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RESEARCH REPORT

Chemokine CXCL1/KC and its Receptor CXCR2 Are Responsible for Neutrophil Chemotaxis in Adenoviral Keratitis

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Epidemic keratoconjunctivitis (EKC), caused by human adenovirus (HAdV), is one of the most common ocular infections and results in corneal inflammation and subepithelial infiltrates. Adenoviral keratitis causes significant morbidity to the patients, and is characterized by infiltration of leukocytes in the corneal stroma, and expression of chemokines. The exact role of these chemokines in adenoviral infection has not been studied due to lack of animal models. Here, we have characterized the role of chemokine CXCL1/KC and receptor CXCR2 in adenoviral keratitis using a novel mouse model. Analysis of chemokine expression, leukocyte infiltration, and development of keratitis was performed by ELISA, flow cytometry, and histopathology, respectively. Deficiency of CXCL1 and CXCR2 resulted in delayed infiltration of neutrophils, but not inflammatory monocytes in HAdV-37 corneal infection. CXCL1$^{-/-}$ mice showed decreased expression of CXCL2/MIP-2, but not CCL2/MCP-1. CXCR2$^{-/-}$ mice showed increased expression of CXCL1 and CXCL2, but not CCL2. Both CXCL1$^{-/-}$ and CXCR2$^{-/-}$ mice demonstrated keratitis similar to wild-type mice. In conclusion, both CXCL1 and CXCR2 play an important role in chemokine expression and neutrophil infiltration following adenoviral corneal infection, but have a redundant role in the development of keratitis.

Introduction

Human adenoviruses (HAdV) cause 3 well-known ocular syndromes, pharyngo-conjunctival fever, follicular conjunctivitis, and epidemic keratoconjunctivitis (EKC). HAdV serotypes 8, 19, and 37 are major etiologic agents of EKC (Butt and Chodosh 2006), which is characterized by acute conjunctivitis and delayed onset keratitis, the latter manifesting as leukocytic infiltrates in the subepithelial corneal stroma. Subepithelial infiltrates can appear in 20%–80% of patients infected with HAdV (Korns and others 1944), and they cause foreign body sensation, pain, and photophobia, all sources of significant visual morbidity in afflicted individuals. The pathophysiology leading to the development of adenoviral keratitis is relatively unknown.

We have developed a novel mouse model to study the keratitis of EKC (Chintakuntlawar and others 2007). Intracorneal injection of HAdV-37 does not result in productive viral replication in this mouse model. However, virus does successfully enter the corneal cells and early viral genes are expressed. The development of subepithelial corneal inflammation is remarkably similar to that in the human patient. Expression of chemokines CXCL1/KC and CCL2/MCP-1 is seen as early as 4-h postinfection (hpi) in adenovirus-injected mouse corneas. Among leukocytes, neutrophils are the first cells to infiltrate the mouse corneal stroma (Chintakuntlawar and others 2007). In human EKC, neutrophils are also the predominant cells in early conjunctival membranes and exudates (Laibson and Green 1970; Jones 1980). Similarly, in the New Zealand white rabbit model of adenoviral keratitis, neutrophils are the initial cells to infiltrate the corneal stroma (Gordon and others 1992).

CXCL1 is one of the major attractants of neutrophils in the mouse and is a functional murine homolog of human CXCL1/Gro-α and CXCL8/IL-8. It binds to the chemokine receptor CXCR2 (Bozic and others 1994; Bozic and others 1995; Van Damme and others 1997) present on neutrophils. CXCL1 expression increases in various experimental corneal infections in the mouse including those due to *Pseudomonas*, *Staphylococcus*, herpes simplex virus-1, and adenovirus

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(Yan and others 1998; Cole and others 2000; Hume and others 2005; Chintakuntlawar and others 2007; Lin and others 2007). However, the precise role of CXCL1 in adenovirus keratitis has not been studied.

CXC chemokines like CXCL1 that have a glutamic acid–leucine–arginine (ELR) sequence immediately preceding the CXC motif (ELR+ CXC chemokines) are potent neutrophil chemoattractants (Baggiolini and others 1989; Baggiolini and others 1991). In mice, CXCR2 binds the (ELR+) CXC chemokines CXCL1, CXCL2/MIP-2, and CXCL6/GCP-2/LIX (Bozic and others 1994; Jerva and others 1997; Van Damme and others 1997). In a variety of tissues, CXCR2 plays an important protective role during bacterial infections by mediating neutrophil chemotaxis (Moore and others 2000; Tsai and others 2000; Olszyna and others 2001; Abu Nabah and others 2007; Montgomery and others 2007). For example, in *Pseudomonas* keratitis, CXCR2 mediates neutrophil extravasation and bacterial clearance (Khan and others 2007). In HSV-1 keratitis, CXCR2 deficiency also restricted neutrophil influx, and resulted in increased severity of clinical keratitis (Banerjee and others 2004). In contrast, in an *Onchocerca volvulus* keratitis model, absence of CXCR2 selectively prevented infiltration of neutrophils (Hall and others 2001) and reduced the clinical signs of keratitis.

HAdV accounts for 15%–70% of all cases of acute conjunctivitis (Woodland and others 1992; Aoki and Tagawa 2002), but not much is known about the pathogenesis of ocular adenoviral infections. Prior microarray studies demonstrated that human CXCL1, CXCL8, and CCL2 are the earliest chemokines to be up-regulated in adenovirus infection of human corneal stromal cells (Natarajan and others 2003). Expression of these chemokines appears to be regulated by an intracellular signaling cascade initiated upon viral binding to the host corneal cells (Natarajan and others 2002; Natarajan and others 2003; Xiao and Chodosh 2005; Rajaiya and others 2008). Furthermore, the early expression of CXCL1 and CCL2 was shown in the mouse model of adenovirus keratitis (Chintakuntlawar and others 2007). However, the relationship between chemokine expression and leukocyte infiltration into the cornea remains to be elucidated. In this study, we use mice deficient in CXCL1 or its receptor CXCR2 to test their respective roles in leukocyte infiltration subsequent to adenovirus infection of mouse cornea. We show that CXCL1 and its receptor CXCR2 play important roles in the neutrophil infiltration in adenovirus keratitis.

Materials and Methods

Virus and animals

Eight to 12-week-old wild-type female C57BL/6J and C3HeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). $CXCL1^{-/-}$ mice on a C57BL/6J background were a kind gift from Dr. Sergio Lira at the Mount Sinai Medical Center, New York. $CXCR2^{-/-}$ mice on a C3HeJ background were the kind gift from Dr. Charles Brown at the University of Missouri, Columbia. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and all experimental protocols were approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee. HAdV-37 (Robinson and others 2008) was obtained from American Type Culture Collection (ATCC, Manassas, VA), propagated and titered using A549 cells, and purified by double cesium chloride gradient.

Experimental infections

Mice were anesthetized by intramuscular injection of ketamine (85 mg/kg) and xylazine (14 mg/kg). Anesthetic drops (0.5% proparacaine hydrochloride, Alcon, Fort Worth, TX) were also applied topically to each eye before injections. One microliter of virus-free dialysis buffer, or HAdV-37 (10^5 TCID [tissue culture infective dose]), was injected in the central corneal stroma with a glass micropipette needle fitted with a gas-powered microinjection system (MDI, South Plainfield, NJ) under an ophthalmic surgical microscope (Carl Zeiss Meditec, Inc., Thornwood, NY). At indicated time points after injection, mice were euthanatized using CO_2 inhalation and corneas removed and processed for further analysis.

ELISA

Mouse corneas were removed at indicated time points ($n = 3$ per time point per group) and flash frozen in liquid nitrogen. Corneas were then homogenized in 400 µL of PBS with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL aprotinin, and 10 µg/mL leupeptin (Sigma-Aldrich, St. Louis, MO, USA). The lysates were centrifuged at 10,000*g* for 10 min at 4°C, and the supernatants were used for ELISA. CXCL1, CXCL2, and CCL2 protein detection was performed with commercially available sandwich ELISA kits with capture and detection antibodies as per the manufacturer's instructions (R&D Systems, Minneapolis, MN). Each sample and standard was analyzed in duplicate. The plates were read on a microplate reader (Molecular Devices, Sunnyvale, CA) and analyzed (SOFTmax software; Molecular Devices). The limits of detection for ELISA were as follows: CXCL1 <2 pg/mL, CXCL2 <1.5 pg/mL, CCL2 <2 pg/mL.

Histopathology

Mouse corneas injected with buffer or HAdV-37 were removed, rinsed in PBS, and fixed with 10% neutral buffered formalin for 24 h at room temperature. After paraffin embedding, whole eyes were cut into 5-µm thick sections, mounted on positively charged slides, and air-dried overnight. After deparaffinization and rehydration, slides were stained with hematoxylin and eosin. Slides were coverslipped using a synthetic resin, and photographed with 10× or 20× objectives (AxiobserverA1; Carl Zeiss Meditec, Inc., Thornwood, NY). The extent to which leukocyte infiltration had progressed from the limbus toward the central cornea was quantified in masked fashion by measuring the proportional distance from the limbus to the central cornea for the central-most leukocytes, with 1.0 representing the presence of infiltrating leukocytes in the geometric center of the cornea.

Flow cytometry

Samples for flow cytometry were prepared and analyzed as described by Carr and coworkers (Carr and others 2008). Corneas were dissected from mouse eyes at indicated time points following infection, cut into small (1–2 mm segments) pieces, and digested with 1 mg/mL collagenase type I (Sigma, St. Louis, MO) for 2 h, with vigorous mixing every 15 min.

Single cell suspensions were washed twice in PBS (300*g*, 5 min/wash) and then incubated on ice for 15 min with 2 µL anti-mouse Fc block (BD Pharmingen, San Diego, CA) in a total volume of 50 µL PBS/1% BSA. Following the incubation, the cells were centrifuged (300*g*, 5 min) and resuspended in 5% normal rat serum (Jackson Immuno Research, West Grove, PA) for an additional 15 min on ice. Cells were then triple labeled with 6 µL of antibody cocktail containing 2 µL FITC-conjugated anti-mouse F4/80 (clone CI:A3-1), 2 µL phycoerythrin-Cy5-conjugated anti-CD45 (clone 30-F11), and 2 µL PE-conjugated anti-mouse Gr-1 (clone RB6-8C5) (all from BD Biosciences, San Jose, CA) and incubated in the dark on ice for 30 min. Following the incubation period, the cells were washed 3 times with PBS/1% BSA (300*g*, 5 min/wash) and resuspended in PBS containing 1% paraformaldehyde. After overnight fixation at 4°C in the dark, cells were pelleted (300*g*, 5 min) and resuspended in PBS/1% BSA. Immediately before analysis, CountBright absolute counting beads (Invitrogen, Eugene, OR) were added (21,600 beads/sample). Cell suspensions were gated on $CD45^{high}$ labeled cells, and the numbers of each cell type were determined at this gate setting. A second gate was established to count the number of beads that passed through during the run (300 s). The absolute number of cells per cornea was determined by calculating the number of input beads/21,600 × number of cells in the $CD45^{high}$-gated sample. Cells that were Gr-1+, F4/80– were defined as neutrophils (Hestdal and others 1991; Del Rio and others 2001). Cells that were Gr-1–, F4/80+ were defined as resident mononuclear cells (Hirsch and others 1981). F4/80+, Gr-1+ cells were defined as inflammatory monocytes (Geissmann and others 2003; Gordon and Taylor 2005).

Statistical analysis

ELISA and flow cytometry experiments were each performed 2 times. Mean values from at least 2 separate experiments were compared by one-way ANOVA with preplanned contrasts. Statistical significance was set at $\alpha \leq 0.05$.

Results

Chemokine CXCL1 mediates leukocyte infiltration in adenoviral keratitis

Neutrophils are the first cells to appear in the preocular tear film of human patients with EKC, and the first cells to infiltrate the corneas of mice and rabbits with experimental adenovirus keratitis (Laibson and Green 1970; Jones 1980; Gordon and others 1992; Chintakuntlawar and others 2007). We infected wild-type and $CXCL1^{-/-}$ mice on a C57BL/6J background with HAdV-37 to determine the role of CXCL1 in the neutrophil infiltration so prominent in adenovirus keratitis.

Histopathology at 1 dpi in adenovirus-infected wild-type mice showed paracentral leukocyte infiltration of all corneas. In $CXCL1^{-/-}$ mice, leukocytes migrated only into the peripheral cornea (Fig. 1). We also measured the degree of leukocyte migration from the limbus toward the central cornea as the proportion of the radius of the cornea occupied by leukocytes. $CXCL1^{-/-}$ corneas showed significantly lesser migration of leukocytes compared to wild type (Table 1, $P < 0.001$). At 4 dpi, both wild-type and $CXCL1^{-/-}$ corneas contained leukocytes throughout the corneal stroma (Fig. 1,

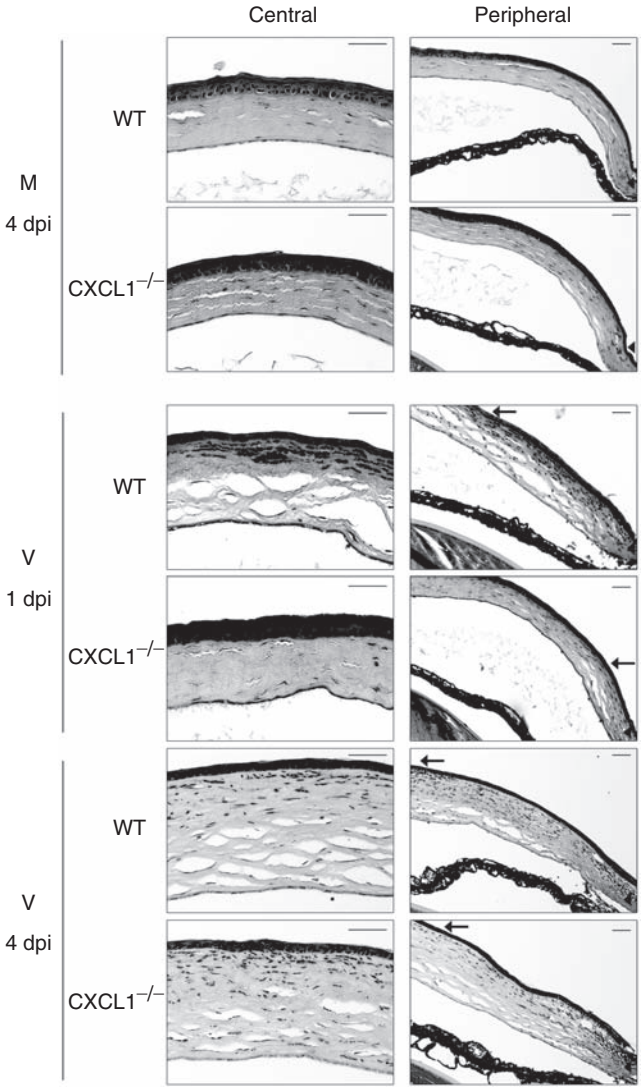

FIG. 1. Leukocyte infiltration in the cornea in $CXCL1^{-/-}$ mice following adenoviral infection. Wild type (WT) and $CXCL1^{-/-}$ were infected with virus-free buffer (M) or 10^5 TCID of HAdV-37 (V), euthanized at 1 or 4 dpi, and stained with hematoxylin and eosin. Arrows denote the anterior-most extension of leukocyte infiltrate and arrowheads denote corneal limbus (periphery of cornea). Images are representative of 6 corneas in each group and were photographed at 20× and 10× magnification for the central and peripheral cornea, respectively ($n = 6$ mice in each group). Scale bars, 50 µm.

Table 1. Proportional Distance From the Limbus to the Central Cornea for the Central-Most Leukocyte Infiltrate in Wild-Type and $CXCL1^{-/-}$ Corneas

	1 dpi	*4 dpi*
WT/buffer	0 ± 0	0 ± 0
$CXCL1^{-/-}$/buffer	0 ± 0	0 ± 0
WT/virus	0.61 ± 0.3	1 ± 0
$CXCL1^{-/-}$/virus	0.11 ± 0.04*	1 ± 0

Data represent mean (±SD) of 6 corneas in each group,
*$P < 0.001$.

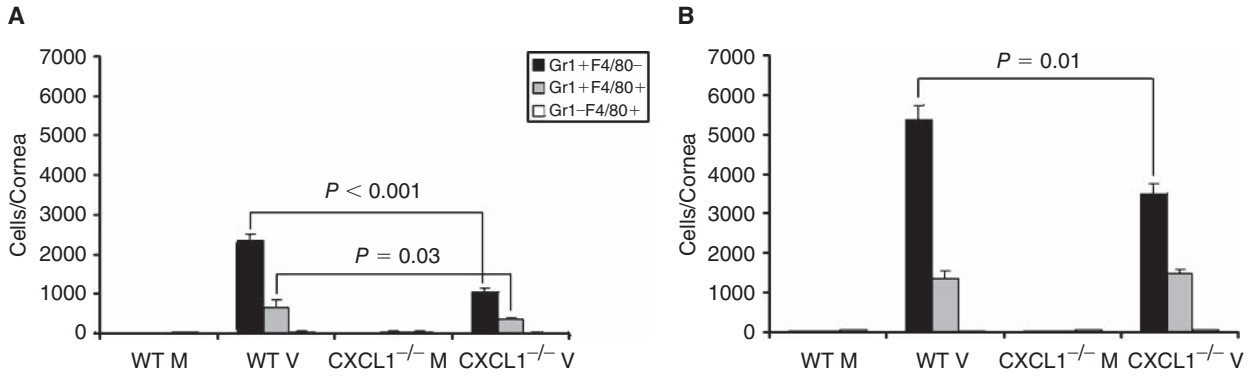

FIG. 2. Flow cytometry of single cell suspensions prepared from wild-type (WT) and CXCL1$^{-/-}$ corneas injected with virus-free buffer (M) or 10^5 TCID of HAdV-37 (V), and stained with antibodies against CD45, F4/80, and Gr-1. Cells were gated on CD45^{high} staining, and the total number of F4/80+ Gr-1–, F4/80+ Gr-1+, and F4/80– Gr-1+ cells were measured in each group at 1 (**A**) and 4 (**B**) dpi. Data represent the mean (±SEM) of at least 2 separate experiments (n = 4 corneas/group).

Table 1). Leukocytes were not seen in buffer-injected corneas at either 1 or 4 dpi. This suggests that there is significant delay of leukocyte migration into the corneas of CXCL1$^{-/-}$ mice following adenoviral infection.

CXCL1 plays an important role in neutrophil, but not mononuclear cell infiltration

We next quantified infiltrating leukocytes by flow cytometry. At 1 dpi, CXCL1$^{-/-}$ mouse corneas infected with 10^5 TCID of HAdV-37 showed 50% less infiltrating neutrophils than wild-type mice ($P < 0.001$). Inflammatory monocytes also decreased in CXCL1$^{-/-}$ corneas at 1 dpi when compared to wild-type corneas ($P = 0.03$) (Fig. 2A). At 4 dpi in the CXCL1$^{-/-}$ mice corneas, we detected significantly less neutrophils than in wild-type corneas infected with adenovirus ($P = 0.01$). However, there was no statistical difference in the numbers of inflammatory monocytes between wild-type and CXCL1$^{-/-}$ corneas at 4 dpi (Fig. 2B). This suggests that mainly neutrophil chemotaxis is affected by loss of CXCL1.

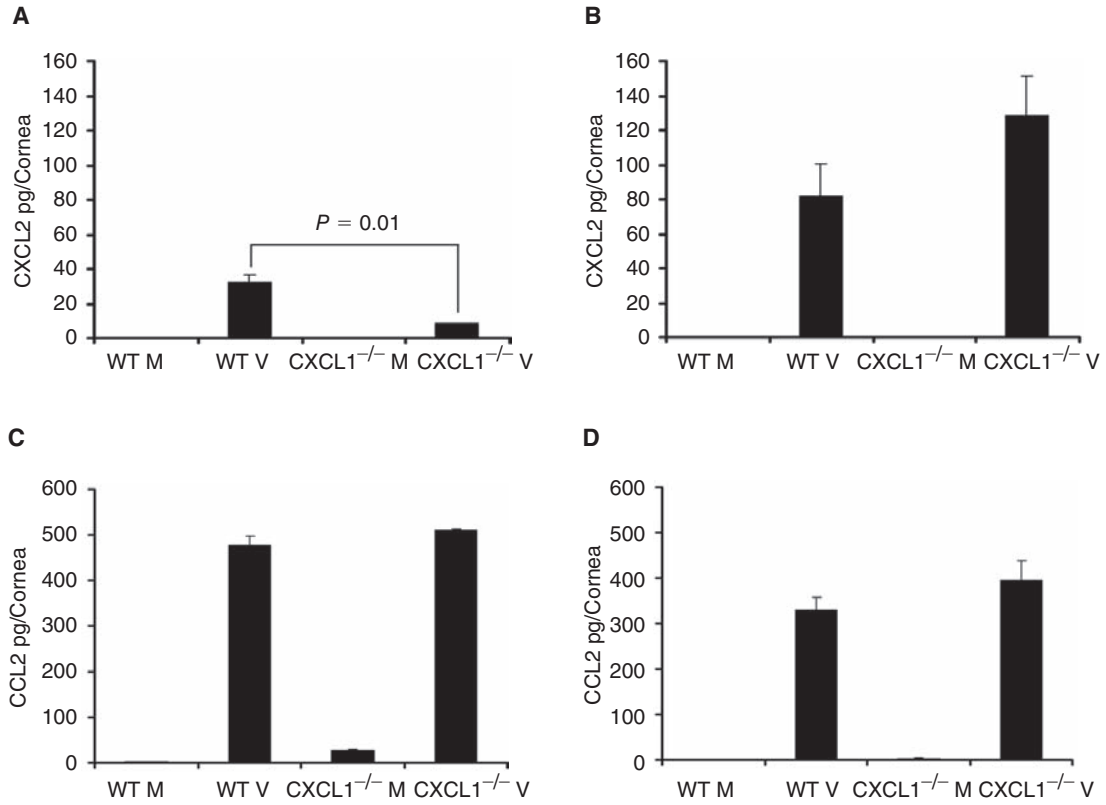

FIG. 3. Chemokine expression in wild-type (WT) and CXCL1$^{-/-}$ corneas following adenoviral infection. Corneas were injected with virus-free buffer (M) or 10^5 TCID of HAdV-37 (V), and analyzed for CXCL2 expression at 16 (**A**) and 48 (**B**) hpi by ELISA. Similarly, CCL2 expression was analyzed at 16 (**C**) and 48 (**D**) hpi. Data represent the mean (±SEM) of at least 2 separate experiments (n = 6 corneas/group).

Differential chemokine expression during adenoviral keratitis in wild-type and CXCL1$^{-/-}$ mice

There is significant redundancy in the actions of ELR+ CXC chemokines for neutrophil chemotaxis (Van Damme and others 1997). In addition to CXCL1, CXCL2 has been shown to be expressed in the cornea and is involved in neutrophil chemotaxis (Yan and others 1998; Kernacki and others 2000). We measured CXCL2 expression by ELISA in virus-infected corneas at 16 and 48 hpi in both wild-type and CXCL1$^{-/-}$ mice. Compared to wild-type corneas, CXCL1$^{-/-}$ corneas showed significantly less CXCL2 expression at 16 hpi ($P = 0.01$) (Fig. 3A), but not at 48 hpi (Fig. 3B). Expression of the mononuclear chemokine CCL2 was not statistically different between wild-type and CXCL1$^{-/-}$ corneas infected with adenovirus at either 16 or 48 hpi (Fig. 3C and D, respectively). Thus, in absence of CXCL1, early in infection, CXCL2 expression is reduced, but not that of CCL2.

To determine if decreases in chemokine expression and leukocyte infiltration affect the development of clinical keratitis, we observed wild-type and CXCL1$^{-/-}$ mice injected with adenovirus up to 4 dpi. Both wild-type and CXCL1$^{-/-}$ mice developed corneal opacities in virus-infected eyes beginning at 1 dpi. The opacities peaked at 4 dpi in both groups and were similar in appearance and size in wild-type and CXCL1$^{-/-}$ corneas (Fig. 4). Buffer-injected corneas in both groups did not develop any opacity up to 4 dpi.

CXCR2 expression contributes to leukocyte infiltration in adenoviral keratitis

CXCR2 is a chemokine receptor that binds to ELR+ CXC chemokines in mice. CXCL1 and CXCL2 belong to this family of chemokines (Van Damme and others 1997). CXCR2 has been shown to be responsible for neutrophil chemotaxis in corneal angiogenic assays (Addison and others 2000; Lu and others 2007), and CXCR deficiency leads to increased virulence and pathology in various tissue infections, including those of the cornea (Tsai and others 2000; Del Rio and others 2001; Banerjee and others 2004; Khan and others 2007). The role of CXCR2 in adenoviral pathogenesis has not been previously tested. Hence, we sought to elucidate the role of CXCR2 in adenovirus keratitis by infecting the corneas of wild-type and CXCR2$^{-/-}$ mice. Chemokine receptor CXCR2$^{-/-}$ mice were on the C3HeJ background. We first compared the characteristics of adenovirus keratitis in wild-type C3HeJ mice and C57BL/6 mice. Intrastromal injection of 10^5 TCID of HAdV-37 into the C3HeJ corneas induced a keratitis clinically and histologically indistinguishable from C57BL/6J corneas (Fig. 5).

At 1 dpi, the corneas of wild-type mice showed infiltration of leukocytes throughout the paracentral region of the corneal stroma. In CXCR2$^{-/-}$ corneas at 1 dpi, leukocyte infiltration was delayed and confined to the peripheral cornea, with the central corneal stroma free of leukocytes (Fig. 6). CXCR2$^{-/-}$ corneas showed significantly lesser migration of leukocytic infiltrates compared to wild type (Table 2, $P < 0.001$). At 4 dpi, the wild-type and CXCR2$^{-/-}$ corneas infected with adenovirus had leukocytes throughout the corneal stroma (Fig. 6, Table 2). Buffer-injected corneas in both groups did not show leukocytes at either 1 or 4 dpi. Thus, CXCR2$^{-/-}$ plays an important role in migration of leukocytes in early adenoviral keratitis, but does not completely prevent infiltration.

Loss of CXCR2 delays but does not prevent infiltration of neutrophils in adenoviral keratitis

We next characterized the infiltrating leukocytes in infected corneas by flow cytometry to phenotype and quantify relative differences between CXCR2$^{-/-}$ and wild-type mice following adenovirus infection. At 1 dpi, CXCR2$^{-/-}$ corneas contained significantly less neutrophils than wild-type corneas ($P = 0.01$), while the numbers of mononuclear cells were comparable between groups (Fig. 7A). By 4 dpi, the numbers of neutrophils were comparable in wild-type and CXCR2$^{-/-}$ corneas infected with adenovirus. The average number of inflammatory monocytes was higher in CXCR2$^{-/-}$ corneas, but the difference between groups was not statistically significant ($P = 0.06$) (Fig. 7B). Our data suggest that CXCR2 plays an important role in infiltration of neutrophils but not inflammatory monocytes.

CXCR2$^{-/-}$ corneas show differences in chemokine expression but similar clinical disease

We observed significant differences in the infiltration of neutrophils and inflammatory monocytes in

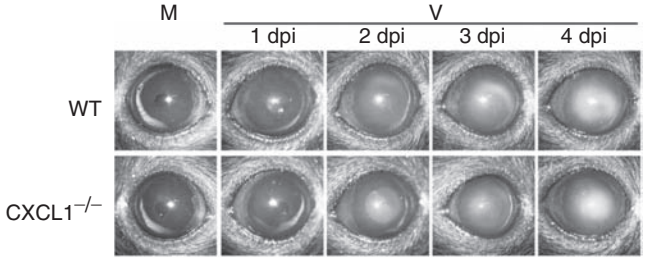

FIG. 4. Clinical appearance of adenoviral keratitis in wild-type (WT) and CXCL1$^{-/-}$ corneas. Corneas were injected with virus-free buffer (M) or 10^5 TCID of HAdV-37 (V) and observed. Representative photographs are shown for buffer-injected corneas at 4 dpi and virus-injected corneas at 1, 2, 3, and 4 dpi ($n = 6$ mice in each group).

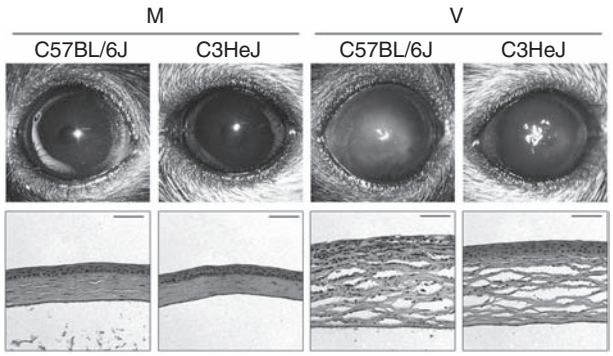

FIG. 5. Adenoviral keratitis in C57BL/6J and C3HeJ mice is indistinguishable. Corneas were infected with virus-free buffer (M) or 10^5 TCID of HAdV-37 (V). Representative pictures of corneas and hematoxylin and eosin–stained sections of central corneas at 4 dpi are shown. Histopathological sections were photographed at 20× magnification ($n = 6$ mice in each group). Scale bars, 50 μm.

Table 2. Proportional Distance From the Limbus to the Central Cornea for the Central-Most Leukocyte Infiltrate in Wild-Type and CXCR2$^{-/-}$ Corneas

	1 dpi	*4 dpi*
WT/buffer	0 ± 0	0 ± 0
CXCR2$^{-/-}$/buffer	0 ± 0	0 ± 0
WT/virus	0.96 ± 0.08	1 ± 0
CXCR2$^{-/-}$/virus	0.3 ± 0.16*	1 ± 0

Data represent mean (±SD) of five corneas in each group, *$P < 0.001$.

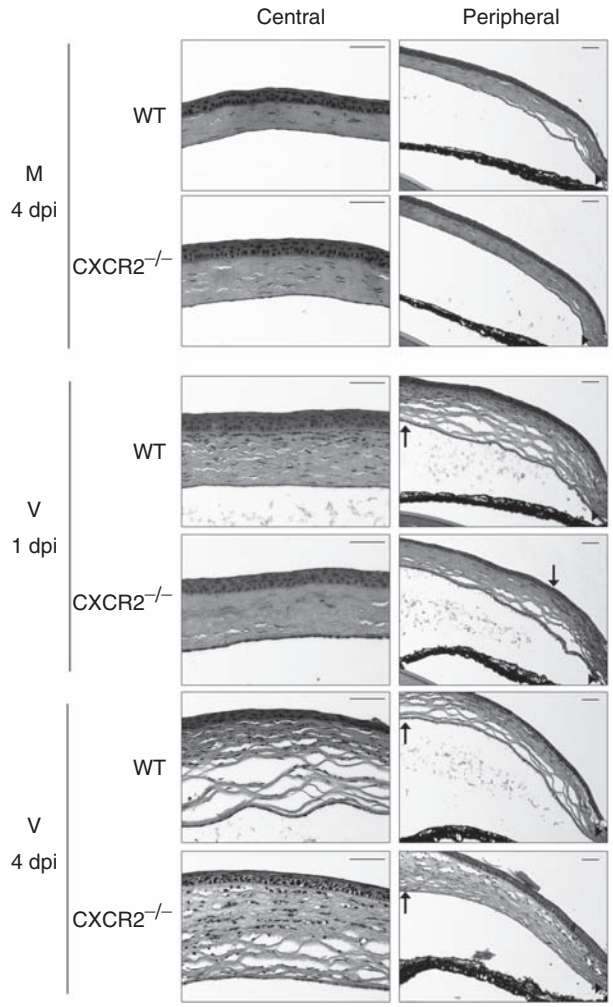

FIG. 6. Infiltration of leukocytes into the corneal stroma of wild-type (WT) and CXCR2$^{-/-}$ mice following adenovirus infection. Corneas were infected with virus-free buffer (M) or 10^5 TCID of HAdV-37 (V), removed after 1 or 4 dpi, and stained with hematoxylin and eosin. Arrows denote the anterior-most extension of leukocyte infiltrate and arrowheads denote the corneal limbus (periphery of cornea). Corneas were photographed at 20× and 10× magnification for the central and peripheral cornea, respectively ($n = 6$ mice in each group). Scale bars, 50 µm.

CXCR2$^{-/-}$ corneas at 1 and 4 dpi, respectively. Therefore, we evaluated the levels of neutrophil chemokines CXCL1 and CXCL2, and mononuclear cell chemokine CCL2 following adenoviral corneal infection (Bozic and others 1994; Van Damme and others 1997; Lu and others 1998). At 16 hpi, CXCL1 and CXCL2 protein were expressed at significantly higher levels in CXCR2$^{-/-}$ corneas when compared to wild-type corneas ($P < 0.001$ and $P = 0.05$, respectively) (Fig. 8A and B). In contrast, expression of CCL2 was comparable in wild-type and CXCR2$^{-/-}$ corneas at 16 hpi (Fig. 8C). This indicates that CXCR2 may negatively regulate the expression of CXCL1 and CXCL2, but not CCL2.

The observed differences in chemokine expression and timing of leukocyte infiltration in CXCR2$^{-/-}$ corneas did not correspond to the clinical appearance of opacity in adenovirus-infected corneas. As with CXCL1$^{-/-}$ corneas, CXCR2$^{-/-}$ corneas at 1 (data not shown) and 4 dpi appeared similar to those of wild-type mice (Fig. 8D). This suggests that development of corneal opacity following adenoviral infection is not directly dependent on the number of infiltrating leukocytes. We also determined viral titers in adenovirus-injected corneas. Viral replication did not occur up to 4 dpi in any wild-type or knockout mouse as assessed by previously reported methods (Chintakuntlawar and others 2007). Viral clearance was modestly reduced in the CXCR2$^{-/-}$ corneas when compared to wild type (data not shown).

Discussion

In vitro infection of various cell lines with adenovirus results in rapid expression of human CXCL8 (Bruder and Kovesdi 1997; Chodosh and others 2000; Alcorn and others 2001; Natarajan and others 2003; Rajaiya and others 2008), a functional homolog of murine CXCL1. In the mouse model of keratitis, HAdV-37 induces expression of CXCL1 within 4 hpi (Chintakuntlawar and others 2007). These observations suggest an important role for CXC chemokines in adenovirus keratitis. In the current study, we investigated the role of chemokine CXCL1 and its receptor CXCR2 in leukocyte infiltration in adenoviral keratitis. We demonstrated that CXCL1 and CXCR2 each play an important role in neutrophil chemotaxis in adenoviral infection. Neutrophil influx following adenoviral infection in CXCL1$^{-/-}$ corneas was significantly delayed when compared to wild-type corneas. Other ELR+ CXC chemokines, such as CXCL2, and some CC chemokines can also contribute to neutrophil chemotaxis (Van Damme and others 1997; Bae and others 2008), and likely explain the observed, albeit reduced, neutrophil infiltration in the absence of CXCL1. The relative contribution of neutrophil chemokines to chemotaxis can vary from one infection to another. In a HSV-1 keratitis model, CXCL2 depletion was more potent than CXCL1 in inhibiting neutrophil chemotaxis into the cornea (Yan and others 1998).

CXCL1 is expressed relatively early in the adenovirus-infected mouse cornea (Chintakuntlawar and others 2007) prior to the infiltration of leukocytes. Therefore, we propose that CXCL1 is expressed principally by resident corneal cells. In contrast, we observed that CXCL1$^{-/-}$ corneas contained less CXCL2 at 16 hpi when compared to wild-type corneas, but was comparable at 48 hpi. The reduced expression of CXCL2 in CXCL1$^{-/-}$ mouse corneas at 16 hpi may arise due to the reduction in infiltrating neutrophils, which themselves express CXCL2 in inflammation (Xing and others 1994). We did not observe any significant decrease in the number of mononuclear cells in CXCL1$^{-/-}$ corneas following adenoviral infection at 4 dpi. This suggests that CXCL1 does not play

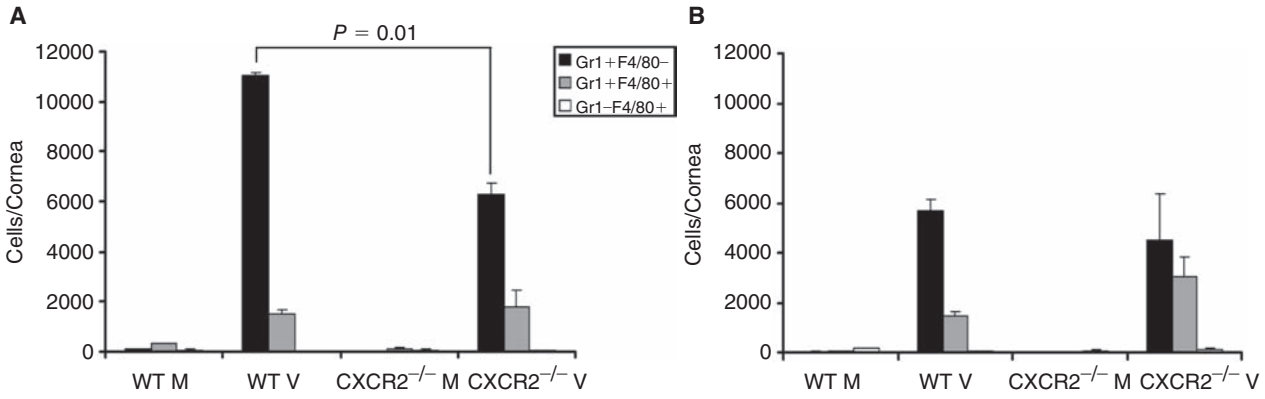

FIG. 7. Decreased neutrophil infiltration in CXCR2$^{-/-}$ versus wild-type (WT) corneas following adenoviral infection. Single cell suspensions were prepared from corneas injected with virus-free buffer (M) or 10^5 TCID of HAdV-37 (V), and stained with antibodies against CD45, F4/80, and Gr-1. Cells were gated on CD45^{high} staining, and the total number of F4/80+ Gr-1–, F4/80+ Gr-1+, and F4/80– Gr-1+ cells measured in each group at 1 (**A**) and 4 (**B**) dpi. Data represent the mean (±SEM) of at least 2 separate experiments (n = 4 corneas/group).

a significant role in the chemotaxis of inflammatory monocytes in adenoviral keratitis.

CXCR2 is thought to be the sole chemokine receptor for ELR+ CXC chemokines in the mouse (Van Damme and others 1997). CXCR2 has been shown to be important during neutrophil infiltration in corneal infections (Hall and others 2001; Banerjee and others 2004; Khan and others 2007), as well as during corneal neovascularization (Addison and

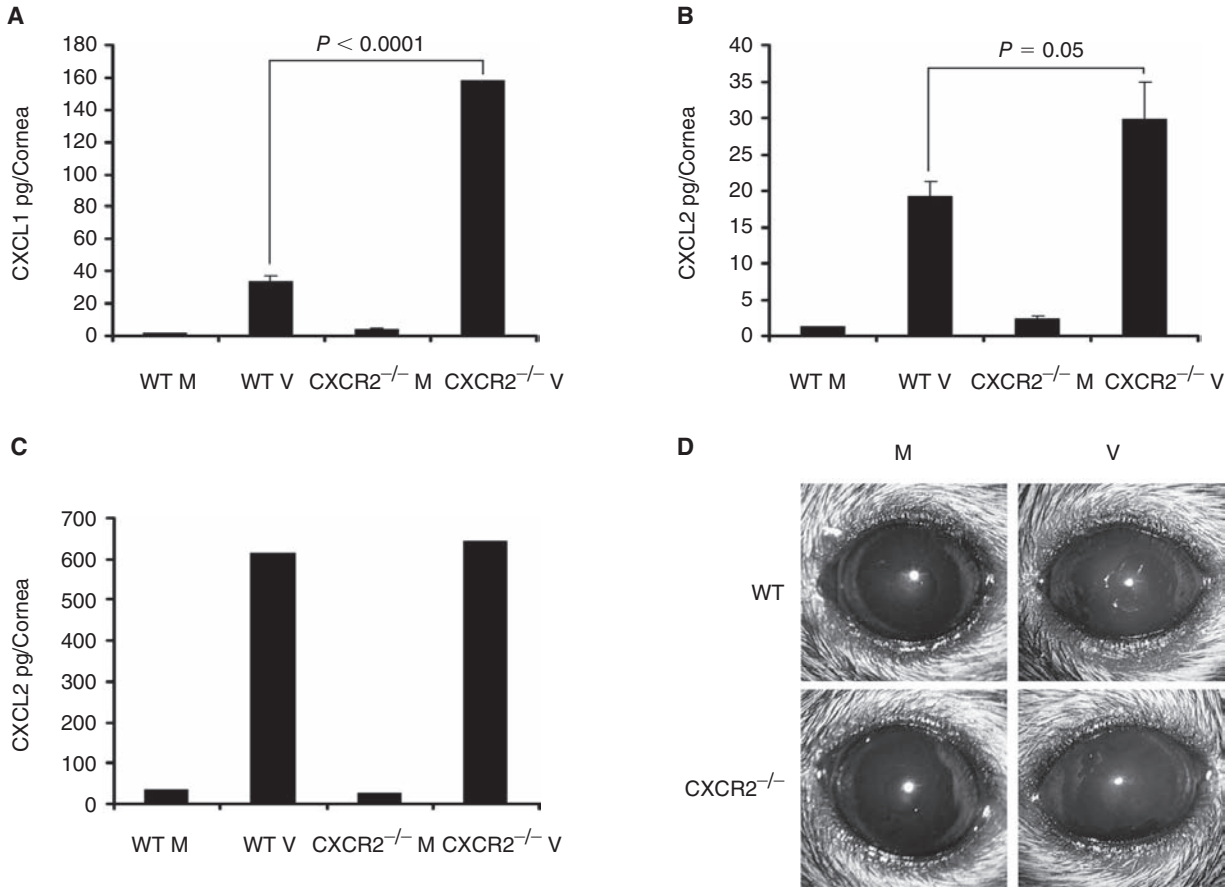

FIG. 8. Differential expression of chemokines in wild-type (WT) and CXCR2$^{-/-}$ corneas following adenoviral infection. Corneas were injected with virus-free buffer (M) or 10^5 TCID of HAdV-37 (V), and analyzed by ELISA for CXCL1 (**A**), CXCL2 (**B**), and CCL2 (**C**) expression at 16 hpi. Data represent the mean (±SEM) of at least 2 separate experiments (n = 6 corneas/group). (**D**) Clinical appearance of adenoviral keratitis in wild-type (WT) and CXCR2$^{-/-}$ corneas at 4 dpi (n = 6 mice in each group).

others 2000; Lu and others 2007). In our study, CXCR2$^{-/-}$ corneas at 1 dpi showed delayed migration of leukocytes into the central corneal stroma. More specifically, the number of neutrophils was decreased at 1 dpi in the CXCR2$^{-/-}$ corneas when compared to wild-type corneas. At 4 dpi, the numbers of infiltrating neutrophils were comparable in wild-type and CXCR2$^{-/-}$ corneas. These data suggest that in adenoviral keratitis, CXCR2 is mainly involved in neutrophil chemotaxis early in infection but is not essential at later time points. The apparent redundancy might be explained by the novel murine receptor CXCR1, which has been shown to bind murine GCP-2/LIX and is functional *in vitro* (Fan and others 2007). Taken together, CXCL1 and CXCR2 play important but not essential roles in neutrophil chemotaxis in adenoviral keratitis.

Similar to CXCL1$^{-/-}$ mice, we noted differential chemokine expression in the corneas of CXCR2$^{-/-}$ mice. At 16 hpi, CXCR2$^{-/-}$ corneas showed significantly increased CXCL1 and CXCL2 expression when compared to wild-type corneas. Similar findings have been reported in helminth and bacterial infections of the cornea (Hall and others 2001; Khan and others 2007). These data suggest that the expression of CXCL1 and CXCL2 is negatively regulated through CXCR2. Such a mechanism would serve to restrict the infiltration of neutrophils and reduce secondary tissue destruction. However, this putative negative feedback loop has not been studied. The monocyte chemokine CCL2 was comparably expressed in wild-type, CXCL1$^{-/-}$, and CXCR2$^{-/-}$ corneas. CCL2 has been previously shown to be important for the chemotaxis of monocytes to foci of inflammation (Gu and others 1998; Lu and others 1998), and has also been proposed as a signal for the constitutive recruitment of bone marrow-derived cells into the corneal stroma (Ebihara and others 2007). However, CCL2$^{-/-}$ mice developed keratitis to a similar degree as wild-type mice, and histopathology was indistinguishable between the 2 types of mice (data not shown). These data suggest that CCL2 does not play a major role in the development of adenoviral keratitis. Other chemokines such as CCL5 and CXCL12 might contribute to mononuclear cell chemotaxis in mouse corneas infected with HAdV-37 (Muller 2001). In a mouse model of HSV-1 keratitis, deficiency of CCL2 resulted in severe keratitis and increased infiltration of neutrophils (Kim and others 2006). In contrast, in the *Pseudomonas* keratitis mouse model, neutralization of CCL2 decreased leukocyte infiltration and the subsequent clinical signs of keratitis (Xue and others 2007). These observations indicate that the role of CCL2 in corneal infections may be pathogen-dependent.

The development of clinical keratitis in CXCL1$^{-/-}$, CCL2$^{-/-}$, and CXCR2$^{-/-}$ mice was similar in appearance as well as progression, suggesting that the genesis of corneal opacity in infectious keratitis depends to some degree on alterations in the corneal stroma other than just leukocyte infiltration. The cornea maintains its clarity by very precise and ordered arrangements of collagen fibrils and stromal cells within a predominantly collagenous matrix (Trelstad and Coulombre 1971; Muller and others 1995). Both monocytes and neutrophils produce matrix metalloproteases (MMPs) upon stimulation, which enzymatically digest and turn over extracellular matrix components (Webster and Crowe 2006). It has also been shown that MMP induction by chemokines in ocular tissues can lead to lens and corneal opacities (Mohan and others 2002; Descamps and others 2005). We did not investigate the role or possible source of MMPs nor the possible contribution of corneal stromal edema to the clinically evident opacity observed in mouse adenovirus keratitis.

In summary, we have demonstrated that CXCL1 and its receptor CXCR2 play an important but not essential role in neutrophil chemotaxis in adenovirus keratitis. Our data further confirm that CXCL1 is one of the major chemokines produced by resident corneal cells to initiate the innate immune response and induce the infiltration of leukocytes, which in turn then amplify this response. There appears to be significant redundancy in the actions of chemokines in adenovirus keratitis, suggesting that targeting one particular chemokine for the treatment or prevention of adenovirus keratitis may not succeed. Further studies to elucidate how adenovirus interactions with corneal cells stimulate expression of inflammatory mediators will lead us to better understanding of molecular pathogenesis of adenovirus keratitis.

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Author Disclosure Statement

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