

## CCL3 protects mice from corneal pathology during recurrent HSV-1 infection

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## 1. ABSTRACT

CCL2 and CCL3 are proinflammatory chemokines that are produced during the early stages of inflammation and are known to stimulate the migration of mononuclear cells to the site of inflammation. Previous studies addressing the role of these chemokines during primary herpetic stromal keratitis (HSK), have suggested that CCL2 is involved in reducing corneal disease and that CCL3 is involved in promoting this disease. We addressed the role that these chemokines play in a recurrent model of HSK. Results from these studies did not demonstrate a significant role for CCL2 except for very early time points following reactivation of virus. Surprisingly, mice deficient in CCL3 did not have significantly reduced recurrent disease, but in fact showed significantly enhanced disease. This argues that CCL3 might play an ameliorative role during recurrent HSK. In addition, we observed that these same CCL3 deficient mice showed increased resistance to viral-induced mortality following infection with HSV-1. Taken together, these results suggest that CCL3 plays a significant protective role during recurrent HSK and is involved in enhancing lethality.

## 2. INTRODUCTION

Herpetic stromal keratitis (HSK) is a potentially blinding corneal inflammation that accompanies herpes simplex virus (HSV) infection of the eye. The disease course in HSK begins with a primary infection by HSV followed by a period during which the virus enters latency in sensory and autonomic ganglia. Many studies have shown that clinical disease is the result of a cocktail of inflammatory cells, consisting of PMN's, macrophages and T cells (both CD4<sup>+</sup> and CD8<sup>+</sup>) (1-5). These cells are recruited to the site of infection by the release of inflammatory factors, including cytokines and chemokines (5-7). Once there, these cells appear to be responsible for the majority of corneal damage (5-7). Key to this process resulting in corneal damage is the presence of CD4<sup>+</sup> Th1 whose cytokines dominate during the preclinical and clinical phases of acute HSK (5-12).

Trafficking of inflammatory cells following infection is governed by the release of chemokines and the expression of various integrin and selectin molecules (13,14). This report is focused on the role that chemokines

play during herpetic disease. Chemokines are a superfamily of structurally related proteins of 8 to 10 kDa that are involved in chemotactic migration of leukocytes to sites of infection and inflammation (15,16). Several reports have demonstrated that chemokines are important factors in viral infections (17) and HSK (6,10). It has been shown that IL-6 stimulates resident corneal cells to produce CCL3 (MIP-1 $\alpha$ ) and CXCL1/2 (MIP-2) (18). Targeting of CCL3 significantly reduces primary HSK (19). These mice displayed a striking paucity of inflammatory cells (both neutrophils and T cells) in the infected corneas, suggesting that CCL3 acts as a chemotactic factor for these cells. Likewise, targeting of MIP-2 led to similar results, suggesting that this chemokine is another important factor in the early stages of primary HSK (20,21). Interestingly, an earlier report targeting CCL2 (MCP-1) did not demonstrate that primary disease was altered (11). However, more recently using CCL2 $^{-/-}$  mice Lee, et al. (22) demonstrated that these mice had significantly greater disease presumably due to the over-production of MIP-2. Eo, et al. (23) co-immunized mice with a plasmid encoding gB of HSV along with various plasmids containing MCP-1, MIP-2, MIP-1 $\alpha$ , and MIP-1 $\beta$  to determine if the adaptive immune response generated was affected by the presence of specific chemokines. Their data indicates that chemokines MCP-1 and MIP-1 $\beta$  biased immunity to a Th2-like response, while MIP-2 and MIP-1 $\alpha$  activated a Th1 response. Mikloska, et al. (24) observed increased levels of MIP-1 $\beta$ , and to a lesser extent, MIP-1 $\alpha$  and RANTES in human vesicle fluid from patients with herpetic corneal lesions. Similar studies evaluating IL-8 expression has shown that infection of corneal-derived cells with HSV-1 leads to significant increases in IL-8 production, which stimulates neutrophil chemotaxis (25,26). The role of IP-10 in primary HSK is more complex. It has been reported that IP-10 (CXCL10) is very important in recruiting activated T cells into sites of inflammation (27). However, IP-10 has been shown to be a mediator of IL-12-mediated anti-angiogenesis during primary HSK (28). Thus it is possible that IP-10 might be involved in both promoting disease, by attracting activated CD4 $^{+}$  T cells and in preventing disease by its ability to inhibit the growth of new blood vessels to the cornea.

Because chemokines play such an important role during acute herpes infections, we decided to investigate the role that two cytokines, CCL2 and CCL3 play during recurrent herpetic stromal keratitis (HSK). These two were chosen because they represent chemokines that interact with different receptors and thus have their activity on somewhat different populations of cells (29-31) and their role in acute disease does not appear to be similar (19-24). To test their role in recurrent disease, we performed studies in mice that are unable to produce these chemokines. Results indicate that mice incapable of producing CCL3 unexpectedly had greater corneal disease while the inability to produce CCL2 did not result in any change in disease phenotype.

## 3. MATERIALS AND METHODS

### 3.1. Virus and cells

The viruses used in these studies were the McKrae strain of HSV-1. A plaque-purified stock was

grown and assayed on Vero cells in minimum essential medium with Earle's balanced salts (MEM-EBS) containing 5% fetal bovine serum, 100U/ml Penicillin and 100  $\mu$ g/ml Streptomycin. (32). Virus titers in eye swabs were determined by standard plaque assay (32).

### 3.2. Mice and primary infection

Investigations with mice conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 (B6) was purchased from NCI. The B6.129S4-*Ccl2*<sup>tm1Roi</sup>/J (B6-CCL2 KO) and B6.129P2-*Ccl3*<sup>tm1Unc</sup>/J (B6-CCL3 KO) mice were purchased from Jackson Labs and maintained in our colony. Eight to twelve week old normal and KO mice were infected as previously described (33). Briefly, following corneal scarification, 2 x 10<sup>5</sup> Plaque Forming Units (PFU) of HSV-1 McKrae strain in 5  $\mu$ l of MEM-EBS was placed onto the surface of the right cornea of C57BL/6, CCL2 KO and CCL3 KO mice.

### 3.3. UV-B inducible model of recurrent herpetic stromal keratitis.

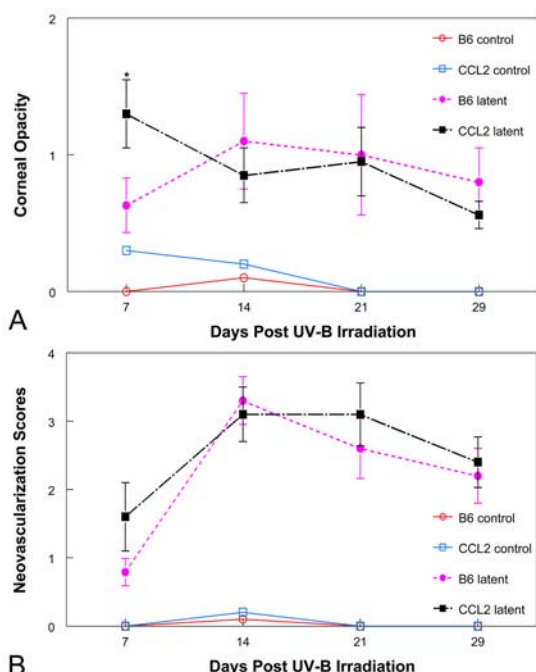
The UV-inducible model of recurrent HSK has been described (34). Briefly, the corneas of 6 week old NIH inbred mice (Harlan Olac Ltd., Oxford, England) (though we have recently used BALB/c and C57BL/6 mice in this model (35)) are infected with 1 X 10<sup>6</sup> pfu HSV-1 McKrae, with concurrent administration of anti-HSV serum to protect corneas from damage during infection. Four+ weeks post infection, the eyes of latently-infected and control mock-infected mice are exposed to 250 mJ/cm<sup>2</sup> of UV-B light. Before (day 0), and on day's 1 to 7 post-UV-B irradiation, the eyes of mice are swabbed and cultured on Vero cells to detect recurrent viral shedding.

### 3.4. Clinical evaluation

On the designated days after viral infection, a masked observer examined mouse eyes through a binocular-dissecting microscope in order to score clinical disease. Stromal opacification was rated on a scale of 0 to 4, where 0 indicates clear stroma, 1 indicates mild stromal opacification, 2 indicates moderate opacity with discernible iris features, 3 indicates dense opacity with loss of defined iris detail except pupil margins, and 4 indicates total opacity with no posterior view. Corneal neovascularization was evaluated as described (32) using a scale of 0-8, where each of four quadrants of the eye is evaluated for the amount of vessels that have grown into them. Periocular disease was measured in a masked fashion on a semiquantitative scale as previously described (36).

### 3.5. Viral titering from tissues

Eye swab material was collected and assayed for virus by standard plaque assay as previously described (33). Trigeminal ganglia and 6-mm biopsy punches of periocular skin were removed and placed in preweighed tubes containing 1-mm glass beads and 1 ml of medium. Trigeminal ganglia and periocular skin homogenates were prepared by freezing and thawing the samples, mechanically disrupting in a Mini-Beadbeater-8 (Biospec Products, Bartlesville, Okla.), and sonicating. Homogenates



**Figure 1.** Absence of CCL2 does not result in changes in disease phenotype during recurrent HSK with the expectation of Day 7. Eyes of latently infected B6 wild-type (n=36) and B6-CCL2 KO (n=32) mice were exposed to UV-B irradiation to elicit viral reactivation. Corneal opacity (A) and corneal neovascularization (B) were measured and compared between these two indicated strains as well as UV-B irradiated, uninfected controls of both B6 (n=10) and B6-CCL2 KO (n=10). Significant virus-induced corneal pathology was observed for all time points for both strains of mice when compared to their respective UV-B controls,  $P < 0.001$  to  $0.05$ . Only Day 7 demonstrated significantly increased pathology for CCL2 KO mice ( $P < 0.01$ ), all other time points did not show significant differences.

were assayed for virus by standard plaque assay, and the amount of virus was expressed as PFU per milliliter of tissue homogenate.

### 3.6. Statistical analysis

All statistical analyses were performed with the aid of Sigma Stat for Windows, version 2.0 (Jandel, Corte Madera, CA). The Student's unpaired *t*-test and the Mann-Whitney rank sum test were used as appropriate to compare corneal disease scores, mortality, and viral shedding data. Fisher's exact or  $\chi^2$  tests were used to compare reactivation rates.

## 4. RESULTS

These studies utilized a mouse model of recurrent HSK that clinically and histologically shows a significant degree of fidelity to that seen during human disease (34,37). In this model, mice undergo corneal infection with HSV-1 in the presence of passively acquired protective antibody to allow for the mice to develop a latent

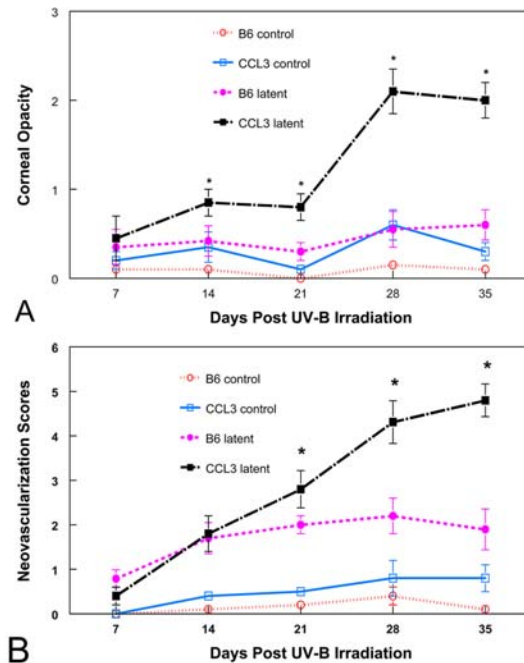
infection of the trigeminal ganglia without significant corneal damage. Once latency is established, these mice are subjected to UV-B irradiation which reactivates viral replication leading to virus being shed in the tear film of the originally infected eye. Viral shedding stimulates an inflammatory response which has been shown to consist of T cells, monocytic cells and PMN's (37,38). Interestingly, both Th1 and Th2 cells are present in the inflammatory infiltrate, even at early time points following reactivation (39,40).

Since both subsets of Th cells are present it is possible that either CCL2 (Th2) or CCL3 (Th1) could be involved in the disease process. We would anticipate, from earlier publications demonstrating the ameliorative role that IL-10 plays in recurrent HSK (35), that inability to produce CCL2 might result in worse corneal disease and that loss of CCL3 might lead to less disease. Results from studies testing this hypothesis only revealed one time point, day 7 that was consistent with that hypothesis (Figure 1A). Thus we conclude that overall, mice incapable of producing CCL2 do not display increased recurrent HSK pathology. On the other hand, opacity scores in for CCL3 KO mice, which we predicted would be less than wild-type B6 mice, were in fact significantly greater (Figure 2A). This same pattern for both B6-CCL2 KO and B6-CCL3 KO mice was also seen when neovascularization was evaluated in these mice (Figure 1B and Figure 2B). Because our observations for B6-CCL3 were the opposite from what has been reported during acute disease, we decided to repeat this study and once again observed the same pattern of corneal pathology following UV-B induced reactivation (Figure 3A and Figure 3B).

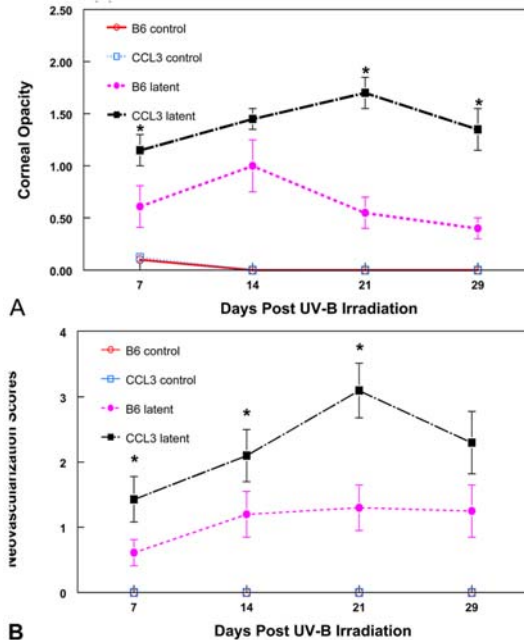
It was possible that increased disease in the corneas of CCL3 KO mice might have been due to impaired viral clearance from the corneas of these mice due to decreased trafficking to the cornea by cells that would be involved in clearance of virus. This could result in higher reactivation rates and persistence of virus in the corneas of these mice. However, that was not the case as the reactivation rates and the kinetics of viral shedding were not significantly greater in CCL3 KO mice (Table 1).

Interestingly, there were significant differences when these parameters were compared between wild-type B6 mice and CCL2 KO mice. The CCL2 KO mice demonstrated significantly reduced reactivation rates and reduced viral shedding (Table 1).

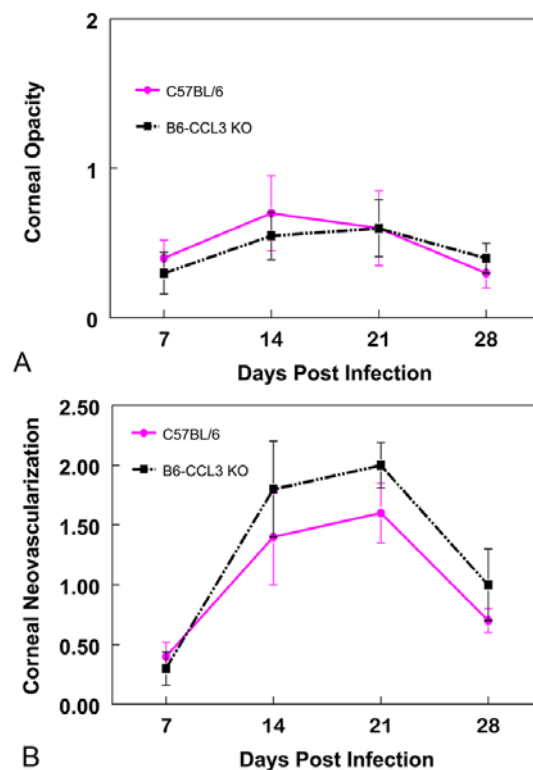
Because our results for CCL3 KO mice were in such stark contrast to what had been reported for this mouse strain during acute HSV-1 infection (19), we decided to perform acute infections in these mice and compared them to wild-type B6 mice. Results from these studies indicated that CCL3 KO mice did not demonstrate significantly less corneal disease than did wild-type B6 mice (Figure 4). In fact the disease profile for both corneal opacity and neovascularization were indistinguishable between these two strains of mice (Figure 4A, 4B). Interestingly however, wild-type mice did experience significantly greater mortality than did CCL3 KO mice (Figure 5).



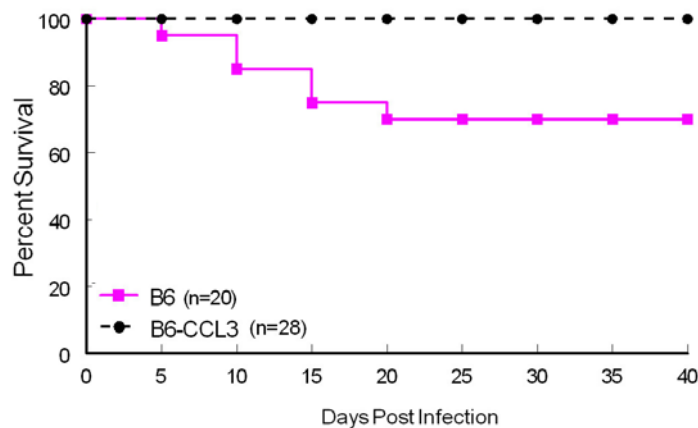
**Figure 2.** Absence of CCL3 leads to significantly worse recurrent HSK disease pathology. Eyes of latently infected B6 wild-type (n=35) and B6-CCL3 KO (n=33) mice were exposed to UV-B irradiation to elicit viral reactivation. Corneal opacity (A) and corneal neovascularization (B) were measured and compared between these two indicated strains as well as UV-B irradiated, uninfected controls of both B6 (n=10) and B6-CCL3 KO (n=10). Significant virus-induced corneal pathology was observed for all time points for CCL3 mice and for days 14 to 35 for wild-type B6 mice when compared to their respective UV-B controls,  $P < 0.0005$  to  $0.05$ . Significantly increased pathology were noted for latently infected CCL3 KO mice as compared to latently infected B6 mice for days 15-35, ( $P < 0.001$  to  $0.02$ ).



**Figure 3.** Absence of CCL3 leads to significantly worse recurrent HSK disease pathology. Eyes of latently infected B6 wild-type (n=27) and B6-CCL3 KO (n=22) mice were exposed to UV-B irradiation to elicit viral reactivation. Corneal opacity (A) and corneal neovascularization (B) were measured and compared between these two indicated strains as well as UV-B irradiated, uninfected controls of both B6 (n=10) and B6-CCL3 KO (n=10). Significant virus-induced corneal pathology was observed for all time points for both wild-type B6 and B6-CCL3 KO mice when compared to their respective UV-B controls,  $P < 0.001$  to  $0.05$ . Significantly increased pathology were noted for latently infected CCL3 KO mice as compared to latently infected B6 mice for days 15-28, ( $P < 0.001$  to  $0.05$ ).



**Figure 4.** No significant differences in corneal pathology were observed following acute infection with  $5 \times 10^5$  pfu of the McKrae strain of HSV-1 in B6 or B6-CCL3 KO mice. Corneal opacity (A) and corneal neovascularization (B) were measured and compared between B6 wild-type mice (n=20) and B6-CCL3 KO (n=30) mice. Figure 5. Significant differences ( $P < 0.02$ ) were observed in survival of B6-CCL3 (n=30) as compared to wild-type B6 (n=20) mice following acute infection.



**Figure 5.** Significant differences were observed in survival of B6-CCL3 (n=30) as compared to wild-type B6 (n=20) mice following acute infection.

## 5. DISCUSSION

Chemokines are a large family of structurally homologous proteins that initiate leukocyte trafficking and regulate the migrations of these cells from the blood to tissues. Hence they are very important in mobilizing an immune response to the site of any microbial infection, including viruses (13-17). It has been reported that following HSV-1 infection, there is an increase in both CCL2 and CCL3 production, both at the site of infection and in the regional nervous

system (41). As indicated earlier CCL2 has been associated with Th2 cells and CCL3 with Th1 cells, though there are reports that CCL2 can also be associated with classical DTH responses that are typically mediated by Th1 cells (42). CCL2 is a potent monocyte chemoattractant secreted by a variety of cell types in response to proinflammatory stimuli (43,44). It has also been reported that mice unable to produce CCL2 displayed significantly increased inflammation by Gr-1<sup>+</sup> and CD11b<sup>+</sup> cells and that these mice experienced

**Table 1.** Reactivation rate and days shedding

Experiment	Shedding <sup>1</sup>	Mouse Strain	Reactivation Rate <sup>2</sup>	Days Shedding <sup>3</sup>	Total Days <sup>4</sup>
One		B6	13/35(37)	2.1± 0.3	27/245
		B6-CCL2 KO	9/32 (28)	1.3± 0.2 <sup>5</sup>	12/224 <sup>6</sup>
		B6-CCL3 KO	12/33 (36)	1.8± 0.4	22/231
Two		B6	10/27 (37)	2.8± 0.6	19/162
		B6-CCL3 KO	9/22 (41)	2.2± 0.3	20/132

<sup>1</sup> Eyes of mice with latent ocular HSV infection were swabbed to detect recurrent viral shedding from days 0 to 7 and again at day 10., <sup>2</sup> Number of eyes shedding virus ÷ total number of UV-B irradiated, latently infected eyes. (% reactivation)., <sup>3</sup> Geometric mean ± SEM., <sup>4</sup> Number of virus positive swabs ÷ total number of swabs performed., <sup>5</sup> P=0.023., <sup>6</sup> P=0.013.

significantly greater opacity and neovascularization (22). These authors proposed that CCL2 was involved in controlling HSK by its ability to reduce the production of MIP-2 in HSV-1 infected corneas (22). However, as we report here, within reactivated disease, CCL2 does not appear to play any substantial role as mice deficient in this chemokine do not demonstrate any more corneal disease than that seen in CCL2 sufficient mice.

These studies also evaluated CCL3, which is another inflammatory chemokine that activates macrophages to secrete TNF- $\alpha$  (30,45) and exhibits chemotactic activities on several cell types, including T cells, monocytes, and to lesser extent neutrophils (46-48). In contrast to what we observed with CCL2 during recurrent HSK, our data indicates that CCL3 plays an important role during recurrent HSK. Much to our surprise, that role for CCL3 is not the same as has previously been reported for it during acute disease (19). In acute disease, it has been reported that mice which lack CCL3 display significantly less disease than that seen in wild-type mice. This was believed to be due to CCL3's role in stimulating the migration of T cells and neutrophils to the corneas of infected mice (19). In contrast to those reports, mice deficient in CCL3 not only did not have less recurrent HSK, but actually displayed significantly worse disease than did CCL3 sufficient mice. This argues that CCL3 plays a protective role during recurrent HSK. This is a very novel observation as, with few exceptions (49), the presence of CCL3 during an immune response is associated with increased inflammation and greater tissue damage (19,30,45-47). The fact that CCL3 is associated with reduced corneal disease during recurrent HSK is perplexing. One possible clue that might begin to unravel this paradox is a report by Trifilo, *et al.* (50) in which they report that CCL3 produced following viral infection not only mediates macrophage chemotaxis, which would tend to promote inflammation, but also has direct effects on the activation and migration of antigen-specific CD8<sup>+</sup> T cells. In addition, our lab reported that CD8<sup>+</sup> T cells were associated with reduced disease during HSV-1 infection of the cornea (51). Thus, it is possible that in mice which are deficient in CCL3 there is reduced activation and mobilization of CD8<sup>+</sup> T cells that are involved in modulating corneal disease leading to an increased disease phenotype in these mice.

Our observation that acute infection in CCL3-/- mice does not result in significantly worse corneal disease than seen in wild-type B6 mice is also at odds with previous reports (19). This might be due to changes that have occurred in

these mouse strains since those studies were performed. None the less, both our results as well as others (19) are not consistent with the phenotype we report here for CCL3's role in recurrent HSK. We believe that this is in part due to the inherent differences that exist between acute and recurrent corneal disease. Comparative studies in mice show that recurrent HSK differs from primary disease in at least four aspects: 1) clinical pathology (34,37); 2) HSV-1 antigen distribution within the cornea (37); 3) macrophages being a greater component of the inflammatory infiltrate than PMN's (37); 4) the differential ability of similar vaccine constructs to ameliorate primary and recurrent HSK (52). Thus it should not be surprising that the relative contribution of CCL3 to corneal responses in a sensitized host (recurrent HSK) would be different from that in a naive host (acute infection). Particularly since CCL3 primarily has its effects on primed CD8<sup>+</sup> T cells (50) as would be the case during recurrent disease. The precise mechanism responsible for our observations remains to be determined and will be the subject of future investigations.

Interestingly, while corneal disease did not demonstrate differences between CCL3 deficient and CCL3 sufficient mice, there was a significant difference in mortality seen in these strains of mice following acute infection. CCL3 sufficient mice displayed a 30% rate of mortality when infected with 5x10<sup>5</sup> pfu of the McKrae strain of HSV-1, while none of the CCL3 deficient mice died. The exact mechanism for this difference in mortality has not been determined. However there are several reports that indicate that CCL3 does play a role in some types of brain pathology. An example of this are reports demonstrating that CCL3, along with several other cytokines are associated with lethal infections by west Nile virus (53). It has also been shown that inflammation of the CNS is promoted by the ability of astrocytes to produce CCL3 which stimulates the migration of dendritic cells to the CNS (54). Likewise, transendothelial migration of both dendritic cells and T cells has been shown in other models to be due to the production of CCL3 (55,56). Thus we would propose that the mortality seen in wild-type B6 mice is due to increased inflammation of the brain following infection of mice with the neurovirulent McKrae strain of HSV-1. CCL3 deficient mice would be somewhat protected from viral-induced encephalitis due to the absence of the CCL3-mediated signal that allows for increased migration of inflammatory cells into the brain. Future studies will test this hypothesis to determine whether there is decreased migration of inflammatory cells into the CNS following HSV-1 infection.

## 6. ACKNOWLEDGEMENTS

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