Protective Roles of the Fractalkine/CX3CL1-CX3CR1 Interactions in Alkali-Induced Corneal Neovascularization through Enhanced Antiangiogenic Factor Expression

Peirong Lu, Longbiao Li, Kouji Kuno, Yu Wu, Tomohisa Baba, Ying-yi Li, Xueguang Zhang and Naofumi Mukaida

*J Immunol* 2008; 180:4283-4291; doi: 10.4049/jimmunol.180.6.4283
http://www.jimmunol.org/content/180/6/4283

References

This article cites 61 articles, 26 of which you can access for free at:
http://www.jimmunol.org/content/180/6/4283.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Protective Roles of the Fractalkine/CX3CL1-CX3CR1 Interactions in Alkali-Induced Corneal Neovascularization through Enhanced Antiangiogenic Factor Expression

Peirong Lu,†‡ Longbiao Li,† Kouji Kuno,§ Yu Wu,‡ Tomohisa Baba,‡ Ying-yi Li,‡ Xueguang Zhang,§ and Naofumi Mukaida‡‡

Macrophages accumulate during the course of corneal neovascularization, but its mechanisms and roles still remain elusive. To address these points, we herein examined corneal neovascularization after alkali injury in mice deficient in fractalkine receptor/CX3CR1, which is normally expressed by macrophages. After alkali injury, the mRNA expression of CX3CR1 was augmented along with accumulation of F4/80-positive macrophages and Gr-1-positive neutrophils in the corneas. Compared with wild-type mice, CX3CR1-deficient mice exhibited enhanced corneal neovascularization 2 wk after injury, as evidenced by enlarged CD31-positive areas. Concomitantly, the accumulation of F4/80-positive macrophages, but not Gr-1-positive neutrophils, was markedly attenuated in CX3CR1-deficient mice compared with wild-type mice. The intraocular mRNA expression of vascular endothelial growth factor (VEGF) was enhanced to similar extents in wild-type and CX3CR1-deficient mice after the injury. However, the mRNA expression of antiangiogenic factors, thrombospondin (TSP) 1, TSP-2, and a disintegrin and metalloprotease with thrombospondin (ADAMTS) 1, was enhanced to a greater extent in wild-type than CX3CR1-deficient mice. A double-color immunofluorescence analysis demonstrated that F4/80-positive cells also expressed CX3CR1 and ADAMTS-1 and that TSP-1 and ADAMTS-1 were detected in CX3CR1-positive cells. CX3CL1 enhanced TSP-1 and ADAMTS-1, but not VEGF, expression by peritoneal macrophages. Moreover, topical application of CX3CL1 inhibited corneal neovascularization at 2 wk, along with enhanced intraocular expression of TSP-1 and ADAMTS-1 but not VEGF. Thus, these observations indicate that accumulation of CX3CR1-positive macrophages intraocularly can dampen alkali-induced corneal neovascularization by producing antiangiogenic factors such as TSP-1 and ADAMTS-1 and suggest the potential therapeutic efficacy of using CX3CL1 against alkali-induced corneal neovascularization. The Journal of Immunology, 2008, 180: 4283–4291.

Corneal neovascularization (CNV) can arise from various causes, including corneal infections, misuse of contact lens, chemical burn, and inflammation (1–3) and frequently leads to impaired vision. Moreover, survival rates of corneal grafts were markedly reduced when placed into vascularized recipient beds or when CNV develops in the transplanted cornea.
accumulation as well as CNV formation, when chemical and mechanical denudation was applied to corneal and limbal epithelium (18, 19). In contrast, several groups have provided evidence of the antiangiogenic activities of infiltrating macrophages in choroidal neovascularization (20, 21).

Mouse monocyttes/macrophages also express CX3CR1 on their surface (22, 23). Moreover, a ligand for CX3CR1, fractalkine, is highly expressed on vascular endothelial cells and therefore is presumed to have a role in angiogenesis (24, 25). Furthermore, the dichotomy of monocytes/macrophages has been proposed based on their expression levels of CCR2 and CX3CR1 (26–30). Thus, it is tempting to speculate that CX3CR1-positive monocytes/macrophages may exhibit distinct activities in CNV, compared with CCR2-positive ones. Hence, to address this assumption, we compared the molecular pathological changes in wild-type (WT) and CX3CR1-deficient mice, involving a frequently used model of ocular corneal neovascularization, namely, alkali injury-induced CNV (18, 19).

Materials and Methods

Reagents and Abs

Recombinant mouse CX3CL1 (571-MF/CF) and recombinant mouse CCL2 were obtained from R&D Systems. Rat anti-mouse F4/80 mAb (clone A3-1) and rat anti-mouse CD68 mAb (clone FA-11) were obtained from Serotec and rabbit anti-mouse CD31 polyclonal Abs (pAbs) were purchased from Abcam (ab28364). Rat anti-mouse-Ly-6G mAb (clone 1A8, catalog no. 551459) and rat anti-mouse pan-NK cell mAb (clone DX5) were obtained from BD Pharmingen. Goat anti-ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin type 1; L-16, sc-31080); goat anti-thrombospondin (TSP) 1 (N-20), and goat anti-CX3CL1 pAbs (M-18) were obtained from Santa Cruz Biotechnology and rabbit anti-ADAMTS-1 pAbs (ALX-210-555) were from Alexis Biochemicals. Alexa Fluor 488 donkey anti-goat IgG (H + L), donkey anti-rat IgG (H + L) as well as Alexa Fluor 594 donkey anti-rabbit IgG (H + L) were purchased from Invitrogen Life Technologies. Rabbit anti-CX3CR1 pAbs were provided by Dr. T. Imai (Kan Research Institute, Kyoto, Japan).

Mice

CX3CR1-deficient mice were prepared as described previously (25) and were a gift from Dr. P. M. Murphy and J.-L. Gao (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The mice were backcrossed to BALB/c mice for more than eight generations. Pathogen-free BALB/c mice were obtained from Clea Japan and were designated as WT mice. Age- and sex-matched CX3CR1-deficient and WT mice were kept under specific pathogen-free conditions in groups of five and fed regular laboratory chow and water ad libitum. A 12-h day and night cycle was maintained during the whole course of the study. All animal experiments were performed at the Institute for Experimental Animals (Kanazawa University Advanced Science Research Center, Kanazawa, Japan) in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University and were approved by the Committee on Animal Experimentation of Kanazawa University.

Alkali-induced corneal injury model

Mice were anesthetized with an i.p. injection of 1.8% (v/v) Avertin at a dose of 0.15 ml/10 g body weight. A 2-mm disc of filter paper saturated with 1 N NaOH was placed onto the right cornea of each mouse for 40 s, followed by rinsing extensively with 15 ml of PBS. The corneal epithelia were removed using a corneal knife in a rotary motion parallel to the limbus by gently scraping over the corneal surface without injuring the underlying corneal stroma (10). Erythromycin ophthalmic ointment was applied to the limbus by gently scraping over the corneal surface without injuring the underlying corneal stroma (10). Erythromycin ophthalmic ointment was followed by rinsing extensively with 15 ml of PBS. The corneal epithelia were removed from each eye. These corneas were placed immediately into RNALatex (Quagen) and kept at −86°C until total RNA extraction. In another series of experiments, mice were killed at the indicated time points (days 0, 2, 4, 14, and 28) after alkali treatment and both eyes were entirely removed from each animal. These eyes were fixed in 10% neutral formalin buffer for histological analysis. The left eye of each mouse was used as an untreated control. In some experiments, recombinant CX3CL1 protein was dissolved in 0.2% sodium hyaluronate (Sigma-Aldrich) at 2.5 μg/μl. Five microliters of CX3CL1 preparation or vehicle was applied topically to the alkali-treated eye twice a day for 7 days. Each experiment was repeated at least three times.

Biomicroscopic examination

Eyes were examined under a Leica MZ16 surgical microscope 14 days after alkali injury. In brief, under anesthesia, photographs of the corneas were obtained using a Leica DFC350FX digital camera that was linked to an operating microscope. Microscopic assessment was done by two independent observers without prior knowledge of the experimental procedures.

Histological and immunohistochemical analysis

The paraffin-embedded tissues were cut into 5-μm-thick slices, mounted on poly-L-lysine-coated slides, and subjected to H&E staining. For immunohistochemical staining, the deparaffinized sections or fixed cryosections (8-μm thick) were used. Endogenous peroxidases were quenched in 0.3% (v/v) hydrogen peroxide for 10 min. For Ag retrieval, sections were treated with 0.1% trypsin in 0.1% CaCl2 for 20 min at 37°C or immersed in 10 mM sodium citrate buffer (pH 6.0) and heated for 20 min in a microwave oven. After washing with PBS, slides were incubated with blocking reagent for 20 min. For the detection of macrophages, the sections were incubated overnight at 4°C with rat anti-mouse F4/80 Ab (1 μg/ml) or rat anti-mouse CD68 Ab (1 μg/ml). Tissue sections were then incubated with biotin-conjugated anti-rat Ig Ab as secondary Abs. To identify neutrophils and NK cells, the sections were incubated overnight at 4°C with purified rat anti-mouse-Ly-6G (2.5 μg/ml) and purified rat anti-mouse pan-NK cell mAbs (2.5 μg/ml), respectively. The sections were further incubated with biotin-conjugated rabbit anti-rat Ig Ab or biotin-conjugated rabbit anti-rat IgM Ab as the secondary Abs. Other sets of sections were incubated overnight at 4°C with rabbit anti-CX3CR1 and goat anti-CX3CL1 pAbs to detect CX3CR1 and CX3CL1 expression, respectively. These slides were incubated with biotin-conjugated anti-rabbit or anti-goat Ig Ab as the secondary Abs. The immune complexes were detected by using an ABC kit and a DAB Substrate Kit from Vector Laboratories according to the manufacturer’s instructions. Slides were then counterstained with hematoxylin and mounted. The numbers of positive cells were counted on five randomly chosen fields of corneal sections in each animal at 200-fold magnification, by an examiner without any prior knowledge of the experimental procedures. The numbers of positive cells per mm2 were calculated.

Enumeration of CNV

The deparaffinized sections (5-μm thick) or fixed cryosections (8-μm thick) were stained using anti-CD31 pAbs and the numbers and sizes of the areas of CNV were evaluated on at least two sections from each eye. The deparaffinized sections or fixed cryosections were analyzed by immunohistochemical analysis at the indicated time points after alkali injury. The numbers of corneal infiltrating inflammatory cells per mm2 were calculated as described in Materials and Methods. All values represent means ± SEM of three to five independent measurements.

![FIGURE 1. Changes with time in each population of intracorneally accumulating inflammatory cells after alkali injury. The harvested corneas were analyzed by immunohistochemical analysis at the indicated time points after alkali injury. The numbers of corneal infiltrating inflammatory cells per mm² were calculated as described in Materials and Methods. All values represent means ± SEM of three to five independent measurements.](http://www.jimmunol.org/graphtext)
Materials and Methods

Total RNAs were extracted from the corneas or cultured peritoneal macrophages with the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The RNA preparations were further treated with RNase-free DNase I (Invitrogen Life Technologies) to remove residual genomic DNA. Two micrograms of total RNA was reverse-transcribed at 37°C for 1 hi n 2 0

turer’s instructions. The RNA preparations were further treated with phages with the RNeasy Mini Kit (Qiagen) according to the manufac-

Total RNAs were extracted from the corneas or cultured peritoneal macrophages 4 days after the injury was determined by a flow cytometric analysis. Purified mononuclear cells were stained with FITC-conjugated rat anti-mouse F4/80 Abs and rabbit anti-CX3CR1 Abs (open heavy-lined histogram) or nonimmunized rabbit IgG (filled histogram) as a negative control. 

**Flow cytometric analysis of intracorneally infiltrating mononuclear cells**

Mononuclear cells were isolated from corneas according to the procedure described previously by Ohshima et al. (33), with some modifications. At 2 days after the alkali injury, corneas were removed, teased away with scissors, and incubated at 37°C for 30 min with constant shaking in the presence of 0.5 ng/ml collagenase type D (Roche Diagnostics). Cell suspensions were then passed over a nylon filter with a 100-

**A double-color immunofluorescence analysis**

Sections were deparaffinized and treated with 0.1% trypsin for 15 min at 37°C for Ag retrieval. Thereafter, the sections were incubated with the combinations of rat anti-mouse F4/80 and rabbit anti-mouse CX3CR1, rat anti-F4/80 and rabbit anti-ADAMTS-1, goat anti-ADAMTS-1 and rabbit anti-mouse CX3CR1, or goat anti-TSP-1 and rabbit anti-mouse CX3CR1 Abs overnight at 4°C. After being rinsed with PBS, for a double-immuno-

**FIGURE 2.** A, Quantitative RT-PCR to assess mRNA expression of CX3CR1 and its ligand CX3CL1 using the obtained total RNAs from corneas at the indicated time points as described in Materials and Methods. Representative results from three independent experiments are shown here. B, CX3CR1 and CX3CL1 protein expression in injured corneas of WT mice. Corneal tissues were obtained 0, 2, 4, and 7 days after the injury from WT mice. Tissues were stained with anti-CX3CR1 Abs (upper panels) and anti-CX3CL1 Abs (lower panels). Representative results from five individual animals are shown here. Original magnification, ×400. Scale bar, 50 μm. C, The numbers of infiltrated CX3CR1-positive cells were determined as described in Materials and Methods and mean and SEM are shown here (n = 5). D, The CX3CR1 expression on the accumulated F4/80-positive macrophages 4 days after the injury was determined by a flow cytometric analysis. Purified mononuclear cells were stained with FITC-conjugated rat anti-mouse F4/80 Abs and rabbit anti-CX3CR1 Abs (open heavy-lined histogram) or nonimmunized rabbit IgG (filled histogram) as a negative control followed by staining with Alexa Fluor 647-conjugated goat anti-rabbit IgG. Most mononuclear cells were positive for F4/80. A representative result from three independent experiments is shown.

**Real-time quantitative RT-PCR**

Real-time quantitative RT-PCR

Total RNAs were extracted from the corneas or cultured peritoneal macrophages with the RNase-free DNase I (Invitrogen Life Technologies) to remove residual genomic DNA. Two micrograms of total RNA was reverse-transcribed at 42°C for 1 h in 20 μl of reaction mixture containing mouse Moloney leukemia virus reverse transcriptase and hexanucleotide random primers (Qiagen). Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR System using the comparative threshold cycle (CT) quantification method. TaqMan Gene Expression Assays (Applied Biosystems) containing specific primers (accession numbers: CX3CL1, Mm00436454_m1; CX3CR1, Mm02620111_s1; TSP-1, Mm00449032_g1; TSP-2, Mm00449036_m1; ADAMTS-1, Mm00477355_m1; VEGF, Mm00477355_m1; TGF-β1, Mm03024053_m1; GAPDH, Mm99999915_m1), and TaqMan MGB probe (FAM dye labeled)/TaqMan Fast Universal PCR Master Mix were used with 10 ng of cDNA to detect and quantify the expression levels of CX3CL1, CX3CR1, TSP-1, TSP-2, ADAMTS-1, VEGF, and TGF-β1. Reactions were performed for 20 s at 95°C, then with 40 cycles of 1 s at 95°C and 20 s at 60°C. GAPDH was amplified as an internal control. C_{T} values of GAPDH were subtracted from C_{T} values of the target genes (ΔC_{T}). ΔC_{T} values of WT mice were compared with ΔC_{T} values of CX3CR1-deficient mice.

**A double-color immunofluorescence analysis**

Sections were deparaffinized and treated with 0.1% trypsin for 15 min at 37°C for Ag retrieval. Thereafter, the sections were incubated with the combinations of rat anti-mouse F4/80 and rabbit anti-mouse CX3CR1, rat anti-F4/80 and rabbit anti-ADAMTS-1, goat anti-ADAMTS-1 and rabbit anti-mouse CX3CR1, or goat anti-TSP-1 and rabbit anti-mouse CX3CR1 Abs overnight at 4°C. After being rinsed with PBS, for a double-immuno-

**FIGURE 3.** Macrophage accumulation in injured corneas of WT and CX3CR1-deficient mice. A, Corneal tissues from WT mice (left panels) or CX3CR1-deficient mice (right panels) obtained 0 (upper panels) or 4 days (middle and lower panels) after the injury were stained with rat anti-F4/80 (upper and middle panels) or rat anti-CD68 (lower panels) mAbs. Original magnification, ×400. Scale bar, 50 μm. B, The numbers of accumulated F4/80-positive or CD68-positive macrophages were determined as described in Materials and Methods and the mean and SEM are shown here (n = 5). *p < 0.05; **p < 0.01, WT vs CX3CR1-deficient mice.
For a double-color immunofluorescence analysis of CX3CR1 and antiangiogenic factors, the sections were further incubated with the combination of Alexa Fluor 488 donkey anti-goat IgG and Alexa Fluor 594 donkey anti-rabbit IgG (1/100) for 40 min at room temperature in the dark. Finally, the sections were washed with PBS and immunofluorescence was visualized in a dual-channel mode on an Olympus fluorescence microscope. Images were processed using Adobe Photoshop software version 7.0.

**Murine peritoneal macrophage isolation and culture**

Specific pathogen-free 8- to 10-wk-old male WT or CX3CR1-deficient mice were injected i.p. with 2 ml of sterile 3% thioglycolate medium (Sigma-Aldrich), and i.p. macrophages were harvested 3 days later as described previously (34). The resultant cell preparation consists of ≥95% macrophages as verified by using flow cytometric analysis on the cell preparation immunostained with anti-F4/80 Ab. The cells were suspended in antibiotic-free RPMI 1640 medium containing 10% FBS, and incubated in a humidified incubator at 37°C in 5% CO2 in 24-well cell culture plates. Two hours later, nonadherent cells were removed and the medium was replaced. The cells were then stimulated with the indicated concentrations of murine CX3CL1 or CCL2 for 12 h. Total RNAs were extracted from the cultured cells and subjected to quantitative RT-PCR as described above. For an immunocytochemical analysis of ADAMTS-1 expression, murine macrophages were seeded onto the wells of a Lab-Tec chamber slide with eight wells (Nalge Nunc) at 5 × 10^5 cells/well. After adhesion, the cells were stimulated with the indicated concentration of murine CX3CL1 for 24 h in a 37°C incubator with 5% CO2 and then subjected to immunocytochemical study as previous described (35).

**Statistical analysis**

The means and SEM were calculated on all parameters determined in the study. Data were analyzed statistically using one-way ANOVA or two-tailed Student’s t test. A value of p < 0.05 was accepted as statistically significant.

**Results**

**CX3CR1-CX3CRL1 expression after alkali-induced corneal injury**

Normal corneas do not contain any leukocytes or vascular structures. In line with our previous observations (10), alkali injury markedly increased the numbers of F4/80-positive macrophages and Gr-1-positive neutrophils in the cornea, reaching maximal levels at 4 days after the injury and decreasing thereafter (Fig. 4).

For a double-color immunofluorescence analysis of CX3CR1 and antiangiogenic factors, the sections were further incubated with the combination of Alexa Fluor 488 donkey anti-goat IgG and Alexa Fluor 594 donkey anti-rabbit IgG (1/100) for 40 min at room temperature in the dark. Finally, the sections were washed with PBS and immunofluorescence was visualized in a dual-channel mode on an Olympus fluorescence microscope. Images were processed using Adobe Photoshop software version 7.0.

**Murine peritoneal macrophage isolation and culture**

Specific pathogen-free 8- to 10-wk-old male WT or CX3CR1-deficient mice were injected i.p. with 2 ml of sterile 3% thioglycolate medium (Sigma-Aldrich), and i.p. macrophages were harvested 3 days later as described previously (34). The resultant cell preparation consists of ≥95% macrophages as verified by using flow cytometric analysis on the cell preparation immunostained with anti-F4/80 Ab. The cells were suspended in antibiotic-free RPMI 1640 medium containing 10% FBS, and incubated in a humidified incubator at 37°C in 5% CO2 in 24-well cell culture plates. Two hours later, nonadherent cells were removed and the medium was replaced. The cells were then stimulated with the indicated concentrations of murine CX3CL1 or CCL2 for 12 h. Total RNAs were extracted from the cultured cells and subjected to quantitative RT-PCR as described above. For an immunocytochemical analysis of ADAMTS-1 expression, murine macrophages were seeded onto the wells of a Lab-Tec chamber slide with eight wells (Nalge Nunc) at 5 × 10^5 cells/well. After adhesion, the cells were stimulated with the indicated concentration of murine CX3CL1 for 24 h in a 37°C incubator with 5% CO2 and then subjected to immunocytochemical study as previous described (35).

**Statistical analysis**

The means and SEM were calculated on all parameters determined in the study. Data were analyzed statistically using one-way ANOVA or two-tailed Student’s t test. A value of p < 0.05 was accepted as statistically significant.

**Results**

**CX3CR1-CX3CRL1 expression after alkali-induced corneal injury**

Normal corneas do not contain any leukocytes or vascular structures. In line with our previous observations (10), alkali injury markedly increased the numbers of F4/80-positive macrophages and Gr-1-positive neutrophils in the cornea, reaching maximal levels at 4 days after the injury and decreasing thereafter (Fig. 4).

**Statistical analysis**

The means and SEM were calculated on all parameters determined in the study. Data were analyzed statistically using one-way ANOVA or two-tailed Student’s t test. A value of p < 0.05 was accepted as statistically significant.

**Results**

**CX3CR1-CX3CRL1 expression after alkali-induced corneal injury**

Normal corneas do not contain any leukocytes or vascular structures. In line with our previous observations (10), alkali injury markedly increased the numbers of F4/80-positive macrophages and Gr-1-positive neutrophils in the cornea, reaching maximal levels at 4 days after the injury and decreasing thereafter (Fig. 4).

**Statistical analysis**

The means and SEM were calculated on all parameters determined in the study. Data were analyzed statistically using one-way ANOVA or two-tailed Student’s t test. A value of p < 0.05 was accepted as statistically significant.

**Results**

**CX3CR1-CX3CRL1 expression after alkali-induced corneal injury**

Normal corneas do not contain any leukocytes or vascular structures. In line with our previous observations (10), alkali injury markedly increased the numbers of F4/80-positive macrophages and Gr-1-positive neutrophils in the cornea, reaching maximal levels at 4 days after the injury and decreasing thereafter (Fig. 4).

**Statistical analysis**

The means and SEM were calculated on all parameters determined in the study. Data were analyzed statistically using one-way ANOVA or two-tailed Student’s t test. A value of p < 0.05 was accepted as statistically significant.
3.0/mm²; day 7, 4.3 and anti-ADAMTS-1 (C) detected after the injury (day 2, undetectable; day 4, 8.8
phological criteria (data not shown). Only a few NK cells were
stained tissue samples were identified as macrophages by mor-
1). Most of the infiltrated mononuclear cells observed in H&E-
stained tissue samples were identified as macrophages by mor-
expression of CX3CR1 and its ligand CX3CL1 in corneas after
expression was enhanced in eyes 2 days after injury and further
rice, by corneal stroma of untreated WT mice. At 2–4 days
expression by murine peritoneal macrophages stimulated with CX3CL1 or
expression by murine peritoneal macrophages stimulated with CX3CL1 or
(C) or anti-CX3CR1 and anti-TSP-1 (D) Abs as described in Materials and Methods and observed under a fluores-
cence microscopy. Original magnification, ×400. Signals were
digitally merged in the right panels. Arrows, The double positively
1). Most of the infiltrated mononuclear cells observed in H&E-
stained tissue samples were identified as macrophages by mor-
expression of CX3CR1 and its ligand CX3CL1 in corneas after
expression was barely detected in untreated eyes, but its
mRNA was detected 7 days after the injury, CNV was not detected microscopically
by macrophages as described in Materials and Methods. The levels of VEGF, ADAMTS-1, and TSP-1 mRNA were determined and normalized to GAPDH mRNA levels. Each value represents the mean and SEM (n = 3). *, p < 0.05; **, p < 0.01, compared with untreated. B, Peritoneal macrophages from WT or
CX3CR1-deficient mice were incubated with CCL2 (10 ng/ml) for 12 h. Quantitative RT-PCR was performed on total RNAs extracted from the macrophages as described in Materials and Methods. The levels of VEGF, ADAMTS-1, and TSP-1 mRNA were determined and normalized to GAPDH mRNA levels. Each value represents the mean and SEM (n = 3). **, p < 0.01, compared with untreated. C–E, Peritoneal macrophages from WT mice were stimulated in the absence (D) or presence of CX3CL1 (10 ng/ml; C and E) for 24 h. The cells were then processed for immunocytochemical analysis using anti-ADAMTS-1 Abs (D and E) or control Abs (C) as described in Materials and Methods. The representative results from three independent experiments are shown. Original magnification, ×400. Scale bar, 50 µm.

**Reduced macrophage accumulation at the wound sites in CX3CR1-deficient mice**

Given the fact that CX3CR1 is expressed on macrophages (22, 23), we examined the effects of CX3CR1 deficiency on leukocyte accumulation in injured corneas. Ly-6G-positive neutrophils accumulated to a similar extent in the corneas of WT and CX3CR1-deficient mice after the injury (data not shown). On the contrary, the number of F4/80-positive cells was markedly depressed in CX3CR1-deficient mice compared with WT mice (Fig. 3). When another macrophage marker, CD68, was used to identify macrophages, similar results were obtained (Fig. 3). Thus, the CXCL1-CX3CR1 interactions may regulate F4/80- and CD68-positive macrophage, but not neutrophil accumulation in damaged corneas.

**Enhanced alkali injury induced CNV in CX3CR1-deficient mice**

We next explored CNV in CX3CR1-deficient and WT mice. Until 7 days after the injury, CNV was not detected microscopically (data not shown). By contrast, CNV was macroscopically evident in WT mice 2 wk after the injury, as we previously reported (10). At this time point, macroscopic CNV was more enhanced in

![FIGURE 6](http://www.jimmunol.org/)

A double-color immunofluorescence analysis of injured corneas. Corneas were obtained from WT mice 2 days after the injury. The samples were immunostained with a combination of anti-F4/80 and anti-CX3CR1 (A), anti-F4/80 and anti-ADAMTS-1 (B), anti-CX3CR1 and anti-ADAMTS-1 (C), or anti-CX3CR1 and anti-TSP-1 (D) Abs as described in Materials and Methods and observed under a fluorescence microscopy. Original magnification, ×400. Signals were digitally merged in the right panels. Arrows, The double positively stained cells. Representative results from three independent experiments are shown.

![FIGURE 7](http://www.jimmunol.org/)

Comparison of the levels of VEGF, TSP-1, and VEGF expression by murine peritoneal macrophages stimulated with CX3CL1 or CCL2. A, Peritoneal macrophages from WT mice were incubated with the indicated concentrations of CX3CL1 for 12 h. Quantitative RT-PCR was performed on total RNAs extracted from the macrophages as described in Materials and Methods. The levels of VEGF, ADAMTS-1, and TSP-1 mRNA were determined and normalized to GAPDH mRNA levels. Each value represents the mean and SEM (n = 3). *, p < 0.05; **, p < 0.01, compared with untreated. B, Peritoneal macrophages from WT or CX3CR1-deficient mice were incubated with CCL2 (10 ng/ml) for 12 h. Quantitative RT-PCR was performed on total RNAs extracted from the macrophages as described in Materials and Methods. The levels of VEGF, ADAMTS-1, and TSP-1 mRNA were determined and normalized to GAPDH mRNA levels. Each value represents the mean and SEM (n = 3). **, p < 0.01, compared with untreated. C–E, Peritoneal macrophages from WT mice were stimulated in the absence (D) or presence of CX3CL1 (10 ng/ml; C and E) for 24 h. The cells were then processed for immunocytochemical analysis using anti-ADAMTS-1 Abs (D and E) or control Abs (C) as described in Materials and Methods. The representative results from three independent experiments are shown. Original magnification, ×400. Scale bar, 50 µm.
CX3CR1-deficient mice than in WT mice (Fig. 4A). An immunohistochemical analysis using anti-CD31 Ab further demonstrated that vascular areas were increased to a larger extent in CX3CR1-deficient mice than in WT mice (Fig. 4, B and C). Moreover, we also observed that macroscopic CNV persisted in CX3CR1-deficient mice 4 wk after the injury, when CNV was resolved in WT mice (Fig. 4, B and C). These observations indicate that CX3CR1 deficiency enhanced the alkali-induced CNV, along with reduced macrophage accumulation.

Reduction in alkali injury-mediated antiangiogenic factor gene expression in CX3CR1-deficient mice

Monocytes/macrophages can be a rich source of both angiogenic and antiangiogenic factors (14, 16, 17, 20, 21, 36–38), and the balance between these two distinct sets of factors can determine the outcome of angiogenesis processes in various situations. Hence, we examined the mRNA expression of angiogenic and antiangiogenic factors in corneas after injury. The mRNA expression of two potent angiogenic factors, VEGF and TGF-β, was marginally augmented and to similar extents in WT and CX3CR1-deficient mice after the injury (Fig. 5, D and E). The intracorneal mRNA expression of the antiangiogenic factors TSP-1 and ADAMTS-1 and, to a lesser extent, TSP-2, was enhanced in WT mice after the injury, but their enhanced expression was depressed in CX3CR1-deficient mice (Fig. 5, A–C). These observations imply that CX3CR1 deficiency reduced antiangiogenic factor expression and favored angiogenesis.

CX3CR1-positive macrophages express antiangiogenic factors

In line with the previous observation that CX3CR1 is expressed on macrophages (22, 23), we observed that a substantial proportion of F4/80-positive macrophages expressed CX3CR1 (Figs. 2D and 6A). ADAMTS-1 protein was detected in F4/80-positive macrophages (Fig. 6B). Moreover, CX3CR1-positive cells, present in injured cornea, expressed antiangiogenic factors such as ADAMTS-1 (Fig. 6C) and TSP-1 (Fig. 6D). These observations imply that CX3CR1-positive macrophages counteracted alkali-induced CNV by expressing antiangiogenic factors such as TSP-1 and ADAMTS-1.

CX3CL1 stimulation enhanced antiangiogenic factor expression by murine peritoneal macrophages

Because the CX3CR1-mediated signal can enhance the functions of macrophages, we next examined the effects of exogenous CX3CL1 on antiangiogenic factor expression by murine peritoneal macrophages. CX3CL1 enhanced TSP-1 and ADAMTS-1, but not VEGF, mRNA expression by peritoneal macrophages (Fig. 7A). CX3CL1-stimulated macrophages consistently exhibited increased ADAMTS-1 protein expression compared with untreated ones (Fig. 7, D and E). By contrast, another macrophage-tropic chemokine, CCL2, enhanced VEGF, but not TSP-1 and ADAMTS-1, mRNA expression by peritoneal macrophages (Fig. 7A). CX3CL1-stimulated macrophages consistently exhibited increased ADAMTS-1 protein expression compared with untreated ones (Fig. 7, D and E). By contrast, another macrophage-tropic chemokine, CCL2, enhanced VEGF, but not TSP-1 and ADAMTS-1, mRNA expression by peritoneal macrophages (Fig. 7A). CX3CL1 can induce CX3CR1-positive macrophages to express antiangiogenic factors such as TSP-1 and ADAMTS-1, while another macrophage-tropic chemokine, CCL2, can induce CX3CR1-negative macrophages to express VEGF.

Topical administration of recombinant CX3CL1 attenuated alkali injury-induced CNV in WT mice

Finally, we examined the effects of topically applied CX3CL1 on alkali-induced CNV. Topical administration of CX3L1 alone
caused little accumulation of F4/80-positive macrophages in corneas (Fig. 8, A and B; day 2, undetectable; day 4, 16.8 ± 8.3). By contrast, topical administration of CX3CL1 augmented alkali injury-induced increases in the numbers of intracorneal macrophages. Similarly, the administration of CX3CL1 after alkali injury enhanced mRNA expression of antiangiogenic factors, TSP-1 and ADAMTS-1, but not VEGF, compared with vehicle treatment (Fig. 8C), whereas CX3CL1 alone failed to increase the mRNA expression of these genes (data not shown). Concomitantly, at 2 wk after the injury, the alkali-induced CNV was significantly attenuated by treatment with CX3CL1, but not with vehicle (Fig. 8, D–I). These results further suggest that CX3CL1 can be effective for prevention and/or treatment for alkali-induced CNV.

Discussion

We previously proved that alkali-induced CNV can develop independently of neutrophil accumulation (10) in contrast to several observations, which indicate the crucial involvement of neutrophils in various types of neovascularization (11, 12, 39–44). We observed that VEGF proteins were detected mainly in the accumulated mononuclear cells in this alkali-induced CNV (10). This may mirror the assumption that macrophages are a rich source of various angiogenic factors. Macrophages utilize various chemokine receptors such as CCR2 or CCR5 for their trafficking. Accordingly, CCR2- or CCR5-deficient mice exhibited reductions in macrophage accumulation and CNV after corneal injury (18, 19). Moreover, several lines of evidence would indicate that monocytes/macrophages can be divided into CCR2highCX3CR1low and CCR2lowCX3CR1high subpopulations (26–30). Indeed, a proportion of intracorneally accumulated macrophages expressed CX3CR1 in the absence of enhanced mRNA expression of its single ligand, CX3CL1, in the injured eyes. CX3CL1 is a membrane-bound chemokine and can be recycled between plasma membrane and a juxtanuclear compartment (45). Thus, it is tempting to speculate that alkali injury could increase the trafficking of CX3CL1 from a juxtanuclear compartment to the plasma membrane and eventually increase the amount of biologically active CX3CL1, which is localized in the plasma membrane. This, in turn, can induce intracorneal accumulation of CX3CR1-expressing macrophages.

These observations prompted us to investigate the roles of the CX3CL1-CX3CR1 interactions in alkali injury-induced CNV by comparing the molecular pathological changes between CX3CR1-deficient and WT mice. We observed that macrophage accumulation was substantially reduced in injured corneas of CX3CR1-deficient mice compared with WT mice, consistent with previous reports that Ab-mediated inhibition of CX3CL1 reduced macrophage migration into the affected organs in experimental allergic myositis and collagen-induced arthritis (46, 47). Thus, the CX3CL1-CX3CR1 interactions may have an important role in macrophage trafficking. However, CX3CR1 deficiency failed to abrogate completely macrophage accumulation into injured cornea, suggesting a contribution of other chemokines and/or other chemotactic factors to macrophage accumulation. Indeed, we observed that alkali injury enhanced intraocular mRNA expression of CCL2 and CCL3 (data not shown), the chemokines that exhibit potent chemotactic activities for macrophages. Thus, it is likely that alkali-induced macrophage accumulation is regulated by the combined action of CX3CL1 with other macrophage-tropic chemokines such as CCL2 and CCL3.

Macrophages are presumed to be proangiogenic in various types of pathological conditions (14, 17, 36, 37). Selective macrophage depletion with clodronate liposomes inhibited laser photocogulation-induced VEGF production and subsequent choroidal neovascularization (13). Furthermore, Ambati et al. (19, 20) reported that CNV and VEGF production was suppressed in mice lacking CCR2 or CCR5, the specific chemokine receptors for CCL2 and CCL3, respectively. Thus, CCR2- and/or CCR5-expressing macrophages can promote neovascularization, probably by producing VEGF. On the contrary, under our present experimental conditions, genetic depletion of CX3CR1 reduced alkali injury-induced macrophage accumulation but promoted CNV with little effects on intraocular VEGF expression. Similarly, Apte et al. (21) demonstrated that inhibition of macrophage accumulation promoted laser photocogulation-induced choroidal neovascularization, whereas direct injection of macrophages suppressed it (21). Considering the proposed hypothesis on the dichotomy of macrophages based on the expression levels of CCR2 and CX3CR1 (26–30), CX3CR1-expressing macrophages may exhibit biological functions distinct from CCR2-expressing ones, in the course of angiogenesis.

Normal cornea lacks any vasculature under physiological conditions. This physiological corneal avascularity is presumed to be maintained by the balance between angiogenic and antiangiogenic factor expression (48–52). Soluble VEGF receptor 1 is presumed to be a major antiangiogenic factor responsible for the maintenance of this physiological corneal avascularity by trapping constitutively expressed VEGF (53). However, it still remains to be established whether soluble VEGF receptor 1 can counteract CNV induced by corneal injury. Although CX3CR1-deficient mice exhibited intracorneal VEGF mRNA expression to a similar extent as WT mice, they still developed a more severe form of CNV than WT mice. Thus, the VEGF/soluble VEGF receptor 1 interactions may not be responsible for enhanced CNV observed in CX3CR1-deficient mice.

Alternatively, antiangiogenic molecules, TSP-1 and TSP-2, which are also constitutively present in cornea, can preclude vasculature development (48, 54, 55). TSP-1 levels in Bruch’s membrane and choroidal vessels was decreased in age-related macular degeneration (AMD) and a reduction in TSP-1 may permit the formation of choroidal neovascularization (56). Moreover, TSP-1 deficiency and, to a lesser extent, TSP-2 deficiency resulted in enhanced CNV induced by suture placement (48). TSP-1 and TSP-2 are processed to release antiangiogenic polypeptides by the action of ADAMTS-1 (57). ADAMTS-1 at a high concentration can also inhibit endothelial migration induced by combined treatment with VEGF and basic fibroblast growth factor (38). Mirroring its antiangiogenic activities, ADAMTS-1-deficient mice exhibited delayed skin wound closure, along with exaggerated neovascularization (57). Because the enhanced expression of TSP-1, TSP-2, and ADAMTS-1 was attenuated in CX3CR1-deficient mice following alkali injury, the reduced expression of these factors may be responsible for enhanced alkali-induced CNV in CX3CR1-deficient mice.

Monocyte/macrophages are a major source of TSP-1 (58), ADAMTS-1 was also detected in infiltrating macrophages in the early phase of skin wound sites (38). TSP-1 and ADAMTS-1 proteins were consistently detected in F4/80-positive and CX3CR1-positive cell populations in this model of alkali-induced cornea injury. Moreover, CX3CL1 augmented TSP-1 and ADAMTS-1 expression by murine peritoneal macrophages. Thus, potent antiangiogenic molecules, TSP-1 and ADAMTS-1, may be expressed by CX3CR1-positive macrophages. This may account for protective roles of the CX3CL1-CX3CR1 interactions in alkali injury-induced CNV.

Chemical burns of the eyes can destroy surface epithelia and cause ischemic necrosis of eye tissues and, if not treated properly, CNV ensues and impairs vision. Alkali, once applied onto eyes, is
difficult to be completely rinsed away due to its rapid penetration into the anterior chamber and, therefore, an effective maneuver(s) is required to prevent alkali injury-induced CNV. Glucocorticoids are widely used for alkali injury of eyes with limited therapeutic benefits (59, 60). In this study, we demonstrated that topical application of CX3CL1 markedly augmented the intraocular expression of antiangiogenic molecules, TSP-1 and ADAMTS-1, without affecting VEGF expression. Moreover, topical application of CX3CL1, even when started after the injury, was very effective in preventing alkali injury-induced CNV. These observations raise the possibility of CX3CL1 as a preventive drug for alkali injury-induced CNV.

Two single nucleotide polymorphisms within the CX3CR1 gene were reported to be associated with AMD and reduce CX3CR1 protein expression (61, 62). Because choroidal neovascularization were reported to be associated with AMD and reduce CX3CR1 gene inhibits choroidal neovascularization.

References

We thank Drs. Philip M. Murphy and Ji-Liang Gao (National Institute of Allergy and Infectious Diseases, National Institutes of Health) for their technical assistance. We express our thanks to Dr. Joost J. Oppenheim (National Cancer Institute, Frederick, MD) for his critical reading of this manuscript.

Disclosures

The authors have no financial conflict of interest.

References


