh ocular tissues. Via immunohistochemistry on frozen sections we found that ocular PE cells, including IPE, CBPE, and RPE from fresh tissues, clearly express TSP-1 whereas other eye epithelial cells such as the cornea epithelium, the conjunctival epithelium, and the lens epithelium express the protein poorly (Fig. 1E). Taken together, the results show that ocular pigment epithelia, primary cultured PE and fresh (i.e., noncultured) PE tissues, each constitutively express TSP-1.

We also asked whether human PE cells produce TSP-1 and found that, as expected, human RPE cells produce TSP-1 protein at robust levels. Moreover, TSP-1 production was reduced when anti-TSP-1 neutralizing Ab was added to the RPE cell culture (Fig. 1F). These results support the idea that, for IPE, TSP-1 is expressed on the cell surface, whereas CBPE and RPE cells produce and secrete soluble forms of TSP-1.

Relationship between TGFβ and TSP-1 produced by ocular PE

We next asked whether exogenous TGFβ is important for expression of the TGFβ and TSP-1 genes in ocular PE cells. To test this, we used recombinant TGFβ2, which is the dominant isoform of TGFβ in the eye. When PE cells were treated with recombinant TGFβ2 in vitro, TGFβ2 and TSP-1 mRNA levels were up-regulated (Fig. 2A). These results imply that TGFβ produced by the pigment epithelium itself may promote the production of TSP-1 and TGFβ.

Next, we looked at the ability of ocular PE obtained from TSP-1-null donors to express TGFβ. PE cell supernatants (from wild-type or TSP-1-null donors) were harvested after 24 h and analyzed via a TGFβ bioassay. IPE cells were also examined by RT-PCR for the expression of TGFβ mRNA. As shown in Fig. 2B, IPE cell supernatants from wild-type donors contain active TGFβ2. In contrast, virtually no TGFβ2 was detected in supernatants from IPE cells obtained from TSP-1-null donors. Additionally, IPE cells from both wild-type and TSP-1-null sources expressed TGFβ2 mRNA at significant levels based on standardization to an internal control, GAPDH (Fig. 2B).

We next examined the influence of TGFβ in vitro on ocular PE cells in the presence of recombinant TGFβ (rTGF-1). The supernatant from RPE cells cultured in the presence of purified TSP-1 protein was analyzed using a TGFβ bioassay. RPE cell supernatants...
significantly suppressed Mv1Lu cell proliferation, whereas anti-TGFβ2-treated RPE supernatants were significantly less able to suppress proliferation (Fig. 2C). As expected, rTSP-1-treated RPE supernatants significantly promoted suppression of the proliferation as well, and this was particularly notable for TGFβ in active form (Fig. 2C).

We next addressed the question whether or not TSP-1 peptide can regulate TGFβ. In cultures containing IPE cells and TSP-1 peptides or 3TSR, IPE cells promoted the expression of TGFβ1 and β2 mRNA (see Fig. 2D). Taken together, the results suggest that TSP-1 is critical for the conversion of the latent form of TGFβ into an active form.

Capacity of IPE cells from TSP-1-null mice to generate T regulators in vitro

We next attempted to determine whether the expression of TSP-1 on ocular PE cells is important in the generation of T regulator cells. In a previous study, we demonstrated that cultured IPE cells can convert CD8⁺ T cells into Treg cells (11, 12). In addition, we have found that RPE cells can convert CD4⁺ T cells but not CD8⁺ T cells into Tregs (S. Sugita, unpublished data). Therefore, we used CD8⁺ IPE-induced Treg cells and CD4⁺ RPE-induced Treg cells in this series of experiments.

For these experiments, ocular PE, IPE and RPE samples were obtained from wild-type C57BL/6 donor mice and mice with TSP-1 gene disruptions, and T cells were exposed in primary cultures to these wild-type or TSP-1-null PE cells. As shown in Fig. 3A, unfractionated or CD8⁺ T cells first exposed to wild-type IPE acquired the capacity to suppress bystander T cell activation. In contrast, when T cells were exposed to IPE from TSP-1-null mice, virtually no suppression was observed (Fig. 3A). Similarly, CD4⁺ T cells exposed to RPE cells or RPE cell supernatant from wild-type donors were able to suppress bystander T cell activation in
secondary culture, whereas CD4+ RPE-induced Treg cells from TSP-1-null RPE and the corresponding supernatants displayed significantly less capacity to suppress T cell activation (Fig. 3B). These results indicate that when PE cells were established from eyes of TSP-1-null donors, ocular PE cells could not convert T cells into Treg cells in vitro.

Next, we conducted an additional experiment using a cytokeratin-positive conjunctiva epithelium (CE) obtained from the ocular surface of normal C57BL/6 mouse eyes. As shown in Fig. 3C, primary cultured CE cells expressed TSP-1 mRNA. However, unlike the intraocular epithelium, these cells failed to induce regulatory T cells in vitro (Fig. 3D). Thus, surface TSP-1 on IPE appears to enable IPE cells to induce regulatory T cells via a cell contact-dependent mechanism. Moreover, soluble TSP-1 secreted by RPE cells enables them to induce a regulatory T cell fate. However, it should be noted that unlike what was observed for IPE, this mechanism does not appear to involve or require direct cell contact.

Expression of TSP-1 by T cell exposed to IPE

It has been reported that T cells constitutively express TSP-1 (38, 39) and thus, we next examined IPE cell-exposed T cells for endogenous expression of TSP-1. First, CD8+ T cells exposed to IPE or unactivated, fresh CD8+ T cells were stained with anti-mouse TSP-1 Abs or an isotype control. The expression of TSP-1 was detectable in CD8+ T cells exposed to IPE, and expression was less robust in fresh unactivated CD8+ T cells (Fig. 4A). Second, T cells from wild-type C57BL/6 or DN TGFβ RI donors were cultured for 24 h in the presence (IPE Treg cells) or absence (control T cells) of IPE, followed by semiquantitative RT-PCR to look at TSP-1 mRNA levels relative to an internal control (GAPDH). Control T cells expressed relatively small amounts of TSP-1 mRNA and IPE Treg cells expressed significantly higher levels of TSP-1 mRNA (Fig. 4B). Additionally, TSP-1 mRNA levels in IPE Treg cells from DN TGFβ RI donors were lower than the levels observed for control T cells (Fig. 4B). These results imply that stimulation of T cells by TGFβ receptor activity initiates or increases the synthesis and secretion of TSP-1, which, in turn, promotes the generation of an active form of TGFβ. We also attempted to determine whether membrane-bound TGFβ+ IPE-induced Treg cells express TSP-1. As shown in Fig. 4C, CD8+ membrane-bound TGFβ+ IPE-induced Treg cells express significantly higher levels of TSP-1 mRNA, but this is not true for membrane-bound TGFβ+ IPE-induced Treg cells. Taken together, the results indicate that TSP-1 promotes the expression of active, membrane-bound TGFβ on T cells exposed to IPE. Thus, the exposure of T cells to IPE up-regulates TSP-1 production, which, in turn, supports TGFβ regulation.

In an attempt to verify these results, it was important to ensure that the TSP-1 mRNA detected in T cells exposed to IPE does not arise from the contamination of TSP-1-expressing IPE cells with another cell type. To help eliminate this possibility, we harvested T cells from TSP-1-null mice and exposed them to wild-type IPE cells for 24 h, followed by assay of TSP-1 mRNA levels in these T cells. The TSP-1 mRNA was not detectable in these cells, strongly supporting the idea that the contamination of the IPE cell population was below the level of resolution (data not shown).

Antibody block of TSP-1 affects the conversion of T cells into regulators

We were interested in learning whether the expression of TSP-1 on IPE and T cells is essential for the conversion of T cells into Treg cells. To address this question, purified T cells were first cultured for 48 h in the presence of IPE cells with or without anti-TSP-1 neutralizing Abs. This was followed by the addition of secondary cultures containing fresh T cells plus anti-CD3 Abs. As shown in Fig. 5A, IPE-induced Treg cells generated in the presence of an
isotype control suppressed T cell activation in secondary cultures, whereas IPE-induced Tregs generated in the presence of anti-TSP-1 Abs had a more limited ability to suppress T cell activation in secondary cultures. Similarly, when anti-TSP-1 Abs were added to secondary cultures containing fresh T cells plus anti-CD3 Abs. Left, Anti-TSP-1/-2 polyclonal Ab or goat IgG was added to primary cultures in which IPE and CD8+ T cells were present for the generation of IPE-induced Treg cells. Right, Anti-TSP-1 Abs were added to cultures in which the irradiated IPE-induced Treg cells were cocultured with the anti-CD3-stimulated T cells. Data shown are the mean counts per minute for triplicate cultures incubated for 72 h ± SEM. **, p < 0.005; ***, p < 0.0005, comparing two groups. B, Left, CD8+ IPE-induced Treg cells from wild type (WT) were first cultured for 48 h in the presence of IPE cells from wild-type mice and then harvested and x-ray irradiated. Center, T cells from TSP-1-null mice were cultured for 48 h with IPE from WT mice. Right, T cells from TSP-1-null mice were cultured for 48 h with IPE from TSP-1-null mice. Control (Cont) T cells were cultured in the absence of IPE. IPE-induced Treg cells (filled bars) and control T cells (open bars) were then added at a concentration of 1 x 10^5/well to fresh responder T cell (T resp) cultures (1 x 10^5/well) in the presence of anti-CD3. Mean counts per minute for triplicate cultures incubated for 72 h are presented ± SEM. *, p < 0.05; ***, p < 0.0005, comparing IPE-induced Treg cells to control T cells.

Next, we used T cells and/or IPE cells from TSP-1-null donors to further address whether the expression of TSP-1 on IPE and/or T cells is important for the efficient conversion of T cells into regulators. In one set of experiments, wild-type CD8+ T cells were exposed in primary cultures to wild-type IPE. In the second set, CD8+ T cells from TSP-1-null donors were exposed to wild-type IPE. After 48 h of incubation, T cells were harvested, x-ray irradiated, and tested for regulatory capacity in secondary cultures containing fresh T cells and anti-CD3. When wild-type CD8+ T cells were first exposed to wild-type IPE, they acquired the ability to suppress the activation of bystander T cells (Fig. 5B). In contrast, IPE-exposed CD8+ T cells from TSP-1-null donors were significantly less able to suppress bystander T cell activation (Fig. 5B). These findings reveal that T cell-derived TSP-1 expression is important in determining whether or not IPE are able to trigger the conversion of T cells into regulators. In a third set of experiments,
primary cultured IPE and CD8+ T cells were established from TSP-1-null mice. The T cells harvested from these cultures were tested for their ability to suppress T cell activation in secondary cultures. TSP-1-null CD8+ T cells exposed to TSP-1-null IPE were significantly less able to suppress bystander T cell activation than control Treg cells (Fig. 5B). These results indicate that the TSP-1 expression on both IPE and T cells is essential when the latter are efficiently converted into regulators.

Expression of thrombospondin family members on ocular PE

A summary of results for the expression of metalloproteinase (MMP) and thrombospondin family members as determined by a GeneChip assay are shown in Table II. MMPs are key effectors of extracellular matrix remodeling, angiogenesis, and cancer progression (40–43); tissue inhibitor of metalloproteinase (TIMP) inhibits the activity of MMPs (41). Of these, MMP-2, MMP-3, TIMP-1, TIMP-2, and TIMP-3 exhibited significant levels of expression (Table II). In addition, MMP-11, -12, -16, -19, -23, and -24 were also detected. A disintegrin-like and metalloproteinase domain with thrombospondin motif (ADAMTS) type 1 motif (ADAMTS1) and F-spondin are members that are included within this family (44). High levels of expression for ADAMTS were noted in three PE cells (ADAMTS-1, -2, and -5); IPE significantly expressed F-spondin as compared with other PEs.

Expression of TSP-1 and Foxp3 transcripts in fresh ocular tissues of uveitis models

As final steps, we asked whether ocular tissues in eyes experiencing an inflammatory response express TSP-1 and if ocular infiltrating cells contain Foxp3+ regulatory T cells. CD25+ regulatory T cells express Foxp3 transcripts robustly (45), and we recently reported that PE-induced Treg cells show strong expression of Foxp3 transcripts (11). EAU was induced via IRBP peptide, and EIU (B) was induced by the addition of LPS. Fresh ocular tissues (including PE cells) were harvested and assayed for changes in the expression of TSP-1 and Foxp3. As a control, fresh ocular tissues from normal C57BL/6 mice were also tested. Fresh iris tissues from an anterior segment of an eye with EAU expressed both TSP-1 and Foxp3 mRNA at high levels as compared with normal ocular tissues from normal donors (Fig. 6A). Similarly, fresh iris tissues from eyes with EIU expressed high levels of both TSP-1 and Foxp3 mRNA (Fig. 6B). These findings suggest that ocular infiltrating cells should contain Foxp3+ Treg cells, as ocular tissues constitutively express immune regulatory molecules such as TSP-1 and TGFβ.

Figure 6. Expression of TSP-1 and Foxp3 mRNA in fresh ocular tissues of uveitis models. EAU (A) was induced by the addition of the IRBP peptide, and EIU (B) was induced by the addition of LPS. Fresh ocular tissues (including PE cells) were harvested and assayed for changes in expression of TSP-1 and Foxp3 transcripts were assayed. Fresh ocular tissues from normal C57BL/6 mice were harvested and used as a control. PCR products were separated by gel electrophoresis and visualized by ethidium bromide staining. The mRNA levels were standardized to GAPDH (internal control).
Discussion

Interactions between T cells and proteins in the extracellular matrix play important roles in several T cell functions, including T cell homing, recruitment to inflammatory sites (48, 49), and regulation of T cell activation (50, 51). As reported in several previous studies, extracellular matrix proteins may have both stimulatory and inhibitory effects on T cell activation (52–56). In the immune system, a family of extracellular matrix proteins referred to as thrombospondins have inhibitory effects on dendritic and thymic epithelial cells (57, 58). Moreover, the thrombospondin TSP-1 inhibits TCR-mediated T cell activation (59). In this study, we have demonstrated that TSP-1 is a potent inhibitor produced by ocular PE and PE-derived regulatory T cells. To the best of our knowledge, this is the first report demonstrating that TSP-1 is essential for the in vitro induction of a regulatory T cell fate and the suppression of bystander T cell fate.

Thrombospondins participate in cell-to-cell and cell-to-matrix communication (14). TSP-1 appears to function at the cell surface via the binding of membrane proteins and cytokines, and at the functional level TSP-1 appears to play a role in the regulation of the extracellular matrix and cellular phenotypes. Membrane proteins present in a thrombospondin-containing complex appear to include integrins and integrin-associated proteins such as CD47, CD36, latency-associated peptide (LAP), proteoglycans, and sulfatides. TGFβ and PDGF also are capable of binding TSP-1. In fact, ocular PE cells expressed thrombospondins and thrombospondin-binding molecules, but not CD36, proteoglycans, or sulfatides (data not shown). In addition, other MMPs genes and members of the thrombospondin family were expressed on ocular PE cells as assayed by GeneChip. These results imply that MMPs and TIMP play essential roles in normal and pathological extracellular matrix degeneration and are involved in the control of angiogenesis in the eye. The thrombospondin type 1 repeat family is a diverse family of extracellular, matrix, and transmembrane proteins, many of which have functions related to regulatory matrix organization and cell-to-cell interaction (42). The ADAMTS motifs have potential roles in embryonic development, angiogenesis, and cartilage degradation (43) and may also play an important role in inflammatory eye and age-related retinal pathologies. A recent study showed that the F-spondin gene, which is expressed in the floor plate, promotes the outgrowth of commissural axons and inhibits the outgrowth of motor axons (44). Interestingly, IPE cells express significant levels of F-spondin as compared with other PE cells. We are now currently investigating whether or not F-spondin is involved in IPE cell functions and ocular immune privilege.

Among thrombospondins, TSP-1 is a member of the thrombospondin family that nonenzymatically converts latent TGFβ into an active form (14–17) and is expressed at high levels in ocular PE cells. By contrast, TSP-2 has not been demonstrated as having activating activity and, moreover, TSP-2 lacks a peptide sequence present in TSP-1 required for activation of TGFβ. TSP-1 also was reported to function as an enzyme inhibitor (60) and an inhibitor of angiogenesis (61), but the full spectrum of functions attributable to TSP-1 remain controversial. Nonetheless, the TSP-1 protein is considered to be important in the regulation of TGFβ. Because ocular PE cells appear to express TSP-1 constitutively (7, 8, 28), we focused our current efforts on the analysis of TSP-1 expression and function in ocular PE and PE-induced Treg cells.

In a previous study, TGFβ was found to be displayed on the cell surface of IPE cells, whereas RPE and CBPE cells secreted soluble TGFβ (7, 12). We found that for TSP-1, the pattern of expression was similar to that observed for TGFβ. TSP-1 was detected on the surface of IPE cells and intracellularly in RPE and CBPE cells. The possibility exists that TSP-1 on the surface of IPE cells binds TGFβ to the plane of the membrane, and this may help to explain how a membrane-associated form of TGFβ can exist. Interestingly, Miyajima-Uchida et al. have reported that human RPE cells produce and release TSP-1 in vitro and that TSP-1 accumulates in the cytoplasm of RPE cells (62). Moreover, TSP-1 itself is constitutively present in the subretinal space and in the aqueous humor (8, 63). Thus, previous results appear to support our finding that cultured RPE and CBPE cells, but not IPE cells, secrete soluble forms of TSP-1. It seems reasonable that production of TSP-1 by RPE cells may be influenced by a TGFβ-rich microenvironment such as that found in the eye.

The functional properties of TSP-1 are relevant to anterior chamber-associated immune deviation (ACAID), which can be induced by APCs (64). Interestingly, TSP-1 is known to bind the latent form of TGFβ and, in the case of ACAID-inducing APCs, TSP-1 promotes conversion of latent TGFβ to an active form (14–17). TSP-1 is also capable of binding to the scavenger receptor CD36, which is constitutively expressed on APC membranes. In both our previous (9) and present reports we found evidence that ocular PE cells express CD36 mRNA and protein. As ocular PE obtained from CD36 deficient mice can convert T cells into regulators (our unpublished data), this implies that the molecules are not relevant to the Treg generation induced by ocular PE cells. We have already noted that CD36 expression on the vascular endothelial cells in the eye is likely to be important for TSP-1 action as an inhibitor of angiogenesis, as TSP-1 inhibits the response of vascular endothelial cells to CD36 (65).

TSP-1 also has a third binding site that enables it to bind to CD47. CD47 is a cell surface glycoprotein that is widely expressed, especially on T lymphocytes. It has recently been reported that TSP-1 treatment of T cells during activation interferes with signal delivery from a CD3/TCR complex, thereby inhibiting T cell activation (59). CD47 has been found to be the T cell surface molecule to which TSP-1 binds to achieve this inhibitory effect. It might be possible that CD47 is constitutively and robustly expressed on ocular PE cells and that PE-induced Treg cells bind to TSP-1 to promote the conversion of responding T cells to regulatory T cells. At the current time we are conducting experiments to determine whether ocular PE cells or PE-induced Treg cells from CD47 knockout donors can bring about conversion to a Treg cell fate.

Murphy-Ullrich et al. first reported that TSP-1 binds and activates TGFβ and, subsequently, several groups have demonstrated a relationship between TGFβ and TSP-1 (14–17). Moreover, TSP-1 production is enhanced by TGFβ (64). Interestingly, TSP-1-null mice have been used to show that TSP-1 activates TGFβ in epithelial tissues (16). Thus, it appears that the activation of TGFβ in normal epithelial cells is a function of TSP-1. In the present study we found that TSP-1 produced by the ocular epithelium exhibits functions related to TGFβ and the ocular microenvironment, revealing a relationship between TGFβ and TSP-1. Specifically, we found that: 1) the expression of TGFβ and TSP-1 mRNA is up-regulated when PE cells are treated with recombinant TGFβ; 2) TSP-1-null PE cells do not secrete active TGFβ; 3) TSP-1-treated ocular PE cell supernatants can induce up-regulation of the secretion of active TGFβ more robustly than that seen for nontreated PE cells; 4) when TSP-1 is expressed in T cells, T cells exposed to IPE-induced Treg cells express significantly higher levels of TSP-1 in contrast to the exposure to IPE-induced Tregs from DN TGFβ RI, a poor expresser of TSP-1; and 5) membrane-bound TGFβ-IPE-induced Treg cells, but not membrane-bound TGFβ-IPE-induced Treg cells, express TSP-1 mRNA, suggesting that
eye-dependent regulatory T cells constitutively express membrane-bound TGFβ and TSP-1, thereby suppressing bystander T cell activation. Taken together, the results indicate that TSP-1 is critical for the conversion of latent TGFβ into an active form. Thus, it appears that TGFβ and TSP-1 produced by PE cells influence the behavior of PE-exposed T cells, leading to further production of TGFβ and TSP-1 by autocrine mechanisms. Therefore, it seems reasonable to suggest a relationship between these molecules and cell types within the ocular microenvironment.

Li et al. reported that endogenous TSP-1 is part of an adhesion-dependent mechanism responsible for controlling cytoplasmic spreading and migration of T cells (38). In addition, Oida et al. reported that CD4+ T cells can suppress colitis via a TGFβ-dependent mechanism dependent on TSP-1 (39). In this work, we found that T cells exposed to PE acquire regulatory properties and strongly suppress the activation of bystander T cells (10, 12). During the conversion of Treg cells, both ocular PE and PE-induced Treg cells express TSP-1. When primary cultures were established in which both CD8+ Treg cells express TSP-1. When primary cultures were established in which both CD8+ T cells and cultured IPE cells were obtained from TSP-1-null mice or IPE-induced Treg cells were added to anti-CD3 stimulated bystander T cells in the presence of an anti-CD4+ T-cell neutralizing Ab, Treg cells failed to suppress the activation of bystander T cells, suggesting that regulatory T cell generation is up-regulated when T cells themselves express TSP-1. Importantly, Zamiri et al. have reported that experimental uveitis from TSP-1-neutralizing Ab, Treg cells failed to suppress the activation of bystander T cells via TGFβ homeostasis and its absence causes pneumonia. J. Clin. Invest. 101: 982–992. Cursiefen et al. 2000. Participation of pigment epithelium of iris and ciliary body in ocular immune privilege; 2: generation of regulatory T cells that suppress bystander T cells via TGFβ. Invest. Ophthalmol. Visual Sci. 41: 3862–3870.


