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CXCL9 and CXCL10 Expression Are Critical for Control of Genital Herpes Simplex Virus Type 2 Infection through Mobilization of HSV-Specific CTL and NK Cells to the Nervous System[1]

Manoj Thapa,* Robert S. Welner,‡ Rosana Pelayo,‡ and Daniel J. J. Carr[2]*†

CXCL9 and CXCL10 mediate the recruitment of T lymphocytes and NK cells known to be important in viral surveillance. The relevance of CXCL10 in comparison to CXCL9 in response to genital HSV-2 infection was determined using mice deficient in CXCL9 ($CXCL9^{-/-}$) and deficient in CXCL10 ($CXCL10^{-/-}$) along with wild-type (WT) C57BL/6 mice. An increased sensitivity to infection was found in $CXCL10^{-/-}$ mice in comparison to $CXCL9^{-/-}$ or WT mice as determined by detection of HSV-2 in the CNS at day 3 postinfection. However, by day 7 postinfection both $CXCL9^{-/-}$ and $CXCL10^{-/-}$ mice possessed significantly higher viral titers in the CNS in comparison to WT mice consistent with mortality (18–35%) of these mice within the first 7 days after infection. Even though $CXCL9^{-/-}$ and $CXCL10^{-/-}$ mice expressed elevated levels of CCL2, CCL3, CCL5, and CXCL1 in the spinal cord in comparison to WT mice, there was a reduction in NK cell and virus-specific $CD8^+$ T cell mobilization to this tissue, suggesting CXCL9 and CXCL10 are critical for recruitment of these effector cells to the spinal cord following genital HSV-2 infection. Moreover, leukocytes from the spinal cord but not from draining lymph nodes or spleens of infected $CXCL9^{-/-}$ or $CXCL10^{-/-}$ mice displayed reduced CTL activity in comparison to effector cells from WT mice. Thus, the absence of CXCL9 or CXCL10 expression significantly alters the ability of the host to control genital HSV-2 infection through the mobilization of effector cells to sites of infection. ***The Journal of Immunology,*** **2008, 180: 1098–1106.**

Type 2 HSV is a significant human pathogen and the most common cause of genital ulcerations in humans (1–5). With over 1.6 million Americans infected annually, HSV-2 is one of the most prevalent sexually transmitted diseases in the United States and worldwide (3, 4). In addition, the viral pathogen can be fatal in newborns and immunocompromised persons (4). Recent studies suggest acquisition of HIV increases 2- to 3-fold in HSV-2-infected individuals underscoring the contribution of this virus in facilitating increased susceptibility to other microbial pathogens (5–7).

The mobilization of effector cells (NK and T cells) to active sites of infection is driven by a number of soluble factors including chemokines, a family of small secreted proteins that are produced in response to viral infection (3–5). However, the role of chemokines in recruitment of effector cells relative to genital HSV-2 infection is largely unknown. It has been found that CXC type chemokines that lack the ELR sequence, including CXCL9 and CXCL10, are potent chemoattractants for activated T cells, NK cells, monocytes, dendritic cells, and B cells (8–11). Both CXCL9 and CXCL10 selectively bind the same G protein-coupled receptor CXCR3 that is highly expressed on activated T cells. Moreover, CXCR3 signaling has been demonstrated to be essential for generating an effective antiviral response against many viral infections by promoting a Th1 response (12–19).

T lymphocytes have been reported to be critical in suppressing viral replication in the mouse model of genital HSV-2 infection (20–26). These effector cells can lyse susceptible virus-infected cells by exocytosis of granzyme containing cytoplasmic granules and perforins or by Fas-mediated apoptosis (27–30). Likewise, NK cells monitor HSV-2 infection in mice operating through similar cytolytic processes in lysing infected cells (31, 32). The cellular processes that control virus infection are complemented by soluble factors including IFN-γ, which is critical for resolution of lesions and clearance of the virus following genital infection (33, 34).

Many chemokines and their receptors are expressed in a tissue-specific manner (35, 36). Previous studies have shown CXCL10 is expressed early in the CNS in response to virus infection (11, 12). We hypothesized the early expression of CXCL10 in infected tissues is critical to the outcome of HSV-2 genital infection by facilitating the recruitment of effector cells (NK and T cells) to the active sites of viral replication. In fact, an initial experiment found CXCL10 but not CXCL9 was significantly elevated above basal levels within 24 h of postinfection (p.i.)[3] in the vaginal tissue. In contrast, CXCL9 but not

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[3] Abbreviations used in this paper: p.i., postinfection; WT, wild type; LN, lymph node.

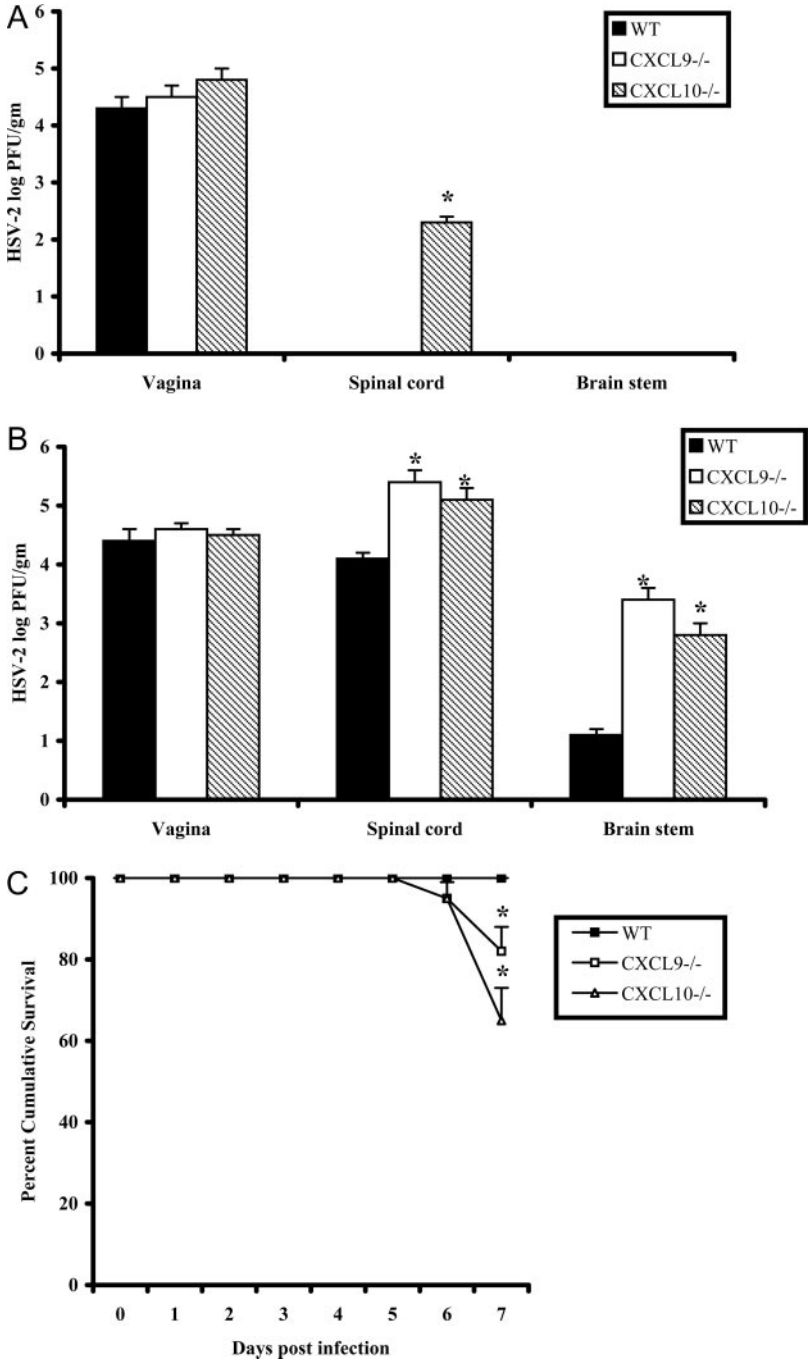

FIGURE 1. CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice are highly susceptible to HSV-2 genital infection. WT, CXCL9$^{-/-}$, and CXCL10$^{-/-}$ mice (n = 6 mice/group) were infected with HSV-2 (2000 PFU/vagina). At indicated times day 3 (*a*), and day 7 (*b*) p.i., mice were exsanguinated and vaginal tissue, brain stem, and spinal cord were processed and assayed for viral titer by standard plaque assay. Viral titer is expressed as mean log PFU ± SEM. *c*, WT, CXCL9$^{-/-}$, and CXCL10$^{-/-}$ mice (n = 44–59 mice/group) were infected with HSV-2 (2000 PFU/vagina) and were monitored and recorded for survival. Each data symbol represent mean ± SEM summarizing the results of three independent experiments. *, $p < 0.05$ comparing WT to CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice.

CXCL10 levels rose significantly within the first 72 h in draining lymph nodes (LNs) following genital HSV-2 infection. Taken together, it would appear the early tissue-specific chemokine response may provide a basis for redundancy is chemokines that operate through the lone receptor for CXCL9 and CXCL10, CXCR3. As a means to compare and contrast the nature of CXCL9 and CXCL10 expression in the immune response to a viral pathogen, mice deficient in CXCL9 (CXCL9$^{-/-}$) and deficient in CXCL10 (CXCL10$^{-/-}$) along with wild-type (WT) mice were evaluated for resistance to HSV-2 genital infection focusing on the inflammatory immune response, phenotypic analysis of leukocytes, and functional analysis of effector cells. The results suggest both CXCL9 and CXCL10 expression are necessary for optimal recruitment of NK cells and CTLs to the spinal cord as well as clearance of HSV-2 from the CNS following genital infection.

Materials and Methods

Virus and cells

A clinical isolate of HSV-2 obtained from Charity Hospital (New Orleans, LA) was propagated in Vero cells (African green monkey kidney fibroblasts, CCL-81; American Type Culture Collection). Virus stock (4.8×10^7 PFU/ml) was stored at −80°C and diluted in RPMI 1640 medium immediately before infection. Vero cells were propagated in RPMI 1640 medium containing 10% FBS, gentamicin, and antimycotic antibiotic solution (complete medium) (Invitrogen Life Technologies) at 37°C in 5% CO_2 and 95% humidity.

Mice and infection

WT C57BL/6 female mice 6- to 8-wk-old (The Jackson Laboratory), and CXCL9$^{-/-}$ (9) and CXCL10$^{-/-}$ (10) female mice backcrossed to the C57BL/6 genetic background for eight to nine generations were used for this study. Depo-Provera (Pharmacia and Upjohn) inoculated mice (2.0 mg/mice) were infected with 2000 PFU HSV-2 (20 μl) intravaginally.

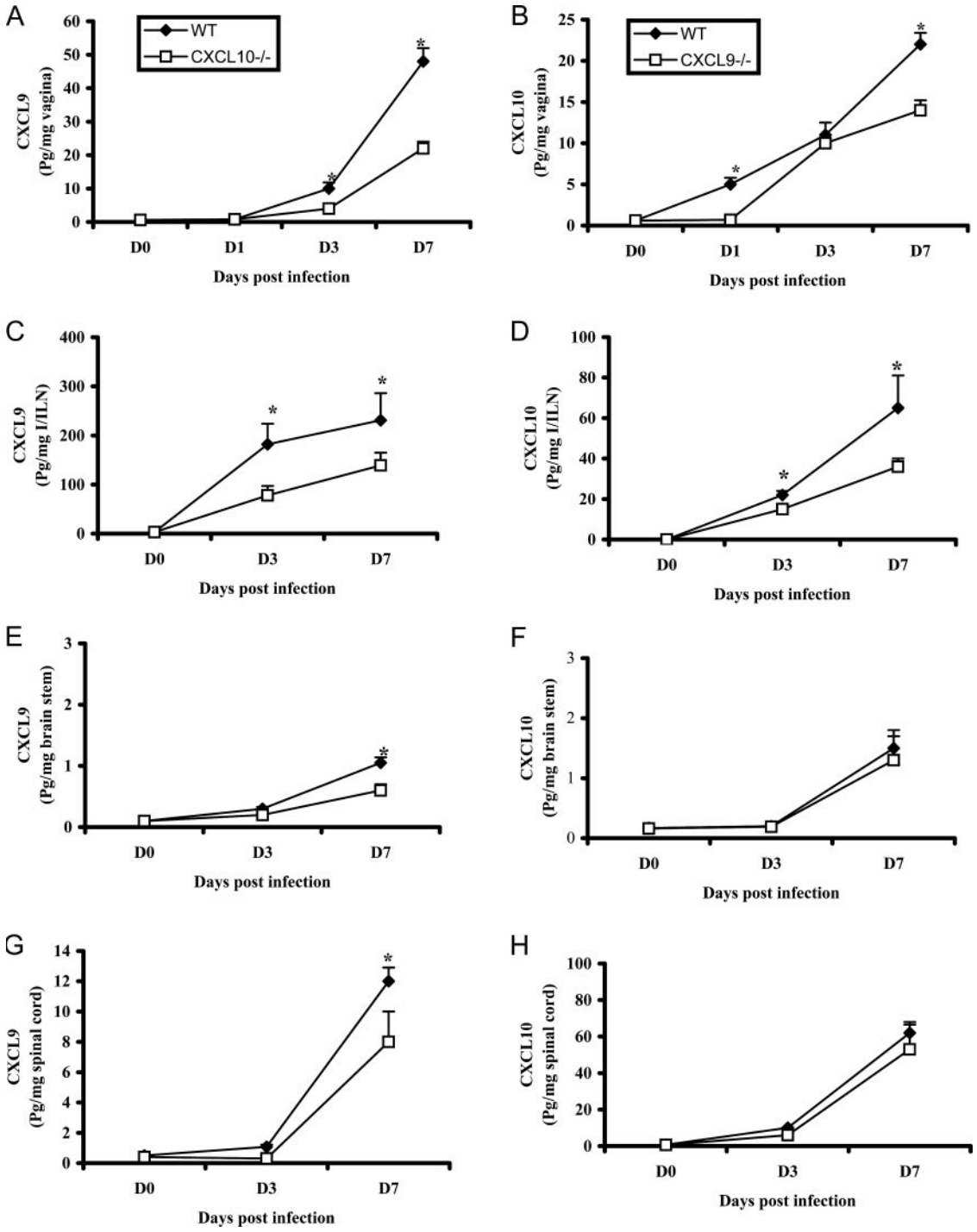

FIGURE 2. CXCL9 and CXCL10 levels are elevated in tissue-specific manner. WT, CXCL9$^{-/-}$, and CXCL10$^{-/-}$ (n = 6/group) were infected with HSV-2 (2000 PFU/vagina). At indicated times, the mice were exsanguinated, and vaginal tissue (*a* and *b*), inguinal/iliac LN (I/ILN) (*c* and *d*), brain stem (*e* and *f*), and spinal cord (*g* and *h*) were removed, processed, and assayed for CXCL9 (*left*) and CXCL10 (*right*) content using ELISA. The weight of the tissue was used to normalize the amount of chemokine per milligram of tissue (expressed as picogram per milligram of tissue). Day 0 time point represents uninfected controls. Each data symbol represents the mean ± SEM summarizing the results of two independent experiments. *, $p < 0.05$ comparing WT to CXCL9$^{-/-}$ and CXCL10$^{-/-}$ groups.

Mice were euthanized at time points p.i. to determine virus titer, phenotypic analysis of leukocytes and cytokine/chemokine content within infected tissues. All procedures were approved by The University of Oklahoma Health Sciences Center and Dean A. McGee Eye Institute Animal Care and Use Committee.

Virus plaque assay

Tissues (vagina, spinal cord, and brain stem) were removed from infected mice at times p.i. (day 3 and day 7), placed into complete medium (500 μl) and homogenized using a tissue homogenizer (Fisher Scientific). Supernatants were clarified (10,000 × *g*, 1 min) and assessed for viral titer by plaque assay as previously described (37).

Suspension array and ELISA

Detection of MCP-1 (CCL2), MIP-1α (CCL3), RANTES (CCL5), KC (CXCL1), IL-12, and IFN-γ were performed using a suspension array system (Bioplex; Bio-Rad). Samples were analyzed in duplicate along with a standard provided. The weight of each tissue was used to normalize amount

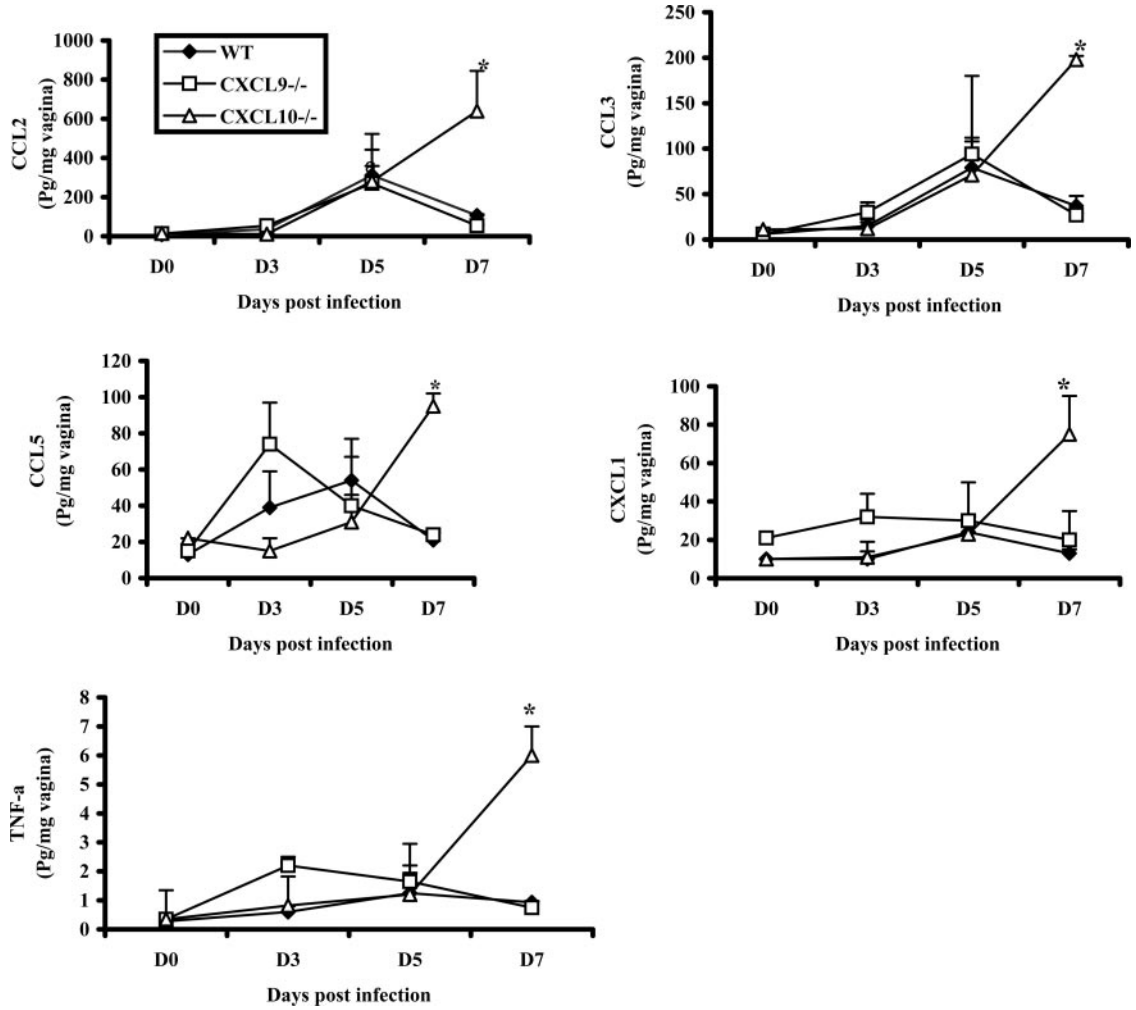

FIGURE 3. Chemokine/cytokine levels are elevated in the vaginal tissue of CXCL10$^{-/-}$ mice. WT, CXCL9$^{-/-}$, and CXCL10$^{-/-}$ mice (n = 6/group) were infected with HSV-2 (2000 PFU/vagina). At indicated times, the mice were exsanguinated, and vaginal tissues were removed, processed, and assessed for CCL2, CCL3, CCL5, CXCL1, TNF-α, and IFN-γ and IL-12p70 (data not shown) content using a suspension array system and ELISA. Samples were analyzed in duplicate along with standard provided to generate standard curves for each analyte. The weight of the tissue was used to normalize the amount of cytokine/chemokine per milligram of tissue weight. Day 0 time point represents uninfected controls. Each data symbol represents mean ± SEM summarizing the results of two independent experiments. *, $p < 0.05$ comparing the WT to CXCL9$^{-/-}$ and CXCL10$^{-/-}$ groups.

of cytokine/chemokine per milligram of tissue weight. Measurement of TNF-α, CXCL9, and CXCL10 was performed by ELISA (R&D Systems). A known amount of each analyte provided was used to generate standard curves to extrapolate the amount of each unknown sample.

Flow cytometry

At the designated time mice were exsanguinated, and vagina, spinal cord, brain stem, spleen, and LNs were removed. Tissues were processed to generate single cell suspensions as described (32) and were transferred into 5 ml of polystyrene round-bottom tubes (BD Biosciences). The cells were incubated with 2 μl of anti-mouse CD16/32 (FcγRIII/RII, 2.4G2; BD Pharmingen) for 20 min on ice. For measuring CD4 T cell, CD8 T cell, and NK cell content, cells were labeled with 1–2 μg of FITC-conjugated anti-CD3 (17A2), and PE-conjugated anti-CD4 (RM4−5) or anti-CD8α (53−6.7) or anti-NK1.1 (PK136), and PE-Cy5 anti-CD45 (30-F11) and allowed to incubate on ice in the dark. After a 30-min incubation, the cells were washed (300 × g, 5 min at 4°C) and resuspended in 1% paraformaldehyde for 60 min. Next, the cells were resuspended in 3 ml of 1× PBS containing 1% BSA. A known number of beads (20,800) (Invitrogen Life Technologies) was immediately added to the sample that was then briefly vortexed and analyzed on a Coulter Epics XL flow cytometer (Beckman Coulter). Cells were gated on CD45^{high} expressing cells, and a second gate was established to capture the number of beads that passed through during the sampling time. The absolute number of leukocytes (CD45^{high}) in the tissue was determined by multiplying the ratio of the number of beads collected per sample to the total number of beads added by the number of CD45^{high} events by sample dilution factor. Isotypic control Abs were included in the analysis to establish background fluorescence levels. Likewise, samples from uninfected mice were also analyzed to determine the degree of contamination from incomplete perfusion. For measuring inguinal/iliac LN and spleen T cell and NK cell content, single cell suspensions (1×10^6 cells) were transferred into 5 ml of polystyrene round-bottom tubes and labeled with Abs and analyzed by flow cytometry. The absolute number of cells was determined by multiplying the percentage of T or NK cell populations found in each lymphoid organ by the total number of cells recovered.

Tetramer staining

For tetramer staining, cells were labeled with 1–2 μg of the HSV peptide-specific $gB_{498-505}$ (SSIEFARL) MHC tetramer (MHC Tetramer Lab, Houston, TX) for 60 min. The cells were washed (300 × g, 5 min at 4°C) and labeled with 1–2 μg of FITC-conjugated anti-CD8 and PE-Cy5-conjugated anti-CD45. Following a 30-min incubation, cells were washed (300 × g, 5 min at 4°C) and resuspended in 1% paraformaldehyde. After a 60-min incubation, the cells were resuspended in 1× PBS. Cells were subsequently analyzed by flow cytometry as described.

CTL assay

MC57G (CRL-2295; American Type Culture Collection) were infected with HSV-2 with a multiplicity of infection of 3 for 8 h at 37°C, in 5% CO_2 and 95% humidity. Following infection, 10^4 MC57G cells were resuspended in prewarmed (37°C) PBS containing 0.25 μM CFSE (Invitrogen

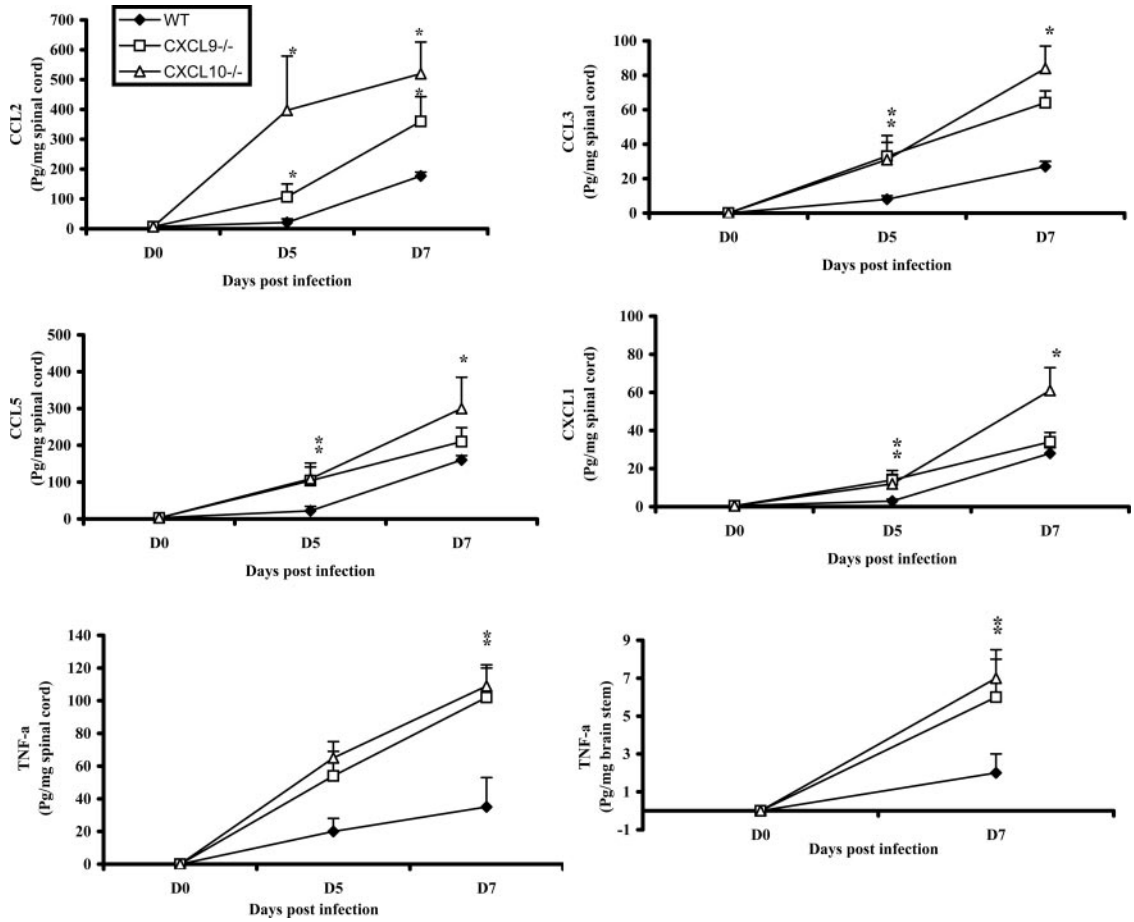

FIGURE 4. Chemokine/cytokine levels are elevated in the nervous system of chemokine knockout mice. WT, CXCL9$^{-/-}$, CXCL10$^{-/-}$ mice (n = 6/group) were infected with HSV-2 (2000 PFU/vagina). At indicated times, the mice were exsanguinated, and the spinal cord and brain stem were removed, processed, and assessed for CCL2, CCL3, CCL5, CXCL1, and TNF-α content using a suspension array system and ELISA. As there were no differences found in other analytes measured, only TNF-α level is shown for brain stem. Samples were analyzed in duplicate along with standard provided to generate standard curves for each analyte. The weight of the tissue was used to normalize amount of cytokine/chemokine per milligram of tissue weight. Day 0 time point represents uninfected controls. Each point represents the mean ± SEM summarizing the results of two independent experiments. *, $p < 0.05$ comparing the WT to CXCL9$^{-/-}$ and CXCL10$^{-/-}$ groups.

Life Technologies) and incubated for 15 min at 37°C. Cells were washed with 1× PBS and resuspended in fresh prewarmed complete medium. The desired number of isolated leukocytes from the processed spinal cord, spleen, and inguinal/iliac LN of mice was added to the CFSE-labeled HSV-2-infected MC57G target cells in 96-well microtiter plate wells in a total volume of 200 μl of complete medium at an E:T cell ratio of 10:1. After a 4-h incubation, 0.5 μl of propidium iodide (0.5 μg) was added to cells followed by a 15-min incubation at 37°C, in 5% CO_2 and 95% humidity. Cells were then washed and resuspended in 1× PBS and immediately analyzed by Epics XL flow cytometry. The gate was set for CFSE-expressing cells. The percentage of cytotoxicity was calculated by dividing the number of propidium iodide-labeled CFSE-expressing cells by the total number of CFSE-expressing cells multiplied by 100. The background level was determined by using target cells without effector cells and target cells incubated with spleen cells from uninfected mice. The assay was repeated using leukocytes from spinal cord of infected CXCL9$^{-/-}$ mice incubated with recombinant CXCL9 (2.0 ng; PeproTech) for the duration of the assay. Leukocytes from the spinal cord of infected WT mice were also included in the assay.

Statistics

All statistical analyses were conducted using the GBSTAT program (Dynamic Microsystems). One-way ANOVA and Tukey's post hoc t test were used to determine significant ($p < 0.05$) differences in WT, CXCL9$^{-/-}$, and CXCL10$^{-/-}$ mice. Mann-Whitney U test was used for analysis of survival to determine significant ($p < 0.05$) differences in WT, CXCL9$^{-/-}$, and CXCL10$^{-/-}$ mice.

Results

CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice are susceptible to genital HSV-2 infection

If both CXCL9 and CXCL10 operate through the only receptor for these chemokines, are there noticeable differences in the sensitivity to infection? To address this question, WT, CXCL9$^{-/-}$, and CXCL10$^{-/-}$ mice were evaluated for virus titer in infected tissue following exposure to HSV-2 (2000 PFU/vagina). CXCL10$^{-/-}$ mice, but not CXCL9$^{-/-}$ or WT mice, were found to harbor infectious virus in spinal cord at day 3 p.i. (Fig. 1*a*). In addition, CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice showed significantly higher viral loads in the spinal cord and brain stem at day 7 p.i. compared with WT controls (Fig. 1*b*). These results are consistent with the incidence of mortality in the CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice during this same time period (Fig. 1c). Specifically, 18% (8/44) of CXCL9$^{-/-}$ and 35% (21/59) of CXCL10$^{-/-}$ mice succumbed to infection in comparison to 0/52 WT mice by day 7 p.i. (Fig. 1*c*). There was no virus recovered from brain stem of WT, CXCL9$^{-/-}$, or CXCL10$^{-/-}$ mice at day 3 p.i. and no significant difference in viral loads found in the vaginal tissue at day 3 or day 7 p.i. comparing all groups of mice (Fig. 1*a*). Taken together, CXCL10$^{-/-}$ mice appear to be more sensitive to genital HSV-2 infection based

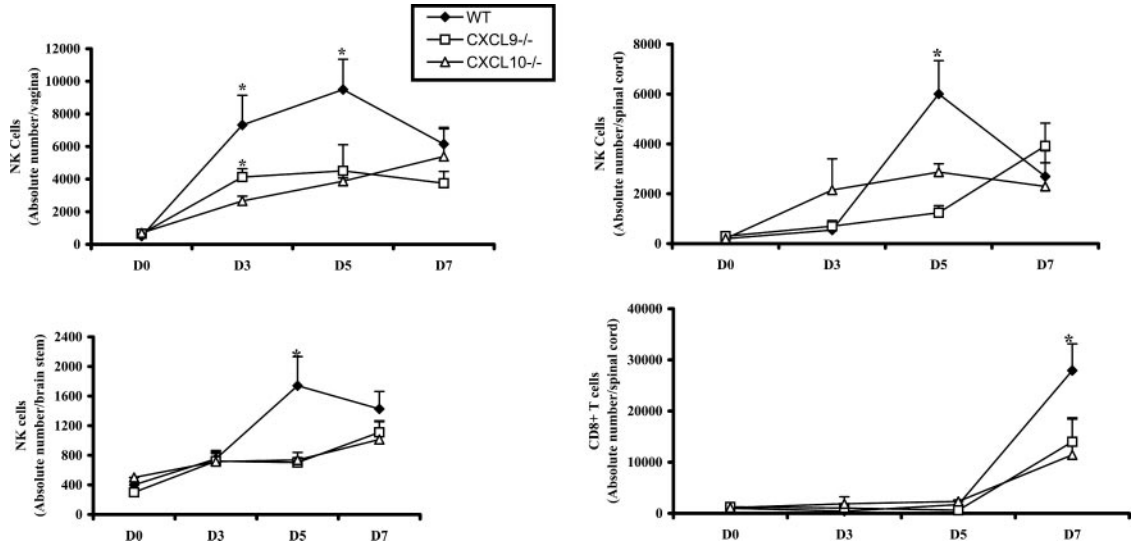

FIGURE 5. NK and CD8$^+$ T cell infiltration into infected tissue of chemokine knockout mice is reduced or delayed. WT, CXCL9$^{-/-}$, and CXCL10$^{-/-}$ mice ($n = 6$/group) were infected with HSV-2 (2000 PFU/vagina) and subsequently exsanguinated at indicated times p.i. Vaginal tissue (*a*), spinal cord (*b*), and brain stem (*c*) samples were processed and analyzed for NK cell (NK1.1$^+$CD3$^-$CD45^{high}) content by flow cytometry. *d*, Similarly, spinal cords were processed and analyzed for CD8$^+$ T (CD3$^+$CD8$^+$CD45^{high}) cells content using flow cytometry. Day 0 time point represents uninfected controls. Each data symbol represents the mean ± SEM summarizing the results of three independent experiments. ∗, $p < 0.05$ comparing WT to CXCL9$^{-/-}$ and CXCL10$^{-/-}$.

on an increase in mortality and appearance of HSV-2 in the spinal cord earlier than in CXCL9$^{-/-}$ mice.

CXCL9 and CXCL10 levels rapidly increase in a tissue-specific manner following infection with HSV-2 in WT mice

Because CXCL10$^{-/-}$ mice were found to be more sensitive to virus infection, we next asked whether there were differences in the expression of CXCL9 and CXCL10 at critical sites following infection that might coincide with an increase in sensitivity. Although both CXCL9 & CXCL10 were constitutively expressed in vaginal tissue, CXCL10 levels increased significantly within 24 h p.i., whereas CXCL9 levels did not change in WT mice (Fig. 2, *a* and *b*). By day 3 p.i., CXCL9$^{-/-}$ mice possessed similar levels of CXCL10 in vaginal tissue compared with WT mice (Fig. 2*b*), whereas CXCL9 levels in CXCL10$^{-/-}$ mice were reduced compared with WT mice (Fig. 2*a*). Both CXCL10 and CXCL9 levels were diminished in CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice, respectively, in the inguinal/iliac LN compared with WT mice at days 3 and 7 p.i. (Fig. 2, *c* and *d*). By comparison, CXCL9 and CXCL10 levels were not significantly elevated in the nervous system of infected mice until the day 7 p.i. (Fig. 2, *e–h*). CXCL9 levels were significantly increased in the brain stem and spinal cord of WT mice compared with CXCL10$^{-/-}$ mice at day 7 p.i. (Fig. 2, *e* and *g*). However, CXCL10 levels in the spinal cord and brain stem did not vary between WT and CXCL9$^{-/-}$ mice (Fig. 2, *f* and *h*). We interpret the results to suggest CXCL10$^{-/-}$ mice cannot respond to an equivalent level with the corresponding CXCR3 chemokine, CXCL9, in the CNS, in comparison to CXCL9$^{-/-}$ mice that are capable of producing similar levels of the corresponding CXCR3 chemokine, CXCL10, in the CNS relative to WT mice. These results demonstrate a deficiency pronounced in the CXCL10$^{-/-}$ mice in comparison to CXCL9$^{-/-}$ mice.

CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice express elevated chemokine/cytokine levels in infected tissues

Many CC type chemokines (i.e., CCL2, CCL3, CCL5), inflammatory cytokines (e.g., IFN-γ) and proinflammatory cytokine (e.g., TNF-α) are expressed locally in response to viral infection (15, 18, 32). To determine whether additional cytokine/chemokine levels were modified in the CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice following HSV-2 infection, candidate cytokines/chemokines were surveyed at times p.i. Even though there was no difference in viral titers found in the vaginal tissue comparing the WT, CXCL9$^{-/-}$, and CXCL10$^{-/-}$ mice at day 7 p.i., there was a significant increase in chemokine expression including CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), and CXCL1 (KC) as well as TNF-α in the vaginal tissue of CXCL10$^{-/-}$ mice in comparison to CXCL9$^{-/-}$ and WT mice (Fig. 3). The IFN-γ and IL-12p70 were also included in the analysis; however, no significant differences were found (data not shown). By comparison, there was an increase in CCL2, CCL3, CCL5, and CXCL1 as well as TNF-α levels in the spinal cord of chemokine knockout mice compared with WT mice at day 5 (except TNF-α) and day 7 p.i. (Fig. 4). TNF-α was the only chemokine or cytokine significantly elevated in the brain stem in both chemokine knockout mice compared with WT controls following viral infection (Fig. 4). Collectively, the results suggest that an increase in cytokine/chemokine expression in the CNS but not vagina is driven by the elevation in infectious virus recovered in this tissue in the CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice.

Effector cell mobilization into the CNS is impaired in CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice

Because chemokine levels were significantly altered in CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice in comparison to WT animals, would these changes influence leukocyte mobilization? In contrast to the predicted outcome, there was no significant difference in the absolute number of CD45^{high}, NKT, CD4$^+$ T, or total CD3$^+$ T cell populations infiltrating the CNS or vagina comparing the WT to chemokine knockout mice in response to acute genital HSV-2 infection (data not shown). However, there was a significant but transient reduction in the number of NK cells infiltrating the CNS and vagina of knockout mice in comparison to WT animals at day 3 (vagina only) or day 5 p.i. (Fig. 5). It should also be noted

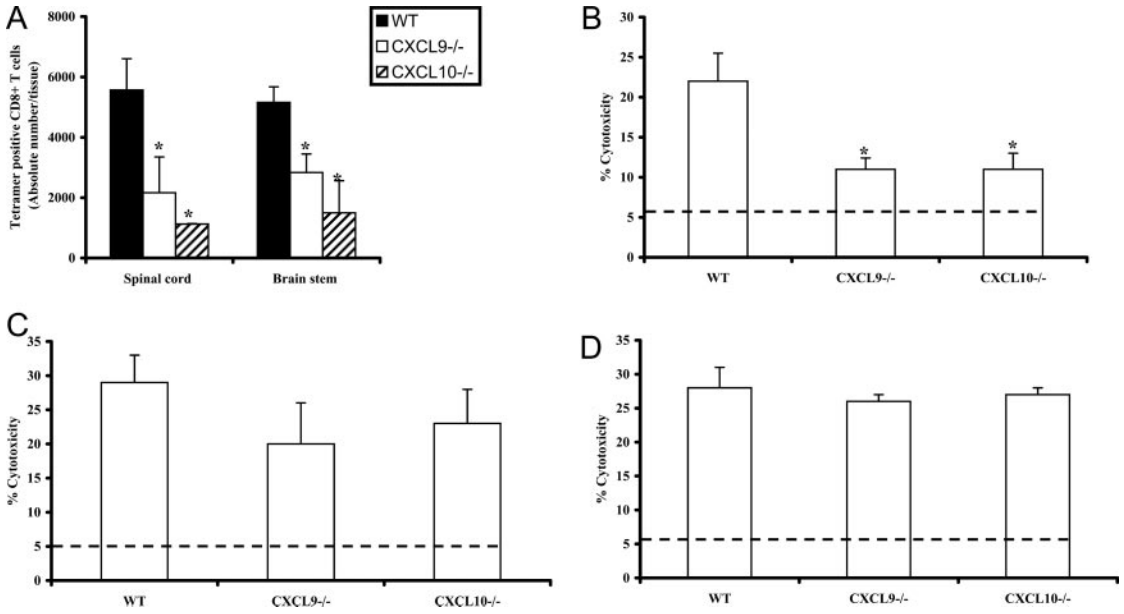

FIGURE 6. Reduced HSV-2-specific $CD8^+$ T cell recruitment is associated with decreased cytolytic activity. WT, $CXCL9^{-/-}$, and $CXCL10^{-/-}$ mice were ($n = 6$/group) were infected with HSV-2 (2000 PFU/vagina). At indicated times, mice were exsanguinated, and spinal cord, spleen, and inguinal/iliac LN were processed and analyzed for tetramer-positive T cells at day 7 (*a*), for CTL activity using Percoll gradient-enriched spinal cord leukocytes (*b*), for CTL activity using inguinal/iliac LN cells (*c*), and for CTL activity using spleen cells (*d*). For tetramer staining, MHC class I tetramer specific for HSV peptide $gB_{498-505}$ (SSIEFARL) was used to assay the total tetramer-positive cells in the spinal cord. For CTL assay, HSV-2-infected target cells (MC57G) were labeled with CFSE dye and incubated with Percoll gradient-enriched spinal cord leukocytes or spleen or LN cells at a E:T ratio of 10:1 for 4 h at 37°C. Propidium iodide was added after the 4 h incubation and the percentage of lysis was determined using flow cytometry. Dashed line (*b–d*) indicates the background p.i. incorporation into CSFE-labeled targets incubated only or in the presence of spleen cells from uninfected mice. Results represent the mean ± SEM summarizing the data of two independent experiments. ∗, $p < 0.05$ comparing WT to $CXCL9^{-/-}$ and $CXCL10^{-/-}$ groups.

$CXCL9^{-/-}$ mice had significantly more NK cells within the vaginal tissue at day 3 p.i. compared with $CXCL10^{-/-}$ mice (Fig. 5). Likewise, total $CD8^+$ T cell numbers were also increased in the spinal cord of WT mice compared with chemokine knockout mice at day 7 p.i. (Fig. 5). A similar trend in $CD8^+$ T cell number was found in the brain stem and vaginal tissue, although the levels did not reach significance (data not shown). Collectively, the results demonstrate that a deficiency in CXCL9 or CXCL10 significantly impacts on the recruitment of $CD8^+$ T cells and NK cells to the spinal cord, whereas the increase in other chemokines observed in this tissue including CCL2, CCL3, and CCL5 do not play a significant role in response to HSV-2 infection (based on their level of expression in the chemokine-deficient mice).

Virus-specific T cell mobilization is reduced in the CNS of $CXCL9^{-/-}$ and $CXCL10^{-/-}$ mice

The ability to identify the Ag-specific $CD8^+$ T cells in a given population of lymphocytes is made possible by the use of tetramer (38). Because a reduction in total $CD8^+$ T cell numbers was found in the spinal cord of $CXCL9^{-/-}$ and $CXCL10^{-/-}$ mice following genital HSV-2 infection, would these changes also be reflected in the number and function of HSV-specific $CD8^+$ effector T cells as well? In the case of HSV-2, we analyzed the HSV-gB-specific tetramer-positive $CD8^+$ T cells residing in the spinal cord, brain stem, vaginal tissue, inguinal/iliac LN, and spleen following HSV-2 infection. There was no detectable HSV-gB^+ $CD8^+$ T cell in tissue before infection. However, there were more tetramer-positive $CD8^+$ T cells residing in the spinal cord and brain stem of WT mice at day 7 p.i. in comparison to $CXCL9^{-/-}$ and $CXCL10^{-/-}$ animals (Fig. 6*a*). In contrast, there was no significant loss of HSV-gB-specific $CD8^+$ T cells found in the vaginal tissue of WT mice in comparison to chemokine knockout mice (data not shown). To establish the functional role of resident $CD8^+$ T cells, leukocytes isolated from spinal cord of WT, $CXCL9^{-/-}$ and $CXCL10^{-/-}$ mice were evaluated for CTL activity. Cells extracted from spinal cord preparations of $CXCL9^{-/-}$ and $CXCL10^{-/-}$ mice were found to exhibit significantly less cytolytic activity against HSV-2-specific target cells in comparison to WT (Fig. 6*b*). By comparison, spleen and inguinal/iliac LN cells from WT, $CXCL9^{-/-}$ and $CXCL10^{-/-}$ HSV-2-infected mice showed similar cytolytic levels against HSV-2-infected targets (Fig. 6, *c* and *d*). To determine whether the reduced cytolytic activity in the chemokine-deficient mice could be corrected with the addition of exogenous chemokine, spinal cord leukocytes obtained from HSV-2-infected $CXCL9^{-/-}$ mice were evaluated for cytolytic activity in the presence or absence of recombinant CXCL9 (2 ng/culture). The results show no difference between effector cells from mock-treated cultures (11% cytolysis of target cells) in comparison to effector cells from CXCL9-treated cultures (12% cytolysis of target cells) relative to effector cells from WT cultures (26% cytolysis of target cells). Taken together, the loss of HSV-specific $CD8^+$ T cells in the CNS of $CXCL9^{-/-}$ and $CXCL10^{-/-}$ mice correlates with a loss in CTL function by the enriched leukocytes from the spinal cord.

Discussion

The present study underscores the role of two specific chemokines CXCL9 and CXCL10 in mounting a host response against genital HSV-2 infection. In the absence of CXCL9 or CXCL10, mice showed heightened sensitivity to genital HSV-2 infection compared with WT controls even though both chemokines operate through the same receptor, CXCR3. Relative to CXCL9 and CXCL10 expression in the vaginal tissue of WT mice, CXCL10 was found to be expressed rapidly within 24 h following HSV-2 infection, whereas CXCL9 expression was not noticeably elevated

until day 3 p.i. Furthermore, CXCL9 levels were significantly reduced in CXCL10$^{-/-}$ mice in comparison to WT mice. We propose the absence of CXCL10 and delay in CXCL9 within the vaginal tissue results in the reduction in NK cell mobilization to the vagina leading to early viral infection of the spinal cord of CXCL10$^{-/-}$ mice. In comparison to these studies, CCR5-deficient mice show no deficiency in NK cell recruitment to the vagina following HSV-2 infection and no difference in virus recovered in the spinal cord early p.i. in comparison to WT animals (32). Consequently, we suggest CXCL10 but not CXCL9 or CCR5 ligand is the principal chemoattractant molecule liberated early postgenital HSV-2 infection that serves to signal NK cell mobilization to the vaginal tissue.

The increase in HSV-2 found in the spinal cord of CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice correlates with elevated chemokine levels including CXCL1, CCL2, CCL3, CCL5 chemokines. Similar observations have also been reported in other CNS virus infections, including vesicular stomatitis virus, lymphocytic choriomeningitis virus, mouse hepatitis virus, as well as experimental autoimmune encephalomyelitis (39–42). Our previous findings suggest that CCL2 or CCL5 levels within the CNS do not correspond to virus-mediated mortality as a result of ocular HSV-1 infection (43, 44), whereas one study reports CNS levels of CCL2 are highly correlative with HSV encephalitis (45). Likewise, CCL3 has been linked to a number of CNS inflammatory infections including dengue virus, progressive multifocal leukoencephalopathy associated with AIDS, and *Listeria* meningoencephalitis (46–48).

We consistently found TNF-α elevated in the vagina, spinal cord, and brain stem of the CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice. Similar results were also reported in CCR5-deficient mice that were also found to have a higher mortality rate compared with the WT controls following genital HSV-2 infection (32). As TNF-α can be neurotoxic (49), it is tempting to speculate the increase in CNS TNF-α levels in the chemokine-deficient mice may be the primary contributory factor resulting in a higher mortality rate in comparison to WT animals. Although mortality rates were not evaluated, early studies suggest the neutralization of TNF-α has no effect on virus resolution following genital HSV-2 infection (50–52).

A significant reduction in NK cell mobilization at days 3 and 5 p.i. and reduction in CD8$^+$ T cell trafficking to the spinal cord at day 7 p.i. were observed in the CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice. We previously reported that depletion of NK cells following genital HSV-2 infection resulted in an elevation in virus recovered in the vaginal tissue and brain stem but not spinal cords of WT mice (32). Furthermore, previous studies have shown that the reduction of NK cell activity through Ab-mediated depletion of IL-12, IL-15, and IL-18 results in higher virus titers and increased mortality following HSV-2 infection (4, 27, 28, 53). Collectively, we interpret these findings to suggest NK cells maintain virus surveillance within the genital tract and brain stem following vaginal HSV-2 infection, whereas CD8$^+$ T cells monitor infection within the spinal cord.

Previous studies have suggested CXCL10 plays a central role in recruiting CD8$^+$ T cells to the lymphocytic choriomeningitis virus-infected CNS controlling virus infection through a perforin-mediated lysis of infected cells (25, 35, 54). Other studies have linked Fas-Fas ligand-mediated apoptosis in control of Theiler's virus, whereas in vaccinia virus, vesicular stomatitis virus, or Semliki virus infections, neither Fas-Fas ligand or perforin pathways were required for CNS clearance (55, 56). Although we have not formally proven the cytolytic process involved in CD8$^+$ CTL effector activity for cells infiltrating the CNS following genital HSV-2 infection, Fas ligand does not appear to be expressed on CD8$^+$ T cells residing in the spinal cord of HSV-2-infected mice (M. Thapa and D.J.J. Carr, unpublished observation). The deficiency in CTL activity in the CNS of CXCL9$^{-/-}$ and CXCL10$^{-/-}$ mice is most likely due to an insufficient number of effector cells (based on HSV-gB-specific tetramer stain) mobilized to the infected tissue as opposed to aberrant cytolytic effector molecules expressed by effector cells because similar CTL levels were observed in the spleen and LN cell populations comparing all genotypes. In addition, recombinant murine CXCL9 did not restore CTL activity by effector cells obtained from the spinal cord of CXCL9$^{-/-}$ mice, suggesting the reduced cytolytic activity is not due to chemokine deficiency.

Collectively, the present study indicates an increase in sensitivity to genital HSV-2 infection in mice deficient in CXCL9 and CXCL10 is associated with a reduction in the recruitment of an optimal number of NK cells and HSV-specific CD8$^+$ T cells to sites of infection. We interpret the nonredundant role of CXCL9 and CXCL10 to be a result of tissue-specific and temporal expression represented by levels measured in the draining LN and infected vaginal tissue of mice. It has yet to be determined whether the source of the chemokines is principally nonhemopoietic or hemopoietic-derived cells. It is also unknown whether CXCR3 expression is modified in the absence of CXCL9 or CXCL10, which could also explain changes in the mobilization of cells. However, the present observation does suggest a hierarchy of CXCR3 ligand expression that influences both HSV-gB-specific CTL mobility and NK cell recruitment to vaginal tissue and the CNS. A rapid expression of CXCL10 in the infected genitalia may augment NK cell mobilization to the vagina ultimately resulting in a reduction or delay in virus trafficking to the sacral ganglia and spinal cord, reducing the likelihood of the establishment of latency.

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Disclosures

The authors have no financial conflict of interest.

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