

ONLINE FIRST

Lack of Thrombospondin 1 and Exacerbation of Choroidal Neovascularization

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Objectives: To assess the impact of thrombospondin 1 (TSP1) deficiency on choroidal neovascularization (CNV) and to determine whether administration of a TSP1 antiangiogenic mimetic peptide attenuates CNV.

Methods: The impact of TSP1 deficiency on laser-induced CNV was assessed using wild-type (TSP1+/+) and TSP1-deficient (TSP1-/-) mice. Three laser burns were placed in each eye of TSP1+/+ and TSP1-/- mice to induce CNV. Intravitreal injection of the TSP1 mimetic peptide was performed on days 1 and 7 postlaser in the mice. For quantitative measurements of neovascularization, intercellular adhesion molecule 2 staining was performed at 14 days postlaser of the choroidal-sclera flat mounts. The recruitment of macrophages to the sites of damage was investigated by immunohistochemistry. The CNV area was measured by intercellular adhesion molecule 2 staining and use of ImageJ software.

Results: The TSP1-/- mice exhibited significantly larger areas of neovascularization on choroidal flat mounts com-

pared with TSP1+/+ mice. This was consistent with enhanced recruitment of macrophages in TSP1-/- mice compared with TSP1+/+ mice 3 days postlaser. The development of CNV was significantly attenuated in mice receiving the TSP1 antiangiogenic mimetic peptide compared with those receiving vehicle alone.

Conclusions: Deficiency of TSP1 contributes to enhanced choroidal neovascularization. This is consistent with the anti-inflammatory and antiangiogenic activity of TSP1. The TSP1 antiangiogenic peptide was effective in attenuation of CNV.

Clinical Relevance: Intravitreal injection of TSP1 antiangiogenic mimetic peptides may provide alternative treatment for CNV.

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AGE-RELATED MACULAR DEGENERATION (AMD) affects millions of individuals worldwide, with approximately 90% of severe vision loss attributed to choroidal neovascularization (CNV).^{1,2} The global prevalence of CNV is expected to double in the next decade because of the aging population. Age-related macular degeneration is characterized by a progressive degeneration of the macula, usually bilateral, leading to a central scotoma and severe decrease in vision. The visual deficit results initially from retinal degeneration called geographic atrophy (dry or nonexudative AMD) often complicated by the secondary effects of CNV (wet or exudative AMD). An early sign of AMD is the appearance of drusen, which are extracellular deposits that accumulate below the retinal pigment epithelium and are known to be a risk factor for developing CNV.²

Angiogenesis, the formation of new capillaries from preexisting capillaries, is associated with the pathogenesis of exudative AMD.^{3,4} Therefore, inhibition of angiogenesis has become a viable strategy to inhibit CNV. Targeting of the proangiogenic factor vascular endothelial growth factor A has been validated in patients with CNV.^{5,6} However, significant improvement in vision is only observed in 30% of patients treated with a vascular endothelial growth factor A antagonist, with 20% of treated patients still progressing to legal blindness. Furthermore, safety concerns about the conditional blockade of vascular endothelial growth factor A, which is constitutively expressed in the normal human retina^{7,8} and glomeruli,^{9,10} are emerging. Thus, additional treatment strategies on the basis of more specific targeting of CNV are desirable.

It is now well accepted that endogenous inhibitors of angiogenesis play a sig-

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nificant role in regulation of angiogenesis and their decreased production may contribute to pathological neovascularization. Thrombospondin 1 (TSP1), a member of the *TSP* gene family, was the first endogenous inhibitor of angiogenesis identified whose expression was decreased during malignant transformation.¹¹ We have shown that TSP1 is an important modulator of retinal vascular homeostasis and its increased production results in attenuation of retinal vascular development and neovascularization.^{12,13} We have also shown that TSP1 is present at very high levels in vitreous samples from various species and its level decreased during diabetes mellitus, perhaps contributing to the pathogenesis of diabetic retinopathy.^{14,15} However, TSP1 expression changes and its contribution to the pathogenesis of AMD need further investigation.

Thrombospondin 1 is synthesized and secreted by retinal pigment epithelial cells and its expression is upregulated by vitamin A.^{16,17} Recent studies also suggest an important role for TSP1 in choroidal vascular homeostasis and the pathogenesis of CNV.¹⁸ Immunohistochemical analysis of sections prepared from human eyes indicated that TSP1 is present in the macula region of the Bruch membrane, choroidal capillaries, and larger choroidal vessels. In addition, a significant decrease in the level of TSP1 was detected in the Bruch membrane and choroidal capillaries from AMD eyes.¹⁹ We, therefore, hypothesized that TSP1 deficiency will exacerbate the pathogenesis of CNV and it may be an important target for inhibition of CNV. Herein, we investigated the impact of TSP1 deficiency on laser-induced CNV using TSP1^{-/-} mice. We also determined the potential therapeutic role of TSP1 in treatment of CNV using a TSP1 antiangiogenic mimetic peptide.

METHODS

ANIMALS

All animal studies were conducted in accordance with an animal protocol reviewed and approved by the University of Wisconsin–Madison Animal Care and Use Committee and in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Six-week-old female wild-type (TSP1^{+/+}) or TSP1-deficient (TSP1^{-/-}) C57BL/6j mice (10 per group) were used in these studies and housed on a 12-hour light-dark cycle, with food and water provided ad libitum.

LASER-INDUCED CNV AND ITS QUANTIFICATION

Various animal models have been instrumental in advancing our understanding of the pathogenesis of AMD and the development and testing of effective treatments.²⁰ An early and important model was the laser-induced CNV model developed by Ryan²¹ in 1979, and it remains one of the commonly used models for wet AMD research. The CNV was induced in mice by laser photocoagulation-induced rupture of the Bruch membrane on day 0 as previously described.^{22,23} Mice were anesthetized with ketamine hydrochloride (100 mg/kg) and xylosine, and the pupils were dilated using a drop of tropicamide, 1%.

Laser photocoagulation (75- μ m spot size; 0.1-second duration; 120 mW) was performed in the 9-, 12-, and 3-o'clock positions of the posterior pole of each eye with the slitlamp delivery system of an OcuLight GL diode laser (Iridex) and a handheld coverslip as a contact lens to view the retina. After 14 days, the eyes were removed and fixed in paraformaldehyde, 4%, at 4°C for 2 hours. Following 3 washes in phosphate-buffered saline (PBS), the eyes were sectioned at the equator, and the anterior half, vitreous, and retina were removed. The remaining eye tissue was incubated in blocking buffer (20% fetal calf serum and 20% normal goat serum in PBS) for 1 hour at room temperature, followed by incubation with anti-intercellular adhesion molecule 2 (1:500 in PBS containing 20% fetal calf serum and 20% normal goat serum; BD Pharmagen) overnight at 4°C. The remaining eye tissue was then washed 3 times with PBS and incubated with the appropriate secondary antibody. The retinal pigment epithelium–choroid–sclera complex was dissected through 5 to 6 relaxing radial incisions and flat mounted on a slide with VectaMount AQ (Vector Laboratories). The samples were viewed by fluorescence microscopy and images were captured in digital format using a Zeiss microscope (Zeiss). ImageJ software (National Institute of Mental Health; <http://rsb.info.nih.gov/ij/>) was used to measure the total area (in micrometers squared) of CNV associated with each burn.

IMMUNOHISTOCHEMISTRY FOR DETECTION OF MACROPHAGES

Eyes were enucleated 3 days after laser injury, when the maximum number of macrophages were observed,²⁴ and fixed in paraformaldehyde, 4%, for 2 hours, and retinal pigment epithelium–choroid–sclera complexes were dissected. Flat mounts were treated with blocking solution (20% fetal calf serum and 20% normal goat serum in PBS) for 1 hour at room temperature, followed by incubation with anti-F4/80 (1:500 in PBS containing 20% fetal calf serum and 20% normal goat serum; BD Pharmagen) overnight at 4°C. The remaining eye tissue was then washed 3 times with PBS and incubated with the appropriate secondary antibody. The tissue was viewed by fluorescence microscopy and images were captured in digital format using a Zeiss microscope. For quantitative analysis of macrophage recruitment, the area of fluorescence and average pixel intensities (mean gray value) were determined using Photoshop software (Adobe). The integrated density was calculated by multiplying the area of fluorescence by the mean gray value.

TSP1 ANTIANGIOGENIC MIMETIC PEPTIDE TREATMENT

The antiangiogenic activity of TSP1 is mapped to peptides from type 1 repeats and the procollagen homology domain.²⁵ An overlapping peptide that expands these regions has shown good efficacy for inhibition of angiogenesis in various tumor models²⁶ and was the basis for development of ABT510 and most recently a newer generation,²⁷ ABT898. The amino acid sequence of ABT898 is *N*-acetyl-glycine-valine-D-alloisoleucine-serine-glutamine-isoleucine-arginine-proline-ethylamid and was synthesized at the University of Wisconsin Biotechnology Peptide Synthesis core facility. The purity and sequence of the peptide were confirmed using standard methods. The peptide was dissolved in dextran solution, 5%, and used for intravitreal injections.

The TSP1^{+/+} mice and TSP1^{-/-} mice were injected intravitreally with the TSP1 antiangiogenic mimetic peptide (2 μ L of 100 μ g/mL) at day 0, right after laser rupture of the Bruch membrane. This dose was found to be most effective. Intravitreal injections were performed with a pump microinjection

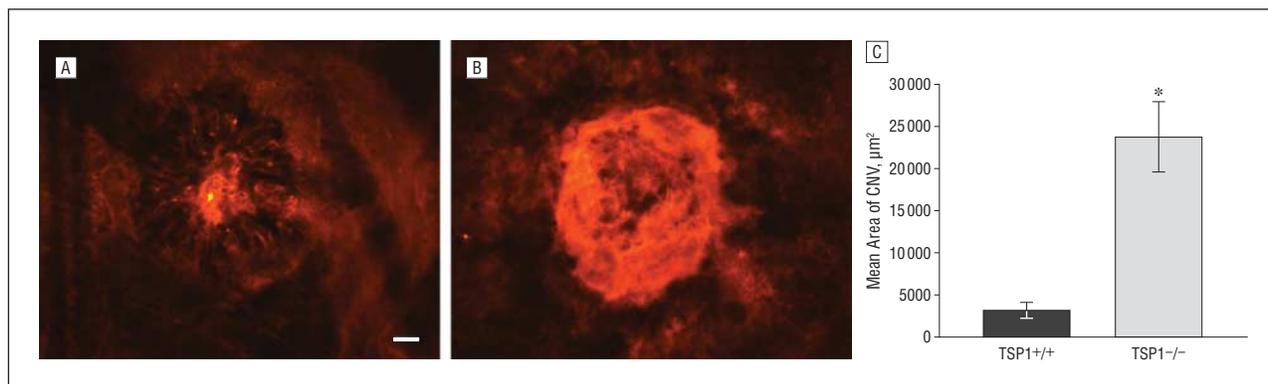


Figure 1. Increased choroidal neovascularization (CNV) lesion size in thrombospondin 1 (TSP1) $-/-$ mice following laser photocoagulation. A and B, Immunohistochemical visualization of the laser scars on retinal pigment epithelium-choroidal intercellular adhesion molecule 2-stained flat mounts 2 weeks after photocoagulation of TSP1 $+/+$ (A) and TSP1 $-/-$ (B) mice. Bar = 50 μm . C, Quantification of the data. A significant increase in the area of CNV was observed in TSP1 $-/-$ mice compared with TSP1 $+/+$ mice ($*P < .05$; $n = 15$ eyes).

apparatus (Harvard Apparatus) and pulled glass micropipettes. Each micropipette was calibrated to deliver 2 μL of vehicle containing the peptide on depression of a foot switch. The mice were anesthetized and the pupils were dilated. Under a dissecting microscope, the sharpened tip of the micropipette was passed through the sclera, just behind the limbus into the vitreous cavity, and the foot switch was depressed. The injections were repeated 7 days after Bruch membrane rupture to replenish the peptide concentration in the vitreous cavity. After 14 days, the samples were prepared for quantification of the area of neovascularization as described earlier.

STATISTICAL ANALYSIS

Statistical differences between groups were evaluated with the unpaired t test (2-tailed). Means and standard deviations are shown. $P \leq .05$ is considered significant.

RESULTS

TSP1 DEFICIENCY RESULTS IN INCREASED AREAS OF CNV

Thrombospondin 1 is a potent inhibitor of angiogenesis and can directly act on endothelial cells, inhibiting their proliferation and migration and promoting their apoptosis. The impact of TSP1 on choroidal endothelial cells, and more specifically on CNV, remains elusive. To determine whether lack of TSP1 expression would impact the degree of neovascularization on laser-induced CNV, 6-week-old C57BL/6 TSP1 $+/+$ and TSP1 $-/-$ female mice underwent laser-mediated photocoagulation and CNV. The areas of neovascularization were assessed as described in the "Methods" section. Images obtained with rat anti-intercellular adhesion molecule 2 staining of the choroidal flat mounts showed the typical morphology of blood vessels in the CNV lesions 2 weeks after laser photocoagulation. The TSP1 $-/-$ mice showed significantly larger neovascular choroidal outgrowths (**Figure 1A** and **B**) compared with TSP1 $+/+$ mice. The average CNV area per eye was significantly increased in TSP1 $-/-$ mice (mean [SD], 23750 [4200] μm^2 ; $n = 15$ eyes) compared with TSP1 $+/+$ mice, approximately 8-fold (mean [SD], 3235 [910] μm^2 ; $n = 15$ eyes) (Figure 1C) ($P < .05$). Thus, enhanced CNV is observed in the absence of TSP1.

ENHANCED INFILTRATION OF MACROPHAGES IN MICE LACKING TSP1 DURING CNV

Recent studies have established an important role for inflammatory processes, particularly recruitment of macrophages, in the pathogenesis of CNV. Macrophages participate in all stages of CNV and modulate the severity of CNV. Thrombospondin 1 is an important modulator of ocular immune privilege, and in its absence, enhanced inflammation has been reported.²⁸ We next analyzed the degree of macrophage infiltration in TSP1 $+/+$ and TSP1 $-/-$ mice subjected to laser-induced CNV by staining choroidal flat mounts with the antibody to macrophage-specific antigen F4/80. We observed a significant increase in the density of F4/80-positive macrophages in TSP1 $-/-$ mice following laser treatment compared with TSP1 $+/+$ mice (**Figure 2**).

ATTENUATION OF CNV BY ADMINISTRATION OF THE TSP1 ANTIANGIOGENIC MIMETIC PEPTIDE

Our results in Figure 1 and Figure 2 coupled with studies demonstrating decreased levels of TSP1 in choroidal membranes prepared from eyes with CNV^{18,19} suggest that TSP1 may suppress CNV. We next determined whether administration of the TSP1 mimetic antiangiogenic peptide can inhibit CNV and/or reduce the area of neovascularization in TSP1 $-/-$ mice. Peptides derived from TSP1 type 1 repeats and the procollagen homology domain, including the peptide used herein, can signal through CD36 (TSP1 receptor) inhibiting angiogenesis.^{29,30} Recent studies have demonstrated that this may also require interaction of TSP1 with CD47 (receptor for C-terminal domain of TSP1).³¹ The TSP1 antiangiogenic mimetic peptide was administered intravitreally to TSP1 $-/-$ mice subjected to the laser-induced CNV. We observed a significant decrease in the mean (SD) area of CNV in TSP1 $-/-$ mice that received the TSP1 mimetic peptide compared with vehicle control (TSP1 $-/-$ mice with peptide: 2254 [1040] μm^2 vs TSP1 $-/-$ mice with vehicle: 11340 [3315] μm^2 ; $n = 15$) (**Figure 3A** and **B**). The mean CNV area in eyes treated with the TSP1 peptide was decreased by approximately 80% compared

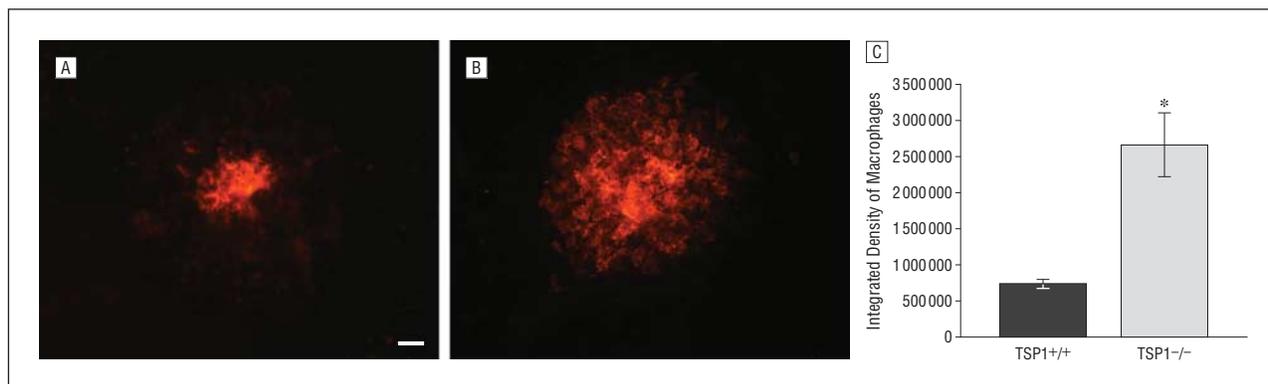


Figure 2. Enhanced infiltration of macrophages in thrombospondin 1 (TSP1) $-/-$ mice after laser injury. A and B, Representative immunohistochemical images of lesions in TSP1 $+/+$ (A) and TSP1 $-/-$ (B) mice following F4/80 staining. Bar=50 μm . C, The density of infiltrating macrophages 3 days after laser application was markedly increased in TSP1 $-/-$ mice compared with TSP1 $+/+$ mice ($*P < .05$; $n = 15$ eyes). The integrated density was calculated by multiplying the area of fluorescence by the mean gray value.

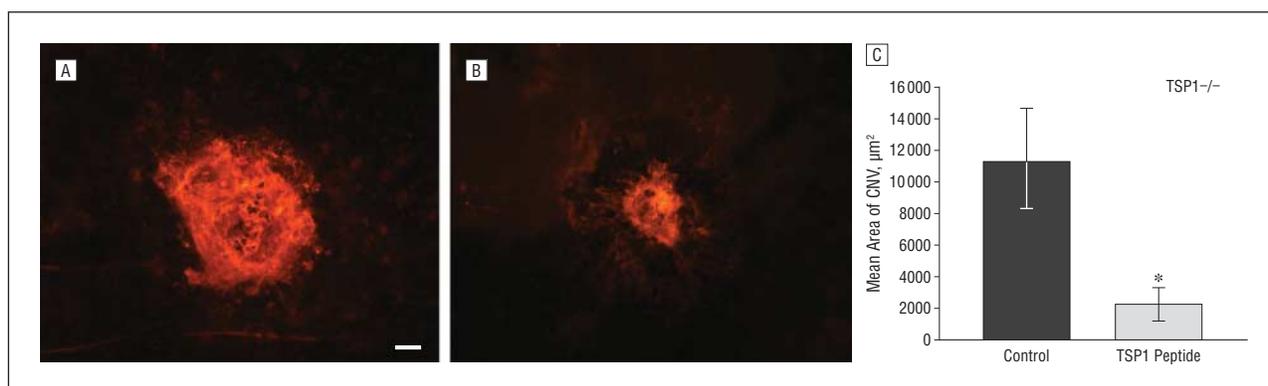


Figure 3. Attenuation of choroidal neovascularization (CNV) following intravitreal administration of the thrombospondin 1 (TSP1) antiangiogenic mimetic peptide in TSP1 $-/-$ mice exposed to laser-induced photocoagulation. Representative choroidal flat mounts after staining with intercellular adhesion molecule 2 two weeks after laser photocoagulation are shown. A and B, The TSP1 $-/-$ mice were subjected to laser photocoagulation and received vehicle (A) or the TSP1 peptide (B) on days 1 and 7 postlaser by intravitreal injection (2 μL of 100 $\mu\text{g}/\text{mL}$ in dextran solution, 5%). Bar=50 μm . C, A significant decrease in the area of CNV was observed in mice that received the TSP1 peptide compared with those that received vehicle ($*P < .05$; $n = 15$ eyes).

with that seen in eyes receiving vehicle alone (Figure 3C) ($P < .05$). Administration of the TSP1 peptide in TSP1 $+/+$ mice also significantly inhibited CNV lesion formation (mean [SD], TSP1 $+/+$ mice with peptide: 1840 [379] μm^2 vs TSP1 $+/+$ mice with vehicle: 3235 [910] μm^2 ; $n = 15$) (**Figure 4**) ($P < .05$). Thus, the TSP1 antiangiogenic mimetic peptide may provide an alternative treatment for CNV.

COMMENT

The studies presented herein demonstrate that lack of TSP1 results in enhanced CNV, and this was associated with a significant increase in the number of macrophages recruited to the sites of laser lesions. These observations are consistent with the important role of inflammation processes in the pathogenesis of CNV and the ocular anti-inflammatory role previously demonstrated for TSP1. In addition, we showed that administration of the TSP1 antiangiogenic mimetic peptide inhibited CNV in both TSP1 $+/+$ and TSP1 $-/-$ mice. Thus, modulation of TSP1 expression or its antiangiogenic mimetic peptides may provide a novel approach for the treatment of CNV associated with AMD.

Histological studies have demonstrated the immediate arrival of macrophages at laser rupture sites within 1 hour of laser application, and macrophages accumulate in areas of disruption of the Bruch membrane.^{24,32-34} Laser-induced CNV is an appropriate model for investigating the relationship between inflammation and angiogenesis. Macrophages secrete interleukin 1 β , among other inflammatory cytokines, in response to tissue injury and inflammation and promote angiogenesis.^{33,34}

There has been considerable discussion of the role of inflammation in promoting ocular angiogenesis, particularly in neovascular AMD. Inflammation is critically involved in the formation of CNV lesions and contributes to the pathogenesis of AMD. Inflammatory cells are found in surgically excised CNV lesions from patients with AMD and in autopsied eyes with CNV. In particular, macrophages have been implicated in the pathogenesis of AMD because of their spatiotemporal distribution in the proximity of the CNV lesions in experimental models and humans, making significant contribution to the pathogenesis of CNV.^{24,32}

The majority of the macrophages found in the proximity of the laser-induced CNV lesions are derived from newly recruited peripheral blood monocytes and are not

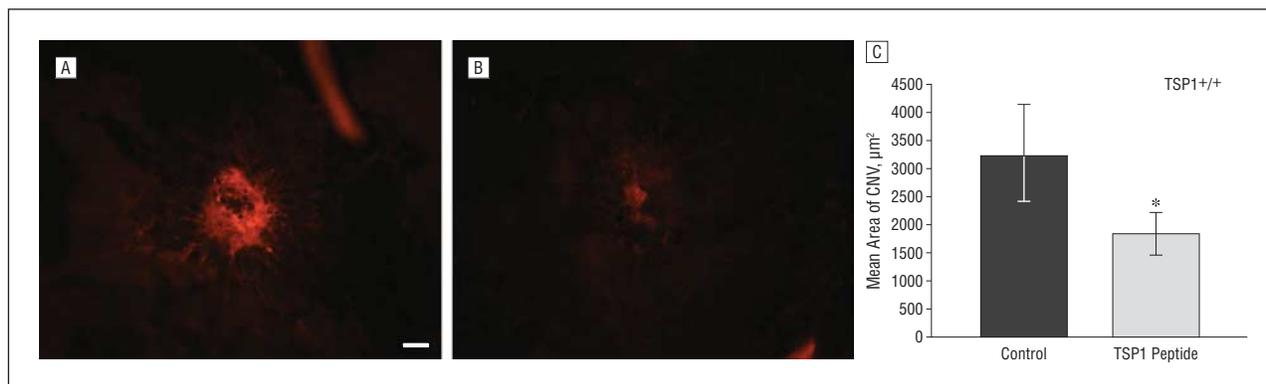


Figure 4. Attenuation of choroidal neovascularization (CNV) following intravitreal administration of the thrombospondin 1 (TSP1) antiangiogenic mimetic peptide in TSP1+/+ mice exposed to laser-induced photocoagulation. Representative choroidal flat mounts after staining with intercellular adhesion molecule 2 two weeks after laser photocoagulation are shown. A and B, The TSP1+/+ mice were subjected to laser photocoagulation and received vehicle (A) or the TSP1 peptide (B) on days 1 and 7 postlaser by intravitreal injection (2 µL of 100 µg/mL in dextran solution, 5%). Bar=50 µm. C, A significant decrease in the area of CNV was observed in mice that received the TSP1 peptide compared with those that received vehicle (* $P < .05$; n = 15 eyes).

resident macrophages.^{34,35} Because macrophages play such a critical role in CNV formation, prevention of monocyte recruitment and infiltration into ocular tissues may ameliorate the development of CNV, a function that could be exploited therapeutically.^{24,32,36,37} Herein we showed that lack of TSP1 exaggerates CNV and this was associated with increased recruitment of macrophages into the sites of lesions. These results are consistent with previous reports of impaired TSP1 expression in the Bruch membrane and choroidal vessels of eyes with AMD^{18,38} and identification of TSP1 as a genetic loci that controls the size of CNV.³⁹

The anti-inflammatory nature of the intraocular environment is critical to the immune privilege of the eye and pathogenesis of CNV. Investigation into the role of inflammation in neovascular eye disease often overlooks the principles of immune privilege in the eye. The ocular environment is generally not proinflammatory and it prohibits inflammation at the expense of certain immune effector mechanisms. It has been suggested that the loss of immune privilege as the eye ages may contribute to the increases in neovascular disease.^{40,41} These concepts challenge the idea that neovascular disease is simply an inflammatory process and support the idea that these diseases may result from the loss or dysfunction of important components of the cellular immune system.²⁴

Inflammatory mechanisms and immune activation have been implicated in the pathogenesis of CNV. Retinal laser burns disrupt the immune privilege in the eye resulting in inflammation.^{42,43} In addition, TSP1 plays a vital role in the modulation of immune privilege such that in its absence the retinal microenvironment is more proinflammatory and supports an activated state of microglia with poor recovery from injury.⁴² This is consistent with the increased recruitment of macrophages and enhanced severity of neovascularization observed herein in TSP1-/- mice.

The anti-inflammatory activity of TSP1 in the eye is mainly attributed to the enhanced levels of active transforming growth factor β (TGF- β) in the presence of TSP1.^{44,45} A major physiological function of TSP1 is activation of latent TGF- β with important function during normal developmental processes.⁴⁶ The TSP1 anti-

angiogenic peptide used herein lacks the TSP1 sequence that is responsible for activation of TGF- β . Thus, the angioinhibitory activity observed herein may be independent of the TSP1 effects on activation of TGF- β and its anti-inflammatory effects on CNV. Thus, the intact TSP1 molecule may impact CNV through both its anti-inflammatory and antiangiogenic activities. The potential synergistic and/or additive effects of these TSP1 activities on CNV associated with AMD are the subject of current investigation in our laboratory.

In summary, TSP1-deficient mice exhibited significantly enhanced choroidal neovascular membrane formation associated with increased inflammation and recruitment of macrophages. Together these results suggest that modulation of TSP1 plays an important role in CNV associated with AMD. Furthermore, TSP1 mimetic peptides could be used as novel therapeutics to inhibit CNV perhaps by modulating both the inflammatory and angiogenic states of choroidal vessels.

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