Sustained Inhibition of Corneal Neovascularization by Genetic Ablation of CCR5

Balamurali K. Ambati,[1,2] *Akshay Anand,*[3] *Antonia M. Joussen,*[2,4] *William A. Kuziel,*[5] *Anthony P. Adamis,*[2,6] *and Jayakrishna Ambati*[2,3]

Purpose. To determine whether genetic ablation of the CC chemokine receptor CCR5 (involved in leukocyte and endothelial chemotaxis) inhibits the development of corneal neovascularization.

Methods. Wild-type C57BL/6J mice and species-specific counterparts with targeted homozygous disruption of the CCR5 gene underwent chemical and mechanical denudation of corneal and limbal epithelium. Corneas were harvested 2 and 4 weeks after injury. Neovascularization was quantified by CD31 immunostaining. Expression of VEGF protein was quantified by ELISA.

Results. The mean percentages of neovascularized corneal area in control mice and CCR5-deficient mice 2 weeks after denudation were 58.3% and 38.5% ($P = 0.05$), respectively. At 4 weeks after denudation, the corresponding percentages were 67.6% and 44.0% ($P = 0.028$). In CCR5-deficient mice, VEGF protein levels were reduced 51.1% at 2 weeks ($P = 0.05$) after injury and 37.3% at 4 weeks ($P = 0.03$).

Conclusions. CCR5-deficient mice showed a persistent 34% to 35% inhibition of corneal neovascularization for up to 4 weeks. This inhibition correlates with reduced expression of VEGF. These data implicate CCR5 as one essential component in the development of corneal neovascularization. (*Invest Ophthalmol Vis Sci.* 2003;44:590–593) DOI:10.1167/iovs.02-0685

Corneal neovascularization (CNV) is a central feature in the pathogenesis of many blinding corneal disorders and a major sight-threatening complication in corneal infections and chemical injury and after keratoplasty, in which neovascularization adversely impacts corneal graft survival.[1]

From the [1]Department of Ophthalmology, Medical College of Georgia, Augusta, Georgia; the [3]Department of Ophthalmology, University of Kentucky, Lexington, Kentucky; the [4]Department of Vitreoretinal Surgery, Center of Ophthalmology, University of Cologne, Cologne, Germany; the [5]Section of Molecular Genetics and Microbiology and Institute for Cellular and Molecular Biology, University of Texas, Austin, Texas; the [2]Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts; and [6]Eyetech Pharmaceuticals, Woburn, Massachusetts.

Supported by the Heed Ophthalmic Foundation (BKA), American Ophthalmological Society-Knapp Testimonial Fund (JA), a Foundation Fighting Blindness Career Development Award (JA), University of Kentucky Physician-Scientist Award (JA), Grant Jo 324/2-1 from the German Research Foundation (AMJ), the National Eye Institute (APA), Massachusetts Lions Eye Research Fund (APA), and the Roberta Siegel Research Fund (APA).

Submitted for publication July 9, 2002; revised August 19, 2002; accepted September 29, 2002.

Disclosure: **B.K. Ambati**, None; **A. Anand**, None; **A.M. Joussen**, None; **W.A. Kuziel**, None; **A.P. Adamis**, Eyetech Pharmaceuticals (E); **J. Ambati**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Jayakrishna Ambati, Department of Ophthalmology, University of Kentucky, 801 Rose Street, Lexington, KY 40536-0284; jamba2@uky.edu.

Although the molecular mechanisms underlying CNV have not been fully deciphered, vascular endothelial growth factor (VEGF) seems to play an indispensable role. It induces CNV when applied exogenously[2] and is the principal endogenous angiogenic force in the neovascularization that follows limbal injury.[3] Transmigrating and invading corneal leukocytes appear to provide much of the requisite VEGF that drives CNV.[3]

The recruitment of leukocytes to inflammatory sites is mediated by chemokines. Chemokines bind to specific receptors that, despite some redundancy, show specificity for individual chemokines. Evidence is emerging that individual chemokines attract specific leukocyte populations through processes determined by ligand specificity and the expression patterns of the corresponding receptors.[4] CCR5 receptor ligands include the macrophage inflammatory proteins (MIPs) 1α and 1β, which recruit both macrophages and neutrophils.[5,6]

Endothelial cell migration and proliferation are also essential to angiogenesis, and these processes are known to be influenced by vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).[7,8] Further, endothelial cell chemotaxis and/or proliferation can be stimulated by chemokines, including interleukin (IL)-8, monocyte chemotactic protein (MCP)-1, regulated on activation normal T-cell expressed and secreted (RANTES), and MIP-1α and -1β.[9,10]

Previous work has confirmed that corneal angiogenesis is driven in large part by inflammatory mediators.[11,12] MIP-1α has been associated with CNV in helminth keratitis,[13] shown to be required for the development of CNV in HSV keratitis[14,15] and demonstrated to be angiogenic in Kaposi's sarcoma–associated herpes virus infection.[16] It has also been associated with angiogenesis in corneal allograft rejection.[17,18] Its receptor, CCR5, also found on endothelial cells, is a receptor for CC chemokines that are involved in angiogenesis, including RANTES and MIP-1α and -1β.[10,19]

We therefore examined the role of CCR5 in a clinically relevant ocular model of corneal injury by studying the neovascular response of animals genetically deficient in these molecules.

Methods

All animal experiments were approved by the Massachusetts Eye and Ear Infirmary Animal Care Committee and the University of Kentucky Animal Care Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized by intramuscular injection of 50 mg/kg ketamine hydrochloride (Abbott Laboratories, N. Chicago, IL) and 10 mg/kg xylazine (Bayer, Shawnee Mission, KS). Animals were killed by a lethal dose of pentobarbital (150 mg/kg).

Animals

Wild-type C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used as controls. To create the CCR-knockout strain, the entire CCR5 coding region was replaced by insertion of a neomycin-resistant expression cassette, as described previously.[20,21] Although the original knockout strains were generated on a mixed C57BL/6 × 129/Ola genetic background, the mice used in the experiments described

herein represent the progeny of the eighth generation backcross of the knockout mutations to inbred C57BL/6 animals. Because the CCR2 and CCR5 genes are very closely linked, a wild-type control strain for both knockout strains was developed by backcrossing the wild-type CCR5 locus from 129/Ola to the C57BL/6 background for eight generations. This control strain minimizes the genetic differences flanking the targeted allele. Wild-type C57BL/6 and 129/Ola CCR5 genes were distinguished by the presence of a *Bgl*II restriction fragment length polymorphism.[20]

Genotype Analysis

The genotype of all experimental animals was determined on tail DNA samples. CCR5 knockout alleles were identified by PCR analysis with a set of four primers. Wild-type CCR5 alleles were identified by synthesis of a 450-bp PCR product with primers complementary to sequences on each side of the crossover site (primer 1, 5′-AAGCAGGAGGATCGT-GAGCTTGAC-3′, and primer 2, 5′-GAAACAAGTTGTTGTGCACCTCAG-3′). Disrupted CCR5 alleles were identified by synthesis of a 350-bp PCR product with a primer that is complementary to a sequence in the neomycin resistance coding region (primer 3, 5′-TTCCATTGCT-CAGCGGTGCT-3′) combined with a primer complementary to a CCR5 sequence downstream of the crossover site (primer 4, 5′-TGTTTCCTC-CTCTAGCCTTCACTATG-3′).

Experimental Design

Both groups of mice (control, CCR5 knockout) underwent corneal injury. Corneas were harvested 2 and 4 weeks after injury. An image analyzer was used by a masked investigator to quantify neovascularization in immunostained corneas.

Model of Corneal Neovascularization

Topical proparacaine and 2 μL of 0.15 M NaOH were applied to the right cornea of each mouse. The corneal and limbal epithelia were removed with a Tooke corneal knife (Katena Products, Denville, NJ) in a rotary motion parallel to the limbus. Erythromycin ophthalmic ointment was instilled immediately after epithelial denudation.

Labeling of Corneal Neovascularization

Immunohistochemical staining for vascular endothelial cells was performed on corneal flatmounts. Fresh corneas were dissected, rinsed in PBS for 30 minutes, and fixed in 100% acetone (Sigma, St. Louis, MO) for 20 minutes. After the corneas were washed in PBS, nonspecific binding was blocked with 0.1 M PBS, 2% albumin (Sigma) for 1 hour at room temperature. Incubation with FITC-coupled monoclonal anti-mouse CD31 antibody (Pharmingen) at a concentration of 1:500 in 0.1 M PBS, 2% albumin at 4°C overnight was followed by subsequent washes in PBS at room temperature. Corneas were mounted with an antifading agent (Gelmount; Biomeda, Inc, San Francisco, CA) and visualized with a fluorescence microscope (Leica, Wetzlar, Germany).

Quantification of Corneal Neovascularization

Digital quantification of corneal neovascularization has been described.[22,23] Images of the corneal vasculature were captured with a CD-330 charge-coupled device (CCD) camera (Dage-MIT, Inc., Michigan City, IN) attached to a fluorescence microscope (MZ FLIII; Leica Microsystems Inc., Deerfield, IL). The images were analyzed on computer (Openlab; Improvision Inc., Lexington, MA), resolved at 624 × 480 pixels, and converted to tagged information file format (TIFF) files. The neovascularization was quantified by setting a threshold level of fluorescence above which only vessels were captured. The entire mounted cornea was analyzed to minimize sampling bias. The quantification of the neovascularization was performed in masked fashion. The total corneal area was outlined, with the innermost vessel of the limbal arcade used as the border. The total area of neovascularization was then normalized to the total corneal area, and the percentage of the cornea covered by vessels was calculated.

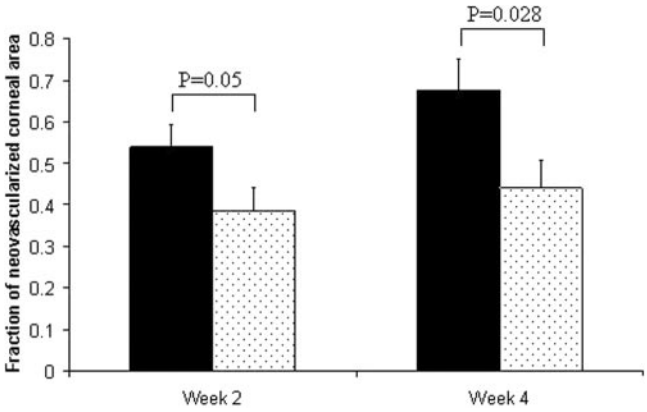

FIGURE 1. Mean ± SEM fraction of corneal neovascularization, 2 and 4 weeks after denudation in control (■) and CCR5-deficient (▨) mice.

Corneal Harvest for ELISA

At 1, 3, 14, and 21 days, scraped corneas were dissected and placed in 60 μL lysis buffer (20 mM imidazole hydrochloride, 10 mM potassium chloride, 1 mM magnesium chloride, 10 mM EGTA, 1% Triton, 10 mM sodium fluoride, 1 mM sodium molybdate, and 1 mM EDTA [pH 6.8]), supplemented with protease inhibitor (Sigma) followed by homogenization. The lysate was cleared of debris by centrifugation at 14,000 rpm for 15 minutes (4°C), and the supernatant was collected. Total protein was determined with a Bradford protein assay (Bio-Rad, Hercules, CA).

VEGF ELISA

VEGF was determined by a commercially available ELISA kit (R&D Systems, Minneapolis, MN) which recognizes the 164-amino-acid splice variant of mouse VEGF. The assay was performed according to the manufacturer's instructions. Briefly, standards or tissue samples (50 μL) were pipetted into an antibody-coated 96-well plate containing 50 μL of assay diluent and incubated for 2 hours at RT on a shaker. The wells were then washed five times with wash buffer, 100 μL of VEGF conjugate was added, and the samples were again incubated for 2 hours at RT. Samples were washed five times, 100 μL substrate buffer was added, the samples were incubated for 30 minutes at RT, the reaction was stopped, and the absorption was measured with an ELISA reader (Emax; Molecular Devices, Sunnyvale, CA) at 450 nm with lambda correction at 570 nm. All measurements were performed in duplicate. The lower limit of ELISA was 3.0 pg/mL. The tissue sample concentration was calculated from the standard curve and corrected for total protein.

Statistics

Data were analyzed by the Kruskal-Wallis test with the Dunn test for multiple comparisons (SPSS, Inc., Chicago, IL). Type I error not exceeding 0.05 was deemed significant.

RESULTS

Data on (mean ± SEM) percentages of neovascularized cornea area in mice 2 and 4 weeks after corneal scraping and implantation are presented in Figure 1.

Percentage Area of Corneal Neovascularization

At 2 weeks after denudation, $CCR5^{-/-}$ mice ($n = 8$) had 34.0% less ($P = 0.05$) neovascularized corneal area than control mice ($n = 13$). At 4 weeks after denudation, $CCR5^{-/-}$ mice ($n = 6$) had 34.9% less neovascularized corneal area than control mice ($P = 0.028$). Representative images are shown in Figure 2.

VEGF Expression

Data are presented in Figure 3. CCR5$^{-/-}$ mice had 51.1% less corneal VEGF than control mice at 14 days after scraping (P = 0.05) and 37.3% less VEGF at 21 days after scraping (P = 0.03).

Discussion

In this study, CCR5-deficient mice experienced a sustained inhibition of corneal neovascularization after chemical and mechanical denudation of corneal epithelium, an effect correlated with reduced VEGF expression. These data implicate CCR5 as one of the components in the development of corneal neovascularization, probably because of the effects of this receptor and ligands on endothelial cells and macrophages. Endothelial cells possess CCR5[19,24,25] and undergo chemotaxis in response to RANTES, MIP-1α, and MIP-1β (ligands for CCR5).[10]

Macrophage involvement in corneal neovascularization is well known[24] and infiltration of these cells, which is closely associated with neovascularization, peaks 2 weeks after corneal injury.[25] Use of manumycin, a Ras farnesyltransferase inhibitor, inhibits CNV and infiltration of macrophages (but not that of neutrophils).[26] Pharmacologic depletion of macrophages dramatically limits CNV in corneal allograft rejection.[27] Increased expression of VEGF after corneal injury has been localized to infiltrating inflammatory cells, especially macrophages, after corneal injury,[3] which probably accounts for the reduced expression of VEGF observed in this study.

CCR5 plays a role in chemotaxis of mature macrophages, because MIP-1α is chemotactic for differentiated macrophages.[28,29] MIP-1α is correlated with corneal infiltration of leukocytes, and antibodies to MIP-1α reduce corneal opacification in herpetic keratitis.[30] Previous reports have found upregulation of MIP-1α after alkali injury, *Pseudomonas* infection, and HSV infection.[30–32] A proximate role of MIP-1α, and thus CCR5, is likely, because it is produced by the corneal stroma in response to injury.[31,33]

Because inhibition of CNV in CCR5-deficient mice is only approximately one third, it is clear that other mechanisms are involved. However, we may speculate as to the reasons for the persistent inhibition of corneal neovascularization observed in CCR5$^{-/-}$ mice. First, CCR5 may be involved in migration and proliferation of endothelial cells. Second, the reduced expression of VEGF in this model (and thus diminished angiogenic stimulus) may have been due to decreased macrophage infiltration.

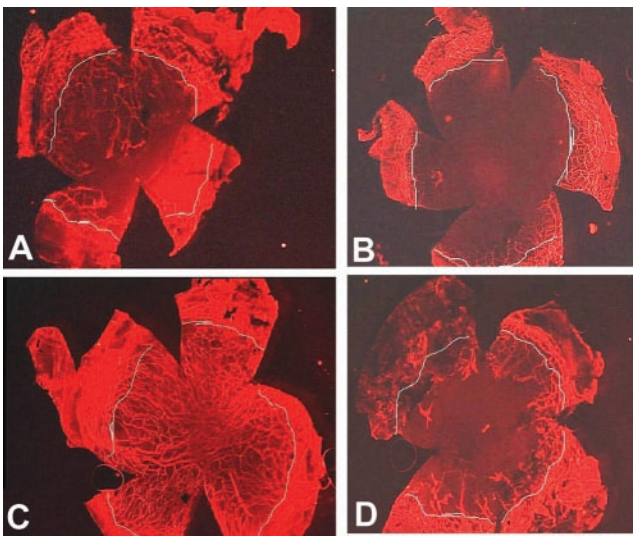

Figure 2. Representative photographs of corneal flatmounts of control (**A, C**) and CCR5-deficient (**B, D**) mouse eyes, 2 (**A, B**) and 4 (**C, D**) weeks after corneal injury, stained with FITC-coupled monoclonal murine anti-CD31 antibody to visualize endothelial cells.

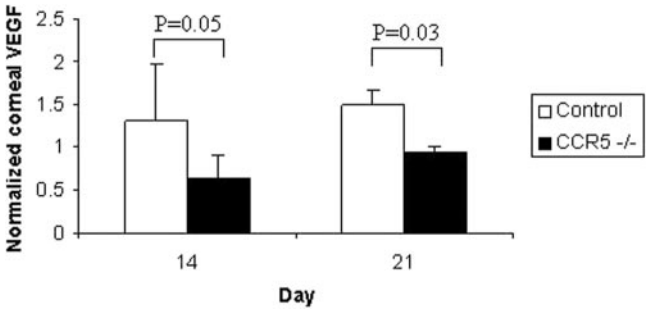

Figure 3. Mean VEGF ± SEM protein expression, normalized to total corneal protein, 2 and 4 weeks after denudation in control (■) and CCR5-deficient (□) mice.

In the present study, neovascularization was persistently inhibited in CCR5-deficient mice, which points the way toward a possible therapeutic target for pharmacologic agents. That this inhibition was demonstrated in a mechanical and chemical corneal trauma increases its clinical relevance. The method of inducing neovascularization affects pharmacologic efficacy—for example, integrin antagonists inhibit bFGF-induced corneal neovascularization but not that caused by chemical injury.[34]

In summary, this finding could advance the management of blinding disorders such as Stevens-Johnson syndrome, cicatricial pemphigoid, corneal allograft rejection, and corneal injury from infection, trauma, or alkali. Future research should also characterize the effect of and the complex molecular interplay among chemokines, leukocytes, and neovascularization and extend this model to other sites of ocular neovascularization.

References

1. Epstein RJ, Stulting RD, Hendricks RL, Harris DM. Corneal neovascularization: pathogenesis and inhibition. *Cornea.* 1987;6:250–257.
2. Kenyon BM, Voest EE, Chen CC, Flynn E, Folkman J, D'Amato RJ. A model of angiogenesis in the mouse cornea. *Invest Ophthalmol Vis Sci.* 1996;37:1625–1632.
3. Amano S, Rohan R, Kuroki M, Tolentino M, Adamis AP. Requirement for vascular endothelial growth factor in wound- and inflammation-related corneal neovascularization. *Invest Ophthalmol Vis Sci.* 1998;39:18–22.
4. Kim CH, Broxmeyer HE. Chemokines: signal lamps for trafficking of T and B cells for development and effector function. *J Leukoc Biol.* 1999;65:6–15.
5. Raport CJ, Gosling J, Schweickart VL, Gray PW, Charo IF. Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1beta, and MIP-1alpha. *J Biol Chem.* 1996;271:17161–17166.
6. Samson M, LaRosa G, Libert F, et al. The second extracellular loop of CCR5 is the major determinant of ligand specificity. *J Biol Chem.* 1997;272:24934–24941.
7. Risau W. Mechanisms of angiogenesis. *Nature.* 1997;386:671–674.
8. Lindner V, Majack RA, Reidy MA. Basic fibroblast growth factor stimulates endothelial regrowth and proliferation in denuded arteries. *J Clin Invest.* 1990;85:2004–2008.
9. Koch AE, Polverini PJ, Kunkel SL, et al. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science.* 1992;258:1798–1801.
10. Berger O, Gan X, Gujuluva C, et al. CXC and CC chemokine receptors on coronary and brain endothelia. *Mol Med.* 1999;5:795–805.

11. Moromizato Y, Stechschulte S, Miyamoto K, et al. CD18 and ICAM-1-dependent corneal neovascularization and inflammation after limbal injury. *Am J Pathol.* 2000;157:1277–1281.
12. Philipp W, Speicher L, Humpel C. Expression of vascular endothelial growth factor and its receptors in inflamed and vascularized human corneas. *Invest Ophthalmol Vis Sci.* 2000;41:2514–2522.
13. Pearlman E, Hall LR, Higgins AW, et al. The role of eosinophils and neutrophils in helminth-induced keratitis. *Invest Ophthalmol Vis Sci.* 1998;39:1176–1182.
14. Chang E, Galle L, Maggs D, Estes DM, Mitchell WJ. Pathogenesis of herpes simplex virus type 1-induced corneal inflammation in perforin-deficient mice. *J Virol.* 2000;74:11832–11840.
15. Tumpey TM, Cheng H, Cook DN, Smithies O, Oakes JE, Lausch RN. Absence of macrophage inflammatory protein-1α prevents the development of blinding herpes stromal keratitis. *J Virol.* 1998;72:3705–3710.
16. Boshoff C, Endo Y, Collins PD, et al. Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines. *Science.* 1997;278:290–294.
17. Torres PF, De Vos AF, van der Gaag R, Martins B, Kijlstra A. Cytokine mRNA expression during experimental corneal allograft rejection. *Exp Eye Res.* 1996;63:453–461.
18. King WJ, Comer RM, Hudde T, Larkin DF, George AJ. Cytokine and chemokine expression kinetics after corneal transplantation. *Transplantation.* 2000;70:1225–1233.
19. Schecter AD, Calderon TM, Berman AB, et al. Human vascular smooth muscle cells possess functional CCR5. *J Biol Chem.* 2000;275:5466–5471.
20. Kuziel WA, Maeda N. CCR2 and CCR5. In: Mak TW, ed. *The Gene Knockout Facts Book.* London: Academic Press; 1998:118–121.
21. Huffnagle GB, McNeil LK, McDonald RA, et al. Cutting edge: role of C-C chemokine receptor 5 in organ-specific and innate immunity to Cryptococcus neoformans. *J Immunol.* 1999;163:4642–4646.
22. Proia AD, Chandler DB, Haynes WL, et al. Quantitation of corneal neovascularization using computerized image analysis. *Lab Invest.* 1988;58:473–479.
23. Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG. Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. *Lab Invest.* 1998;78:1385–1394.
24. Polverini PJ, Cotran PS, Gimbrone MA Jr, Unanue ER. Activated macrophages induce vascular proliferation. *Nature.* 1977;269:804–806.
25. Ding Z, He Y, Wei H. A morphological study of experimental corneal neovascularization [in Chinese]. *Chung Hua Yen Ko Tsa Chih.* 1995;31:49–51.
26. Sonoda K, Sakamoto T, Yoshikawa H, et al. Inhibition of corneal inflammation by the topical use of Ras farnesyltransferase inhibitors: selective inhibition of macrophage localization. *Invest Ophthalmol Vis Sci.* 1998;39:2245–2251.
27. Van der Veen G, Broersma L, Dijkstra CD, Van Rooijen N, Van Rij G, Van der Gaag R. Prevention of corneal allograft rejection in rats treated with subconjunctival injections of liposomes containing dichloromethylene diphosphonate. *Invest Ophthalmol Vis Sci.* 1994;35:3505–3515.
28. Kaufmann A, Salentin R, Gemsa D, Sprenger H. Increase of CCR1 and CCR5 expression and enhanced functional response to MIP-1α during differentiation of human monocytes to macrophages. *J Leukoc Biol.* 2001;69:248–252.
29. Fantuzzi L, Borghi P, Ciolli V, Pavlakis G, Belardelli F, Gessani S. Loss of CCR2 expression and functional response to monocyte chemotactic protein (MCP-1) during the differentiation of human monocytes: role of secreted MCP-1 in the regulation of the chemotactic response. *Blood.* 1999;94:875–883.
30. Tumpey TM, Cheng H, Yan XT, et al. Chemokine synthesis in the HSV-1-infected cornea. *J Leukoc Biol.* 1998;63:486–492.
31. Sosne G, Szliter EA, Barrett R, et al. Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. *Exp Eye Res.* 2002;74:293–299.
32. Kernacki KA, Barrett RP, McClellan S, et al. MIP-1alpha regulates CD4+ T cell chemotaxis and indirectly enhances PMN persistence in Pseudomonas aeruginosa corneal infection. *J Leukoc Biol.* 2001;70:911–919.
33. Hall LR, Diaconu E, Patel R, et al. CXC chemokine receptor 2 but not C-C chemokine receptor 1 expression is essential for neutrophil recruitment to the cornea in helminth-mediated keratitis (river blindness). *J Immunol.* 2001;166:4035–4041.
34. Klotz O, Park JK, Pleyer U, et al. Inhibition of corneal neovascularization by alpha(v)-integrin antagonists in the rat. *Graefes Arch Clin Exp Ophthalmol.* 2000;238:88–93.