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*J Immunol* 2014; 192:1762-1767; Prepublished online 17 January 2014;
doi: 10.4049/jimmunol.1302957
http://www.jimmunol.org/content/192/4/1762

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CXCL1 but Not IL-6 Is Required for Recurrent Herpetic Stromal Keratitis

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Herpetic stromal keratitis (HSK) is characterized by an inflammatory response that includes neutrophils, macrophages, NK cells, and T cells. The factors that are responsible for this inflammation are proinflammatory cytokines and chemokines. Many of these factors have been defined for primary disease, but relatively few have been investigated during recurrent HSK. The present study was designed to determine the role that two of these factors, IL-6 and CXCL1, play during recurrent HSK. Results clearly indicate that unlike primary disease, IL-6 plays no role in recurrent HSK. However, the presence of CXCL1 is required for recurrent HSK as evidenced by the lack of corneal disease in mice treated with anti-CXCL1 Ab. This was confirmed using mice lacking the primary receptor for CXCL1, CXCR2. Corneal disease in this strain was significantly reduced compared with wild-type C57BL/6 controls. Unexpectedly, lack of disease occurs even though CXCL1 knockout mice display increased viral shedding at the cornea. The primary mechanism that CXCL1 plays during disease is its ability to stimulate neutrophils to infiltrate the cornea following reactivation. This paper provides further evidence that primary HSK and recurrent HSK possess overlapping yet distinct disease mechanisms. The Journal of Immunology, 2014, 192: 1762–1767.

Herpetic stromal keratitis (HSK) is an infection of the cornea with HSV-1 and is the leading cause of infectious blindness in the Western world with one study determining a prevalence of HSV keratitis of 149 of 100,000 people (1). As with other herpes viruses, there are both primary and recurrent forms of the disease.

In humans, primary disease is rare, occurring mostly in children and the immunosuppressed. Primary disease is most often clinically asymptomatic, although in 1–6% of cases it presents as blepharo-conjunctivitis that heals without scarring (2). Primary disease begins typically by exposure through corneal or oral epithelium. The virus replicates in these cells and then travels via retrograde axonal transport in sensory neurons to the sensory ganglia (most often trigeminal) where it establishes latency. During latency, the viral genome is present, but few active virions are detected in these latently infected neurons (3). The dominant form of clinical disease is the result of reactivation of virus that is typically “triggered” by immunosuppressive events such as fever, menses, sunlight (UV), irradiation, stress, and trauma (4). Following reactivation, the virus travels via anterograde axonal transport back to the epithelial surface, and its replication and subsequent host immune response are responsible for observed symptoms that define most cases of corneal keratitis (5).

Recurrent disease in the cornea is an immunopathologic condition that is initiated by renewed presence of virus in the cornea that restimulates the immune response leading to inflammation of the cornea resulting in damage to the cornea. In humans, the inflammatory infiltrate in HSK is characterized by an influx of a phenotypically diverse population of leukocytes consisting of lymphocytes, neutrophils, and mononuclear phagocytes (6, 7). Animal studies have shown that the cell type found in greatest numbers in corneas displaying disease are neutrophils (8).

Typically, neutrophils follow chemokine and cytokine cues as to when and where to enter tissues in response to pathogens. In 2008, this laboratory demonstrated that proinflammatory cytokines CCL2 and CCL3 were unimportant in the pathogenesis of recurrent HSK. In fact, CCL3-deficient mice were shown to experience worse disease than wild-type mice (9). In 2007, Lin et al. (10) showed that neutrophils very quickly infiltrate the cornea in response to LPS administration and that CXCL1/keratinocyte-derived chemokine (KC), produced by corneal stromal cells, increased in parallel with neutrophil infiltration. Previous studies also demonstrated that CXCL1 is upregulated in HSV-1 cornea infection and that it is crucial to this neutrophil infiltrate (10–13). One of the receptors for CXCL1, CXCR2 (14), has been shown to be important in controlling viral infection of the cornea (15). Banerjee et al. (15) reported that in the absence of CXCR2 there was minimal neutrophil influx during the first 7 d and that these mice exhibited increased IL-6 production that appeared to induce vascular endothelial factor production leading to worse HSK than was observed in wild-type mice. An additional chemokine, CXCL10, more recently has been reported to restrict viral replication in the cornea and to reduce the severity of primary HSK in a model using the RE strain of HSV-1 for infection (16).

In addition to CXCL1, HSV-1–infected human corneal epithelial cells also produce increased levels of the proinflammatory cytokine IL-6 following primary infection with HSV-1 (17). This mechanism responsible for increased proinflammatory cytokine production has been proposed to be through sequential activation of TLRs (11). In support of IL-6’s role in primary HSK, Fenton et al. (12) demonstrated that IL-6 knockout (KO) mice experience significantly decreased corneal opacity in primary HSK when compared with wild-type mice. Furthermore, they demonstrated that administration of exogenous IL-6 at time of infection restored...
disease to the same level as that experienced by wild-type mice, confirming that IL-6 was crucial to developing primary HSK (12).

As previously described, the predominant form of HSK that affects humans is recurrent disease (1–7). Consequently, we decided to investigate whether CXCL1 and IL-6, which are so important during acute HSK, play similar roles during recurrent HSK. Our results demonstrate that CXCL1 is critical in developing recurrent HSK in mice. However, in contrast to what is seen in primary HSK (12, 15–17), IL-6 has no role during recurrent HSK.

Materials and Methods

**Mice**

Investigations with mice conformed to the Association for Research in Vision and Ophthalmic Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 (B6) were purchased from the National Cancer Institute, and National Institute of Health-inbred mice were obtained from Harlan OLAC (Blackthorn, U.K.). The B6.129S2(C)-Cxcr2tm1Mwm/J (B6-CXCR2 KO) mice and the B6.129S2-IL6tm1Kopf/J (B6-IL-6 KO) mice were obtained initially from The Jackson Laboratory (Bar Harbor, ME) and bred in our colony.

**Infection of mice**

Mice (6–12 wk old) were infected on the scarified cornea with 10^6 PFU HSV-1 McKrae strain as described previously (18). Each mouse received an i.p. injection of 0.5 ml pooled human serum (ED50 for virus neutralization = 1:1600; Sigma-Aldrich, St. Louis, MO) concurrent with infection. Administration of anti-HSV Abs at the time of ocular infection has been shown to protect mice from death and corneal disease during primary infection while allowing for the establishment of latency and subsequent reactivation of virus after corneal UV-B exposure. These Abs are undetectable at the time of UV-B irradiation 5 wk after primary infection. HSV-positive eye swabs obtained 3 d after application of virus confirm primary infection.

**UV-B irradiation and virus reactivation**

Mice were reactivated from latency as described previously (18). Briefly, the eyes of all latently infected mice were examined for corneal opacity before irradiation, and only animals with clear corneas were used. At least 5 wk after primary infection, the eyes of latently infected and control mock–infected mice were exposed to 250 mJ/cm^2 UV-B light using a TM20 5 wk after primary infection, the eyes of latently infected and control mock–infected mice were exposed to 250 mJ/cm^2 UV-B light using a TM20 5 wk after primary infection, the eyes of latently infected and control mock–infected mice were exposed to 250 mJ/cm^2 UV-B light using a TM20.

**Flow cytometric analysis**

Cells were isolated from corneas as described previously (21). Briefly, corneas were excised at 18 and 23 d postinfection and incubated in PBS-EDTA at 37˚C for 15 min at 37˚C. Stromas were separated from overlying epithelium and digested in 84 U collagenase type 1 (Sigma-Aldrich) per cornea for 2 h at 37˚C and then were triturated to form a single-cell suspension. Suspensions were filtered through a 40-μm cell strainer cap (BD Labware, Bedford, MA) and washed and then stained. Suspensions were filtered through a 40-μm cell strainer cap (BD Labware, Bedford, MA) and washed and then stained. Suspensions were stained with PerCP-conjugated anti-CD45 (clone 30-F11) and Alexa Fluor 700-Gr-1 (clone RB6-8C5) (from BioLegend, San Diego, CA); FITC-conjugated anti-CD4 (clone RM4-5), PE-conjugated anti-CD8 (clone 53-6.7), and PE-Cy7–conjugated anti-CD11c (clone HL3) (all BD PharMingen); and eFluor450-conjugated CD11b (clone M1/70) (from eBiosciences, San Diego, CA). Cells were then analyzed on a flow cytometer (FACS4流.ia with FACSData data analysis software; BD Biosciences).

**Statistics**

All statistical analyses were performed with the aid of Sigma Stat for Windows, version 2.0 (Jandel, Corte Madera, CA). The log-rank test was used to compare disease scores. A Student unpaired *t* test was used to compare virus titer data. Fisher’s exact χ² tests were used to compare limiting dilution assay data.

**FIGURE 1.** Recurrent HSK in anti–CXCL1-treated mice was significantly less than in anti–IL-6–treated and control Ab–treated mice. Eyes of B6 mice were infected with 10^6 PFU HSV-1, McKrae strain. Six weeks following infection, mice were irradiated with UV-B to reactivate the latent infection. Mice were then treated with anti–CXCL1 (*n* = 19) or anti–IL-6 (*n* = 20) or treated with control Ab (*n* = 18). Corneal opacity (A) and corneal neovascularization (B) were measured and compared between these different treatments. Mice treated with anti–CXCL1 displayed significantly reduced corneal opacity and neovascularization than did either anti–IL-6–treated or control Ab–treated mice from day 7 until day 35 (*p* < 0.01–0.001). No significant differences were noted between anti–IL-6 and control Ab–treated mice.
Results

Previous studies evaluating the early production of chemokines and cytokines during primary HSK have indicated that several of them are critical to the development of disease (22). Some but not all of these factors have been evaluated in recurrent HSK (23). The results of these later studies have been to illustrate that cytokines and chemokines that are important to the development of primary HSK may have very little to do with the development of recurrent HSK (23). As a consequence of our ongoing interest in evaluating these factors in recurrent HSK, we evaluated the role of CXCL1 (KC in mice) and IL-6 during recurrent HSK.

Inflammation is critical to the development of clinical disease in HSK. By blocking the factors that promote inflammatory cell migration, we hypothesized that this would lead to decrease the total inflammation in the eye a consequence would be the presumed decrease in clinical disease. We chose CXCL-1 and IL-6 precisely because they have proinflammatory properties and as such provide logical targets for therapeutic intervention.

We and others have demonstrated that the presence of a significant infiltration of neutrophils into the cornea is associated with clinical disease in both primary (22, 24, 25) and recurrent (21) models of HSK. Consequently, our first molecular target was CXCL1, a potent neutrophil chemokine (9–12). Our data demonstrate that Ab neutralization of CXCL1 in vivo resulted in significantly lower clinical scores in both opacity and neovascularization during each week of clinical observation following reactivation as compared with treatment with a control Ab preparation (Fig. 1). At the same time, we also targeted IL-6 with a neutralizing Ab toward this cytokine. Unlike what has been reported during primary HSK (11, 17), neutralizing IL-6 did not lead to a decrease in recurrent HSK (Fig. 1). When these mice were assayed for shedding virus, as a measure of apparent reactivation rate, more mice treated with anti-CXCL1 had detectable virus than either anti–IL-6 or control Ab–treated mice; however, there were no differences in viral persistence between these different treatment groups (Table I). This suggests that in the absence of CXCL1, more viruses are produced in the cornea following UV-B–induced reactivation, but that virus is eventually cleared with the same kinetics.

Because the effect of targeting CXCL1 was so dramatic, we wished to confirm these results by performing similar studies in a different strain of mouse. We chose the inbred NIH strain, because this mouse strain has been used very successfully in recurrent models of HSK (23). As was seen in B6 mice, these mice also failed to generate significant levels of recurrent HSK disease when treated with a neutralizing Ab directed against CXCL1 (Fig. 2). It should be noted that there were no differences in viral shedding between anti–CXCL and control Ab–treated mice; however, there were no differences in viral persistence between these different treatment groups (Table I).

To provide further confirmation, we decided to test gene-targeted mice who do not express the primary receptor for CXCL1, namely CXCR2 (13, 14), in our model of recurrent HSK. Results from these studies further emphasized that CXCL1 is needed for recurrent HSK, because B6-CXCR2 KO mice displayed significantly reduced recurrent HSK than did wild-type B6 mice (Fig. 3).

Because our results for neutralizing IL-6 were so unexpected, we evaluated recurrent HSK in mice genetically incapable of producing this cytokine, B6-IL-6 KO mice. Results were similar to neutralization studies (Fig. 1), because recurrent HSK in B6-IL-6 KO mice was similar to that observed in wild-type B6 mice (Fig. 4).

In fact, if anything, the trend, though not statistically significant for virtually all time points, seemed to be toward increased recurrent HSK in these mice. Consequently, these data support the notion that IL-6 plays little, if any, critical role in the development of recurrent HSK.

Because mice treated with anti-CXCL1 demonstrated its critical role during recurrent HSK, we decided to characterize the in-

Table I. HSV-1 shedding following UV-B reactivation

<table>
<thead>
<tr>
<th>Parameter Analyzed</th>
<th>IgG</th>
<th>Anti–IL-6</th>
<th>Anti–CXCL1</th>
</tr>
</thead>
<tbody>
<tr>
<td>% positive swabs</td>
<td>7%</td>
<td>6%</td>
<td>12%</td>
</tr>
<tr>
<td>Total shedding days</td>
<td>6</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Days shedding/mouse</td>
<td>1.2 ± 0.11</td>
<td>1.0 ± 0.00</td>
<td>1.2 ± 0.10</td>
</tr>
<tr>
<td>% reactivation rate</td>
<td>27.80%</td>
<td>30%</td>
<td>47.40%</td>
</tr>
<tr>
<td>Final day shedding</td>
<td>Day 5</td>
<td>Day 5</td>
<td>Day 5</td>
</tr>
<tr>
<td>n</td>
<td>18</td>
<td>20</td>
<td>19</td>
</tr>
</tbody>
</table>

*The percent positive swabs is the percentage of virus-positive eye swabs (140–286 eye swabs/group) over the 10-d period following UV-B irradiation (p < 0.01 for anti–CXCL1-treated mice).

†Total shedding days is the number of days swab positive (p < 0.01 for CXCL1-treated mice).

‡Days shedding/mouse is the number of days that a positive mouse shed virus (no significance).

§Percent reactivation rate is the percentage of mice who reactivated (p < 0.02 for CXCL1-treated mice).

A: Recurrent HSK in anti–CXCL1-treated NIH mice was significantly less than in control Ab–treated mice. Eyes of NIH mice were infected with 106 PFU HSV-1, McKrae strain. Six weeks following infection, mice were irradiated with UV-B to reactivate the latent infection. Mice were then treated with anti-CXCL1 (n = 20) or treated with control Ab (n = 20). Corneal opacity (A) and corneal neovascularization (B) were measured and compared between these different treatments. Mice treated with anti-CXCL1 displayed significantly reduced corneal opacity and neovascularization than did control Ab treated mice from day 7 until day 35 (p < 0.01–0.001).

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Inflammatory infiltrate of B6 mice treated with anti-CXCL1 versus B6 mice treated with control IgG via flow cytometry. This analysis revealed two basic differences, the first being that the CD45+ infiltrate was less in anti–CXCL1-treated mice (4,678 ± 1,046/cornea) than in B6 control Ab–treated mice (36,723 ± 8,439/cornea), indicating that overall inflammation by CD45+ cells is significantly affected by targeting CXCL1. The second difference was that analysis of the CD45+ infiltrate in mice treated with anti-CXCL1 displayed a significantly reduced percentage of Gr-1+ neutrophils (p = 0.019; Fig. 5) and a nonstatistically significant trend toward increased percentages of CD4+ T cells than did mice treated with control Ig (Fig. 5). These data confirm the important role that CXCL1 plays in directing migration of neutrophils to corneas experiencing recrudescence of HSV-1.

**Discussion**

At present, the treatment of choice for HSK is a combination of antitherpetic drugs to limit the replication of virus and steroids to prevent, or at least restrict, inflammation. In most cases, this is effective in controlling the disease. However, such treatment comes with certain costs. One is that not all individuals respond the same to these treatments (1–6). In one study, up to 20% of patients retained some degree of corneal clouding (26), whereas in another study, 30% had lingering corneal inflammation (27). Another is that it is possible for the virus to become refractory to the antiviral treatment (5, 28). And finally, steroid treatment itself has the potential of causing its own unwanted side effects (29–31). We have been studying recurrent HSK to better understand the factors that control disease such that we might identify more specific targets of therapeutic intervention. In this paper, we demonstrate that at least two strains of mice treated with anti-KC (CXCL1) displayed significant and strikingly lower incidence and severity of disease when compared with control Ab–treated animals. Because of CXCL1/KC’s established role as a chemoattractant for neutrophils, these results again confirm the importance of neutrophils in recurrent HSK pathology. They also support the idea that the majority of corneal disease pathology can be prevented with neutralization of CXCL1/KC. Given the fact that there are few effective treatments for ocular HSV-1 infections, these data suggest a possible new target for medical treatment of a disease that continues to be responsible for significant morbidity worldwide.

We further confirmed the importance of CXCL1 by using mice who do not express the CXCR2 chemokine receptor for which CXCL1 is known to bind. These mice also displayed significantly less disease than did wild-type B6 mice. Consequently, the case for CXCL1 being a critical factor leading to significant recurrent HSK disease is very compelling.

So although the case for targeting CXCL1 during recurrent HSK is strong, we did not observe similar results when targeting IL-6.
This is in contrast to what has been reported during primary HSK (12, 15, 32, 33) where IL-6 production appears to play a significant role during acute HSV-1 infection of the cornea and thus is involved in the disease pathology associated with primary HSK. Our study used both Ab neutralization and IL-6 gene–targeted mice as a means of targeting IL-6. The results for both of these methods indicate that lack of functional IL-6 does not affect recurrent HSK disease incidence or progression in B6 mice. Although it is possible IL-6 might play a more critical role in mice who display greater disease than do B6 mice, such as NIH mice, we have not observed that for other factors that we have tested in our recurrent model of HSK (Refs. 19 and 21 and P. Stuart and T. Keadle, unpublished observations when assessing IFN-γ and IL-10). That said, these results concerning IL-6 were surprising, because this cytokine is found in abundance during both primary (12, 15, 32, 33) and recurrent (34) HSK, and thus, it has been assumed that it is important in both forms of the disease. However, none of these studies actually neutralized IL-6 and thus did not evaluate its importance to the disease process in animals that can and do generate a normal IL-6 response. Another possible implication of these data concerns the potential role that Th17 cells might play in recurrent HSK. It is known that IL-6 is involved in the generation of Th17 cells (35), which have been implicated in primary HSK (25, 36). Consequently, if Th17 or IL-17 itself is playing a significant role in recurrent HSK and IL-6 is not involved in recurrent HSK, this implies that either Th17 cells are generated by a pathway that does not involve IL-6 or some other cell in the cornea is producing IL-17. Future studies need to be performed to address the role that IL-17 plays in recurrent HSK, particularly because Suryawanshi et al. (25) reported that IL-17A leads to increased production of CXCL1 during primary HSK, which they demonstrated led to increased neutrophilic infiltration into the cornea.

It is interesting to note that, although we did not observe an increase in the time that mice shed virus, we did note that the number of mice shedding virus was significantly greater in mice treated with neutralizing anti–CXCL1 Ab (47%) than in B6 mice treated with a control Ab (28%). One would have predicted that this might lead to increased corneal disease because of greater levels of virus in these CXCL1-neutralized mice as compared with control Ab–treated B6 mice. This was precisely the argument made for results reported for CXCL10 KO mice who displayed increased viral titers, which they argued led to increased primary HSK (16). That said, that argument is not supported by our data for neutralizing CXCL1 nor did we see that in CXCR2 KO mice who also displayed increased numbers of mice shedding virus despite reduced recurrent HSK (data not shown). We would argue that the primary reason for our observations is that although targeting CXCL1 leads to increased virus shedding in the corneas, the fact that the numbers of neutrophils infiltrating the cornea is significantly reduced will lead to less disease. Particularly because we know that HSK requires a significant neutrophilic infiltrate of the cornea (21, 24), less disease will be the consequence of fewer neutrophils. That without the recruitment of neutrophils to the cornea it does not matter whether viral production is not controlled as efficiently because these corneas lack a critical cell required for HSK. In fact, reports have indicated that the dominant cells that remove virus from the cornea are T cells and NK cells and not neutrophils (37–39); thus, viral clearance is not dependent on a robust recruitment of neutrophils.

Taken together, our results clearly indicate that CXCL1 is a major player in recurrent HSK. That therapeutic targeting of this chemokine significantly reduces recurrent disease. These results also indicate that IL-6 is not intimately involved in recurrent HSK and thus is not a useful target for therapeutic intervention.

Acknowledgments
We thank Stephanie Zobell and Chloe Potter for technical assistance in this work. We thank Dr. Robyn Klein (Department of Internal Medicine and Infectious Diseases, Washington University, St. Louis, MO) for helpful discussions.

Disclosures
The authors have no financial conflicts of interest.

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