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CXCL1/KC and CXCL5/LIX are selectively produced by corneal fibroblasts and mediate neutrophil infiltration to the corneal stroma in LPS keratitis

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Abstract

The severity of corneal inflammation depends on the activity of infiltrating neutrophils responding to chemotactic factors such as CXC chemokines. This study examines the relative contribution of CXCL1/keratinocyte-derived chemokine (KC), CXCL2/monocyte-inhibitory protein-2 (MIP-2), and CXCL5/LPS-induced chemokine (LIX) in neutrophil recruitment to the corneal stroma during LPS keratitis, where neutrophils infiltrate the corneal stroma at 6 h after LPS injection and peak at 24 h. Consistent with this timeframe, KC was detected after 3 h, reached peak levels at 24 h, and decreased thereafter. In contrast, LIX production was not detected until 8 h after injection and peaked at 24 h. MIP-2 was detected at 3 h but did not reach the levels of KC and LIX. Cell types associated with corneal inflammation produced markedly different chemokines in vitro: Murine corneal fibroblasts (MK/T-1) produced LIX and KC in response to LPS but did not produce MIP-2, whereas peritoneal macrophages and neutrophils produced MIP-2 and KC but did not produce LIX. To determine the role of these chemokines in neutrophil recruitment to the cornea, anti-LIX, anti-KC, or anti-MIP-2 was injected into the corneal stroma of enhanced GFP chimeric mice prior to LPS, and total cell and neutrophil infiltration was examined. Antibody to LIX and KC, injected individually or in combination, significantly inhibited neutrophil recruitment to the cornea, whereas anti-MIP-2 had no inhibitory effect. Together, these findings demonstrate cell-specific production of CXC chemokines and show that LIX and KC mediate neutrophil recruitment into the cornea during LPS keratitis.

Keywords

chemokine; macrophage; inflammation; MIP-2

INTRODUCTION

Neutrophils play a critical role in bacterial clearance during corneal infections; however, neutrophil infiltration and activation in the cornea also cause tissue damage, which can result in loss of corneal clarity, visual impairment, and even blindness. Pathogens that cause keratitis, in which neutrophils infiltrate the cornea, include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Onchocerca volvulus*, HSV-1, and fungal pathogens such as *Fusarium solani*. This recruitment of neutrophils to the cornea stroma is mediated in part by CXC chemokines [1–7].

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The chemokine family is divided into four main groups based on their structure and chemotactic activity for specific leukocyte populations: C, CC, CXC, and CX_3C. The subset of CXC chemokines containing a glycine-leucine-arginine (ELR) motif, immediately preceding the CXC residues, selectively target neutrophils. Although there are seven ELR^+ CXC chemokines in the human genome, only four have been identified in the murine genome: CXCL1/keratinocyte-derived chemokine (KC), CXCL2/monocyte-inhibitory protein-2 (MIP-2), CXCL5/LPS-induced chemokine (LIX), and CXCL15/lungkine [8–12]. KC was first identified from a cDNA library of a platelet-derived growth factor-induced fibroblast line [12]. MIP-2 was initially isolated from a LPS-stimulated macrophage cell line, RAW 264.7 [9]. LIX, originally identified from LPS-stimulated fibroblasts, was shown to have a role in neutrophil recruitment in rodent models of cardiac ischemia-reperfusion, colitis, and LPS-induced acute lung inflammation [8, 10, 13–16]. Although ELR^+ CXC chemokines mediate the regulation of neutrophil recruitment, migration, and activation at sites of infection/injury, the relative contribution of each CXC chemokine to these functions remains unclear.

To elucidate the role of these chemokines in neutrophil recruitment into the cornea, we examined the kinetics of CXC chemokine production in a mouse model of LPS-induced keratitis [17, 18] and also examined the production of these chemokines in corneal fibroblasts and peritoneal macrophages and neutrophils. In addition, we used neutralizing antibody to determine the role of these chemokines in corneal inflammation.

MATERIALS AND METHODS

Animals and reagents

C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Highly purified LPS (TLR4-specific) from *Escherichia coli* K12 was purchased from InVivoGen (San Diego, CA). Rat antimouse NIMP-R14 antibody was supplied by Achim Hoerauf (Bernard Nocht Institute of Tropical Medicine, Hamburg, Germany). Rat IgG_{2a}, rat IgG_{2b}, rat antimouse KC, rat antimouse LIX, and rat antimouse MIP-2 antibodies and mouse KC, MIP-2, and LIX ELISA kits were obtained from R&D Systems (Minneapolis, MN, USA). Dr. Robert Gendron (Memorial University of Newfoundland, Canada) [19] kindly provided MK/T-1 corneal stromal fibroblasts. Casein was purchased from Sigma-Aldrich (St. Louis, MO, USA). Percoll was obtained from GE Healthcare Biosciences (Piscataway, NJ, USA).

Murine model of LPS keratitis

Four- to 8-week-old female C57BL/6 mice were anesthetized by i.p. injection with 0.4 ml of a 1.2% solution of 2,2,2-tribromoethanol (Sigma-Aldrich) containing 2.5% 2-methyl-2-butanol (tertiary amyl alcohol, Sigma-Aldrich) dissolved in distilled water. After creation of a tunnel into the corneal stroma using a 33-gauge needle, 1 μg LPS in 2 μl HBSS (Invitrogen, Carlsbad, CA, USA) was injected into the central corneal stroma via a separate 33-gauge needle attached to a 5-μl glass syringe (Hamilton Co., Reno, NV, USA) as described previously [17]. Control corneas were injected with 2 μl HBSS. All mice were treated in accordance with The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Histology and immunohistochemistry

Mice were killed, and eyes were enucleated and snap-frozen in OCT (Tissue-Tek, Sakura, Torrance, CA, USA) using liquid nitrogen. Cryosections (5 μm) were stained for neutrophils using the rat antimouse mAb NIMP-R14, which is specific for neutrophils [20]. Briefly, sections were fixed in 4% formaldehyde for 30 min and rinsed in 0.05 M Tris buffer solution, pH 7.6. Sections were then incubated with the 8-μg/ml NIMP-R14 for 2 h. After

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washing, sections were incubated for 45 min with 5 μg/ml FITC-conjugated antirat IgG (H + L, Vector Laboratories, Burlingame, CA, USA). Sections were mounted with VectaShield mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories). Neutrophils, per 5 μm section, were counted (from limbus to limbus at 400×) by fluorescence microscopy (Olympus Optical Co. Ltd., Tokyo, Japan).

Cell culture and activation

MK/T-1 cells were maintained in DMEM/low glucose containing 10% FBS and 50 μg/ml hygromycin (Invitrogen) at 37°C with 5% CO_2. Cells were then stimulated with 100 ng/ml LPS in DMEM without hygromycin and FBS. For controls, cells were incubated with media alone. Supernatants collected at the indicated time-points were analyzed for KC, MIP-2, and LIX by ELISA.

Neutrophil isolation and activation

Neutrophils were isolated from the peritoneal cavity as described previously [21, 22]. Mice were injected twice i.p. with 1% casein solution containing 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$. Three hours after the second casein injection, a peritoneal lavage with 10 ml HBSS was performed to collect cells. After washing with HBSS, a cell count was performed using trypan blue, and the cells were brought to a concentration of 3–5 × 10^7 cells/ml in 1× PBS. Cell solution (1 ml) was added to a Percoll gradient solution (GE Healthcare Biosciences) and centrifuged at 31,500 rpm in an ultracentrifuge (Beckman-Coulter, Fullerton, CA, USA) with a 50-Ti rotor for 20 min at 4°C. The neutrophil cell layer was collected, washed with HBSS, and resuspended in DMEM containing 10% FBS. Diff-Quik stain (VWR, Bridgeport, NJ, USA) showed >98% neutrophil purity. Cells (1×10^5 per well) were preincubated in GM-CSF for 18 h at 37°C, with or without LPS (100 ng/ml). Supernatants were collected and assayed for the presence of chemokines by ELISA.

Macrophage isolation and activation

Macrophages were isolated from the peritoneal cavity using casein. Briefly, mice were injected i.p. with 1% casein solution containing 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$. After 24 h, a peritoneal lavage with 10 ml HBSS was performed to collect cells. After lysis of RBC using RBC lysis buffer (Sigma-Aldrich), remaining cells were washed with HBSS. A cell count was performed using trypan blue, and cells were resuspended in DMEM containing FBS, penicillin, and streptomycin (DMEM/FBS/P/S). Cells were allowed to adhere to the tissue-culture plate for 2 h. After removal of adherent cells, cells were incubated overnight at 37°C, 5% CO_2, in DMEM/FBS/P/S. Cells were then incubated with or without 100 ng/ml LPS in DMEM alone. Supernatants were collected at indicated time-points and assayed for the presence of chemokines by ELISA.

Quantification of CXC chemokines by ELISA

After LPS or HBSS treatment as indicated above, corneas were excised at the indicated time-points, the epithelium was removed following an incubation in 20 mM EDTA for 20 min, and the stroma were suspended in 200 μL RPMI 1640. As described previously, samples were sonicated for 88 s with 50% duty cycle (VibraCell, Sonics and Materials, Danbury, CT, USA) [6]. Tissue and in vitro LPS-stimulated, cell-free supernatant chemokine levels (collected as described above) were assessed by a sandwich ELISA according to the manufacturer's protocol (R&D Systems). The limit of detection of the assays was 15 pg/ml.

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Generation of enhanced GFP (eGFP) chimeric mice

eGFP chimeric mice were prepared as described previously [17]. Briefly, isolated bone marrow from the femur and tibia of C57Bl/6-transgenic mice (ACTbGFP, Jackson Laboratories) was reconstituted in sterile HBSS, incubated briefly in RBC lysis buffer (eBioscience, San Diego, CA, USA), and resuspended in DMEM. Bone marrow cells (2–5×10^6) were injected i.v. (tail-vein) in 0.2 ml into recipient C57Bl/6 mice, which had been exposed to 2 × 600 γ-doses of whole-body radiation 3 h apart. The eGFP chimeric mice were then used for subsequent experiments 2 weeks later.

In vivo inhibition of eGFP inflammatory cells

Corneas of eGFP chimeric mice were injected with 2 ul containing 15 ug neutralizing antibody to KC, LIX, MIP-2, control rat IgG_{2a}, or control rat IgG_{2b} 2 h prior to intrastromal injection of 200 ng LPS in 0.5 μl HBSS. [The anti-MIP-2 antibody 50% neutralizing dose (ND_{50}) is 1–4 ug/ml for 2 ng/ml recombinant murine (rm)MIP-2; anti-KC ND_{50}, 1–4 ug/ml antibody for 0.5 ug/ml rmKC; and anti-LIX ND_{50}, 0.5–2.5 ug/ml antibody for 0.2 ug/ml rmLIX.] Twenty hours after LPS injection, in vivo fluorescence stereomicroscopic images were captured using a MZFLIII microscope and Spot RT Slider KE camera (Leica Microsystems Inc., Bannockburn, IL; Diagnostics Instruments, Sterling Heights, MI, USA) as described previously [17]. The number of eGFP cells was quantified in a constant-defined area of the image (ImagePro Plus, Media Cybernetics, Carlsbad, CA, USA). Mice were killed, eyes were enucleated, and neutrophils were assessed as described above. Difference in the means was calculated, and percent inhibition was derived using Prism Graph-Pad software (San Diego, CA, USA).

Statistical analysis

Statistical analysis was performed with an unpaired *t*-test (Prism, GraphPad Software). $P < 0.05$ was considered significant.

RESULTS

CXCL1/KC, CXCL2/MIP-2, and CXCL5/LIX have distinct temporal expression in comparison with neutrophil infiltration in LPS-injected corneas

To examine the temporal production of CXC chemokines in the corneal stroma in relation to neutrophil infiltration, 1 μg LPS was injected into the corneal stroma of C57BL/6 mice. After 3, 8, 24, 48, and 72 h, corneas were dissected, and the corneal stroma was disrupted to measure KC, LIX, and MIP-2 production by ELISA, or eyes were processed for immunohistochemistry to quantify neutrophils in the cornea.

As shown in Figure 1, neutrophils were detected as early as 3 h after LPS injection and were mostly in the peripheral cornea (not shown). By 8 h, neutrophil numbers had increased and were detected throughout the cornea. Total neutrophil numbers peaked at 24 h and declined by 48 h. For CXC production, KC levels increased 800-fold within 3 h of injection and continued to increase to ~1500 pg/ml at 24 h. After this time, KC levels decreased in parallel with neutrophil numbers. MIP-2 was also detected within 3 h but remained at ~100 pg/ml for 24 h. In contrast, LIX was not detected until 8 h postinjection and gradually increased to 500 pg/ml at 24 h. By 48 h, KC and LIX levels had decreased and remained low or undetectable. In this model of corneal inflammation, chemokine production by naïve corneas and by corneas injected with saline was below the limit of detection (not shown).

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CXCL1/KC, CXCL2/MIP-2, and CXCL5/LIX are differentially expressed by corneal fibroblasts, macrophages, and neutrophils

During wound-healing or inflammation, keratocytes are activated, differentiate into fibroblasts, and produce a number of cytokines in response to LPS [23–28]. In addition, resident macrophages and infiltrating neutrophils are a potential source of cytokines [29–32]. To determine the relative CXC chemokine production by each cell type, corneal fibroblasts (MK/T-1) [19], peritoneal macrophages, and peritoneal neutrophils were stimulated in vitro with LPS. After stimulation, supernatants were examined for the production of each chemokine by ELISA.

As shown in Figure 2, LPS-stimulated corneal stromal fibroblasts produced LIX and KC with distinct temporal patterns. KC was detected within 2 h after stimulation and peaked within 6 h, whereas LIX production increased gradually, reaching similar concentrations after 18 h. MIP-2 was not detected (<15 pg/ml), even after 18 h. These findings demonstrate clearly that corneal fibroblasts produce KC and LIX but not MIP-2 in response to LPS treatment.

In contrast to the corneal fibroblasts, LIX was not detected in supernatants from LPS-stimulated neutrophils or macrophages, even after 18 h. However, MIP-2 production was elevated within 18 h in neutrophils and macrophages, respectively. In these cells, KC was also detected within 3–5 h but at lower levels compared with MIP-2 during this incubation period. Chemokine production by neutrophils incubated in the absence of LPS was less than 15 pg/ml.

Taken together, these results demonstrate a distinct cellular pattern of CXC chemokine production, where corneal fibroblasts are a major source of KC and LIX, but not MIP-2, and neutrophils and macrophages are a major source of MIP-2 and a minor source of KC but do not produce LIX.

CXCL1/KC and CXCL5/LIX mediate neutrophil infiltration to the corneal stroma

To determine which CXC chemokines mediate neutrophil recruitment to the cornea and migration through the corneal stroma, neutralizing antibodies to KC, LIX, or MIP-2 were injected into the corneal stroma of eGFP chimeric mice 2 h prior to LPS injection. These conditions are consistent with other studies [17, 18, 33] and are within the neutralizing capacity of these antibodies as described in Materials and Methods. Infiltration of total eGFP bone marrow-derived cells into the corneal stroma of live mice was detected by fluorescence microscopy, and images of the whole cornea were captured for analysis. After sacrifice, the eyes were then sectioned, and corneas were examined by immunohistochemistry to determine the number of neutrophils in the cornea.

Figure 3A shows representative images of whole corneas, flattened images (background fluorescence equalized), and analysis of cellular infiltrate at 20 h post-LPS injection. Figure 3B shows quantitative analysis of four corneas per group. Corneas of eGFP chimeric mice at 20 h post-LPS injection in the presence of control antibody showed an intense infiltrate of bone marrow eGFP cells throughout the cornea. Infiltration of these cells was significantly lower in corneas injected with anti-LIX or anti-KC (58±19.8% and 64±13.5% reduction, respectively; Fig. 3B). In contrast, there was no significant difference in the number of bone marrow-derived cells between anti-MIP-2 and control IgG isotype-treated corneas. Figure 3C shows that the number of neutrophils was also decreased significantly in corneas treated with anti-KC (54±5.6%) or anti-LIX (56±16%) compared with IgG isotype control-treated corneas, whereas anti-MIP-2-treated corneas did not show a significant decrease in neutrophil infiltration.

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As injection of neutralizing antibodies only had a partial inhibitory activity, we next determined if there was an additive or synergistic effect of these chemokines in neutrophil infiltration. Corneas were injected with a combination of anti-KC and anti-LIX or with anti-KC, anti-LIX, and anti-MIP-2 and compared with control IgG (final antibody concentrations were the same as injection of individual antibodies). Pretreatment with a mixture of anti-KC and anti-LIX suppressed the infiltration of eGFP bone marrow-derived cells (25±10%) and neutrophils (40±6.5%) into the cornea compared with control IgG (Fig. 4A). The combination anti-KC, anti-LIX, and anti-MIP-2 also significantly decreased eGFP cells (40±7.6%) and neutrophils (60±21.7%; Fig. 4B) but not more than anti-LIX or anti-KC individually (Fig. 3). In addition, there was no significant difference between neutrophil numbers in corneas treated with anti-KC and anti-LIX (Fig. 4A) and corneas injected with all three antibodies (Fig. 4B) (P=.31). Taken together, we conclude that KC and LIX mediate neutrophil recruitment to the corneal stroma in LPS keratitis.

DISCUSSION

Neutrophil migration to the sites of inflammation involves the initial adhesion of circulating neutrophils to activated vascular endothelium, extravasation from the vessels into the tissue, and movement through the extracellular matrix toward the inflammatory stimulus. If the stimulus is bacterial, neutrophil killing of microorganisms occurs through phagocytosis, generation of reactive oxygen species, and the release of cytotoxic antimicrobial products and proteolytic enzymes [34]. In corneal inflammation, neutrophils extravasate from the peripheral limbal vessels and migrate through the stroma where they become activated. Concurrently, the cytotoxic mediators produced by the activated neutrophils can cause local tissue damage to the cornea, disruption of the highly organized stromal matrix, and subsequent loss of corneal clarity. This migration of neutrophils relies on the coordinated action of several chemoattractants, including local production of chemokines, which regulate directional movement, adhesion, and activation.

In the current study, we focused on the murine ELR^+ chemokines CXCL1/KC, CXCL5/LIX, and CXCL2/MIP-2, which bind CXCR2 on neutrophils. Our findings demonstrate that chemokine expression occurs in a temporal pattern; KC is expressed prior to neutrophil infiltration and at high levels, and LIX production is detected later and at lower levels (threefold less than KC) but parallels neutrophil infiltration to the corneal stroma.

In addition to the temporal pattern of chemokine expression, we observed cell-specific expression of CXC chemokines. Whether resident keratocytes express TLR4 or respond to LPS is uncertain; however, keratocytes differentiate into fibroblasts under inflammatory conditions and can produce a number of cytokines in response to LPS [23–28]. Similarly, the MK/T-1 stromal fibroblast line used in the current study responds to highly purified LPS. The corneal fibroblasts in culture selectively express KC and LIX, and neutrophils produce MIP-2 and KC. Based on this differential production of chemokines, we anticipate that the KC and LIX detected in vivo originate from corneal fibroblasts. This is consistent with a mouse model of surgical injury in the skin in which KC is expressed primarily in dermal fibroblasts and endothelial cells, whereas MIP-2 is restricted to infiltrating leukocytes [35]. Similarly, a rat model of ischemia-reperfusion showed that LIX was expressed by resident myocardial cells, whereas MIP-2 localized primarily to infiltrating cells [14]. Our findings that LIX is produced selectively by corneal fibroblasts and that infiltrating neutrophils and macrophages serve as the predominant source of MIP-2 support Hamilton's proposal that there is a nonmyeloid and myeloid pattern of chemokine production and also introduce the possibility that LIX is a "nonmyeloid" chemokine [35]. Further studies remain to determine whether LIX is produced exclusively by nonmyeloid cells. These findings are distinct from a report where G-CSF-stimulated human neutrophils

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increase mRNA expression of CXCL5/epithelial-derived neutrophil-activating factor-78 (ENA-78), a human homologue of LIX, and anti-ENA-78 antibodies abrogate neutrophil chemotaxis [36]. Although the argument remains that murine neutrophils could produce LIX under certain stimulatory conditions, the same study found no expression of CXCL6/granulocyte chemotactic protein-2 (GCP-2) from stimulated or unstimulated human neutrophils. Although LIX shares 55% sequence homology to human CXCL5/ENA-78, it also shares 61% homology to human CXCL6/GCP-2 and is therefore no more closely related to ENA-78 than to GCP-2 [37]. In contrast to LIX, we showed that corneal fibroblasts did not produce MIP-2 in vitro, although in vivo results show the detection of MIP-2 as early as 3 h. Bone marrow-derived cells are present in the normal murine cornea, and activated macrophages in vitro produce MIP-2, as we and others have shown [38–40]. Therefore, the MIP-2, detected prior to recruitment of neutrophils, likely originates from resident macrophages. Once present in the corneal stroma, release of prestored MIP-2 from neutrophils likely contributes to its production in the cornea [29].

Although all of these chemokines bind CXCR2, the cell-specific production of CXC chemokines and their temporal expression could indicate a complementary network of chemokine function in the cornea. Based on these results, we propose a sequence of events regarding the chemokine network and its contribution to neutrophil infiltration and inflammation: Upon interaction with LPS, resident stromal cells and macrophages produce KC and MIP-2. These chemokines, in turn, recruit neutrophils from peripheral vessels into the corneal stroma. Once present in the corneal stroma and exposed to LPS, neutrophils release MIP-2 to continue the cycle of neutrophil recruitment and chemotaxis. Simultaneously, the corneal fibroblasts can up-regulate LIX and KC production to further mediate recruitment of neutrophils into the inflamed cornea. Although this sequence of events is speculative, we tested in vivo the biological role of KC, LIX, and MIP-2 in neutrophil recruitment by treating corneas with neutralizing antibodies prior to LPS challenge. Use of anti-LIX or anti-KC antibodies significantly inhibited neutrophil recruitment to the cornea, thereby identifying a functional role for these chemokines in LPS keratitis. Moreover, our data show that only corneal fibroblasts produce LIX, indicating that these cells play a direct role in neutrophil infiltration. Other models of corneal inflammation and wound-healing have shown production of KC and MIP-2 and more recently, LIX in the cornea, but the role of LIX in mediating neutrophil infiltration to the cornea has not been examined [41– 43]. In the present study, CXCL1/KC is the predominant CXC chemokine produced, and neutralization significantly inhibits recruitment of neutrophils to the corneal stroma. Xue et al. also observed that KC neutralization decreased neutrophil emigration in *P. aeruginosa*-induced keratitis [44]. Their study and others also showed a role for MIP-2 in infiltration of neutrophils to the cornea in models of microbial keratitis [17, 18, 33, 45]. In contrast, we found that although MIP-2 is produced, local administration of anti-MIP-2, alone or in combination with anti-KC and anti-LIX, had no significant effect on neutrophil recruitment. The absence of significant neutralizing activity was not a result of lack of antibody function, as we found that it bound MIP-2 in vitro (data not shown). The difference between these reports and our findings has yet to be determined but may relate to the initial wave of neutrophil recruitment to the corneal stroma versus migration through the corneal stroma. Although we also found that simultaneous injection of antibodies to KC, LIX, and MIP-2 did not completely inhibit neutrophil recruitment and migration to the cornea, it is possible that additional neutrophil chemoattractants, including monocyte-inhibitory protein-1α (MIP-1α), acetylated-proline glycine-proline (acPGP)-containing collagen fragments generated during the inflammatory process, or complement mediators such as C3a and C5a may contribute to recruitment [46–48].

Our study focuses on the role of chemokines in neutrophil migration; however, ELR^+ CXC chemokines have differential effects on neutrophils and can regulate other cells, although

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they all signal through CXCR2. These functions include nonneutrophil-related functions such as angiogenesis and monocyte arrest [43, 49, 50]. Studies indicating that these chemokines can have distinct functions contributing to neutrophil infiltration as well as the inflammatory response include elastase release, NF-κB activation, integrin expression, selectin shedding, and selective transport into the systemic circulation [51–55]. Although these mechanisms have not been elucidated in the cornea, results from the present study clearly demonstrate an important role for KC and LIX in mediating neutrophil recruitment and infiltration to the corneal stroma and may provide the basis for a more effective strategy for the treatment of bacterial keratitis.

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REFERENCES

1. Carr DJ, Harle P, Gebhardt BM. The immune response to ocular herpes simplex virus type 1 infection. Exp. Biol. Med. (Maywood). 2001; 226:353–366. [PubMed: 11393165]
2. Girgis DO, Sloop GD, Reed JM, O'Callaghan RJ. A new topical model of *Staphylococcus* corneal infection in the mouse. Invest. Ophthalmol. Vis. Sci. 2003; 44:1591–1597. [PubMed: 12657597]
3. Hazlett LD. Corneal response to *Pseudomonas aeruginosa* infection. Prog. Retin. Eye Res. 2004; 23:1–30. [PubMed: 14766315]
4. Wu TG, Keasler VV, Mitchell BM, Wilhelmus KR. Immunosuppression affects the severity of experimental *Fusarium solani* keratitis. J. Infect. Dis. 2004; 190:192–198. [PubMed: 15195260]
5. Hume EB, Cole N, Khan S, Garthwaite LL, Aliwarga Y, Schubert TL, Willcox MD. A *Staphylococcus aureus* mouse keratitis topical infection model: cytokine balance in different strains of mice. Immunol. Cell Biol. 2005; 83:294–300. [PubMed: 15877608]
6. Hall LR, Diaconu E, Patel R, Pearlman E. 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. [PubMed: 11238651]
7. Tang Q, Hendricks RL. Interferon γ regulates platelet endothelial cell adhesion molecule 1 expression and neutrophil infiltration into herpes simplex virus-infected mouse corneas. J. Exp. Med. 1996; 184:1435–1447. [PubMed: 8879215]
8. Smith JB, Herschman HR. Identification of inflammatory mediators by screening for glucocorticoid-attenuated response genes. Methods Enzymol. 1997; 287:250–265. [PubMed: 9330327]
9. Wolpe SD, Sherry B, Juers D, Davatelis G, Yurt RW, Cerami A. Identification and characterization of macrophage inflammatory protein 2. Proc. Natl. Acad. Sci. USA. 1989; 86:612–616. [PubMed: 2643119]
10. Wuyts A, Haelens A, Proost P, Lenaerts JP, Conings R, Opdenakker G, Van Damme J. Identification of mouse granulocyte chemotactic protein-2 from fibroblasts and epithelial cells. Functional comparison with natural KC and macrophage inflammatory protein-2. J. Immunol. 1996; 157:1736–1743. [PubMed: 8759763]
11. Rossi DL, Hurst SD, Xu Y, Wang W, Menon S, Coffman RL, Zlotnik A. Lungkine, a novel CXC chemokine, specifically expressed by lung bronchoepithelial cells. J. Immunol. 1999; 162:5490–5497. [PubMed: 10228029]
12. Bozic CR, Kolakowski LF Jr, Gerard NP, Garcia-Rodriguez C, von Uexkull-Guldenband C, Conklyn MJ, Breslow R, Showell HJ, Gerard C. Expression and biologic characterization of the murine chemokine KC. J. Immunol. 1995; 154:6048–6057. [PubMed: 7751647]
13. Kwon JH, Keates AC, Anton-Gay P, Botero M, Goldsmith JD, Kelly CP. Topical antisense oligonucleotide therapy against LIX an enterocyte-expressed CXC chemokine, reduces murine

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colitis. Am. J. Physiol. Gastrointest. Liver Physiol. 2005; 289:G1075–G1083. [PubMed: 16099872]

14. Chandrasekar B, Smith JB, Freeman GL. Ischemia-reperfusion of rat myocardium activates nuclear factor-κB and induces neutrophil infiltration via lipopolysaccharide-induced CXC chemokine. Circulation. 2001; 103:2296–2302. [PubMed: 11342480]
15. Jeyaseelan S, Chu HW, Young SK, Worthen GS. Transcriptional profiling of lipopolysaccharide-induced acute lung injury. Infect. Immun. 2004; 72:7247–7256. [PubMed: 15557650]
16. Madorin WS, Rui T, Sugimoto N, Handa O, Cepinskas G, Kvietys PR. Cardiac myocytes activated by septic plasma promote neutrophil transendothelial migration: role of platelet-activating factor and the chemokines LIX and KC. Circ. Res. 2004; 94:944–951. [PubMed: 14988231]
17. Carlson EC, Drazba J, Yang X, Perez VL. Visualization and characterization of inflammatory cell recruitment and migration through the corneal stroma in endotoxin-induced keratitis. Invest. Ophthalmol. Vis. Sci. 2006; 47:241–248. [PubMed: 16384969]
18. Khatri S, Lass JH, Heinzel FP, Petroll WM, Gomez J, Diaconu E, Kalsow CM, Pearlman E. Regulation of endotoxin-induced keratitis by PECAM-1, MIP-2, and Toll-like receptor 4. Invest. Ophthalmol. Vis. Sci. 2002; 43:2278–2284. [PubMed: 12091428]
19. Gendron RL, Liu CY, Paradis H, Adams LC, Kao WW. MK/T-1, an immortalized fibroblast cell line derived using cultures of mouse corneal stroma. Mol. Vis. 2001; 7:107–113. [PubMed: 11344338]
20. Lopez AF, Strath M, Sanderson CJ. Differentiation antigens on mouse eosinophils and neutrophils identified by monoclonal antibodies. Br. J. Haematol. 1984; 57:489–494. [PubMed: 6743568]
21. Gillette-Ferguson I, Hise AG, McGarry HF, Turner J, Esposito A, Sun Y, Diaconu E, Taylor MJ, Pearlman E. Wolbachia-induced neutrophil activation in a mouse model of ocular onchocerciasis (river blindness). Infect. Immun. 2004; 72:5687–5692. [PubMed: 15385467]
22. Gregory MS, Repp AC, Holhbaum AM, Saff RR, Marshak-Rothstein A, Ksander BR. Membrane Fas ligand activates innate immunity and terminates ocular immune privilege. J. Immunol. 2002; 169:2727–2735. [PubMed: 12193747]
23. Fini ME. Keratocyte and fibroblast phenotypes in the repairing cornea. Prog. Retin. Eye Res. 1999; 18:529–551. [PubMed: 10217482]
24. Jester JV, Barry PA, Lind GJ, Petroll WM, Garana R, Cavanagh HD. Corneal keratocytes: in situ and in vitro organization of cytoskeletal contractile proteins. Invest. Ophthalmol. Vis. Sci. 1994; 35:730–743. [PubMed: 8113024]
25. Kumagai N, Fukuda K, Fujitsu Y, Lu Y, Chikamoto N, Nishida T. Lipopolysaccharide-induced expression of intercellular adhesion molecule-1 and chemokines in cultured human corneal fibroblasts. Invest. Ophthalmol. Vis. Sci. 2005; 46:114–120. [PubMed: 15623762]
26. Fukuda K, Kumagai N, Yamamoto K, Fujitsu Y, Chikamoto N, Nishida T. Potentiation of lipopolysaccharide-induced chemokine and adhesion molecule expression in corneal fibroblasts by soluble CD14 or LPS-binding protein. Invest. Ophthalmol. Vis. Sci. 2005; 46:3095–3101. [PubMed: 16123407]
27. Rodriguez-Martinez S, Cancino-Diaz ME, Cancino-Diaz JC. Expression of CRAMP via PGN-TLR-2 and of α-defensin-3 via CpGODN- TLR-9 in corneal fibroblasts. Br. J. Ophthalmol. 2006; 90:378–382. [PubMed: 16488966]
28. Rodriguez-Martinez S, Cancino-Diaz ME, Jimenez-Zamudio L, Garcia-Latorre E, Cancino-Diaz JC. TLRs and NODs mRNA expression pattern in healthy mouse eye. Br. J. Ophthalmol. 2005; 89:904–910. [PubMed: 15965176]
29. Scapini P, Lapinet-Vera JA, Gasperini S, Calzetti F, Bazzoni F, Cassatella MA. The neutrophil as a cellular source of chemokines. Immunol. Rev. 2000; 177:195–203. [PubMed: 11138776]
30. Cassatella MA. The production of cytokines by polymorphonuclear neutrophils. Immunol. Today. 1995; 16:21–26. [PubMed: 7880385]
31. Sosnova M, Bradl M, Forrester JV. CD34+ corneal stromal cells are bone marrow-derived and express hemopoietic stem cell markers. Stem Cells. 2005; 23:507–515. [PubMed: 15790772]
32. Brissette-Storkus CS, Reynolds SM, Lepisto AJ, Hendricks RL. Identification of a novel macrophage population in the normal mouse corneal stroma. Invest. Ophthalmol. Vis. Sci. 2002; 43:2264–2271. [PubMed: 12091426]

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33. Yan XT, Tumpey TM, Kunkel SL, Oakes JE, Lausch RN. Role of MIP-2 in neutrophil migration and tissue injury in the herpes simplex virus-1-infected cornea. Invest. Ophthalmol. Vis. Sci. 1998; 39:1854–1862. [PubMed: 9727408]
34. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell. 1994; 76:301–314. [PubMed: 7507411]
35. Armstrong DA, Major JA, Chudyk A, Hamilton TA. Neutrophil chemoattractant genes KC and MIP-2 are expressed in different cell populations at sites of surgical injury. J. Leukoc. Biol. 2004; 75:641–648. [PubMed: 14704366]
36. Smith JB, Herschman HR. Glucocorticoid-attenuated response genes encode intercellular mediators, including a new C-X-C chemokine. J. Biol. Chem. 1995; 270:16756–16765. [PubMed: 7622488]
37. Suzuki S, Kobayashi M, Chiba K, Horiuchi I, Wang J, Kondoh T, Hashino S, Tanaka J, Hosokawa M, Asaka M. Autocrine production of epithelial cell-derived neutrophil attractant-78 induced by granulocyte colony-stimulating factor in neutrophils. Blood. 2002; 99:1863–1865. [PubMed: 11861308]
38. Rovai LE, Herschman HR, Smith JB. Cloning and characterization of the human granulocyte chemotactic protein-2 gene. J. Immunol. 1997; 158:5257–5266. [PubMed: 9164944]
39. Nakamura T, Ishikawa F, Sonoda KH, Hisatomi T, Qiao H, Yamada J, Fukata M, Ishibashi T, Harada M, Kinoshita S. Characterization and distribution of bone marrow-derived cells in mouse cornea. Invest. Ophthalmol. Vis. Sci. 2005; 46:497–503. [PubMed: 15671274]
40. Cavaillon JM. Cytokines and macrophages. Biomed. Pharmacother. 1994; 48:445–453. [PubMed: 7858154]
41. Flesch IE, Barsig J, Kaufmann SH. Differential chemokine response of murine macrophages stimulated with cytokines and infected with *Listeria monocytogenes*. Int. Immunol. 1998; 10:757–765. [PubMed: 9678756]
42. Li Z, Burns AR, Smith CW. Two waves of neutrophil emigration in response to corneal epithelial abrasion: distinct adhesion molecule requirements. Invest. Ophthalmol. Vis. Sci. 2006; 47:1947–1955. [PubMed: 16639002]
43. Rudner XL, Kernacki KA, Barrett RP, Hazlett LD. Prolonged elevation of IL-1 in *Pseudomonas aeruginosa* ocular infection regulates macrophage-inflammatory protein-2 production, polymorphonuclear neutrophil persistence, and corneal perforation. J. Immunol. 2000; 164:6576–6582. [PubMed: 10843717]
44. Xue ML, Thakur A, Willcox MD, Zhu H, Lloyd AR, Wakefield D. Role and regulation of CXC-chemokines in acute experimental keratitis. Exp. Eye Res. 2003; 76:221–231. [PubMed: 12565810]
45. Cole N, Krockenberger M, Stapleton F, Khan S, Hume E, Husband AJ, Willcox M. Experimental *Pseudomonas aeruginosa* keratitis in interleukin-10 gene knockout mice. Infect. Immun. 2003; 71:1328–1336. [PubMed: 12595449]
46. Kernacki KA, Barrett RP, Hobden JA, Hazlett LD. Macrophage inflammatory protein-2 is a mediator of polymorphonuclear neutrophil influx in ocular bacterial infection. J. Immunol. 2000; 164:1037–1045. [PubMed: 10623854]
47. Weathington NM, van Houwelingen AH, Noerager BD, Jackson PL, Kraneveld AD, Galin FS, Folkerts G, Nijkamp FP, Blalock JE. A novel peptide CXCR ligand derived from extracellular matrix degradation during airway inflammation. Nat. Med. 2006; 12:317–323. [PubMed: 16474398]
48. Mondino BJ, Sundar-Raj CV, Brady KJ. Production of first component of complement by corneal fibroblasts in tissue culture. Arch. Ophthalmol. 1982; 100:478–480. [PubMed: 7065969]
49. 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. [PubMed: 9557652]
50. Huo Y, Weber C, Forlow SB, Sperandio M, Thatte J, Mack M, Jung S, Littman DR, Ley K. The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. J. Clin. Invest. 2001; 108:1307–1314. [PubMed: 11696575]

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51. Barcelos LS, Talvani A, Teixeira AS, Cassali GD, Andrade SP, Teixeira MM. Production and in vivo effects of chemokines CXCL1-3/KC and CCL2/JE in a model of inflammatory angiogenesis in mice. Inflamm. Res. 2004; 53:576–584. [PubMed: 15597153]

52. Bajt ML, Farhood A, Jaeschke H. Effects of CXC chemokines on neutrophil activation and sequestration in hepatic vasculature. Am. J. Physiol. Gastrointest. Liver Physiol. 2001; 281:G1188–G1195. [PubMed: 11668027]

53. Call DR, Nemzek JA, Ebong SJ, Bolgos GL, Newcomb DE, Remick DG. Ratio of local to systemic chemokine concentrations regulates neutrophil recruitment. Am. J. Pathol. 2001; 158:715–721. [PubMed: 11159209]

54. Chandrasekar B, Melby PC, Sarau HM, Raveendran M, Perla RP, Marelli-Berg FM, Dulin NO, Singh IS. Chemokinecytokine cross-talk. The ELR+ CXC chemokine LIX (CXCL5) amplifies a proinflammatory cytokine response via a phosphatidylinositol 3-kinase-NF-κ B pathway. J. Biol. Chem. 2003; 278:4675–4686. [PubMed: 12468547]

55. Quinton LJ, Nelson S, Zhang P, Boe DM, Happel KI, Pan W, Bagby GJ. Selective transport of cytokine-induced neutrophil chemoattractant from the lung to the blood facilitates pulmonary neutrophil recruitment. Am. J. Physiol. Lung Cell. Mol. Physiol. 2004; 286:L465–L472. [PubMed: 14617513]

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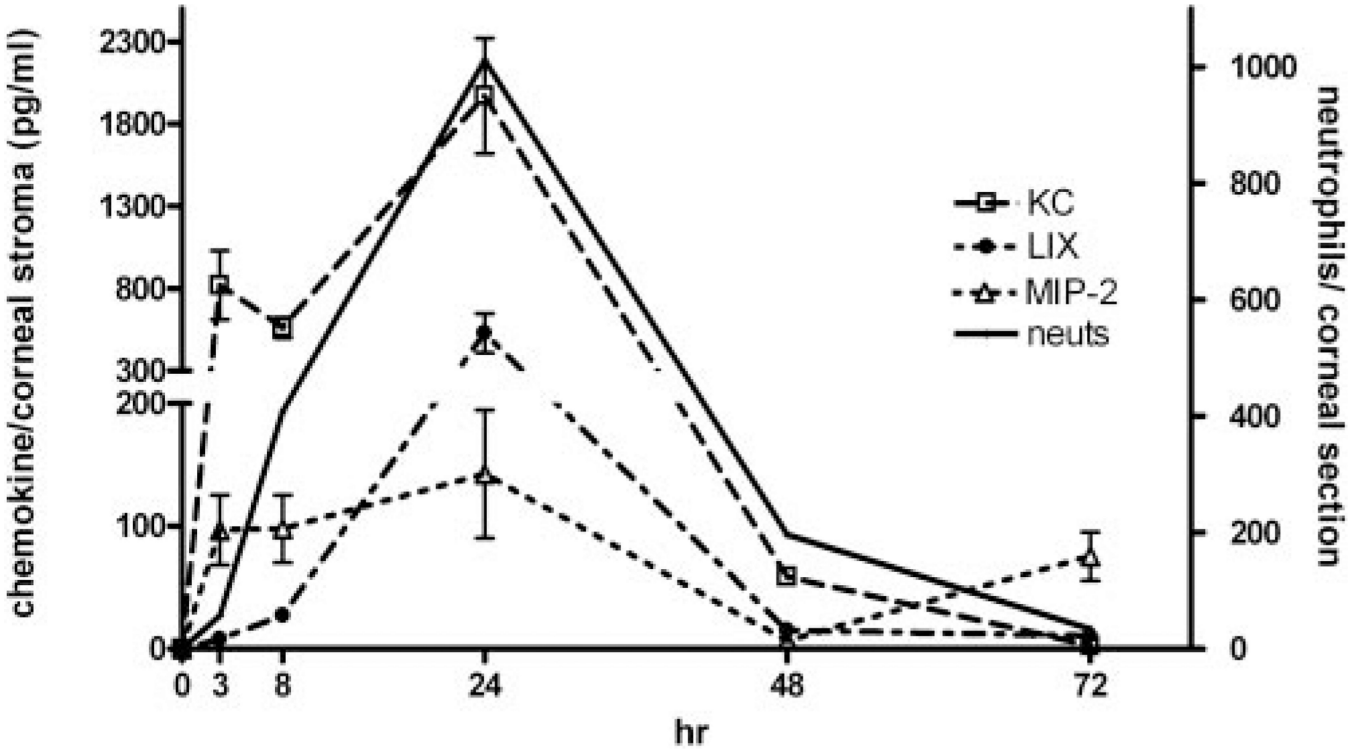

Fig. 1.

Neutrophil infiltration and CXC chemokine production in the corneal stroma during LPS keratitis. Corneas were injected intrastromally with 1 ug LPS. After 0, 3, 8, 24, 48, and 72 h, the corneas were sonicated, and KC (□), LIX (●), and MIP-2 (△) levels were determined by ELISA. Limit of detection was 15 pg/ml. At the indicated time-points, additional eyes were enucleated, sectioned, and immunostained with anti-NIMP-R14. The number of neutrophils (solid line) was determined by direct counting in 5 μm sections. Results are mean ± SEM of six individual corneas per time-point and are representative of four experiments.

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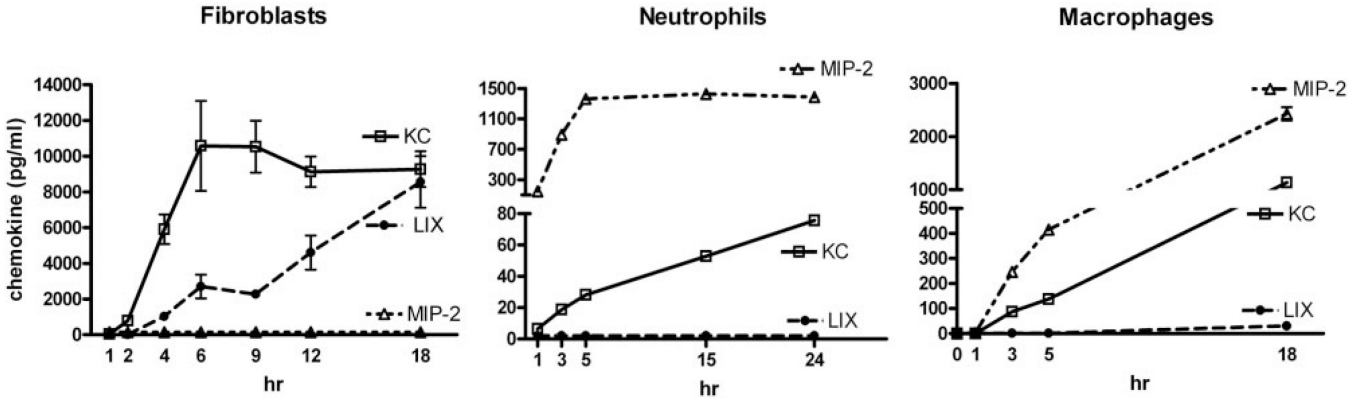

Fig. 2.

Production of KC, LIX, and MIP-2 by corneal fibroblasts, neutrophils, and macrophages after LPS stimulation. MK/T-1 corneal fibroblasts, peritoneal neutrophils, and macrophages were stimulated with 100 ng/ml *E. coli* LPS. At each time-point, supernatants were collected and analyzed for the presence of KC (□), LIX (●), and MIP-2 (△) by ELISA. Limit of detection was 15 pg/ml. The results are representative of one of three experiments performed in triplicate (mean±SEM).

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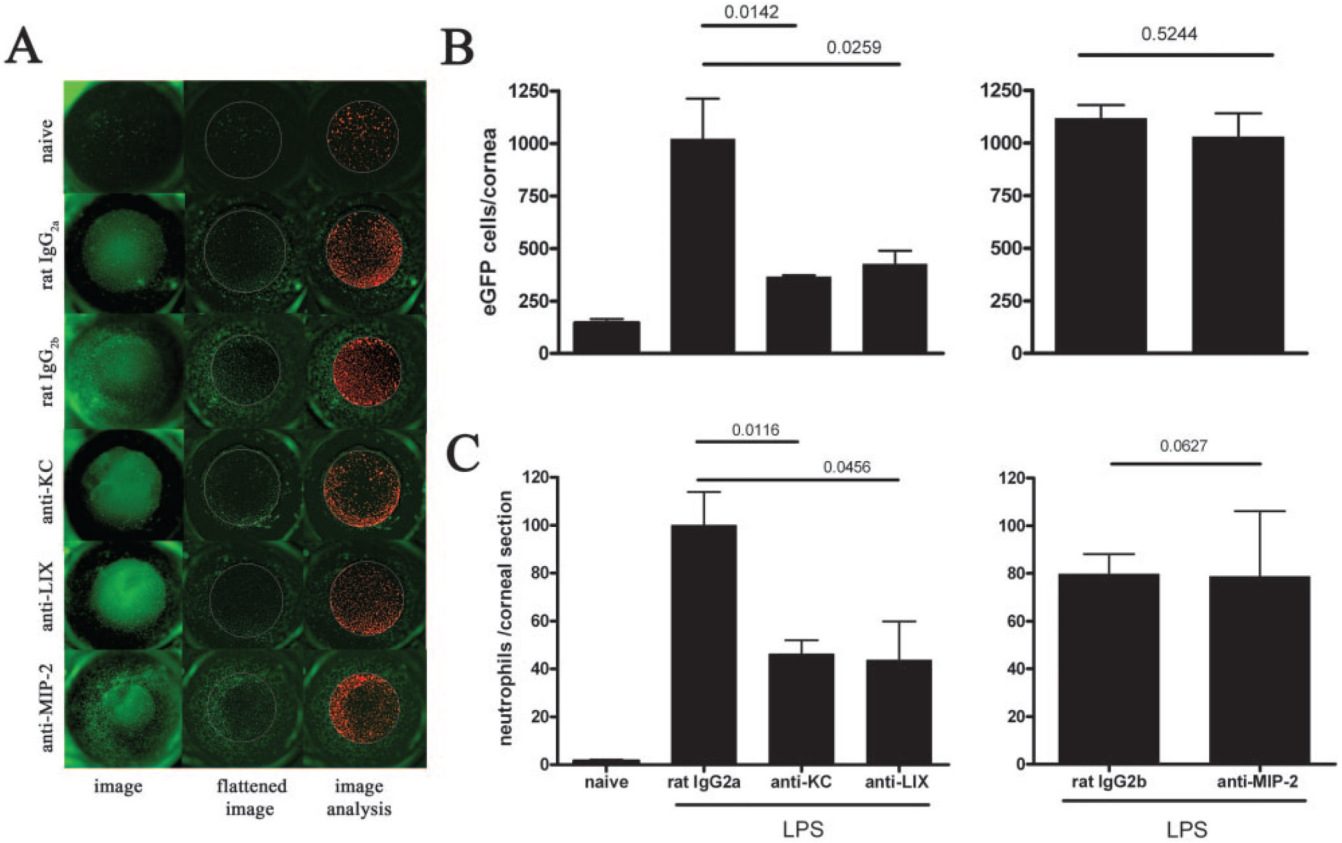

Fig. 3.

Effect of KC, LIX, or MIP-2 neutralization on eGFP cellular and neutrophil infiltration in the cornea. Antibodies to KC, LIX, and MIP-2 or isotype controls were injected in corneas of eGFP chimeric mice. After 1 h, LPS (200 ng) was injected into the corneal stroma, and eyes were examined by fluorescence microscopy after 20 h. (A) Left panel shows representative in vivo fluorescence images (25× magnification; 3 s exposure) of eGFP-positive cells in corneas after 20 h; middle panel depicts flattened image with decrease background fluorescence; and right panel shows quantification of eGFP-positive cells (in red) in a defined region on the image. (B) Mean ± SEM of eGFP cells per group (n=4). (C) Mean ± SEM of neutrophils per group (n=4). These data are representative of two experiments.

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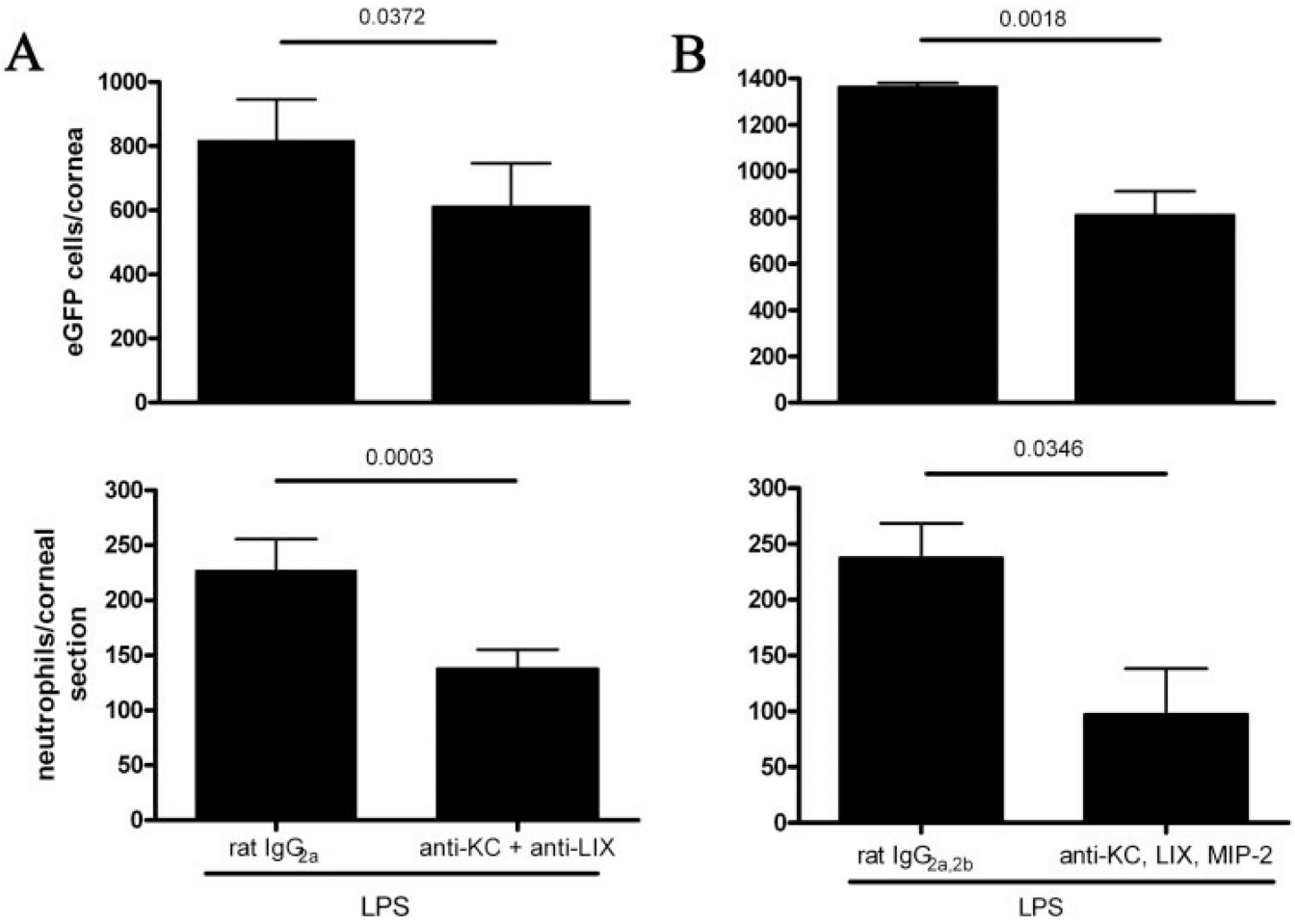

Fig. 4.

Effect of combined KC, LIX, and MIP-2 neutralization on eGFP cellular and neutrophil infiltration in the cornea. LPS (200 ng) was injected into the corneal stroma of eGFP chimeric mice 2 h following injection of isotype IgG control, a combination of anti-KC and anti-LIX, or a combination of anti-KC, anti-LIX, and anti-MIP-2 mAb (all at the same final concentration). (A) eGFP cells and neutrophils in corneas given anti-KC and anti-LIX or control IgG antibody treatment. (B) eGFP cells and neutrophils in the corneas given anti-KC, anti-LIX, and anti-MIP-2 or control IgG antibody treatment. Data are representative of one of two experiments with four corneas per group (mean±SEM).