

# Activation of CD36 Inhibits and Induces Regression of Inflammatory Corneal Neovascularization

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**PURPOSE.** This study was undertaken to investigate the role of the antiangiogenic receptor CD36 during inflammatory corneal neovascularization (CNV).

**METHODS.** In a murine model of inflammatory CNV, CD36 expression was evaluated by RT-PCR and immunofluorescence. Mice subjected to CNV were treated topically (thrice daily) with CD36 functionally neutralizing antibodies against the oxidized low-density lipoprotein (oxLDL) and thrombospondin (TSP)-1 sites (clones JC63.1 and FA6-152, respectively). Neovascularization was analyzed by CD31-immunostained corneal flatmounts. The role of the less characterized oxLDL site during angiogenesis was elucidated by using the CD36 ligand 1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine (POVPC; 50, 100  $\mu\text{g}/\text{mL}$ ) 24 hours after corneal injury for 7 days, whereas in angioregressive studies, POVPC treatments were initiated 10 days after induction of CNV. In this process, VEGF expression was also studied. Effects of CD36 activation were further examined *ex vivo* using the mouse aortic ring assay.

**RESULTS.** CD36 expression was upregulated after corneal injury; CD36 was expressed in corneal epithelium, limbus, invading microvessels, and stromal macrophages. Blocking CD36 activity with FA6-152 significantly increased CNV ( $P < 0.001$ ). Conversely, activating CD36 with POVPC dose dependently inhibited CNV ( $P = 0.003$ ); this effect was blocked by JC61.3. POVPC also significantly regressed preformed blood vessels ( $P < 0.001$ ). *Ex vivo* experiments on aortic rings confirmed the angioinhibitory and -regressive effects of POVPC. Because corneal macrophages express CD36 and may partake in angiogenesis via VEGF-A secretion, we surmised that VEGF-A could be modulated by CD36. Indeed, POVPC downregulated VEGF-A

expression in a time-dependent fashion ( $P < 0.001$ ), whereas FA6-152 induced its expression ( $P < 0.05$ ).

**CONCLUSIONS.** CD36 is involved both physiologically and pharmacologically in inhibition and regression of CNV, by direct effect on endothelial cells and partly by negatively regulating VEGF expression in macrophages. (*Invest Ophthalmol Vis Sci.* 2006;47:4356–4364) DOI:10.1167/iovs.05-1656

Angiogenic stimulators and inhibitors are the counterbalancing systems that tightly control corneal angiogenesis.<sup>1</sup> The healthy cornea is devoid of vascular elements and is maintained as an immune-privileged site.<sup>2</sup> This immunity is thought to be due to the plethora of antiangiogenic factors present in this tissue.<sup>1</sup> These include pigment epithelium-derived factor (PEDF); maspin; thrombospondin; endostatin, a proteolysis product of type XVIII collagen; and angiostatin, a proteolysis product of plasminogen.<sup>3–8</sup>

Although vascular endothelial growth factor (VEGF) is known to be a potent stimulator of corneal neovascularization (CNV),<sup>9,10</sup> the molecular basis of this condition remains poorly defined. It is well known, however, that transmigrating and invading macrophages are closely associated with neovascularization and provide much of the requisite VEGF that drives this process.<sup>11,12</sup>

The class B scavenger receptor CD36 is a transmembrane glycoprotein that has been identified as the critical receptor for thrombospondin (TSP)-1, a potent endogenous inhibitor of angiogenesis,<sup>13–15</sup> including that which occurs in the cornea.<sup>16</sup> CD36 also binds to a variety of other ligands including oxidized low-density lipoproteins (oxLDLs),<sup>17,18</sup> oxidized phospholipids (oxPLs),<sup>19–22</sup> *Plasmodium falciparum*-infected erythrocytes,<sup>23</sup> collagen,<sup>24</sup> and apoptotic cells.<sup>25</sup> Expression of CD36 is broad and encompasses microvascular endothelial cells (ECs), monocytes/macrophages, platelets, conjunctival dendritic cells, and the retinal pigment epithelium.<sup>25–28</sup> Furthermore, CD36 has been implicated in a wide variety of normal and abnormal biological functions, including angiogenesis, atherosclerosis, phagocytosis, inflammation, lipid metabolism, and removal of apoptotic cells.<sup>25</sup>

With respect to its angiostatic functions, CD36 is essential for inhibiting *in vitro* EC migration and the formation of capillary-like structures by TSP-1.<sup>15</sup> CD36 plays a critical role *in vivo*, as demonstrated by the inability of TSP-1 to inhibit angiogenesis in CD36 null mice.<sup>29</sup> We have also recently demonstrated the specific involvement of CD36 and TSP-1 in mediating antiangiogenic signals in ischemic proliferative retinopathy.<sup>30,31</sup> Nevertheless, the involvement of CD36 and the relative role of its less well-characterized oxidized lipid-binding site in regulating pathologic corneal angiogenesis has not yet been fully elucidated. We herein report that expression of CD36 in macrophages and microvascular endothelial cells after corneal injury suppresses CNV. This effect can be ascribed to the proapoptotic antiangiogenic property of CD36 on endothelial cells, as well as partly to a downregulation of VEGF from CD36-expressing macrophages.

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## MATERIALS AND METHODS

### Animals

Male C57BL/6 mice were purchased from Harlan (Indianapolis, IN). Animals were given food and water ad libitum, maintained in pathogen-free, 12-hour light-dark conditions. All animal experiments followed the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care Committee of Sainte-Justine Hospital, Montreal.

### Murine Model of CNV

The CNV model used in our studies is characterized by the removal of the corneal and limbal epithelium by means of a chemical and mechanical injury to the cornea. This causes resurfacing of the cornea by a conjunctiva-like epithelium replete with blood vessels. The neovascularization persists for at least 8 weeks and is accompanied by inflammation.<sup>9</sup>

All mice used for inflammation-induced CNV experiments were between 6 and 8 weeks of age. Each mouse was anesthetized before all surgical procedures with isoflurane (Abbott, Montréal, Québec, Canada). Topical proparacaine (Alcon Canada, Mississauga, Ontario) and 2  $\mu$ L of 0.15 M NaOH were applied to the central cornea of each mouse. The corneal and limbal epithelia were removed by scraping with a no. 23 scalpel. Gentamicin sulfate ophthalmic solution (Sabex, Inc., Boucherville, Québec, Canada) was instilled immediately after epithelial denudation, three times daily for 2 days. Buprenorphine (0.05 mg/kg; Schering-Plough, Ltd., Pointe Claire, Québec, Canada) was administered after surgery for analgesia.

### Pharmacological Treatment of Mice with CNV

C57BL/6 mice undergoing inflammation-induced angiogenesis were randomly divided into four groups. Twenty-four hours after corneal injury, one group received treatment with 100  $\mu$ g/mL of an anti-CD36 monoclonal antibody (mAb) against the  $\alpha$ LDL binding site (clone JC63.1 IgA mouse; Cayman Chemical, Ann Arbor, MI) or an isotype control antibody (100  $\mu$ g/mL anti-mouse IgA; Sigma-Aldrich, St. Louis, MO). A second group was treated with 200  $\mu$ g/mL of an anti-CD36 mAb against the TSP-1-binding site (clone FA6-152 IgG<sub>1</sub> mouse; Beckman Coulter, Fullerton, CA) or 200  $\mu$ g/mL anti-mouse IgG<sub>1</sub> (Sigma-Aldrich). The third group was administered vehicle (99% 0.9% NaCl and 1% ethanol) or 50 or 100  $\mu$ g/mL POVPC (1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine; Cayman Chemical). In the fourth group, vehicle (99% 0.9% NaCl and 1% ethanol) or 100  $\mu$ g/mL POVPC treatments were administered 10 days after surgery for angiogenesis studies. All treatments were administered topically three times daily for 7 days, after which corneas were harvested for immunostaining. In another set of experiments, one group of mice underwent vehicle (99% 0.9% NaCl and 1% ethanol) or 100  $\mu$ g/mL POVPC treatments for 2 and 4 days, whereas a second group was treated with 200  $\mu$ g/mL IgG<sub>1</sub> or 200  $\mu$ g/mL FA6-152 for 4 days. The corneas were subsequently dissected and processed for RNA extraction and RT-PCR analysis. In all experiments, the treatment groups consisted of 10 mice per group, and each set of experiments was repeated at least two times.

### Labeling and Quantification of CNV

Visualization of vascular endothelial cells was performed by immunostaining corneal flatmounts with FITC-conjugated anti-CD31, as previously described,<sup>6</sup> or with FITC-conjugated ICAM-1, to demonstrate activated vascular endothelial cells. Fresh corneas were dissected, rinsed in 0.1 M PBS for 30 minutes, and fixed in 100% ice-cold acetone for 25 minutes. After the specimens were washing in 0.1 M PBS, nonspecific binding was blocked with 0.1 M PBS, 2% bovine serum albumin (BSA; Sigma-Aldrich) for 1 hour at room temperature. Incubation with FITC-conjugated anti-CD31 (1:300; BD Pharmingen, San Diego, CA) or ICAM1-FITC (1:100, Abcam Plc, Cambridge, UK) in 0.1 M PBS, 2% BSA at 4°C overnight was followed by subsequent washes in

0.1 M PBS at room temperature. Corneas were mounted with an anti-fade agent (Gelmount; Biomed, Inc., San Francisco, CA) and observed with an epifluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan). Images were captured with a digital camera (DXM 1200, with ACT 1, ver. 2.62 software; Nikon).

The CNV was quantified in a masked fashion with image-analysis software (Photoshop 7.0; Adobe, Mountain View, CA). The entire flatmounted cornea was analyzed, to minimize sampling bias. The total corneal surface area was outlined with the innermost vessel of the limbal arcade as the border and the ratio [(neovascularized area/total cornea area)  $\times$  100] was used to provide a measure of the percentage of vascularized cornea.<sup>6</sup>

### Immunostaining of Corneal Frozen Sections

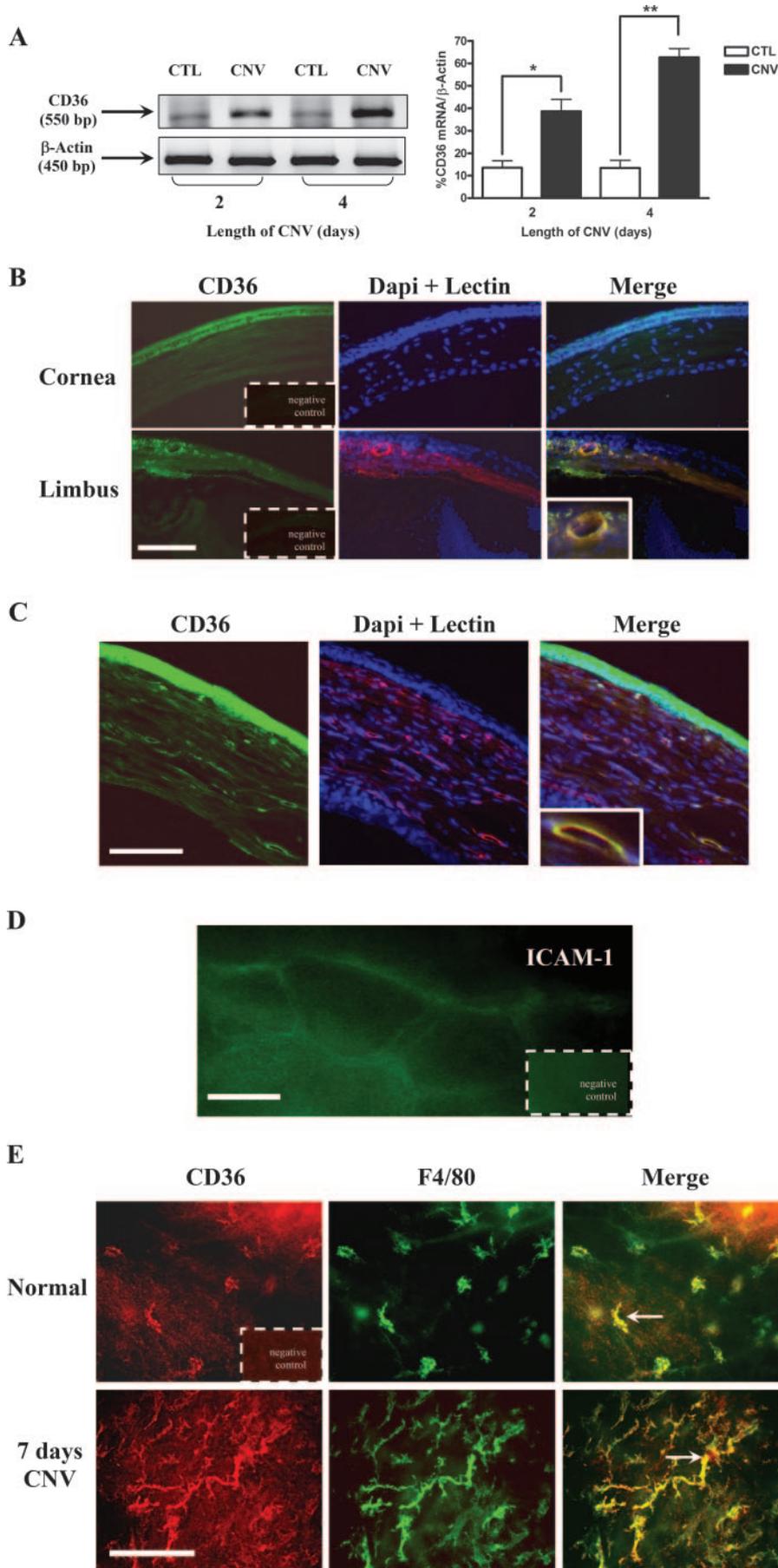
Mice were killed 7 days after corneal injury. Enucleated eyes were fixed in 4% paraformaldehyde, transferred to 30% sucrose/PBS overnight at 4°C, washed with PBS, and embedded in optimal cutting temperature (OCT) medium (Sakura Finetek, Torrance, CA). Sixteen-micrometer frozen sections were washed with 0.1% Triton X-100/PBS and blocked for 1 hour with 2% BSA before overnight incubation with rabbit polyclonal CD36 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA). The sections were subsequently incubated with a goat anti-rabbit secondary antibody (1:1000; Invitrogen-Molecular Probes, Eugene, OR), preceded by a 1-hour incubation with a TRITC-conjugated lectin endothelial cell marker from *Griffonia simplicifolia* (1:100, Sigma-Aldrich, Inc.). Cell nuclei were labeled with the nucleic acid stain 4',6-diamidino-2-phenylindole (DAPI, 300 nM; Invitrogen-Molecular Probes). In negative control experiments, the CD36 primary antibody was omitted, and sections were incubated with 0.1% Triton X-100/PBS followed by the goat anti-rabbit secondary antibody and DAPI. Images were visualized by epifluorescence microscopy.

### RNA Extraction and RT-PCR Analysis

The eyes were enucleated and the corneas dissected and immediately placed in stabilization solution (RNAlater; Ambion, Inc., Austin, TX). Total RNA ( $n = 10$  per group) was extracted with a standard RNA isolation protocol (TRIzol; Invitrogen, Inc., Carlsbad, CA). cDNA was synthesized from 1  $\mu$ g RNA with M-MLV reverse transcriptase (Promega, Inc., Madison, WI) according to the manufacturer's instructions. The following primers were used for PCR from 5' to 3': CD36 sense, GATGACGTGGCAAAGAACAG; CD36 antisense, AAAGGAGGCTGCGTCTGTG; VEGF-A sense, ACTGGACCTGGCTTACTG; VEGF-A antisense, TATGTGCTGGCTTTGGTGAG; JNK-1 sense, TGTGGAATCAAGCACCTTCACTCTGCTG; JNK-1 antisense, GCAAAC CATTTC-CCATAATGCACCC; c-JUN sense, ATGCCCTCAAGCGCTCTCCTCC; c-JUN antisense, CTGCTCGTCCGTCACGTTCTTGGG;  $\beta$ -actin sense, AGCCATGTACGRAGCCATCC; and  $\beta$ -actin antisense, ATGCCACAGGATTCCATACC. 18S (Ambion, Inc.) also served as an internal control. PCR (*Taq* DNA polymerase; Invitrogen, Inc.) was performed under the following conditions: denaturation at 94°C, annealing at 56°C (CD36,  $\beta$ -actin; VEGF-A: 65°C; JNK-1, cJUN: 64°C; 18S: 60°C), and extension at 72°C. The predicted sizes of PCR products are 550, 350, 300, 350, 450, and 315 bp for CD36, VEGF-A, JNK-1, cJUN,  $\beta$ -actin, and 18S respectively. Densitometry values were measured in terms of pixel intensity (Image-Pro Plus software, ver. 4.1; Media Cybernetics, Silver Spring, MD).

### Aortic Ring Angiogenesis Assay

This assay was performed as described previously by us and others.<sup>31,32</sup> In brief, thoracic aortas were removed from 6-week-old mice killed by CO<sub>2</sub> asphyxiation and immediately transferred to a culture dish containing ice-cold endothelial cell basal medium (EGM-2; Cambrex Bio Science, Walkersville, MD). The periaortic fibroadipose tissue was carefully removed with fine microdissecting forceps and scissors, paying special attention not to damage the aortic wall. One millimeter-long aortic rings (12 per aorta) were sectioned and rinsed extensively in eight consecutive washes of EGM-2. The rings were then individually



**FIGURE 1.** CD36 expression and immunolocalization in the normal and neovascularized cornea. **(A)** RT-PCR analysis of CD36 mRNA expression in normal and injured corneas after 2 and 4 days of injury (\* $P < 0.01$  vs. control at day 2; \*\* $P < 0.001$  vs. control at day 4). Immunofluorescence on **(B)** normal and **(C)** inflamed corneas revealed CD36 immunoreactivity (green) in the corneal epithelium, and coexpression with lectin (red) in vessels from the normal limbus, as well as in newly formed vessels in the stroma of the inflamed cornea. **(D)** Vascular endothelial cells of the neovascularized cornea stained positive for ICAM-1, a marker of activated endothelial cells. **(E)** Expression of CD36 by stromal macrophages from normal and injured corneas. Fluorescence micrographs illustrate CD36 (red) coexpression with the microglial/macrophage marker F4/80 (green) in both the normal and 7-day neovascularized cornea as indicated by arrows. Yellow: merged fluorescent labeling. Insets: images taken at higher magnification. Scale bars: **(B)** 50  $\mu$ m; **(C, D, E)** 30  $\mu$ m.

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embedded in 48-well plates previously coated with 50  $\mu$ L synthetic basement membrane (Matrigel; BD Biosciences, Bedford, MA) per well. Next, an additional 50  $\mu$ L of Matrigel was placed over each ring. After 1 hour, 500  $\mu$ L EGM-2 was added to each well, and the cultures were incubated at 37°C for 5 days. The culture medium was changed on day 3 and the test compounds added. The test compounds and their concentrations were: vehicle (99% 0.9% NaCl and 1% ethanol) and POVPC (20  $\mu$ g/mL), in the absence or presence of anti-CD36 antibodies (JC61.3, 6  $\mu$ g/mL; FA6-152, 10  $\mu$ g/mL). The aortic rings were photographed on day 5 at 4 $\times$  magnification with an inverted microscope (Eclipse TE300; Nikon). For neovessel-regression experiments, the rings were cultured without drugs until day 6, after which the rings were treated with the test compound and allowed to grow until day 7. The angiogenic response was determined by measuring the area of neovessel formation on computer (Image Pro Plus software; Media Cybernetics, Inc.).

### Immunostaining of Wholmount Corneal Stromas

Mice were subjected to corneal injury, after which corneal and limbal tissue were excised at day 7 and subsequently prepared for staining as wholmounts, as previously described.<sup>35</sup> Briefly, the corneal epithelium was separated after a 20-minute incubation at 37°C in 20 mM EDTA (Sigma-Aldrich). The resultant corneal stromas were then fixed for 30 minutes at 4°C in 1% paraformaldehyde-PBS followed by extensive washing in PBS. After fixation, the corneal tissue was blocked for 1 hour in PBS-GEN (PBS containing 3% BSA, 0.25% gelatin, 5 mM EDTA, and 0.025% Nonidet-P40) and then processed for double immunofluorescence with rabbit polyclonal CD36 (1:100, Santa Cruz Biotechnology), rabbit anti-mouse vascular endothelial growth factor A (VEGF-A; 1:100; Chemicon International, Inc.), and the monocyte/macrophage marker rat anti-mouse F4/80 (1:100, Serotec, Oxford UK). Primary antibodies were visualized using appropriately tritiated Alexa Fluor-conjugated secondary antibodies (1:1000, goat anti-rabbit for CD36 and VEGF-A, goat anti-rat for F4/80). Negative control experiments were conducted in parallel by incubating sections with PBS-GEN alone followed by the secondary antibodies. Sections were visualized by epifluorescence microscopy.

### Isoprostane Measurements

Isoprostanes (8-Iso-PGF2 $\alpha$ ) were measured in homogenized normal ( $n = 10$ ) and 4-day postinjury corneas ( $n = 10$ ) by enzyme immunoassay (Cayman Chemical, Inc.), as previously described.<sup>34</sup> The levels of 8-isoprostane were quantified and normalized to the protein content of the corneal tissue.

### Statistical Analysis

Results are expressed as the mean  $\pm$  SEM. Statistical analyses were performed by using ANOVA with comparison among means performed by the appropriate post hoc test, unless otherwise stated. Statistical significance was set at  $P < 0.05$ .

## RESULTS

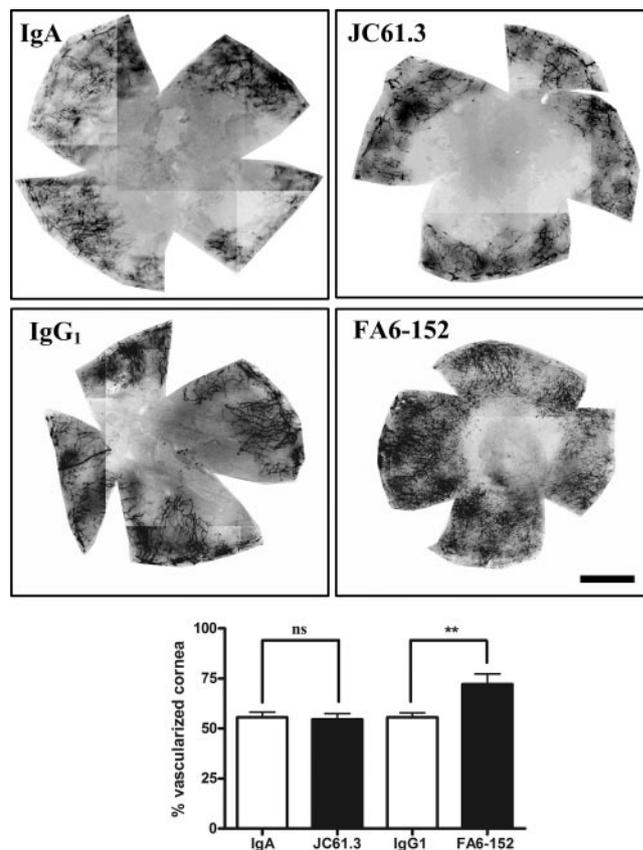
### Expression of CD36 in Normal and Neovascularized Corneas

We examined the expression of CD36 (2 and 4 days after injury) in the normal and neovascularized cornea. As revealed in Figure 1A, the CD36 gene was transcriptionally active, and its expression was substantially induced during the course of neovascularization ( $P < 0.001$ ). CD36 immunoreactivity was largely localized in the corneal epithelium and limbal vessels of the normal cornea (Fig. 1B) and was highly expressed by infiltrating vessels in the stroma of the neovascularized cornea (Fig. 1C). By immunostaining neovascularized corneas with ICAM-1, we further illustrated that the vascular endothelial cells were activated, an observation that has been corroborated

by others.<sup>35</sup> Because of the significant induction of CD36 gene expression observed in the injured corneas, we hypothesized that inflammatory cells such as macrophages also significantly express CD36. Immunofluorescence analysis revealed CD36 expression by dendritiform cells in the normal corneal stroma (Fig. 1D). Double staining for CD36 and F4/80, a monocyte/macrophage marker, demonstrated that CD36<sup>+</sup> cells were also uniformly F4/80<sup>+</sup>. In addition, as early as 3 days after induction of CNV, the cells coexpressing CD36 and F4/80 were present in the central areas of the cornea and increased in number through day 7, when the maturational state of these cells also changed (Fig. 1D).

### Effect of Blocking CD36 on CNV

CD36 is a potent antiangiogenic receptor activated on separate binding sites by oxLDL and TSP-1.<sup>13-15,17,18</sup> We postulated that a blocking antibody would exacerbate inflammation-induced CNV. Twenty-four hours after corneal injury, mice treated for 7 days with the anti-TSP-1 binding site antibody (FA6-152) but not with the anti-oxLDL antibody (JC61.3) exhibited a 23% greater vascularized corneal area ( $72.1\% \pm 5.2\%$  vs.  $55.6\% \pm 2.2\%$ , respectively;  $P < 0.001$ ) and a more pronounced vessel density (Fig. 2); an isotype-control antibody had no significant effect.



**FIGURE 2.** Blocking CD36 activity enhanced CNV. Mice subjected to inflammation-induced CNV were treated three times daily for 7 days with vehicle, 100  $\mu$ g/mL of an anti-CD36 monoclonal antibody (CD36 mAb) against the oxLDL site (JC61.3), or 200  $\mu$ g/mL of a CD36 mAb against the thrombospondin-1 binding site (FA6-152). Corneas were harvested and flatmounts were labeled with murine CD31-FITC. Quantification of the vascularized area (\*\* $P < 0.001$  vs. IgG<sub>1</sub>). Scale bar, 200  $\mu$ m.

### Effect of Activation of CD36 by Oxidized Phospholipids on CNV and Preformed Blood Vessels

Because the anti-oxLDL-binding site antibody did not affect CNV in response to corneal injury (Fig. 2), we postulated that oxidized phospholipids may not be abundantly generated; indeed concentrations of isoprostanes, a marker of oxidized lipid generation, were not increased 4 days after injury (Fig. 3A). Accordingly, if stimulated with a synthetic oxidized phospholipid—namely, 1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine (POVPC)—CNV should be decreased. This assumption was confirmed, as topical application of POVPC (50 and 100  $\mu\text{g}/\text{mL}$ ) decreased CNV in a concentration-dependent manner ( $P = 0.003$ ; Fig. 3B); as expected, this effect was abrogated by JC61.3 ( $P = 0.02$ ; Fig. 3B).

We also tested whether activation of CD36 would cause regression of existing CNV, a clinically desirable effect.<sup>36</sup> Topical application of POVPC (100  $\mu\text{g}/\text{mL}$ ) in vascularized corneas (10 days after corneal injury) reduced the neovascularized area by 24% ( $P < 0.001$ ; Fig. 3C).

### Effect of Oxidized Phospholipids on CNV and Existing Microvessels in an Aortic Ring Angiogenesis Assay

To ascertain whether the effects of POVPC are CD36-dependent, we used the mouse aortic ring angiogenesis assay. Aortic rings were treated on day 3 with saline, POVPC (20  $\mu\text{g}/\text{mL}$ ), or a combined treatment of POVPC and JC61.3 (6  $\mu\text{g}/\text{mL}$ ), or FA6-152 (10  $\mu\text{g}/\text{mL}$ ). POVPC inhibited neovessel formation ( $P < 0.001$ ; Fig. 4A). This angiostatic effect of POVPC was blocked by the anti-oxLDL site antibody ( $P < 0.001$ ). Effects of POVPC were hardly affected by the anti-TSP-1 site antibody. POVPC also induced regression of new vessels grown for 6 days ( $P < 0.0001$ ; Fig. 4B).

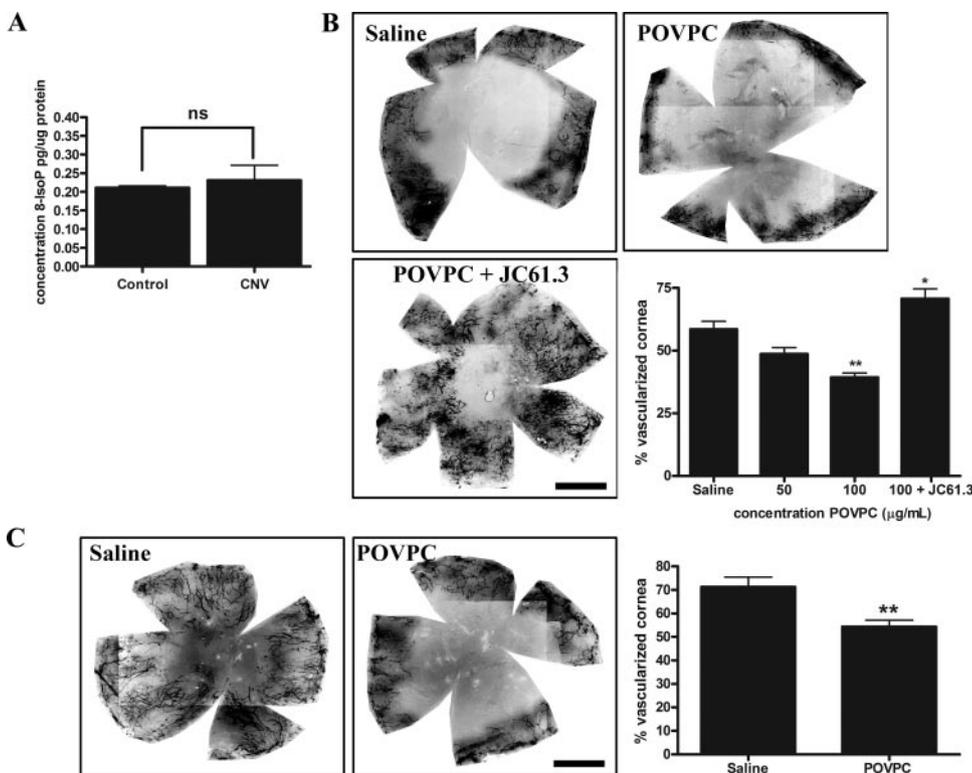
### Effect of Activation of CD36 on VEGF-A Expressed by Macrophages in Inflamed Cornea

Aortic ring angiogenesis does not involve a role for macrophages. Hence, to elucidate the role of macrophage-expressing CD36 in CNV (Fig. 1D), we surmised that VEGF expression is modulated by CD36. This inference is supported by evidence that macrophages are a significant source of VEGF-A.<sup>11,37,38</sup> VEGF-A colocalized with the F4/80 macrophage marker (Fig. 5A). Corneal injury resulting in CNV was associated with an increase in VEGF-A (mRNA) expression (RT-PCR; Fig. 5B). POVPC significantly diminished VEGF-A expression in a time-dependent fashion ( $P < 0.001$ ), whereas treatment with FA6-152 significantly induced expression of VEGF-A mRNA ( $P < 0.05$ ; Fig. 5C).

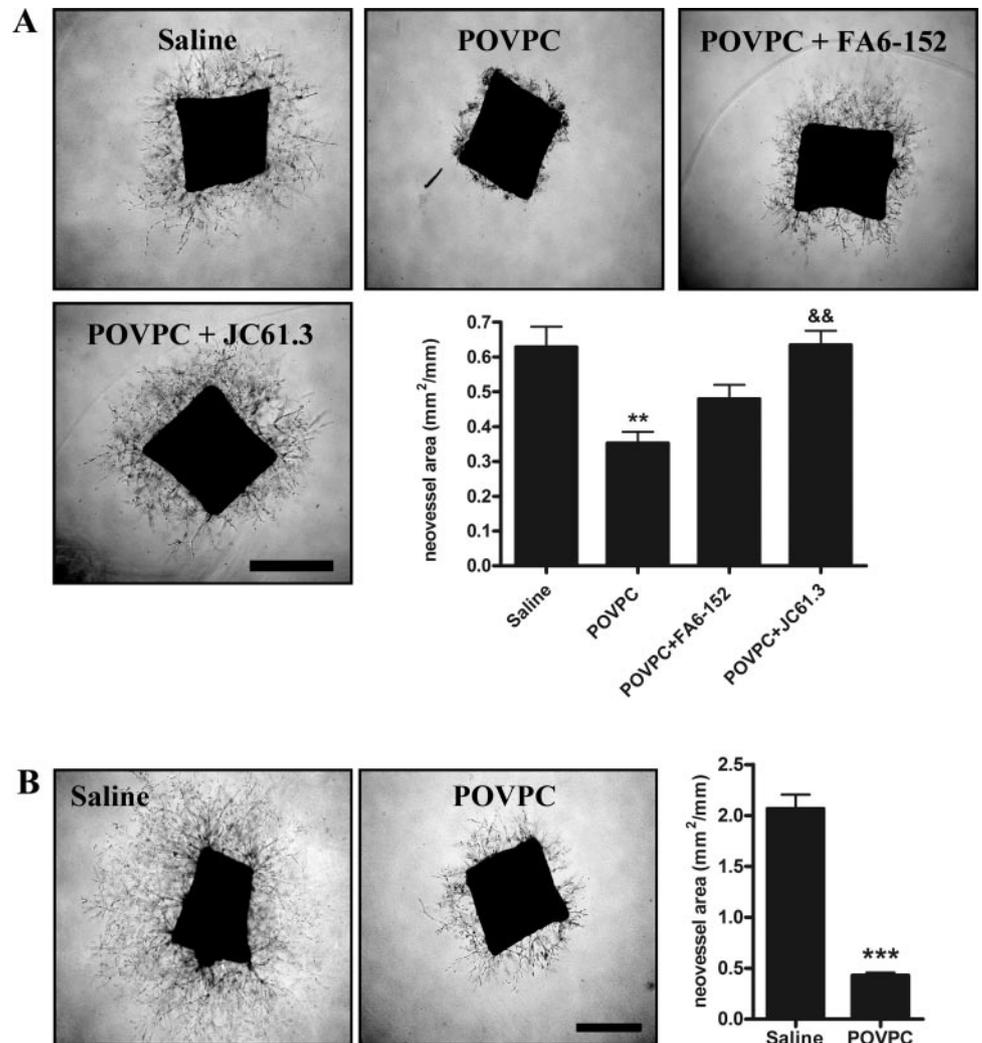
We conducted further experiments to explore the mechanisms underlying the FA6-152 induced upregulation of VEGF-A. Activation of the mitogen-activated protein kinase (MAPK) JNK-1 has been shown to play a critical role in the inhibition of bFGF-induced corneal angiogenesis in a CD36-dependent manner.<sup>39</sup> In our model, the effects of FA6-152 were associated with a marked increase in the mRNA expression of JNK-1 ( $P < 0.001$ ) and its phosphorylation substrate c-JUN ( $P < 0.05$ ; Fig. 5D).

### DISCUSSION

CD36 is a potent anti-angiogenic receptor, and its angiostatic effects have been widely documented.<sup>15,25,29</sup> However, to date the expression profile and specific involvement of CD36 in the cornea and during CNV have not been fully elucidated. Our studies revealed that the CD36 gene was transcriptionally active in the normal cornea, its expression was substantially upregulated during the course of CNV (Fig. 1A), and, in this context, it was present largely in microvessels and macrophages. Interference of its action enhanced neovascularization (Fig. 2), and, conversely, activation of CD36 diminished angio-



**FIGURE 3.** CD36 ligation by an oxidized phospholipid ligand inhibits and induces regression of CNV. (A) Levels of 8-isoprostane were measured in normal and 4-day postinjury corneas. (B) Mice underwent corneal injury and received three treatments daily of 50 or 100  $\mu\text{g}/\text{mL}$  of the oxidized phospholipid 1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine (POVPC), or a cotreatment of POVPC (100  $\mu\text{g}/\text{mL}$ ) and JC61.3 (100  $\mu\text{g}/\text{mL}$ ) for 7 days. Corneal flat-mounts were labeled with murine CD31-FITC. \* $P = 0.02$ ; \*\* $P = 0.003$  vs. saline. (C) Treatments with saline or 100  $\mu\text{g}/\text{mL}$  POVPC were initiated 10 days after corneal injury for a period of 7 days, and the vascularized corneal area was subsequently determined and quantified (\*\* $P < 0.001$  vs. saline). Scale bar, 200  $\mu\text{m}$ .



**FIGURE 4.** CD36 activation using POVPC inhibits microvessel outgrowth from aortic ring explants. Mouse aortas were seeded on Matrigel and on day 3 incubated in the presence of (A) saline, the oxidized phospholipid 1-palmitoyl 2-(5'-oxovaleroyl) phosphatidylcholine (POVPC; 20  $\mu\text{g}/\text{mL}$ ), or POVPC plus JC61.3 (6  $\mu\text{g}/\text{mL}$ ) or FA6-152 (10  $\mu\text{g}/\text{mL}$ ). Photographs were taken on day 5. (B) For regression analysis, aortic rings were treated with POVPC (20  $\mu\text{g}/\text{mL}$ ) on day 6 and photographs taken on day 7. \*\* $P < 0.001$  vs. saline; && $P < 0.001$  vs. POVPC. Scale bar, 200  $\mu\text{m}$ .

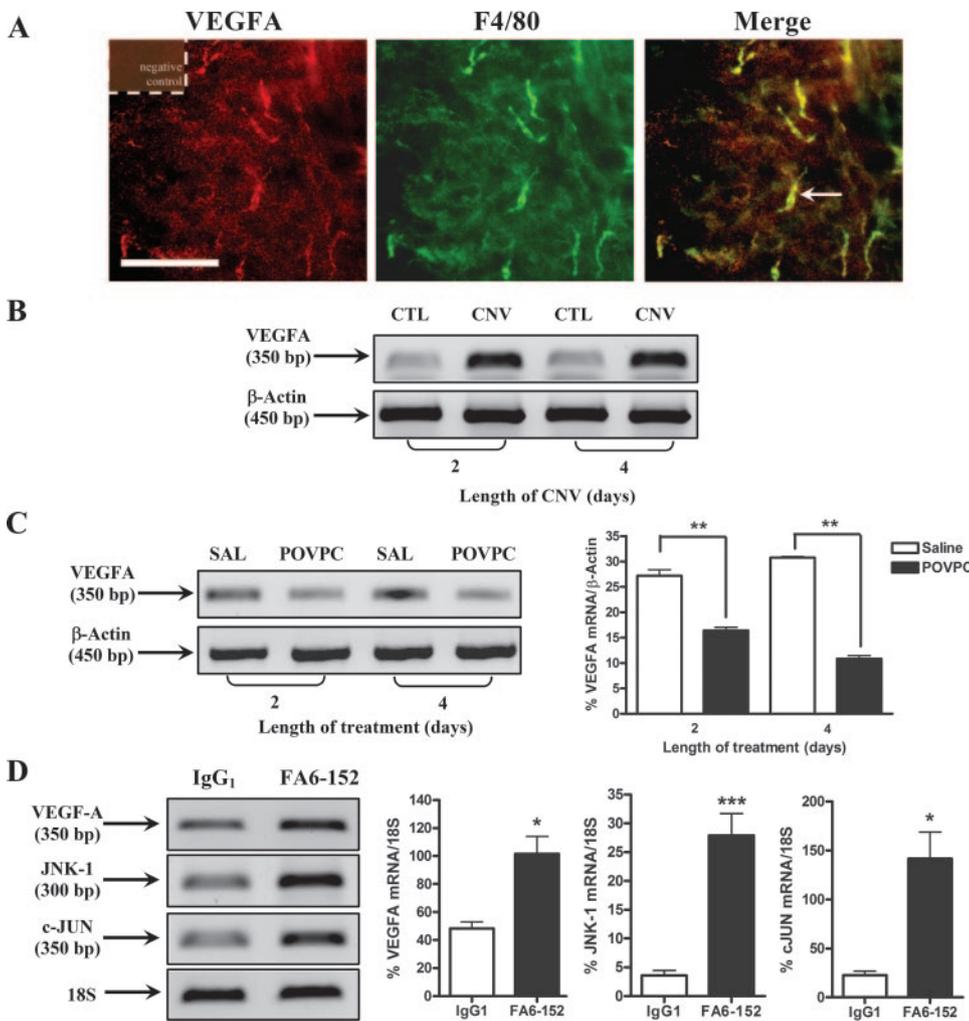
genesis and induced regression of existing corneal microvessels (Fig. 3). The antiangiogenic effects of CD36 stimulation appear to be dependent on direct actions on microvessels (Fig. 4) and likely indirectly by diminishing concentrations of macrophage-derived VEGF-A (Fig. 5). Hence, CD36 limits corneal neovascularization in response to injury, and thus provides a major role in attempting to maintain corneal avascularity, a prominent feature of the normal cornea.

In the pathophysiological setting of corneal injury, stimulation of CD36 seems largely mediated through its TSP-1 site (Fig. 2), consistent with the already reported role of TSP-1 in CNV.<sup>16</sup> However, CD36 can also be activated by several oxidized lipids and LDLs<sup>14,15,17-20</sup>. Numerous studies have identified oxidized phospholipids as high-affinity CD36 ligands,<sup>19-22</sup> but we could not find increased levels of markers of oxidized lipids, namely isoprostanes, in injured corneas (Fig. 3). On the other hand, activation of the CD36 receptor using an oxPL ligand (POVPC) significantly suppressed CNV, and this effect was prevented by a cognate antibody, confirming the specificity of POVPC for CD36 (Fig. 3).<sup>19-22</sup> In vivo effects were corroborated using the ex vivo aortic ring angiogenesis assay. Collectively, our results, together with those reported on TSP-1,<sup>16</sup> provide conclusive in vivo and ex vivo evidence of the efficacy of CD36 as a major target in CNV.

Our in vivo observations after corneal injury pointed to expression of CD36 in macrophages (Fig. 1D). Macrophages play an important role in angiogenesis,<sup>11,12</sup> in that their selec-

tive depletion markedly limits pathologic neovascularization.<sup>12,37</sup> Macrophages are also a significant source of VEGF-A (Fig. 5A) as reported,<sup>38,40</sup> and abundant evidence points to a dominant role for VEGF in inflammation-induced CNV.<sup>9,10,37</sup> VEGF also amplifies inflammatory CNV by further recruiting macrophages/monocytes.<sup>37</sup> We therefore determined whether CD36 could modulate VEGF expression in the injured cornea and found that stimulation of CD36 with POVPC diminished VEGF-A expression, whereas blocking CD36 expression with FA6-152 substantially induced VEGF-A mRNA levels (Fig. 5). This is consistent with recently documented effects of another CD36 ligand, TSP-1.<sup>41</sup>

The mechanisms of action of angiogenesis inhibitors have been a subject of considerable attention. The prevailing mode of action of CD36 in inhibition of angiogenesis is believed to be through sequential activation of p59<sup>l<sup>y</sup>n</sup>, caspase-3-like proteases, and p38 MAPKs, by targeting newly formed endothelial cells.<sup>15,29,42</sup> In an attempt to explore the signaling pathway on binding of the anti-TSP1 CD36 mAb, we evaluated the expression of the stress-activated MAPK, JNK-1, which was found to be significantly induced in FA6-152-treated corneas. This finding is corroborated by a study reporting an activation of the p38 and p42/44 MAPKs in an inflammatory model of CNV.<sup>43</sup> We propose that the induction of JNK-1 may be attributed to the upregulation of VEGF-A observed after CD36 blockade. Consistent with this hypothesis, there has been a report that VEGF stimulates MAPK activity in various settings.<sup>43</sup> Other



**FIGURE 5.** VEGF-A is expressed by macrophages in the inflamed cornea and is downregulated after activation of CD36. (A) Immunofluorescent staining of corneal stromal flat-mounts revealed that VEGF-A (red) colocalizes with the microglial/macrophage marker F4/80 (green) in the inflamed cornea 7 days after injury. Yellow: merged image of fluorescent labeling. Scale bar, 30  $\mu$ m. (B) Top: VEGF-A mRNA expression 2 and 4 days after corneal injury; bottom:  $\beta$ -actin. (C) VEGF-A mRNA expression after 2 and 4 days of POVPC treatment (\*\* $P < 0.001$  vs. saline). (D) VEGF-A, JNK-1, and c-JUN mRNA expression after 4 days of FA6-152 treatment (\* $P < 0.05$ , \*\* $P < 0.001$  vs. IgG<sub>1</sub>).

signaling mediators such as the Sonic hedgehog (Shh) pathway, whose inhibition was recently reported to reduce ocular neovascularization,<sup>44</sup> were not activated in our model nor were they implicated in the antiangiogenic effects of CD36 (data not shown). Of noteworthy mention, it may have been interesting to investigate whether the CLESH-containing protein, histidine rich glycoprotein (HRGP), modulates CD36 interactions in our studies, seeing that HRGP has been shown to abrogate the CD36-dependent signaling of TSP-1.<sup>45</sup> Taken together, antiangiogenic effects of CD36 are mediated, not only through a direct effect on microvessels (Fig. 4) but also apparently by inhibiting macrophage-derived VEGF-A expression (Fig. 5).

Current therapies for CNV, such as thermal laser or photodynamic therapy, induce only temporary closure of blood vessels,<sup>46</sup> whereas a clinically more important aspect of CNV therapy is regression of established blood vessels. In the present study, we investigated this limitation by delaying POVPC treatments until 10 days after corneal injury and observed a significant reduction in existing CNV (Fig. 3B); a similar effect was observed ex vivo on aortic ring explants (Fig. 4B). Therefore, activating CD36 not only suppressed but also induced regression of blood vessels. The mechanisms of blood vessel regression are not fully characterized. Nonetheless, certain inferences can be made based on available evidence. For instance, it has been proposed that in the ovary, blood vessel regression involves endothelial cell detachment and blood vessel occlusion.<sup>47</sup> Pericyte loss also determines the susceptibility

of vessels to regression.<sup>48</sup> Likewise, increased levels of angiopoietin-2, along with a downregulation of VEGF can destabilize mature capillaries and induce their regression.<sup>49</sup> These mechanisms may operate in response to CD36 stimulation.

Taken together, the current findings provide the first demonstration of the protective involvement of CD36 in limiting inflammatory CNV. Other antiangiogenic factors such as endostatin, thrombospondin, PEDF, and maspin have been found in the uninjured cornea, reaffirming the importance of these types of factors in maintaining transparency of the healthy cornea.<sup>2,3,50</sup> Our observations have significant implications for the treatment of ocular neovascularization, in that CD36 stimulants would be effective not only in patients with ongoing CNV, but also in those with established CNV. Findings may not only apply to corneal inflammation after injury or infection, but may also be relevant in corneal graft failure which involves an inflammatory immune rejection. Finally, stimulation of CD36 with simple agonists such as oxPLs may provide insights into inexpensive therapies for CNV secondary to commonly encountered infections (such as trachoma) in developing countries.

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