Targeting Immune Privilege to Prevent Pathogenic Neovascularization

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PURPOSE. Current studies suggest that the immune system plays a critical role in blinding eye disorders. The eye is an immune-privileged site, and FasL expression is a major part of that mechanism because Fas/FasL interactions regulate inflammation and neovascularization, preventing damage to delicate ocular structures. These studies were undertaken to test the idea that modulating immune privilege might be an effective therapeutic approach to pathogenic angiogenesis in the eye.

METHODS. C57BL/6 mice or FasL-defective B6-*gld* mice were laser treated to induce choroidal neovascularization (CNV). Mice were injected with cytotoxic FasL in the vitreous cavity or were treated with oral doxycycline in the drinking water. They were evaluated for CNV 7 days later. In some experiments eye tissue was harvested and evaluated for FasL expression, macrophage influx by immunohistochemistry, and release of sFasL.

RESULTS. Injection of cytotoxic FasL successfully prevented neovascularization in a mouse model of CNV. Oral doxycycline increased functional FasL in the eye and substantially inhibited neovascularization. Doxycycline treatment increased FasL expression on the RPE cells and reduced circulating and tissue-associated sFasL. Treatment was ineffective in B6-*gld* mice, demonstrating that CNV inhibition was mediated by FasL.

CONCLUSIONS. Targeting immune privilege using cytotoxic molecules or by increasing expression of the proapoptotic protein FasL may be a viable approach to treating neovascular eye disease. (*Invest Ophthalmol Vis Sci.* 2010;51:3560–3566) DOI: 10.1167/iops.09-3890

Immune privilege is a term applied to organs that have a unique relationship with the immune response. These sites prohibit the spread of inflammation at the expense of immune effector mechanisms because even minor episodes can threaten organ integrity. Once thought to be a passive process relying on physical barriers, immune privilege is now viewed as an active process that uses multiple anti-inflammatory mechanisms to maintain organ function (reviewed in Refs. 1, 2). The prototypic organ of immune privilege is the eye, in which the

spread of inflammation can lead to blindness. An important part of immune privilege is the constitutive expression of the death-inducing ligands FasL and TNF-related apoptosis inducing ligand (TRAIL), which inhibit inflammation and tumor growth by inducing apoptosis in cells expressing the Fas or TRAIL receptors.^{3–5} Although initial studies focused on the control of inflammation³ and the impact of these molecules on the systemic immune response,^{6,7} more recent studies have extended this concept to pathogenic neovascularization. These studies demonstrated an important role for FasL in controlling neovascular diseases of the cornea,⁸ retina,⁹ and choroid.¹⁰ It is now known that FasL expression on the parenchymal tissues of the eye regulates the extent of neovascularization by inducing apoptosis in Fas⁺ endothelial cells.^{10,11}

In the eye, neovascularization is a major component of several ocular disorders, including diabetic retinopathy, retinopathy of prematurity, and age-related macular degeneration (AMD).¹² In these diseases, the growth of new vessels can impair vision and threaten quality of life. AMD is a progressive disease that causes irreversible visual impairment and blindness in nearly 50 million people globally.^{13,14} Neovascular AMD is the more aggressive form and accounts for 90% of blindness from this disease. It is characterized by choroidal neovascularization (CNV), which is the development of abnormal blood vessels under the retina. Neovascular AMD is a progressive disease that can eventually result in blindness. Consequently, identifying therapies that can control neovascularization and prevent progression to vision loss is an area of intense study. Presently, only anti-VEGF therapy has proven effective at slowing the progression of neovascular AMD¹⁵ but may not permanently prevent blindness from this disease.

Recent work^{16–18} has focused on the role of chronic inflammation in the development of pathologic processes such as cancer, heart disease, and eye disorders. Because inflammation can promote angiogenesis there is considerable discussion of the role of inflammation in promoting ocular angiogenesis, particularly in neovascular AMD.^{12,13,18–22} Investigations into the role of inflammation and neovascular eye disease often overlook the principles of immune privilege whereby the ocular environment is not proinflammatory but tends to prohibit inflammation at the expense of certain immune effector mechanisms.¹ It has been suggested that the loss of immune privilege as the eye ages may contribute to the increases in neovascular disease,^{1,10,23} but this has not been studied. Given that FasL expression is an important component of immune privilege, we tested the idea that targeting this molecule would have therapeutic value in the treatment of eye disease. Our results showed that injection of a cytotoxic form of FasL can successfully prevent the progression of blood vessels into the eye. In addition, we showed that augmenting FasL expression by oral treatment with doxycycline leads to ablation of the neovascular response. Thus, modulation of immune privilege may be a successful adjunct therapy for blinding neovascular eye disorders.

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METHODS

Mice

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). B6-*gld* (C57BL/6 background) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animal experiments were approved by the Animal Studies Committee at Washington University School of Medicine and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experiments contained at least three mice per group and were repeated a minimum of three times.

Laser-Induced Murine Model of CNV

CNV was induced by rupture of the retinal pigment epithelium (RPE) and underlying Bruch's membrane with a krypton laser in 7-week-old mice, as described.^{1,10,23} Mice were anesthetized using intraperitoneal ketamine hydrochloride (86.9 mg/kg) and xylazine (13.4 mg/kg), and their pupils were dilated. Using krypton red laser, four laser burns were placed around the optic nerve (0.05 seconds, 50 μ m, 150 mW). After 7 days, the animals were perfused with 3% FITC-conjugated high-molecular weight dextran (2000 kDa). Eyes were immediately enucleated and fixed in 4% paraformaldehyde for 1 hour. A dissecting microscope was used to remove the cornea and lens and gently separate the retina from the underlying choroid and sclera. Microscissors were used to make four radial incisions in the sclero-choroidal eyecup to prepare choroidal flatmounts on glass slides. A drop of gel-mount fixative and a glass coverslip were placed on each slide. Choroidal flatmounts were analyzed for the presence of CNV by confocal microscopy. The extent of choroidal neovascularization was quantified by image acquisition and analysis software (MetaMorph; Molecular Devices, Sunnyvale, CA) and was reported as volume of fluorescence.

Treatment with sFasL

Recombinant human sFasL was purchased from R&D Systems (Minneapolis, MN), and recombinant mouse soluble TRAIL (sTRAIL) was purchased from Biomol International (Plymouth Meeting, PA). On day 2 after laser, 3 μ L containing 30 or 300 ng sFasL were injected into the vitreous cavity of each mouse eye with a 33-gauge needle fitted to a Hamilton syringe. Control animals were treated with either phosphate-buffered saline (PBS) or sTRAIL.

Treatment with Oral Doxycycline

To study the effect of doxycycline treatment on laser-induced CNV, doxycycline (Sigma, St. Louis, MO) was administered starting on the day of laser treatment at 0.5, 1, 2, or 4 mg/mL doses with drinking water. The samples were prepared in concentrations estimated to provide 20 mg/kg, 40 mg/kg, 60 mg/kg, or 160 mg/kg, respectively, of doxycycline daily on the basis of the average B6 mouse water intake. The solution was changed twice weekly. These doses have been used successfully and without side effects in several mouse models.^{15,24–26} Another group of age-matched animals was given water alone. On various days after treatment animals were killed after anesthesia with ketamine/xylazine for immunohistochemical staining (days 1, 3, or 7) or CNV lesion area determination (day 7), as indicated in the figure legends.

Immunohistochemistry

Sclero-choroidal flatmounts were prepared 1, 3, or 7 days after laser treatment. Tissues were fixed for 45 minutes at 4°C in 4% paraformaldehyde-PBS, followed by extensive washing with PBS. Sections were treated for 30 minutes at 37°C with PBS containing 3% goat serum to block nonspecific binding. Staining for membrane FasL was performed by incubating the flatmount with biotin-conjugated anti-CD178 antibody (1:400; BD Bioscience, San Jose, CA). The tissue was then washed five times for 5 minutes each with cold PBS, followed by incubation

with Cy5 labeled streptavidin (1:500; Rockland Immunochemicals, Boyertown, PA) for 30 minutes. CD11b cells were visualized with Alexa 488-conjugated anti-CD11b antibody (Biolegend, San Diego, CA). The mounts were then washed with PBS three times and analyzed by three-dimensional confocal microscopy. Isotype-matched antibodies were included in each experiment on identical whole mount tissue, and staining was not observed.

sFasL Levels in Serum

Mice were pre-bled 48 hours before laser treatment, after which they were laser treated and administered water or doxycycline. Mice were then serially bled at 24 hours and 48 hours by tail vein. Blood samples were centrifuged at 3000 rpm for 5 minutes, and serum was frozen at –70°C for subsequent sFasL assays. Serum concentrations of sFasL were measured with commercially available enzyme linked immunoassay (ELISA) kits (R&D Systems) according to the manufacturer's instructions. The concentration in serum samples was determined from the standard curve and had a detection limit of 3.6 pg/mL. Levels below the detection limit were coded as 0 pg/mL and were included in the analysis. All assays were conducted in duplicate.

sFasL Levels in Ocular Tissue

Mice were laser treated and administered water or doxycycline. They were anesthetized with ketamine (86.9 mg/kg) and xylazine (13.4 mg/kg) before they were killed by cervical dislocation. Eyes were enucleated and placed in ice-cold PBS buffer with protease inhibitor cocktail (pH 7.5; Roche Chemicals, Basel, Switzerland). A circumferential incision was performed posterior to the ciliary body, and the anterior segment, including the cornea, iris, ciliary body, and lens, were discarded. Posterior eyecups consisting of the retina, choroid, and RPE were washed repeatedly (by up and down pipetting for 30 seconds) with a small volume of PBS (0.1 mL). The buffer was then collected, pooled (8–10 eyes), centrifuged at 400g, and stored at –20°C. Protein concentration was then determined. Concentrations of tissue-derived sFasL were measured with commercially available ELISA kits. All assays were performed in duplicate, and two independent experiments were performed.

Quantification of CD11b⁺ Cells per Lesion

Sclero-choroidal flatmounts were prepared 1, 3, and 7 days after laser treatment. Alexa 488-conjugated anti-CD11b antibody (Biolegend, San Diego, CA) was used to stain the mounts for 1 hour at room temperature, which were then washed with PBS three times and analyzed by three-dimensional confocal microscopy. Numbers of macrophages (CD11b⁺) were counted per lesion (per 20 $\times$ field centered on the laser lesion), and average numbers were represented.

Real-time PCR and Gene Expression Analysis

Total RNA was prepared from sclero-choroidal flatmounts 3 days after laser injury (RNeasy mini kit; Qiagen, Valencia, CA). Non-laser-treated ocular tissue was harvested as control. cDNA was prepared with a transcription kit (High Capacity cDNA Reverse; Applied Biosystems, Foster City, CA). Relative levels of target gene expression were measured (7500 Real-time RT-PCR system; Applied Biosystems). For fluorescence measurement and assay preparation, VIC-based gene expression assay mix (*TaqMan*; Applied Biosystems) specific for each gene of interest and universal mix (*TaqMan* Universal Master Mix; Applied Biosystems) were used. Relative quantification PCR analysis was performed (ABI 7500 SDS Software; Applied Biosystems). GAPDH expression was used to serve as the loading control. Data are represented as gene expression increase or decrease in laser-treated lesions from C57BL/6 and B6-*gld* mice after injury compared with non-laser-treated tissue within the same group.

Statistical Analysis

Differences between the control and treatment groups in CNV experiments were evaluated on a pairwise basis using a generalized linear

modeling approach adjusting for correlation between the samples taken from each mouse using a repeated-measures method. We report *P* values for these comparisons, unadjusted for multiple comparisons, because the comparisons were established a priori. All statistical analyses were conducted with commercial software (SAS Proc GLM, SAS 9.1.3; SAS Institute; Cary, NC). *P* < .01 was considered significant.

RESULTS

Local Injection of sFasL Inhibits CNV

The extent of CNV in the laser model is regulated by the expression of FasL on RPE cells and macrophages.^{1,10,23} Studies show that this is mediated by the induction of apoptosis after the interaction of FasL with Fas on growing endothelial cells.^{10,11} A practical approach to therapy in AMD has been the local injection of agents that inhibit neovascularization¹⁵; therefore, we tested whether a local injection of cytotoxic sFasL, which induces Fas-mediated apoptosis, would have therapeutic efficacy. Mice were laser treated and on day 2 received intravitreal injections of PBS, sFasL, or sTRAIL. On day 7, CNV was evaluated as described.^{1,10,23} Data in Figure 1A demonstrate that injection of PBS or sTRAIL had no effect on CNV, whereas intravitreal injection of 30 or 300 ng sFasL significantly reduced CNV volume. Figures 1B to 1E show typical CNV lesions from these treated mice, demonstrating substantial CNV in the PBS-treated (Fig. 1B) and sTRAIL-treated (Fig. 1E) groups but reduced CNV in the sFasL-treated animals (Figs. 1C, 1D). In fact, mice treated with 300 ng sFasL had barely detectable CNV lesions (Fig. 1C). We conclude that targeting endothelial cells with cytotoxic sFasL can significantly suppress CNV.

Oral Doxycycline Increases FasL Expression on RPE Cells

We observed no systemic effects after local injection of sFasL (e.g., liver and kidney toxicity), or any disruption in normal retinal architecture of sFasL-injected mice (data not shown). However, we reasoned that a less invasive method to modulate the Fas/FasL system without side effects from intraocular injection might be more efficacious. Thus, we considered a method to increase naturally occurring FasL with the idea that this might be a way to control neovascularization. One such approach is to prevent cleavage of FasL from the cell surface with matrix metalloproteinase (MMP) inhibitors, as we have previously demonstrated in corneal grafting.²⁷ Consequently, we tested whether oral treatment with an MMP inhibitor might be effective in increasing FasL expression. For these studies we chose doxycycline, an MMP inhibitor that is well tolerated at high doses.^{25,28,29} Mice were laser-treated, and doxycycline (2 mg/mL) was placed in the drinking water for the duration of the experiment. On days 1 and 3, retinal whole mounts were prepared and stained for FasL expression. Surface FasL staining was not observed in the non-laser-treated eyes (Fig. 2A); however, in mice that received water, FasL staining is evident in the area of the laser lesion on day 1 (Fig. 2B) and day 3 (Fig. 2C). When mice were treated with oral doxycycline (2 mg/mL in drinking water), FasL expression was significantly increased 1 day after laser treatment (Fig. 2E) and on day 3 (Fig. 2F). Oral doxycycline did not increase surface FasL expression when there was no laser treatment (Fig. 2D).

Oral Doxycycline Reduces Laser-Induced CNV

We then examined the effect of the MMP inhibitor on the development of CNV after laser treatment. Doxycycline was placed in the drinking water of mice beginning on the day of laser treatment, and the animals were maintained on doxycy-

cline for the duration of the experiment (7 days). Data in Figure 3A show the effect of decreasing doses of doxycycline and demonstrate that oral treatment with 4 mg/mL or 2 mg/mL significantly reduced CNV, though treatment with 1 mg/mL showed a lesser effect and treatment with 0.5 mg/mL had no effect. Figures 3B to 3E showed representative CNV lesions, confirming that oral treatment with 2 mg/mL (Fig. 3C) virtually abolished the laser-induced CNV response compared with control mice (Fig. 3B). Treatment with 1 mg/mL (Fig. 3D) doxycycline was less effective, and mice given 0.5 mg/mL doxycycline (Fig. 3E) had results similar to those of control.

Doxycycline Requires Functional FasL to Inhibit CNV

Based on our previous observations²⁷ and data described here (Figs. 1, 2), we hypothesized that doxycycline might inhibit CNV through its effect on the FasL protein. We tested this idea

FIGURE 1. Intravitreal sFasL inhibits CNV. C57Bl/6 mice were laser treated and on day 2 were injected in the vitreous cavity with PBS, sFasL (300 or 30 ng), or sTRAIL (300 ng). CNV area was evaluated on day 7 by confocal microscopy. (A) CNV lesion volume (expressed as volume of fluorescence) in treated mice. **P* < 0.01; significant difference from PBS control. (B–E) Representative CNV lesions from mice treated with PBS, 300 ng sFasL, 30 ng sFasL, or 300 ng sTRAIL, respectively.

FIGURE 2. FasL staining on RPE cells. C57BL/6 mice were laser treated and administered water (A–C) or doxycycline (2 mg/mL) (D–F). Sclero-choroidal flatmounts were prepared 1 and 3 days after laser treatment and were stained for surface FasL. Micrographs were taken, centered on the laser lesions. Circles: approximate locations of the laser-induced lesions.

using FasL-defective B6-*gld* mice, which do not express functional FasL.^{5,30} Previous studies using this strain helped identify FasL as an important inhibitor of neovascularization in the retina, whereas FasL expressed on RPE cells induced apoptosis in Fas⁺ choroidal endothelial cells.¹⁰ C57BL/6 mice or C57BL/6-*gld* (B6-*gld*) mice were laser treated and administered water or doxycycline (2 mg/mL); on day 7 the extent of CNV was quantified. Figure 4A shows that though CNV was inhibited in C57BL/6 (B6) mice by doxycycline therapy, B6-*gld* mice were unaffected. Thus, the effect of this MMP inhibitor on the CNV model is likely mediated through the observed increase in surface FasL expression because CNV in mice without functional FasL (B6-*gld* mice) were unaffected by doxycycline treatment.

Data in Figure 2 demonstrate increased FasL expression on RPE cells in mice treated with doxycycline. These data, along with results showing that doxycycline treatment inhibits CNV in C57BL/6 mice (Fig. 3) but not in B6-*gld* mice (Fig. 4A), suggest that MMP inhibitors prevent the cleavage of FasL from the cell surfaces of the RPE, leading to increased inhibition of CNV volume. One prediction would be that higher levels of cleaved FasL (i.e., soluble FasL, sFasL) should be detected in untreated mice and that these levels should be reduced in doxycycline-treated animals. We tested this by examining the level of sFasL in serum and tissue after laser treatment. Mice were laser treated, and, 24 hours and 48 hours later, sera were obtained and the levels of sFasL were determined by ELISA. Data in Figure 4B show that mice treated with water had increased levels of sFasL in sera at 24 and 48 hours; however, when treated with doxycycline, serum-borne sFasL was not detected. Similarly, sFasL was detectable in the tissue of laser-treated mice given water; however, in mice exposed to doxycycline, sFasL was undetectable (Fig. 4C). We conclude that treatment with this MMP inhibitor prevents the cleavage of FasL, leading to increased inhibition of CNV by the membrane-associated protein.

Direct Inhibition of VEGF Production Is Not Responsible for the Effects of Doxycycline

Tetracycline derivatives have been shown to have inhibitory effects on VEGF-induced functions through inhibition of RNA transcription and the Erk1/2 and PI3K pathways.^{25,28} Therefore, it is possible that some effects of doxycycline may be

FIGURE 3. Doxycycline inhibits CNV. C57BL/6 mice were laser treated and administered water or varying doses of doxycycline. CNV was evaluated on day 7 by confocal microscopy. (A) Volume of CNV lesions (expressed as volume of fluorescence) in treated mice. * $P < 0.01$; significant difference from water control. Representative CNV lesions from mice treated with water (B); 2 mg/mL doxycycline (C); 1 mg/mL doxycycline (D); 0.5 mg/mL doxycycline (E).

FIGURE 4. Doxycycline requires functional FasL to inhibit CNV. C57BL/6 or B6 *gld* mice were laser treated and administered water or doxycycline (2 mg/mL). (A) CNV volume (expressed as volume of fluorescence) was evaluated on day 7 by confocal microscopy. * $P < 0.01$; significant decrease from PBS control. ** $P < 0.01$; significant increase from PBS control. (B) C57BL/6 (B6) mice were pre-bleed 48 hours before laser treatment. They were then administered water or doxycycline (2 mg/mL) and were laser treated. Mice serially bled at 24 and 48 hours by tail vein and serum concentrations of sFasL were measured with a commercially available ELISA. Levels below the detection limit were coded as 0 pg/mL and were included in the analysis. All assays were conducted in duplicate. NS, not significantly different from the pre-bleed group. (C) C57BL/6 (B6) mice were administered water or doxycycline (2 mg/mL) and then were laser treated. Tissue from the posterior segment of the eye was harvested at 48 and 72 hours, and the soluble proteins were extracted. Concentrations of sFasL were measured with a commercially available ELISA and are expressed as picogram per milligram of protein.

Levels below the detection limit were coded as 0 pg/mg protein and were included in the analysis. Each point represents the value from 8 to 10 pooled eyes. NS, not significantly different from the untreated control. (D) C57BL/6 or B6 *gld* mice were laser treated and administered water or doxycycline (2 mg/mL). On day 3 after laser treatment, eyes were harvested, mRNA was isolated, and qRT-PCR for VEGF was performed.

attributed to the modulation of VEGF production or activity. Consequently, we explored the effect of doxycycline on VEGF production in the laser model of CNV. Mice were treated with doxycycline beginning on the day of laser treatment. On day 3 eyes were harvested and qRT-PCR was performed for VEGF. Figure 4D shows that C57BL/6 (B6) mice treated with doxycycline had decreased expression of VEGF compared with non-laser-treated controls, suggesting an effect on VEGF; however, when an identical experiment was performed on B6-*gld* mice, no inhibition of VEGF was observed. Thus, the lack of VEGF in doxycycline-treated wild-type mice was likely caused by the decreased angiogenesis induced by FasL, not a direct effect of the MMP inhibitor on VEGF production. Increased expression of VEGF in B6-*gld* mice was expected because these animals have significantly elevated levels of CNV in this model.¹⁰

Doxycycline-Based Therapy Is Independent of Macrophage-Derived FasL

FasL expression that modulates CNV in this model has been found on RPE cells¹⁰; however, macrophage expression of the protein has been shown to be important when these cells are injected into the vitreous cavity.^{1,23} Therefore, we also tested whether doxycycline treatment increased the number of macrophages found in the CNV lesions and whether FasL expression on CD11b⁺ macrophages was increased. Mice were laser treated, and on days 1, 3, and 7 retinal whole mounts were prepared and the number of CD11b⁺ macrophages in the lesions were quantified as described.¹ These data show that similar numbers of macrophages were found in the lesions for water- and doxycycline-treated mice (Fig. 5A). Immunohistochemical staining for FasL and CD11b were performed on day 3. FasL (Fig. 5B) and CD11b (Fig. 5C) were detected in water-

fed animals, and no costaining was observed (Fig. 5D, merge). In doxycycline-treated animals, FasL (Fig. 5E) and CD11b (Fig. 5F) were also detected, but only minimal costaining was observed (Fig. 5G). We conclude that the dominant effect of doxycycline in this model is on the RPE cell-derived FasL.

DISCUSSION

AMD is a significant disease affecting millions of persons worldwide. Although the neovascular (wet) form of AMD represents only 10% of reported cases, more than 90% of the blindness from this disorder is caused by this form. Consequently, treatments that target neovascularization have important implications for the quality of life of many patients. Current anti-VEGF therapies have been effective,³¹ but there is no therapy that successfully targets other proinflammatory components of this disorder. Because the eye is an immune-privileged site and contains constitutive mechanisms to prohibit inflammation and angiogenesis,^{2,30} we tested the idea that modulating immune privilege might have important therapeutic implications. Our results show that we can prevent blood vessel growth by injection of cytotoxic sFasL into the vitreous cavity. In addition, we can inhibit neovascularization by increasing RPE cell expression of FasL by systemic treatment with doxycycline. We conclude that targeting the Fas/FasL pathway may be a viable treatment for pathogenic neovascularization in the eye.

FasL is an important component of the immune privilege, and loss of this protein can have significant consequences for inflammation,³ corneal transplantation,^{32,33} and neovascularization.^{8–10} FasL is expressed on parenchymal cells throughout the eye and forms a barrier to inflammatory cells and sprouting

FIGURE 5. Doxycycline does not increase macrophage infiltration or FasL expression. (A) Sclero-choroidal flatmounts were prepared 1, 3, and 7 days after laser treatment and stained for CD11b. Numbers of macrophages (CD11b⁺) were counted per lesion (per 20× field centered on the laser lesion), and average numbers are represented. (B–G) C57BL/6 mice were laser treated and administered water (B–D) or doxycycline (2 mg/mL) (E–G). Sclero-choroidal flatmounts were prepared and costained for FasL (red) (B, E) and CD11b (green) (C, F) and were examined by confocal microscopy. FasL⁺ and CD11b⁺ images were merged to detect coexpression (D, G).

endothelial cells.^{10,11,34} In the retina, FasL is expressed in the RPE cells.^{3,6,34,35} Studies have shown that FasL expression is tightly regulated in both lymphoid and nonlymphoid cell populations, thereby preventing unwanted damage to Fas⁺ cells and tissues throughout the body.^{1,36} Thus, cells that use FasL as a barrier to cellular invasion or as an effector molecule typically do not display the protein on their surfaces. It is either stored in secretory vesicles for rapid mobilization to the cell surface^{37,38} or produced de novo after cellular activation, stress, or injury.^{1,36} Our data support this idea because we have shown that FasL is not expressed on the surfaces of RPE cells; rather, surface expression is rapidly increased on laser injury. Interestingly, even FasL upregulation in response to laser injury does not provide an absolute barrier to penetration of the eye by endothelial cells because laser-treated mice with increased surface FasL expression develop neovascularization. One possible explanation for this is the sensitivity of cell surface FasL to the activity of MMPs, which can cleave the protein from the cell surface, thereby rapidly downregulating its activity.^{27,37} As a result, increased surface expression is offset by enzymatic activity resulting in less efficient FasL function. Our results show that the use of a potent MMP inhibitor can increase FasL expression and effectively reduce FasL cleavage, thereby eliminating the neovascular response.

Doxycycline is an effective antimicrobial agent that has potent anti-inflammatory effects in a number of experimental models.^{25,28,39} Besides being a potent MMP inhibitor, doxycycline can inhibit IL-1 β production,²⁹ decrease nitric oxide production,⁴⁰ inhibit stress kinase activation,²⁸ and prevent cellular proliferation.¹⁰ These properties can easily explain the potent effect of doxycycline on CNV in the laser model be-

cause angiogenesis can depend on an initial inflammatory response.^{16–18} However, our data suggest that though these functions of doxycycline may be important to its antiangiogenic properties, the ultimate effect of doxycycline on the CNV response is mediated by its effect on FasL expression. That doxycycline treatment is ineffective in mice without functional expression of this proapoptotic protein (i.e., in B6-*gld* mice) supports this idea.

Doxycycline has also been shown to have effects on several VEGF-related functions.^{25,28} In the current studies we observed a decrease in VEGF after doxycycline treatment, suggesting that this may be a potential mechanism. However, VEGF production was unaffected in FasL-deficient *gld* mice, suggesting that the reduction in VEGF production may be related to the decreased angiogenesis in doxycycline-treated mice, not the result of a direct effect on VEGF production.

Recently, we demonstrated that FasL was also expressed on macrophages and that these cells were capable of inhibiting laser-induced CNV. Consequently, we examined FasL expression on the macrophage infiltrates in doxycycline-treated mice. We observed little FasL expression on CD11b⁺ cells, further supporting our previous study demonstrating that RPE cell-derived FasL was the major effector in the antiangiogenic properties of FasL in the eye. In addition, doxycycline treatment did not alter the infiltration of CD11b⁺ cells into the laser lesions. We conclude that the major effect of FasL in this model is likely the inhibition of endothelial cell function through apoptosis, as has been demonstrated previously.^{8–10}

As a final point, we used doses of doxycycline that are much higher than the appropriate therapeutic doses of the compound.²⁶ We found that these levels were well tolerated in

short-term experiments, but we do not have data on any long-term effects of doxycycline treatment. However, we are not specifically proposing doxycycline as a long-term treatment for CNV but are suggesting that targeting an important molecule involved in immune privilege with the use of a potent MMP inhibitor has therapeutic efficacy. Thus, targeting the Fas-FasL pathway alone, or in combination with other treatments, may be a viable clinical approach to the treatment of neovascular eye disease.

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