

# Thrombospondin Plays a Vital Role in the Immune Privilege of the Eye

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**PURPOSE.** The role of thrombospondin (TSP)-1 in TGF- $\beta$  activation and T-cell suppression was studied in the retinal pigment epithelial (RPE) cells, a monolayer of pigmented cells that line the subretinal space, an immune-privileged site in the eye.

**METHODS.** Posterior eyecups were prepared by excising the anterior segment, lens, and retina from enucleated eyes of C57BL/6, thrombospondin-1 knockout (TSP-1KO), and TGF- $\beta$ 2 receptor II double-negative (TGF- $\beta$ 2 RII DN) mice, leaving behind a healthy monolayer of RPE resting on choroid and sclera. Serum-free medium was added to these RPE eyecups, and, after various time intervals, supernatants (SNs) were removed and tested.

**RESULTS.** SNs of an ex vivo culture of RPE cells from C57BL/6 mice were shown to inhibit both antigen and anti-CD3 activation of T cells, partially due to constitutive production of TGF- $\beta$  and to the ability of RPE to activate the latent form of TGF- $\beta$ . Activation of TGF- $\beta$  was entirely dependent on TSP-1, also produced by RPE. SNs of RPE from TSP-1KO mice failed to inhibit T-cell activation. Ovalbumin (OVA)-specific delayed hypersensitivity (DH) was not impaired when OVA was injected either into the subretinal space or into the anterior chamber of TSP-1KO mice before OVA immunization. Moreover, experimental autoimmune uveoretinitis was significantly more intense in eyes of TSP-1KO mice and failed to undergo spontaneous resolution unlike wild-type mice.

**CONCLUSIONS.** Production of both TSP-1 and active TGF- $\beta$  by RPE is essential to the creation and maintenance of immune privilege in the subretinal space and that the immune privilege limits the severity and duration of retinal inflammation due to autoimmunity. (*Invest Ophthalmol Vis Sci.* 2005;46:908–919) DOI:10.1167/iovs.04-0362

Ocular immune privilege is believed to be an important physiological adaptation of the eye that prevents innate and adaptive immune responses from destroying precious ocular cells that are essential for vision and are incapable of regeneration. The immune privilege also prevents immunologically induced inflammation from clouding the ocular media and distorting the visual axis, for when the ocular cells die

and/or the visual axis is disrupted, loss of vision (blindness) is the inevitable consequence. Some of the mechanisms that allow the presence of immune privilege in the eye are understood. These include (1) immune deviation to antigens injected into all ocular compartments, the anterior chamber, the subretinal space, and the vitreous cavity; (2) the presence of an immunosuppressive microenvironment in these compartments; and (3) the prolonged survival of solid-tissue allografts placed within ocular compartments.<sup>1</sup> Failure of ocular immune privilege has been associated with disease formation and progression. A notable example is herpes simplex keratitis,<sup>2</sup> an immunopathogenic disease that leads to corneal scarring.

Most studies of ocular microenvironments have concentrated on the properties of the aqueous humor that fills the anterior chamber of the eye. Less attention has been paid to the parenchymal cells in the eye and their contribution to immunosuppressive microenvironments. Recently, our laboratory demonstrated that ocular pigment epithelia of iris, ciliary body, and retina inhibits T-cell proliferation in vitro. Unlike the pigment epithelium of the iris, retinal pigment epithelium (RPE) inhibited T-cell proliferation through soluble factors.<sup>3,4</sup> In the experiments to be described, we sought to determine some of the factors and mechanisms that are responsible for the ability of RPE to inhibit T-cell activation in vitro. We further wanted to determine whether there is a correlation between the T-cell inhibitory capability of RPE and the presence of immune privilege in the posterior segment of the eye.

RPE is a neuroepithelial layer of pigmented cells that lines the outermost boundary of the retina and forms the posterior border of the subretinal space, an immune privileged site in the posterior segment of the eye. The RPE is strategically placed, not only to act as an immunologic barrier by providing the outer blood-retinal barrier, but also by expressing cell surface molecules and by secreting soluble mediators that influence the immune system.<sup>5</sup>

To evaluate the immunomodulatory properties of RPE, we have devised an in vitro experimental system composed of an intact monolayer of RPE (RPE eyecup) resting on the choroid and the sclera. The reasons for this approach are several: (1) The presence of immune privilege in the subretinal space is largely dependent on the presence of an intact and healthy monolayer of the RPE<sup>6</sup>; (2) RPE cells are polarized in vivo and secrete many factors in a polarized manner<sup>5</sup>; and (3) cultured monodisperse RPE cells can behave differently from RPE in intact monolayers, both in physical characteristics and in secretory behavior.<sup>7,8</sup>

Our results indicate that the apical surface of RPE in a posterior eyecup constitutively elaborated immunosuppressive factors such as TGF- $\beta$  that profoundly inhibit T-cell activation in vitro. Thrombospondin (TSP)-1, also produced by RPE, appeared to play a central role in converting latent TGF- $\beta$  into its active, immune inhibitory form. Eyes of TSP-1 knockout (TSP-1KO) mice with genetic deficiency of TSP-1 failed to promote immune deviation and displayed exaggerated and unresolved experimental autoimmune uveoretinitis (EAU).

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## METHODS

### Animals

Adult male C57BL/6 and DO11.10 mice aged 6 to 10 weeks were obtained from Taconic Laboratories (Germantown, NY) and Jackson Laboratories (Bar Harbor, ME), respectively. TSP-1KO mice were a generous gift from Jack Lawler (Beth Israel Deaconess Medical Center, Boston, MA) and transforming growth factor- $\beta$  receptor II double negative (TGF- $\beta$  RII DN) mice were kindly donated by Ron Gress (National Cancer Institute, Bethesda, MD). Mice were housed in a common room of the vivarium. All experimental procedures conformed to the ARVO Statement for the Use of Animals for Ophthalmic and Vision Research.

Inoculations, injections, and clinical examinations were performed under anesthesia induced by intraperitoneal injection of ketamine (Ketalar; Parke Davis, Paramus, NJ) at 0.075 mg/g of body weight, and xylazine (Rompun; Phoenix Pharmaceutical, St. Joseph, MO) at 0.006 mg/g of body weight. Enucleation of eyes and removal of spleen and lymph nodes were performed after the animals were euthanized.

### Preparation of Posterior Eyecups

Eyes from euthanized C57BL/6 or TSP-1KO mice were enucleated and placed in  $\text{Ca}^{2+}/\text{Mg}^{2+}$  free HBSS on ice for 30 minutes. After excision of the muscles, connective tissue, and conjunctiva, a circumferential incision was performed below the level of the ciliary body, and the remainder of the anterior segment (cornea, iris, ciliary body, and lens) was discarded. The remaining tissue was placed in 0.01 U/mL of chondroitinase ABC<sup>9</sup> for 30 minutes at 37°C, placed on ice, and washed three times in HBSS. The neural retina was gently lifted off the RPE layer by microsurgical forceps. Neural retina-deficient posterior eyecups, consisting of sclera, choroid, and a healthy monolayer of RPE, were studied by placing them in individual wells of microculture plates (S plate; Nunc Laboratories, Naperville, IL). The RPE eyecups were partially submerged in serum-free medium (SFM; RPMI 1640, 0.1 M HEPES, 0.1% bovine serum albumin, 1  $\mu\text{g}/\text{mL}$  iron-free transferrin, 10 ng/mL linoleic acid, 0.3 ng/mL  $\text{Na}_2\text{Se}$ , and 0.2  $\mu\text{g}/\text{mL}$   $\text{Fe}(\text{NO}_3)_3$  for further experimentation. Into each eyecup 10  $\mu\text{L}$  of the SFM medium was placed. Unless otherwise stated, the RPE supernatant (SN) from within the eyecup was removed 24 hours after incubation and further diluted 1:10 in serum-free medium. The diluted SN was then used in the assays. In all studies, the final dilution of SN was 1:40.

### Cultures of Monodisperse RPE Cells

Eyes from adult C57BL/6 mice were enucleated and placed in HBSS on ice for 3 hours. The anterior segment and neural retina were then excised and discarded. The remainder of the posterior segment of the eye was incubated with 0.2% trypsin (BioWhittaker, Walkersville, MD) for 1 hour at 37°C. The eyecups were washed, and the RPE microaggregates were removed. A single-cell suspension, formed by repeated pipetting of microaggregates of RPE, was seeded on six-well plates (Corning-Costar, Corning, NY) in modified DMEM (with 0.1 M HEPES, 20% FCS, 1% L-glutamine, 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, and 1% nonessential amino acid [NEAA]), passed once, and cultured until confluence.

### DO11.10 T-Cell Proliferation Assay

T cells from DO11.10 mice express a transgenic T-cell receptor that recognizes ovalbumin (OVA) peptide fragment 323-339 in the context of I-A<sup>d</sup>.<sup>10</sup> DO11.10 mice were euthanized, and their lymph nodes were removed. DO11.10 lymph node cells (LNCs;  $4 \times 10^5$ ) in a volume of 25  $\mu\text{L}$  were placed in wells of a 96-well round-bottomed plate (Corning-Costar). SNs of RPE eyecups were collected after appropriate intervals and diluted 1:10 in SFM. DO11.10 LNCs were suspended in the diluted SN, plus 50  $\mu\text{g}/\text{mL}$  OVA (Sigma-Aldrich, St. Louis, MO) in a volume of 25  $\mu\text{L}$  and incubated for 96 hours at 37°C. [<sup>3</sup>H]thymidine (0.5  $\mu\text{Ci}$ ; NEN Life Sciences Products, Boston, MA) was added to each

well on day 4 and incubated overnight. Thymidine uptake was assessed as a measure of T-cell proliferation. Positive control wells contained DO11.10 LNCs and OVA, and negative control wells contained only DO11.10 LNCs. To assess the role of TGF- $\beta$ , some experimental wells also received either anti-pan TGF- $\beta$  antibody (R&D Systems, Minneapolis, MN) alone or in combination with soluble TGF- $\beta$  receptor II (R&D Systems). Three replicate wells were used for each time point, and the experiment was repeated three times.

### Anti-CD3 T-Cell Activation Assay

Spleens and lymph nodes were removed from naive C57BL/6 mice. T cells ( $4 \times 10^5$ ) were added to the wells of 96-well round-bottomed plates at the volume of 25  $\mu\text{L}$  per well (Corning-Costar) together with anti-TCR antibody (2C11; 1  $\mu\text{g}/\text{mL}$  [ATCC]) and 50  $\mu\text{L}$  of 1:10 diluted SN of RPE eyecups prepared from the eyes of C57BL/6 or TSP-1KO mice. Some of the cultures were incubated for 24 hours, and 0.5  $\mu\text{Ci}$  [<sup>3</sup>H]thymidine (NEN Life Sciences Products) was added to the wells. The cultures were incubated for a further 24 hours and [<sup>3</sup>H]thymidine uptake was then measured. Other culture wells were incubated for 48 hours, after which their SNs were removed and used in IFN- $\gamma$  ELISA assays to measure the amount of IFN- $\gamma$  produced by T cells.

### Quantification of TGF- $\beta$

The concentration of TGF- $\beta$  in the SNs of RPE eyecups was measured with the standard Mv1Lu (CCL-64; ATCC, Manassas, VA) mink lung epithelial cell proliferation inhibition assay.<sup>11</sup> Before the assay, aliquots of RPE SN underwent transient acidification by addition of 5  $\mu\text{L}$  of 1.0 N HCl to every 100  $\mu\text{L}$  of 1:10 diluted SN of RPE eyecups, lowering the pH to  $\sim 2$ . The acid-treated SN was incubated for 1 hour at 4°C. The acid was neutralized by adding 10  $\mu\text{L}$  of a 1:1 mixture of NaOH (0.01 M) and HEPES (0.04 M, pH 7.4). Acid neutralization was confirmed by samples of the SN's turning the pH indicator paper to the color that indicates 7.4 pH.

Mv1Lu cells from subconfluent cultures were incubated with a serial dilution of unmodified or acid-treated SN of RPE eyecups in 96-well flat-bottomed microtiter plates (Fisher Scientific, Pittsburgh, PA) in EMEM (Invitrogen-Gibco, Gaithersburg, MD) supplemented with 0.05% fetal calf serum. Cultures were incubated for 20 hours at 37°C. At this time 0.5  $\mu\text{Ci}$  [<sup>3</sup>H]thymidine was added to each culture, and 4 hours later the amount of incorporated label was measured. The concentration of TGF- $\beta$  was determined by comparing the half-maximum inhibition ( $\text{IC}_{50}$ ) of the cultures treated with diluted SN of RPE eyecups with a standard curve from the cell cultures treated with known amounts of TGF- $\beta$ . Specificity of the assay was monitored by treating the cells with the SNs of RPE eyecups in the presence of neutralizing anti-pan-TGF- $\beta$  antibody (R&D Systems).

### IFN- $\gamma$ ELISA

IFN- $\gamma$  production was assayed by using a sandwich ELISA. In brief, a 96-well microtiter plate was coated with capturing mAb to IFN- $\gamma$  (BD Biosciences, Franklin Lakes, NJ) and incubated overnight at 4°C and blocked with PBS containing 1% BSA. Sample and standard recombinant IFN- $\gamma$  were applied to the plate and incubated for 3 hours at room temperature. Biotinylated detecting antibody (BD Biosciences) specific to IFN- $\gamma$  was added for 1 hour. Streptavidin- $\beta$ -galactosidase was added to the wells and incubated for 30 minutes. The substrate chlorophenylred- $\beta$ -D-galactopyranoside was then added to the wells. A standard ELISA plate reader was used to read the optical density of the color change at a wavelength of 570 nm.

### RNA Isolation

Total RNA was isolated from RPE cells harvested from eyecups or from the confluent second passage of cultured RPE cells (RNA STAT-60 kit; Tel-Test Inc., Friendswood, TX) according to the manufacturer's in-

structions. This kit utilizes a single-step method by acid guanidinium thiocyanate-phenol-chloroform extraction.

### Reverse Transcription–Polymerase Chain Reaction

cDNA was synthesized by reverse transcribing RNA using random hexamers and AMV RT (Promega). For PCR amplification of TSP, cDNAs were amplified using the following primers: (5′–3′ sequences) forward TSP, GTT CGT CGG AAG GAT TGT TA; reverse TSP, TCT ATT CCA ATG GCA ACG AG (733 bp). The TSP intron-spanning primers for specific amplification of selected genes were designed with gene sequences from the public database and a software program (Oligo Primer Analysis software 6.0; Molecular Biology Insights, Inc., Plymouth, MN). PCR reactions were performed in a 50- $\mu$ L amplification mixture containing 1 $\times$  polymerase buffer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 1  $\mu$ M forward and reverse primers, 1.25 U *Taq* polymerase (Applied Biosystems, Inc. [ABI], Foster City, CA). After PCR thermal profile was performed in a thermal cycler (GeneAmp PCR System 2400; ABI). One cycle of 5 minutes at 94°C and 5 minutes at 60°C; 40 cycles of 2 minutes at 72°C and 1 minute at 94°C, 1 minute at 58°C; and 1 cycle of 10 minutes at 72°C, hold at 4°C, PCR products were separated by 1.5% agarose gel electrophoresis.

### Immunoblot Analysis

The protein content of RPE eyecup SNs was determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Commercially available purified platelet-derived TSP protein MW 450,000; Haematologic Technology, Inc., Essex Junction, VT) was used as a positive control. In the presence of reducing agent, equivalent amounts of RPE eyecup SN protein were subjected to SDS-PAGE in 3% to 8% Tris-acetate gradient gel (Invitrogen, Inc., Carlsbad, CA) followed by electrophoretic transfer of the separated proteins to nitrocellulose membranes (Pierce). Immunoblot analysis was performed using anti-TSP-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibodies bound to proteins on the membrane were identified using horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) and detected with chemiluminescent substrate (ECL detection reagents; Amersham Pharmacia Biotech, Piscataway, NJ). The immunoblot membranes were then exposed to autoradiograph film (Biomax Light Film; Kodak, Rochester, NY) to detect the chemiluminescent signal. Under reducing conditions TSP-1 polypeptides are known to migrate at 185 and 160 kDa. Both forms of TSP polypeptides were detectable as shown in Figure 5.

### Injections into the Anterior Chamber and Subretinal Space

For anterior chamber injection, anesthetized mice received a 0.3-mm penetrating wound in the peripheral portion of the cornea, 1 mm anterior to the limbus, using a 30-gauge needle. OVA in a volume of 2  $\mu$ L, was slowly injected into the anterior chamber with a glass micropipette connected to a 10- $\mu$ L syringe. The subretinal injections were performed according to the procedure of Whiteley et al.<sup>12</sup> on anesthetized animals that, in addition, received topical proparacaine to anesthetize the ocular surface and tropicamide 1% to dilate the right pupil. Injection into the subretinal space was performed using very fine, bevelled, pulled glass micropipettes that were connected to a 10- $\mu$ L syringe (Hamilton, Reno, NV) through a fine polyethylene tube. The entire apparatus was filled with HBSS, but an airlock was produced before the volume to be injected, thus preventing dilution of the injected material. The bore of the glass needle was coated with silicon (Sigma coat) to prevent adherence of the cells. The injections were made via a transscleral approach under direct visualization, using a binocular surgical microscope and a coverslip held on the cornea. The glass needle was advanced carefully until it reached the subretinal space where 1  $\mu$ L OVA (50 ng) was injected.

### Induction and Assessment of Immune Deviation

The expression of delayed hypersensitivity (DH) to OVA antigen was evaluated in mice that received an anterior chamber or subretinal injection of 50  $\mu$ g/mL OVA. Seven days after intracameral injections, the animals, together with a group of naïve animals (positive control), were immunized subcutaneously with 1:1 mixture of 100  $\mu$ g of OVA and complete Freund's adjuvant (CFA). An ear-swelling analysis was performed 7 days later on all experimental animals, together with a group of naïve animals that served as the negative control. Each group consisted of five mice. Delayed hypersensitivity was measured based on ear swelling, as previously described.<sup>13</sup> Briefly, 200  $\mu$ g of OVA in 10  $\mu$ L of HBSS were injected into the right ear pinnae of the mice. The left ear served as the untreated control. Both ear pinnae were measured with an engineer's micrometer (Mitutoyo, Tokyo, Japan) immediately before and 24 hours after the ear injection. The measurements were performed in triplicate. Anterior chamber-associated immune deviation (ACAID) was detected as the suppression in DH, which was measured as change in ear swelling [(24-hour measurement – 0-hour measurement in the experimental ear) – (24-hour measurement – 0-hour measurement in the negative control ear)] of experimental groups was expressed relative to positive control animals. A two-tailed Student's *t*-test was used, with significance assumed at  $P \leq 0.05$ .

### Induction and Evaluation of EAU

To induce EAU in C57BL/6 and TSP-1KO mice (H-2<sup>b</sup>) mice, five mice in each group were immunized subcutaneously in the back with 200  $\mu$ g human interphotoreceptor retinoid-binding protein (IRBP) peptide 1-20 (GP<sub>THLF</sub>QPSLVLDMAKVVLD; Invitrogen, Inc.) emulsified into CFA, which contains 6.0 (final 3.0) mg/mL *Mycobacterium tuberculosis* (H37RA; Difco Laboratories, Detroit, MI). The mice were also injected intraperitoneally with 0.1  $\mu$ g purified *Bordetella pertussis* toxin (PTX; Sigma-Aldrich).<sup>14</sup> Disease severity was clinically assessed by ocular fundus examinations performed in animals under anesthesia. Pupils were dilated with 1% tropicamide ophthalmic solution (Akorn, Buffalo Grove, IL) before examination. Clinical scoring of EAU was based on retinal vessel dilatation; the number of white, focal, perivascular lesions; and the extent of retinal vessel exudates, hemorrhage, and detachment. Clinical severity was graded on a 0 to 5, as described previously.<sup>15</sup>

The difference between the clinical scores of the two groups was analyzed statistically by the nonparametric Mann-Whitney test for the comparison of two independent populations. Differences were deemed significant when  $P < 0.05$ .

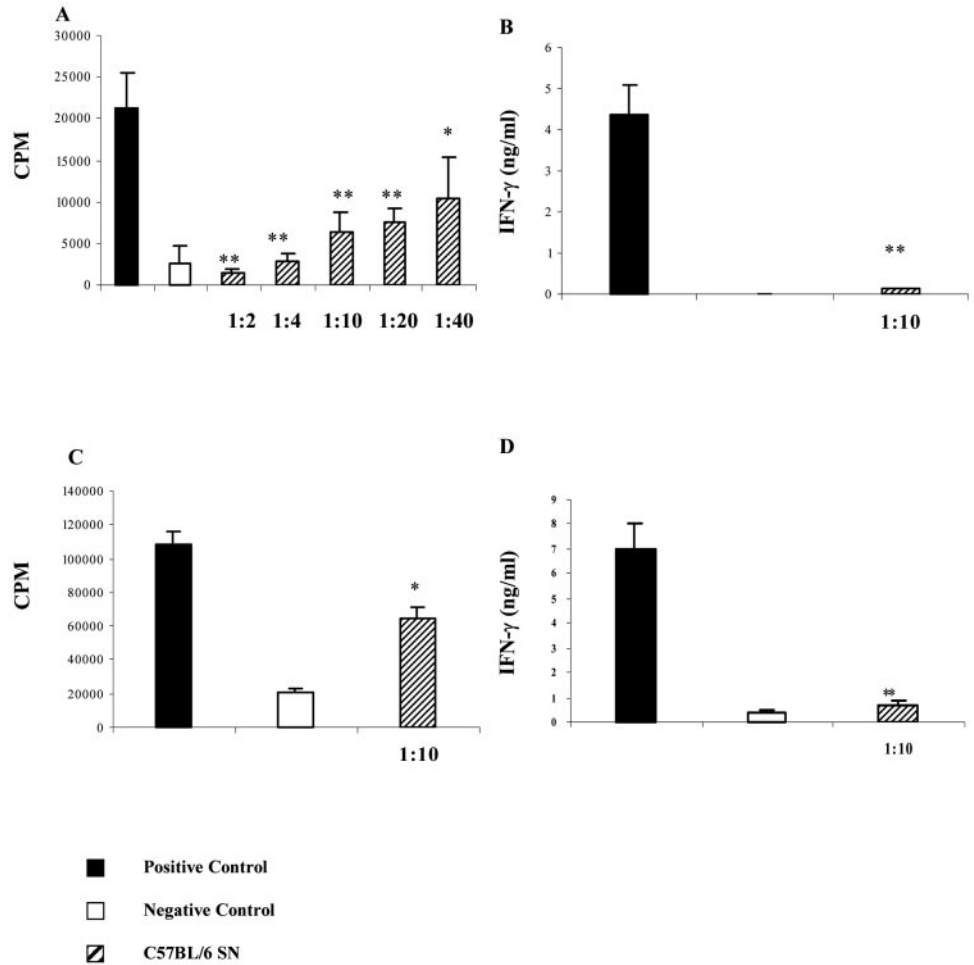
### Histologic Examinations

The C57BL/6 or TSP-1KO mice were euthanatized 120 days after they were immunized with IRBP, and their eyes were enucleated, fixed immediately in 10% formalin, and embedded in methacrylate. Five-micrometer sections were cut and stained with hematoxylin and eosin. Tissue sections were then examined by light microscopy.

## RESULTS

### Ability of SNs of RPE Eyecups to Suppress T-Cell Activation In Vitro

SNs from cultured RPE cells have been shown to inhibit T-cell proliferation by elaborating soluble factors.<sup>3</sup> However, proliferation is only one parameter that expresses the state of T-cell activation. Ocular immune privilege has been found to suppress T cells that secrete Th1 type cytokines, such as IFN- $\gamma$ ,<sup>15</sup> and we wondered whether the SN harvested from RPE eyecups is capable of inhibiting both proliferation and IFN- $\gamma$  production by antigen-activated T cells. LNCs from DO11.10 mice, with T cells carrying a *Tcr* transgene specific for OVA, were incubated with diluted 24-hour RPE SN and OVA. T-cell proliferation and



**FIGURE 1.** Effect of SN from C57BL/6 RPE eyecups on T-cell activation. (A, B) DO11.10 LNCs were incubated with OVA and serially diluted SNs of RPE eyecups for 96 hours. Positive control cultures were DO11.10 LNCs and OVA and negative control cultures were DO11.10 LNCs alone. T-cell proliferation (A) and IFN- $\gamma$  production (B) were measured at 96 hours. (C, D) Twenty-four-hour SNs of eyecups from eyes of C57BL/6 mice were incubated with enriched T cells and anti-CD3 Ab for 24 hours. T-cell proliferation (C) and IFN- $\gamma$  production (D) were measured after 48 hours. Positive control wells contained T cells and anti-CD3 Ab, and negative control wells contained T cells only. Data represent the mean  $\pm$  SEM of results in triplicate wells in a representative experiment. \* $P < 0.05$  and \*\* $P < 0.01$ : significantly less than the positive control.

IFN- $\gamma$  production were measured at 96 hours. Positive control cultures contained DO11.10 LNC and OVA and negative control cultures contained only DO11.10 LNC. Figure 1A shows that DO11.10 T cells are capable of mounting strong proliferative responses to OVA and that RPE SN inhibited this proliferation in a concentration-dependent manner. Even at a dilution of 1:40 the antiproliferative capacity of RPE SN was significant. Our results displayed in Figure 1B reveal that DO11.10 T cells are capable of producing appreciable amounts of IFN- $\gamma$  in response to OVA, and that IFN- $\gamma$  production is significantly suppressed when the T cells are stimulated in the presence of SN of RPE eyecups. Thus, SN from RPE eyecups contains a soluble factor(s) that suppresses T-cell activation in vitro by two parameters.

In the DO11.10 T-cell activation assay just described, unfractionated LNCs, containing both T cells and antigen-presenting cells (APCs), were used as responders. To determine the extent to which RPE-derived immunosuppressive factors act directly on T cells, we tested RPE SN on T cells activated in vitro by mitogenic anti-CD3 antibodies (Ab). In this assay, enriched T cells prepared from spleens of normal C57BL/6 mice were stimulated in vitro with anti-CD3 Ab in the presence of 24-hour SN obtained from RPE eyecups of normal C57BL/6 mice. T-cell proliferation and IFN- $\gamma$  production were measured at 48 hours. Cultures with only T cells and anti-CD3 antibody served as positive controls, and those containing T cells alone served as negative controls.

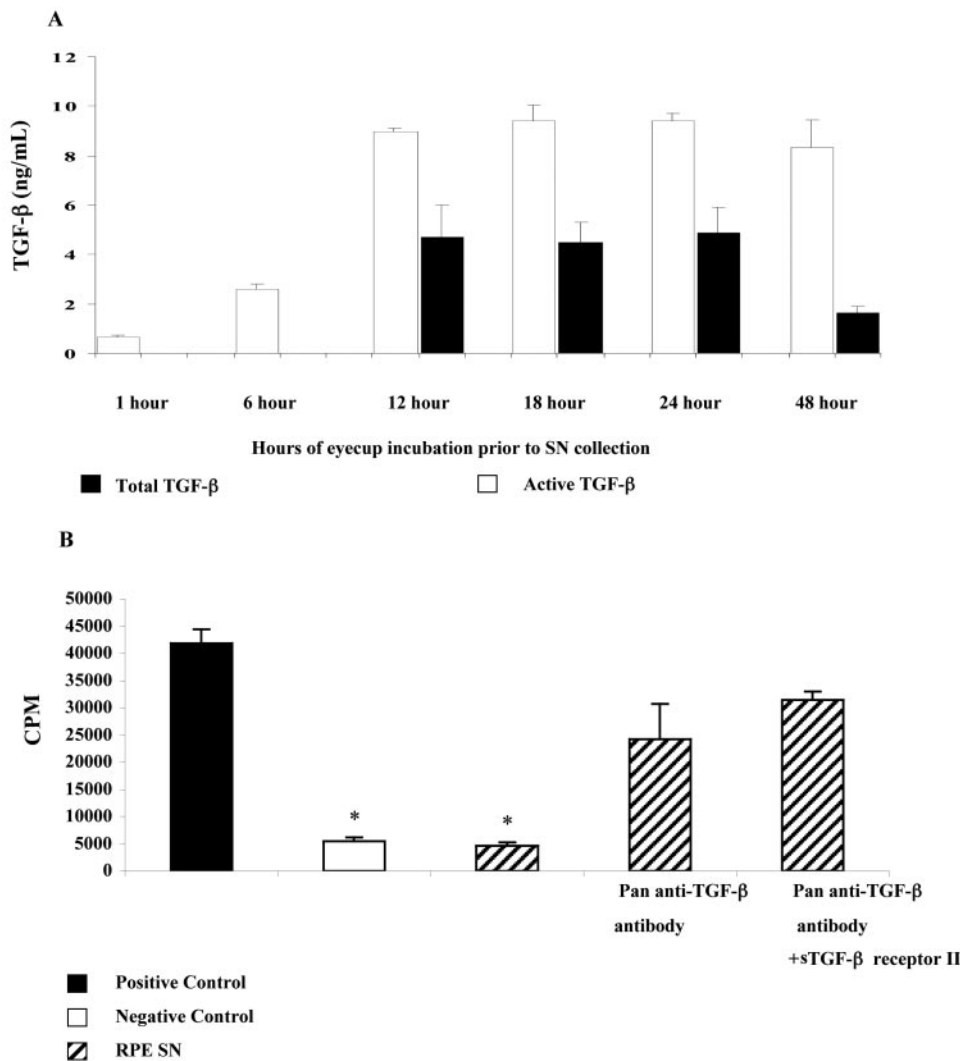
Stimulated T cells mounted an appreciable proliferative response to anti-CD3 Ab (Fig. 1C) and produced significant amounts of IFN- $\gamma$  (Fig. 1D). T cells stimulated by anti-CD3 Ab

in the presence of SN from normal C57BL/6 RPE eyecups proliferated poorly and produced virtually no IFN- $\gamma$ . These results reveal that factors produced by normal RPE eyecups directly suppress T-cell activation, independent of their capacity to alter APC function.

### Role of TGF- $\beta$ in T-Cell Suppression by RPE Eyecup SN

Because RPE cells constitutively express mRNA for TGF $\beta$ <sup>16</sup> and, when activated, TGF- $\beta$  profoundly inhibits both proliferation and lymphokine production by T cells,<sup>17</sup> we wanted to determine whether TGF- $\beta$  is present in RPE eyecup SN, and if so, whether it was responsible for the capacity of these SNs to suppress T-cell activation.

To identify the presence of TGF- $\beta$ , we used the mink lung epithelial cell proliferation inhibition assay. The SN was harvested from RPE eyecups after 0, 1, 6, 12, 24, and 48 hours of incubation. Mink lung cells were incubated with transiently acidified SN (to reveal the total amount of TGF $\beta$  present) or untreated SN (to reveal the amount of active TGF- $\beta$  present) diluted SN for 20 hours. Then, [<sup>3</sup>H]thymidine was added, and the amount of isotope incorporated was assessed. Figure 2A demonstrates that within 1 hour of incubation of RPE eyecups in SFM, latent (but not active) TGF- $\beta$  was detected. Maximum levels of TGF- $\beta$  were found in RPE eyecup SN at 12 hours. Active TGF- $\beta$  was first detected at 12 hours, with the peak levels at 24 hours of incubation and declining at 48 hours. We interpret these results to mean that RPE eyecups elaborate



**FIGURE 2.** Kinetics of TGF- $\beta$  production and activation in SN of RPE eyecups and role of TGF- $\beta$  in DO11.10 T-cell proliferation in the presence of RPE SN. (A) Posterior eyecups were incubated with SFM for 1, 6, 12, 18, 24, and 48 hours. Mv1Lu cells were incubated with unmodified or acid-treated SNs of the RPE eyecups. A mink lung cell proliferation inhibition assay was performed by measuring incorporated [ $^3$ H]thymidine. The final dilution of SN in all wells was 1:40. (B) Experimental wells contained 24-hour untreated RPE SN, SN treated first with anti-pan-TGF- $\beta$  antibody, or SN treated first with both anti-pan-TGF- $\beta$  and sTGF- $\beta$ RII. Positive control wells contained LNCs and OVA, and negative control wells contained only LNCs. Thymidine uptake was measured at 96 hours. Data represent the mean  $\pm$  SEM of results in triplicate wells in a representative experiment. \*Significantly less than the positive control ( $P < 0.05$ ).

latent TGF- $\beta$ , which they then convert into the active form by a mechanism yet to be revealed.

To determine whether the active TGF- $\beta$  present in RPE eyecup SN was responsible for inhibiting T-cell activation in vitro, we next added a pan-anti-TGF- $\beta$  Ab reagent to 24-hour RPE eyecup SN before suspending DO11.10 lymph node cells in the medium in the presence of OVA. As a control, DO11.10 lymph node cells were incubated with OVA alone, with SFM alone, or with OVA and untreated SNs of RPE eyecups. Figure 2B demonstrates that neutralization of active TGF- $\beta$  by the antibody reversed the inhibitory effect of 24-hour RPE SN on DO11.10 T-cell proliferation, but only partially. In case the anti-TGF- $\beta$  Ab failed to neutralize all TGF- $\beta$ , we performed an additional experiment in which soluble TGF- $\beta$  receptor II (sTGF- $\beta$ RII) was also added to SN containing anti-TGF- $\beta$  antibodies. As revealed in Figure 2B, addition of sTGF- $\beta$  RII further neutralized the suppression inherent in the RPE SN, but still not completely. These results confirm that TGF- $\beta$  contributes to the ability of RPE eyecup SN to suppress T-cell activation.

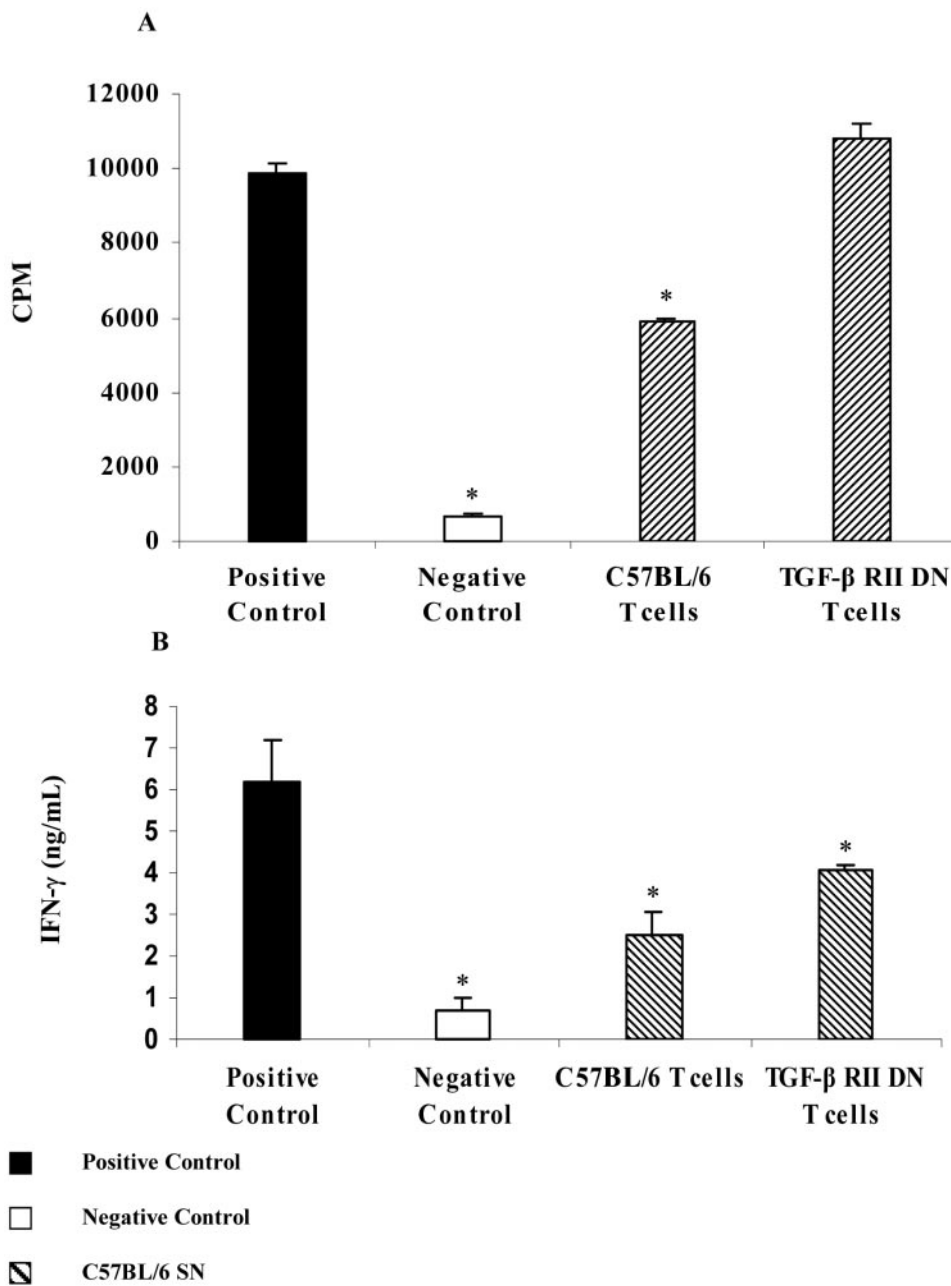
#### Effectiveness of SN of RPE Eyecup in Inhibiting Activation of T Cells from TGF- $\beta$ RII DN Mice

To confirm the role of the TGF- $\beta$  present in the SN of RPE in T-cell inhibition, we used T cells from TGF- $\beta$  RII DN mice (Fig. 3). These transgenic mice express a dominant negative TGF- $\beta$  type II receptor selectively on T cells, allowing direct study of

the role of TGF- $\beta$  in T-cell function.<sup>18</sup> T cells enriched from lymph nodes and spleens of naïve C57BL/6 or TGF- $\beta$  RII DN mice were cocultured with anti-CD3 Ab and 24-hour SN of eyecups from C57BL/6 eyes. T-cell proliferation and IFN- $\gamma$  production were measured, as described earlier. The positive control was cultures of T cells and anti-CD3 Ab without RPE eyecup SN, and the negative control was cultures containing only T cells. T cells from both wild-type and TGF- $\beta$  RII DN mounted comparable proliferative responses to anti-CD3 Ab (Fig. 3A), and produced similar levels of IFN- $\gamma$  (Fig. 3B). Whereas SN of RPE eyecups significantly inhibited both proliferation and IFN- $\gamma$  production by wild type C57BL/6 T cells, the SN failed to inhibit proliferation of TGF- $\beta$  RII DN T cells (Fig. 3A). The ability of RPE SN to inhibit IFN- $\gamma$  production was less affected. We conclude that the TGF- $\beta$  present in the SN is the major inhibitor of T-cell proliferation, but that factors in addition to TGF- $\beta$  suppress IFN- $\gamma$  production.

#### Correlation of the Capacity of RPE SN to Suppress T-Cell Activation with the Presence of Active TGF- $\beta$

To ascertain what role the activation of TGF- $\beta$  plays in T-cell inhibition by RPE SN, SNs were collected from the eyecups at 0, 1, 6, and 24 hours after incubation. These SN were diluted and placed in T-cell proliferation and IFN- $\gamma$  production assays as previously described. As shown in Figure 4A, the SN of RPE eyecups collected after 0 and 1 hour of incubation failed to



**FIGURE 3.** Effect of SNs of RPE eyecups from C57BL/6 and TGF- $\beta$  RII DN mice on anti-CD3 activation of T cells. T cells from either C57BL/6 or TGF- $\beta$  RII DN mice were incubated with 24-hour SN of the C57BL/6 RPE eyecup and anti-CD3 Ab. Positive control wells contained TGF- $\beta$  RII DN T cells and anti-CD3 Ab, and negative control wells contained only TGF- $\beta$  RII DN T cells. T-cell proliferation (A) and IFN- $\gamma$  production (B) were measured after 48 hours. Data represent the mean  $\pm$  SEM of results in triplicate wells in a representative experiment. \*Significantly less than the positive control ( $P < 0.05$ ).

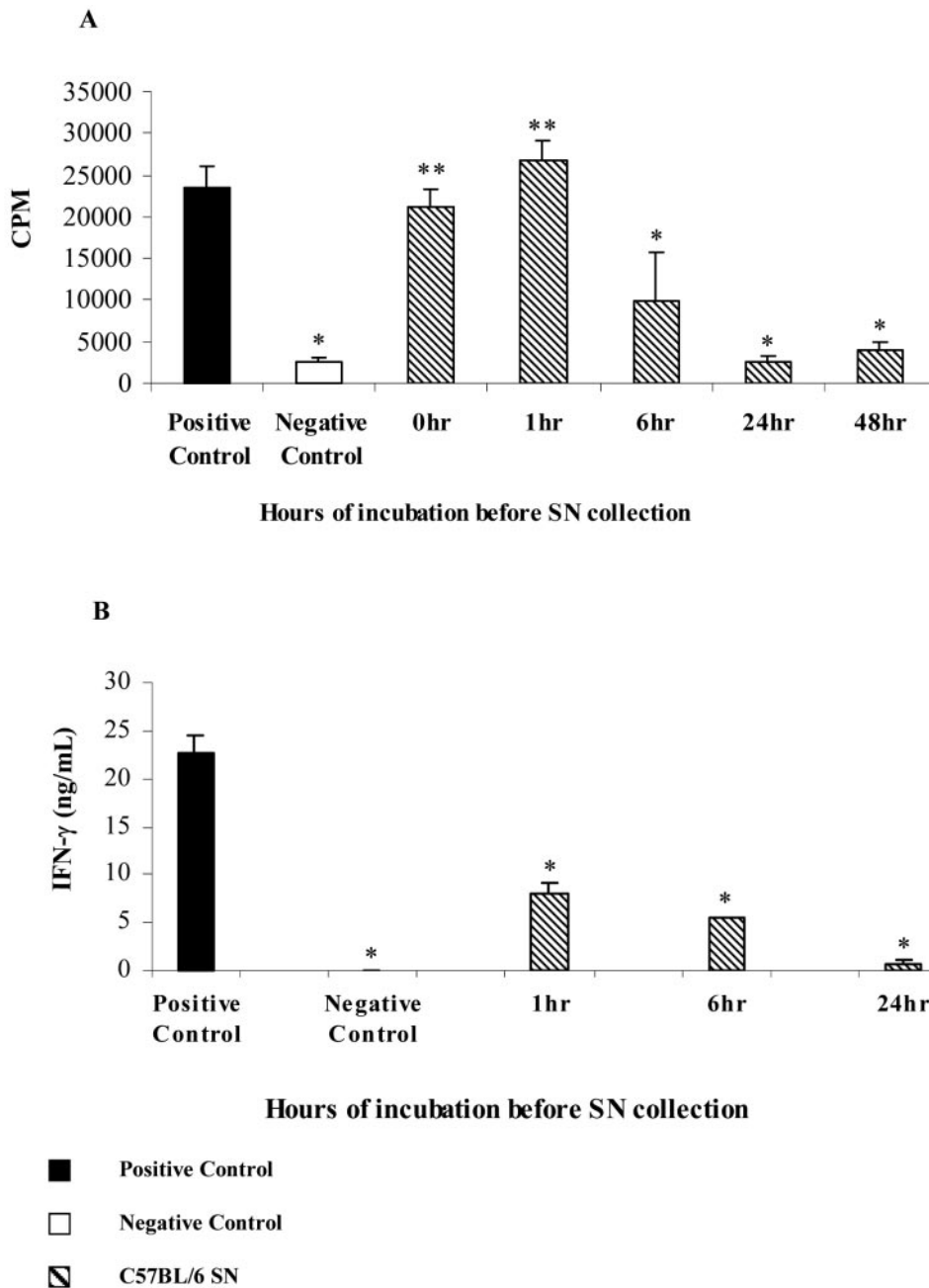
inhibit T-cell proliferation. Only the RPE SN collected after 6 hours and thereafter suppressed T-cell proliferation, and these time points correspond to the presence of active TGF- $\beta$ . IFN- $\gamma$  production was also inhibited by the RPE SN collected after 6 hours of incubation and beyond, but it was also reduced at 1 hour compared with the positive control (Fig. 4B). These results provide some evidence for the view that the activation of TGF- $\beta$  in RPE SN is necessary for T-cell proliferation to be suppressed. However, the results also imply that another immunosuppressive agent(s) is present to inhibit IFN- $\gamma$  production.

#### Effect of TSP-1 Produced by RPE on Latent TGF- $\beta$ in Eyecup SN

Our attention was drawn to TSP-1 as the activator of TGF- $\beta$  in RPE eyecup SN, because (1) TSP-1 is the major activator of

TGF- $\beta$ , both in vivo and in vitro<sup>19</sup>; (2) cultured RPE cells express mRNA for TSP<sup>20</sup>; (3) cultured human RPE cells secrete TSP-1<sup>21</sup>; and (4) TSP-1 has the ability to alter directly the functional properties of Th1 cells otherwise destined to become Th1-like.<sup>22</sup> To demonstrate that RPE eyecups contain mRNA for TSP-1 and secrete TSP-1 protein, RT-PCR and immunoblot analysis for TSP-1 was performed. The RPE eyecups of C57BL/6 mice contained easily detectable TSP mRNA transcripts and TSP-1 protein (Fig. 5).

To determine whether TSP-1 plays an important role in activating TGF- $\beta$  found in RPE SN, eyecups were prepared from eyes of TSP-1KO mice (TSP-1KO). The SN was collected from the eyecups after 24-hour incubation and assayed for the presence of active and total TGF- $\beta$ . The results presented in Figure 6 indicate that SN of RPE eyecups of TSP-1KO mice contained amounts of total TGF- $\beta$  comparable to that found in



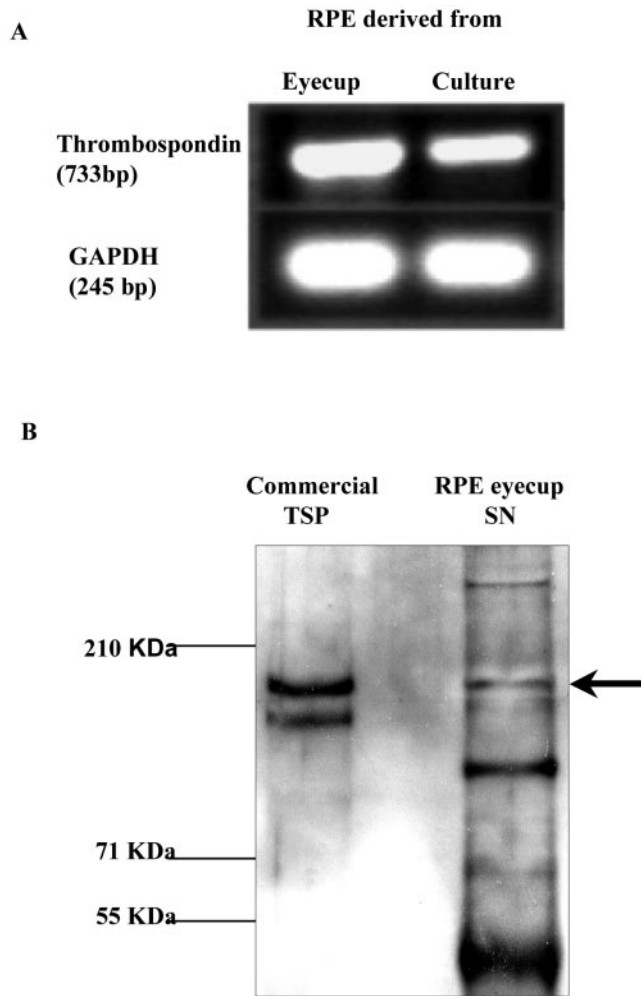
**FIGURE 4.** Correlation between the duration of RPE eyecup incubation and ability of SNs to suppress T-cell activation. RPE eyecups were incubated with serum-free medium for 1, 6, 24, and 48 hours. DO11.10 LNCs were incubated with OVA and different SN. Positive control cultures contained DO11.10 LNCs and OVA and negative control cultures contained only DO11.10 LNCs. T-cell proliferation (A) and IFN- $\gamma$  production (B) were measured at 96 hours. Data represent the mean  $\pm$  SEM of triplicate wells from a representative experiment. \* $P < 0.05$  significantly less than positive control and \*\* $P < 0.05$  significantly greater than 24-hour-SN effect.

SN of wild-type C57BL/6 RPE eyecups. However, virtually no active TGF- $\beta$  was found in TSP-1KO SN.

To reveal the functional importance of TSP-1-dependent TGF- $\beta$  activation in these studies, TSP-1KO RPE SNs were tested for their capacity to suppress the activation of DO11.10 T cells as well as anti-CD3 stimulated T cells in vitro, as described earlier. The RPE SN from wild-type C57BL/6 eyecups suppressed T-cell proliferation and IFN- $\gamma$  production (Fig. 7). The RPE SN from TSP-1KO eyecups did not inhibit anti-CD3 driven T-cell proliferation or IFN- $\gamma$  production and were significantly less effective, compared with the RPE SN from wild-type C57BL/6 eyecups, in suppressing proliferation and IFN- $\gamma$  production by DO11.10 T cells (Fig. 7). However, compared with the positive control, the SN from TSP-1KO eyecups still inhibited partially both proliferation and IFN- $\gamma$  production by DO11.10 T cells (Fig. 7).

#### Ability of Eyes from TSP-1KO Mice to Promote Immune Deviation

Immune deviation, an essential component of immune privilege, diminishes DH responses mediated by Th1 cells.<sup>1</sup> Because the RPE SN of TSP-1KO eyecups did not inhibit T-cell activation to the same degree as that of wild-type eyecups, we wanted to see whether the eyes of these mice would support the induction of immune deviation. Immune deviation was elicited according to the procedures described in the Methods section. Figure 8 demonstrates that the positive control TSP-1KO mice that received a subcutaneous injection of OVA and CFA mounted intense DH responses. In contrast to wild-type C57BL/6 mice, TSP-1KO mice that first received an inoculation of OVA in the subretinal space or the anterior chamber of the eye also displayed an intense DH responses. Our results demonstrate that lack of TSP-1 production by RPE in TSP-1KO mice



**FIGURE 5.** Expression of TSP-1 mRNA and presence of TSP-1 protein by RPE eyecups. **(A)** TSP-1 mRNA: total RNA extracted from freshly isolated or cultured RPE cells was subjected to RT-PCR analysis using primers for TSP-1 and GAPDH. PCR products were detected by ethidium bromide/agarose gel electrophoresis. **(B)** TSP-1 protein in 24-hour RPE SN was determined by immunoblot analysis. Total protein from SN samples and commercial TSP (positive control) was used for this analysis. TSP protein was detected with anti-TSP-1 Ab and chemiluminescence detection. *Arrow*: TSP-1 band in SN from the eyecup.

prevents their eyes from promoting immune deviation via the anterior chamber and the subretinal space.

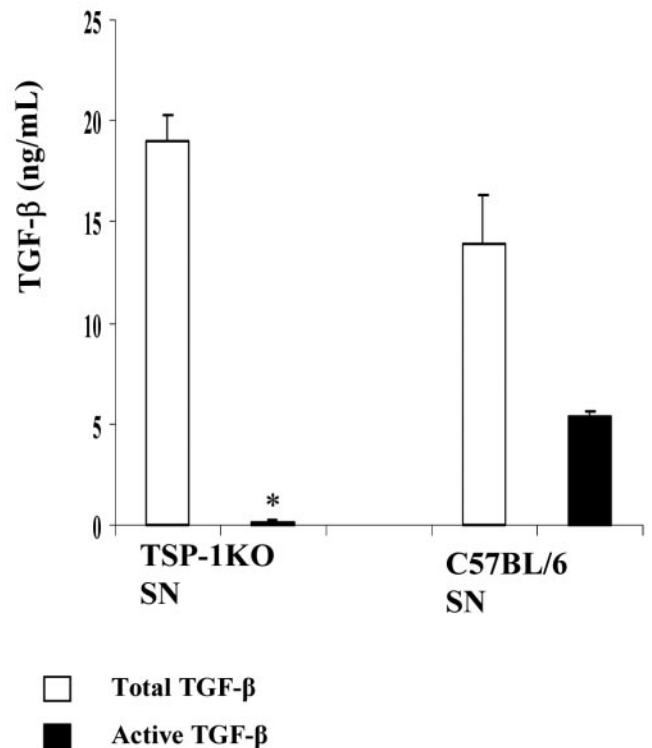
#### Development of Experimental Autoimmune Uveoretinitis in C57BL/6 and TSP-1KO Mice

We next explored the role of TSP-1 and active TGF- $\beta$  in the ability of C57BL/6 mice to withstand autoantigen-induced inflammation in the ocular posterior segment. Given that experimental autoimmune uveitis (EAU) is mediated by Th1 cells,<sup>23</sup> and because we found that the RPE from TSP-1KO mice did not inhibit IFN- $\gamma$  production by activated Th1 cells, we hypothesized that TSP-1KO mice might experience exaggerated EAU compared with control mice. Both wild-type C57BL/6 and TSP-1KO mice were immunized subcutaneously with IRBP peptide and CFA and intraperitoneally with PTX. Their retinas were examined clinically at regular intervals, and the degree of uveitis was measured and scored. Wild-type C57BL/6 mice displayed significant retinal inflammation with the average maximum score reaching  $2.5 \pm 0.6$  on day 18 after immuniza-

tion (Fig. 9, circles). One hundred twenty days after immunization, the disease abated and eyes reached the score of zero. In contrast, TSP-1KO mice had a significantly higher average score of  $3.5 \pm 0.5$  at the peak of EAU, and these mice continued to display severe inflammation throughout the examination period (Fig. 9, squares). Our results indicate that expression of TSP is essential in diminishing the severity and duration of uveitis in EAU.

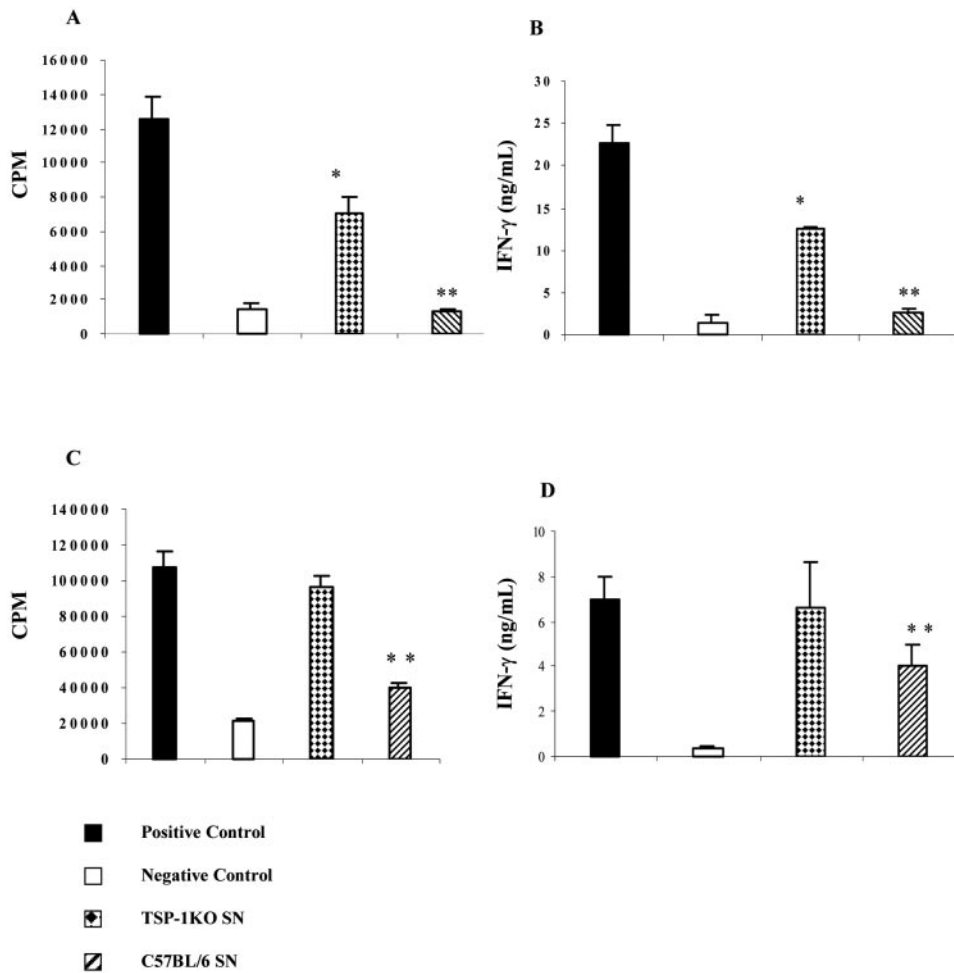
#### Retinal Recovery of C57BL/6 and TSP-1KO Mice from EAU

To examine the histologic status of the retina in eyes of C57BL/6 mice and TSP-1KO mice, immunized mice from both groups were euthanized after 120 days, and their eyes were prepared for histologic evaluation. Figure 10D demonstrates a retina taken from a naïve 6-week-old TSP-1KO mouse. All retinal layers are present in normal thickness and configurations with an intact RPE layer. The retinas of C57BL/6 mice that recovered clinically from EAU, retained normal retinal architecture, although minor irregularities were detected over a small section of the RPE and there was slight thinning of the outer and inner nuclear layers (Fig. 10A). In contrast, the retina of immunized TSP-1KO mice displayed severe abnormalities (Figs. 10B, 10C): The RPE layer was discontinuous, and in fact totally destroyed in places. There were few photoreceptor outer segments remaining, and the inner nuclear layer was reduced to half of its original thickness. There was a complete loss of the outer plexiform layer with disorganization of the inner plexiform and ganglion cell layers. Areas of hemorrhage

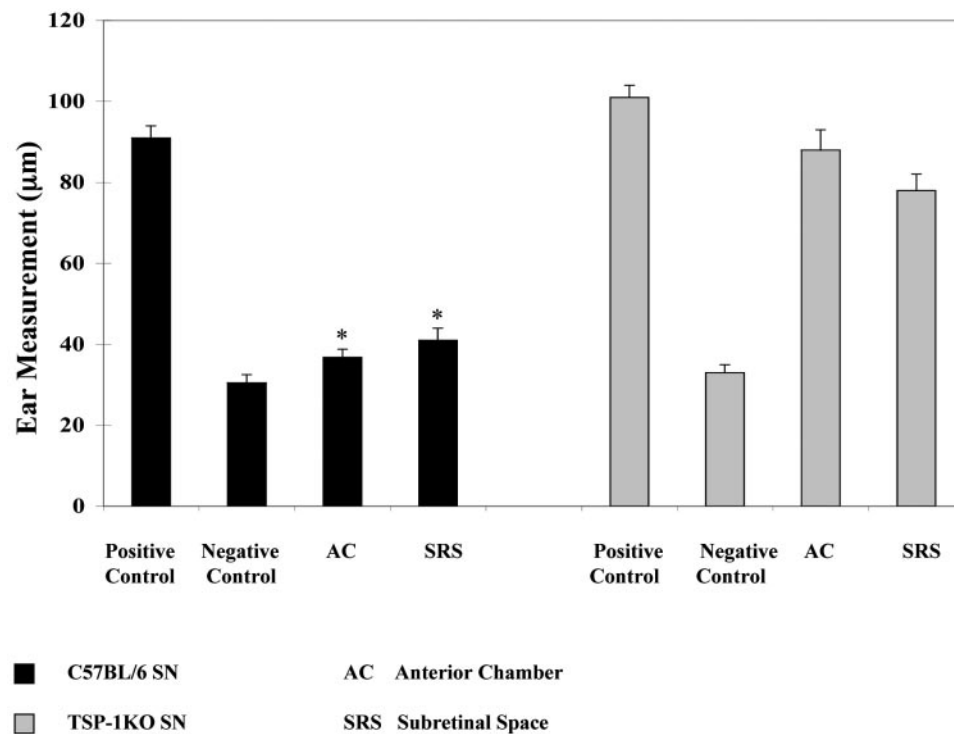


**FIGURE 6.** Total and active TGF- $\beta$  content of SN of eyecups prepared from C57BL/6 and TSP-1 KO mice. Mv1Lu cells were incubated with unmodified or acid treated 24-hour SN of eyecups prepared from either C57BL/6 or TSP-1KO mice. [<sup>3</sup>H]thymidine was added to each well, and 4 hours later the amount of incorporated label was measured. Data are expressed as the mean  $\pm$  SEM of results in triplicate wells in a representative experiment. \*Significantly less than the SN effect in C57BL/6 mice ( $P < 0.01$ ).

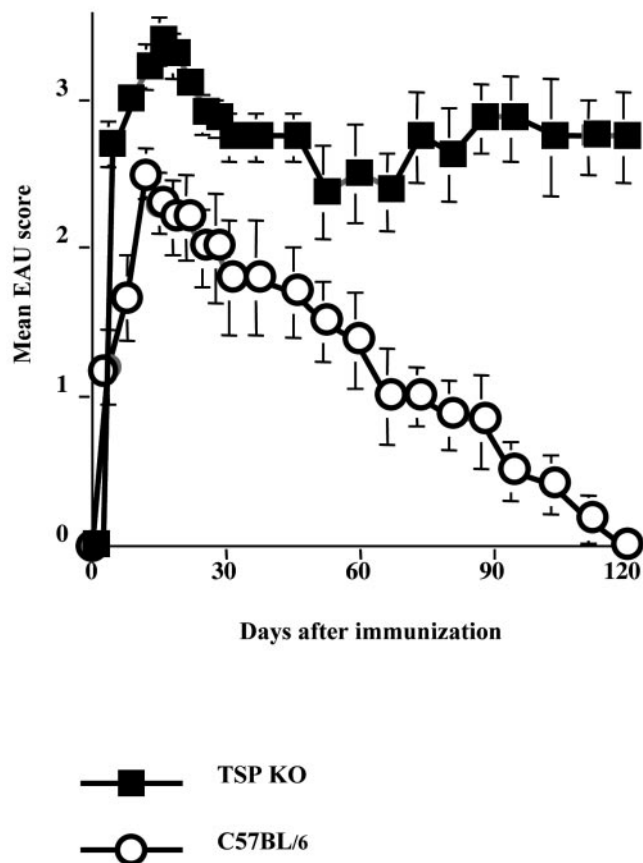




**FIGURE 7.** Effects of SNs of RPE eyecups from TSP-1KO and C57BL/6 mice on antigen and anti-CD3 stimulated T-cell proliferation. After 24 hours of incubation of RPE eyecups from TSP-1KO or C57BL/6 mice, SNs were collected. (A, B) DO11.10 LNCs were incubated with OVA and serially diluted SNs of RPE for 96 hours. Positive control cultures contained DO11.10 LNCs and OVA, and negative control cultures contained only DO11.10 LNCs. T-cell proliferation (A) and IFN-γ production (B) were measured at 96 hours. (C, D) Twenty-four-hour SNs from eyecups prepared from eyes of C57BL/6 mice were incubated with purified T cells and anti-CD3 Ab for 24 hours. T-cell proliferation (C) and IFN-γ production (D) were measured 48 hours later. Positive control cultures contained T cells with anti-CD3 Ab, and negative control wells contained only T cells. Data are expressed as the mean ± SEM of results in triplicate wells in a representative experiment. \**P* < 0.05, \*\* *P* < 0.01; significantly less than the positive control.



**FIGURE 8.** Capacity of eyes of TSP-1KO and C57BL/6 mice to support immune deviation to intracameral OVA antigen. All control and experimental animals underwent an ear challenge on day 14, and ear thickness was measured by micrometer 24 hours later. Experimental TSP-1KO and C57BL/6 mice received injections of OVA into the anterior chamber or the subretinal space on day 1, followed by subcutaneous immunization with OVA and CFA 7 days later. Positive control groups also received subcutaneous immunization. The data depicted are expressed as a percentage of the positive control percentage. \*Significant difference between ear swelling in either anterior chamber- or subretinal space-injected animals and the positive control (*P* < 0.05).



**FIGURE 9.** Expression of EAU in C57BL/6 and TSP-1KO mice. Groups of C57BL/6 and TSP-1KO mice were immunized subcutaneously with IRBP peptide and CFA and received simultaneous intraperitoneal injection of PTX. Retinas were examined at regular intervals throughout a 120-day period. The extent of uveitis is represented as the mean EAU score for both eyes on the day of examination.

were found within the outer retina. New vessels were seen to stretch between the ganglion cell layer and the inner nuclear layer (Fig. 10B) as well as between the choroid and the RPE layers (Fig. 10C).

Our results reveal that remarkable histologic recovery from inflammation occurred in the retinas of C57BL/6 mice that recovered clinically from EAU. Alternatively, mice deficient in TSP-1 displayed a devastating and prolonged inflammatory response that destroyed the organization of the retina and many cells in the various layers.

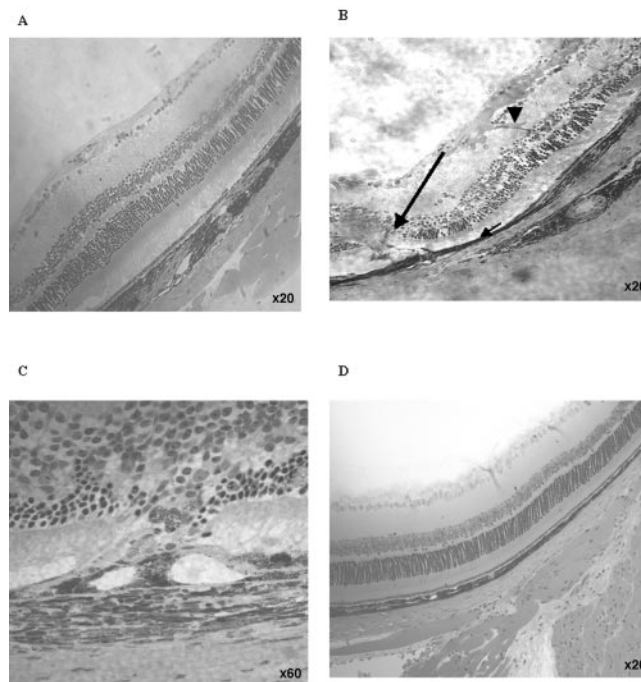
## DISCUSSION

Data recently published<sup>4</sup> and our results (Fig. 1) indicate that T cells that receive activation signals, either through APCs or through their T-cell receptors for antigen, fail to respond in the presence of soluble factors produced by the RPE—that is, these T cells fail to proliferate and secrete proinflammatory cytokines such as IFN- $\gamma$ . In the present experiments, active TGF- $\beta$  played an essential role in conferring immunosuppressive properties on RPE SNs. A sensitive bioassay showed latent TGF- $\beta$ , produced constitutively by the monolayer of RPE and present in the SN of RPE eyecups. However, latent TGF- $\beta$  in RPE SN did not inhibit T-cell proliferation; only on activation did TGF- $\beta$  suppress T-cell activation. We have also demonstrated that SN of RPE did not inhibit activation of purified T cells taken from

TGF- $\beta$  RII DN mice, illuminating the fundamental role that TGF- $\beta$  plays in T-cell inhibition.

As alluded to in the Results section, inhibition of TGF- $\beta$  by its neutralizing antibody or by TGF- $\beta$  receptor II did not fully reverse the effectiveness of the SN of RPE in inhibiting T-cell activation. In addition, inhibition of IFN- $\gamma$  production persisted when there was no active TGF- $\beta$  (Fig. 4B) and T cells from TGF- $\beta$  RII DN mice were used (Fig. 3B). We conclude that although active TGF- $\beta$  has an essential role in inhibition of T-cell activation, it is not the sole factor responsible. A search for other factors that may play a part in conferring immune-privilege properties on RPE SN is under way.

Other laboratories have shown that RPE cells inhibit T cells.<sup>24,25</sup> Liversidge et al.<sup>24</sup> have reported that RPE cells use both soluble and membrane-bound mechanisms to suppress lymphocyte proliferation stimulated by antigen, mitogen, and IL-2. They did not find TGF- $\beta$  in the SN of cocultured T cells and RPE by Western blot, leading them to conclude that TGF- $\beta$  did not play a role in suppression of T-cell activation. Rezaei et al.<sup>25</sup> subsequently corroborated the ability of human fetal RPE (HFRPE) cells to suppress, through apoptosis, T-cell activation across a migration assay membrane (Transwell; Corning-Costar). Their search for the factor secreted by HFRPE cells excluded TGF- $\beta$ , TNF- $\alpha$ , and IL-10, since these factors were not found by ELISA, and their addition to T-cell cultures did not lead to T-cell apoptosis.<sup>26</sup> It is possible that in these studies,



**FIGURE 10.** Histology of eyes from C57BL/6 and TSP-1KO mice 120 days after induction of EAU. (A) Retina of C57BL/6 mouse at day 120 after induction of EAU. Punctate neuron loss was noted in both the inner nuclear and photoreceptor cell layers. The normal architecture of the retina was preserved. (B) Retina from TSP-1KO mouse 120 days after IRBP immunization, at which time the average clinical uveitis score was 3. The RPE layer appeared discontinuous and was totally destroyed in places (*short arrow*). Photoreceptor outer segments were barely visible, and there was complete loss of the outer plexiform layer and profound disorganization of the inner plexiform and ganglion cell layers. An area of hemorrhage (*long arrow*) and a new vessel (*arrow-head*) stretched between the ganglion cell and inner nuclear layers. (C) A higher magnification of TSP-1KO retina 120 days after immunization. A new vessel arose from the choroidal circulation incorporating the RPE. (D) Retina of a naive 6-week-old TSP-1KO mouse.

TGF- $\beta$  was present but was latent or that an intact monolayer of RPE is essential for RPE production of appreciable levels of active TGF- $\beta$ .

We have also demonstrated that eyecups from TSP-1KO mice were ineffective in inhibiting T-cell activation. RPE SN from TSP-1KO animals contained levels of latent TGF- $\beta$  comparable to that of wild-type C57BL/6 eyecups, but lacked active TGF- $\beta$ . The mechanisms of TGF- $\beta$  activation in the eye are not fully understood. Activation of TGF- $\beta$  in vitro is achieved by treating the latent TGF- $\beta$  with extremes of heat and pH, whereas, in vivo the activation of TGF- $\beta$  is largely enzymatic through proteolytic activities of plasmin, cathepsin, and others.<sup>26</sup> TSP can activate TGF- $\beta$  by binding the latent TGF- $\beta$  complex,<sup>27</sup> and the effectiveness of TSP in activating TGF- $\beta$  is usually enhanced in the presence of proteolytic enzymes. Although RPE produce plasminogen activators<sup>28</sup> and display receptors for urokinase,<sup>29</sup> there was no indication that RPE from TSP-1KO mice mediates TGF- $\beta$  activation through enzymatic mechanisms alone. To our knowledge, this is the first report to show that RPE activates TGF- $\beta$  and to demonstrate that the mechanism of this activation is solely through TSP-1. SNs of eyecups from TSP-1KO mice failed entirely to inhibit T-cell activation if the purified T-cell populations were stimulated through anti-CD3, but when lymph node cells were stimulated with OVA, SN of RPE from TSP-1KO mice inhibited T-cell activation partially. The reason for this partial response could be that lymph node mixtures contain other factors, such as proteases, that can activate TGF- $\beta$  despite the absence of TSP-1, leading to inhibition of T-cell activation. We conclude that it is essential that TSP-1 be expressed by RPE to create and maintain the immunosuppressive microenvironment of the subretinal space, probably through the ability of TSP to activate latent TGF- $\beta$ .

Both the anterior chamber and the subretinal space of eyes of TSP-1KO mice, in contrast to the wild-type mice, failed to support induction of OVA-specific immune deviation. This result demonstrates that the presence of TSP-1 is essential in the ability of the eye to divert the immune response away from a proinflammatory Th1 course. To our knowledge, this is the first report to demonstrate an essential role for TSP-1 in promoting ocular immune deviation.

Moreover, TSP-1KO mice that were immunized with IRBP to induce EAU experienced significantly enhanced uveitis that failed to resolve. Our findings in TSP-1KO mice that were immunized with IRBP reveal that the absence of TSP, and therefore of a potential mechanism of TGF- $\beta$  activation, resulted in destructive and sustained autoimmune inflammation of the uveal tract and retina. Our laboratory has shown that preemptive induction of IRBP-specific ACAID can prevent the onset of EAU, and that induction of ACAID once EAU is established abruptly terminates the disease.<sup>30</sup> Others have shown that regulatory mechanisms come into play as EAU wanes after its initial clinical expression.<sup>11</sup> Because we also demonstrated that the subretinal space of TSP-1KO mice does not support immune deviation, we infer that induction of immune deviation is responsible, at least in part, for the suppression of autoimmunity that limits both the extent and the duration of EAU in normal mice.

Unlike the retina of normal C57BL/6 mice, the retina of TSP-1KO mice subjected to EAU revealed new vessel formation from both the choroidal and retinal circulations. Under normal circumstances, spontaneous neovascularization was not noted in the retinas of TSP-1KO mice, but once their retinas received an insult, such as EAU, there were multiple new vessels in evidence. TSP-1 has been shown to inhibit angiogenesis in vivo and in vitro assays,<sup>31</sup> and overexpression of TSP-1 in tumor cells leads to inhibition of angiogenesis.<sup>32</sup> The antiangiogenic activity of TSP has been attributed by various investigators to

its ability to activate latent TGF- $\beta$ ,<sup>33</sup> to bind to CD36,<sup>34</sup> or to interact with heparan sulfate proteoglycans.<sup>35</sup> The presence of new vessels in the retina of TSP-1KO mice that had uveitis points to an important role that TSP-1 plays as an antiangiogenic factor in the retina.

We have demonstrated that TSP-1, possibly through activation of TGF- $\beta$ , provides a molecular mechanism by which the immune privilege of the posterior segment of the eye can be maintained. We have also shown a direct link between the recovery of the retina from a severe inflammatory insult and the presence or resurgence of immune privilege in the posterior segment of the eye. Therapeutic attempts to restore immune privilege may aid recovery from autoimmune diseases and limit inflammation-induced damage.

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