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Linda N. Stiles, Jenny L. Hardison, Chris S. Schaumburg,
Lucia M. Whitman and Thomas E. Lane

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T Cell Antiviral Effector Function Is Not Dependent on CXCL10 Following Murine Coronavirus Infection¹

Linda N. Stiles,² Jenny L. Hardison,² Chris S. Schaumburg, Lucia M. Whitman, and Thomas E. Lane³

The chemokine CXCL10 is expressed within the CNS in response to intracerebral infection with mouse hepatitis virus (MHV). Blocking CXCL10 signaling results in increased mortality accompanied by reduced T cell infiltration and increased viral titers within the brain suggesting that CXCL10 functions in host defense by attracting T cells into the CNS. The present study was undertaken to extend our understanding of the functional role of CXCL10 in response to MHV infection given that CXCL10 signaling has been implicated in coordinating both effector T cell generation and trafficking. We show that MHV infection of CXCL10^{+/+} or CXCL10^{-/-} mice results in comparable levels of T cell activation and similar numbers of virus-specific CD4⁺ and CD8⁺ T cells. Subsequent analysis revealed no differences in T cell proliferation, IFN- γ secretion by virus-specific T cells, or CD8⁺ T cell cytolytic activity. Analysis of chemokine receptor expression on CD4⁺ and CD8⁺ T cells obtained from MHV-immunized CXCL10^{+/+} and CXCL10^{-/-} mice revealed comparable levels of CXCR3 and CCR5, which are capable of responding to ligands CXCL10 and CCL5, respectively. Adoptive transfer of splenocytes acquired from MHV-immunized CXCL10^{-/-} mice into MHV-infected RAG1^{-/-} mice resulted in T cell infiltration into the CNS, reduced viral burden, and demyelination comparable to RAG1^{-/-} recipients of immune CXCL10^{+/+} splenocytes. Collectively, these data imply that CXCL10 functions primarily as a T cell chemoattractant and does not significantly influence T cell effector response following MHV infection. *The Journal of Immunology*, 2006, 177: 8372–8380.

The CXCL 10 (also known as IFN- γ -inducible protein at 10 kDa) exerts a potent chemotactic effect on activated T lymphocytes and NK cells by binding to its cognate cell surface receptor, CXCR3. Although initially identified to be an IFN- γ -responsive gene, a number of different cytokines including IFN- $\alpha\beta$ are capable of inducing CXCL10 gene expression in diverse cell types during periods of inflammation (1–5). Initial studies suggested a potentially important role for CXCL10 in the pathogenesis of various inflammatory diseases by attracting CXCR3-expressing leukocytes to sites of inflammation. However, CXCL10 is also detected in secondary lymphoid tissue suggesting a role in generating effector T cells during Ag-specific activation, expansion, and egress (6–8). Indeed, several studies have demonstrated that when either CXCL10 and/or CXCR3 signaling is impaired, specific T cell functions including proliferation and IFN- γ secretion are affected, indicating that CXCL10 is capable of directing effector T cell generation in addition to lymphocyte trafficking (9–12).

Previous studies from our laboratory have highlighted the functional relevance of CXCL10 and CXCR3 signaling in host defense and disease after viral infection of the CNS (9, 13–15). Intracere-

bral (i.e.)⁴ infection of susceptible mice with neurotropic strains of mouse hepatitis virus (MHV, a member of the Coronaviridae family) results in an acute encephalomyelitis characterized by widespread viral replication throughout the parenchyma (16–18). Mice that survive the acute stage of disease often develop an immune-mediated demyelinating disease in which viral RNA and protein are present in white matter tracts accompanied by focal areas of myelin damage (19, 20). Virus-specific T cells and macrophages are the primary mediators of demyelination, whereas autoreactive T cells recognizing defined myelin epitopes are not considered important in contributing to disease (21–25). Although the outcome from infection is dictated by many factors, it is evident that an effective immune response defined by the infiltration of virus-specific CD4⁺ and CD8⁺ T cells into the CNS and the subsequent reduction in viral burden through secretion of IFN- γ and cytolytic activity is essential in providing optimal protection (26–28).

In response to MHV infection, there is an orchestrated expression of chemokines that precedes and accompanies leukocyte infiltration into the CNS (29). Both resident cells of the CNS as well as infiltrating leukocytes are capable of secreting chemokines, which serves to amplify inflammation and protection by attracting T cells. Indeed, blocking CXCL10 during acute disease by administering neutralizing Abs results in increased mortality accompanied by reduced infiltration of T cells into the CNS and a corresponding increase in CNS viral titers (13). Moreover, MHV infection of CXCL10^{-/-} mice results in a similar outcome as Ab treatment, further supporting the importance of CXCL10 in host defense against MHV infection of the CNS (9). Presumably, inhibiting CXCL10 signaling prevents attraction of virus-specific T cells bearing the CXCL10 receptor, CXCR3, into the CNS (30, 31). However, accumulating evidence also implicates CXCL10 in

Department of Molecular Biology and Biochemistry and Center for Immunology, University of California, Irvine, CA 92697

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² L.N.S. and J.L.H. contributed equally to this work.

³ Address correspondence and reprint requests to Dr. Thomas E. Lane, Department of Molecular Biology and Biochemistry, 3205 McGaugh Hall, University of California, Irvine, Irvine, CA 92697-3900. E-mail address: tlane@uci.edu

⁴ Abbreviations used in this paper: i.c., intracerebral; MHV, mouse hepatitis virus; p.i., postinfection; M, transmembrane protein; S, surface glycoprotein.

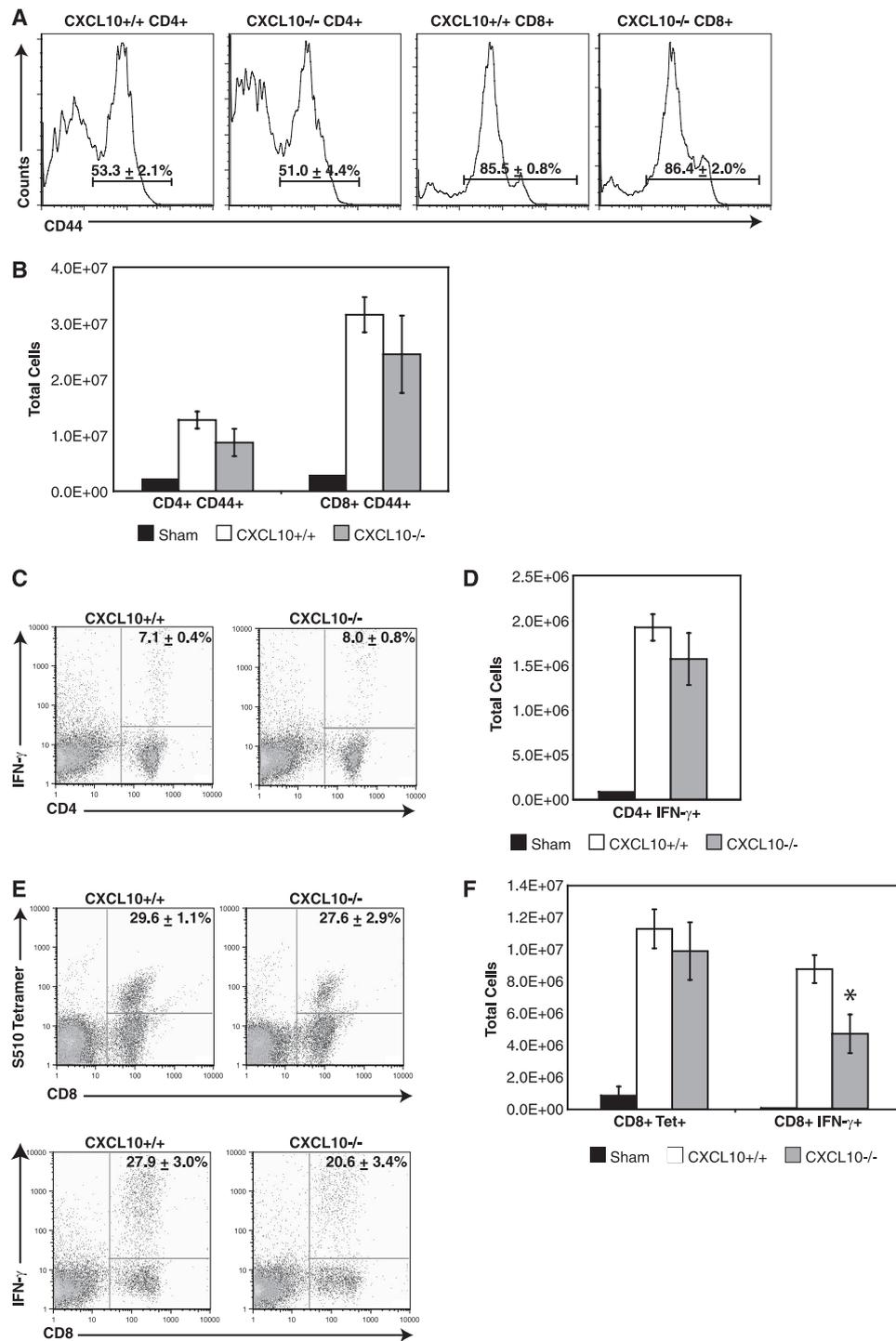


FIGURE 1. No defects in the generation of virus-specific T cells in MHV-infected CXCL10^{-/-} mice. CXCL10^{+/+} and CXCL10^{-/-} mice were infected i.p. with MHV, and splenocytes were harvested at 7 days p.i. to determine the CD4⁺ and CD8⁺ T cell response. There were no differences in either the frequencies (A) or total numbers (B) of CD4⁺ or CD8⁺ T cells expressing the activation marker CD44. Representative histograms are shown in A, and the frequency (average \pm SEM) is indicated. Cells were stained for CD4 or CD8, and IFN- γ expression was evaluated by intracellular cytokine staining following stimulation with the CD4 epitope M₁₃₃₋₁₄₇ or the CD8 epitope S₅₁₀₋₅₁₈. No differences in either the frequency (C) or total numbers (D) of virus-specific CD4⁺ T cells were detected between MHV-infected CXCL10^{+/+} and CXCL10^{-/-} mice. Similarly, there were no differences in either the frequency (E) or total numbers (F) of virus-specific CD8⁺ T cells as determined by S₅₁₀₋₅₁₈ tetramer staining; however, intracellular IFN- γ staining showed a decrease (*, $p < 0.02$) in CXCL10^{-/-} mice compared with CXCL10^{+/+} mice. Representative dot blots are shown in C and E, and the frequency (average \pm SEM) of dual-positive cells in infected mice is indicated in the upper right quadrant. Data presented in B, D, and F represent the average \pm SEM. Experiments shown in A and B were performed a minimum of two separate times with at least three mice per experiment. Data presented in C and D are derived from two separate experiments with $n = 9$ for both CXCL10^{+/+} and CXCL10^{-/-} mice. CD8⁺ T cell S₅₁₀₋₅₁₈ tetramer (Tet) data presented in E and F reflect four separate experiments with $n = 15$ for CXCL10^{+/+} and CXCL10^{-/-} mice. CD8⁺ T cell intracellular IFN- γ staining in response to S₅₁₀₋₅₁₈ in E and F are derived from two separate experiments with $n = 9$ for CXCL10^{+/+} and CXCL10^{-/-} mice.

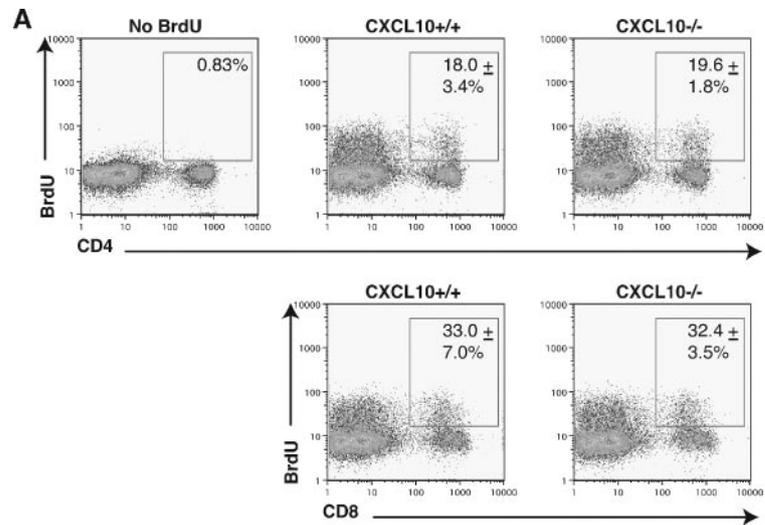
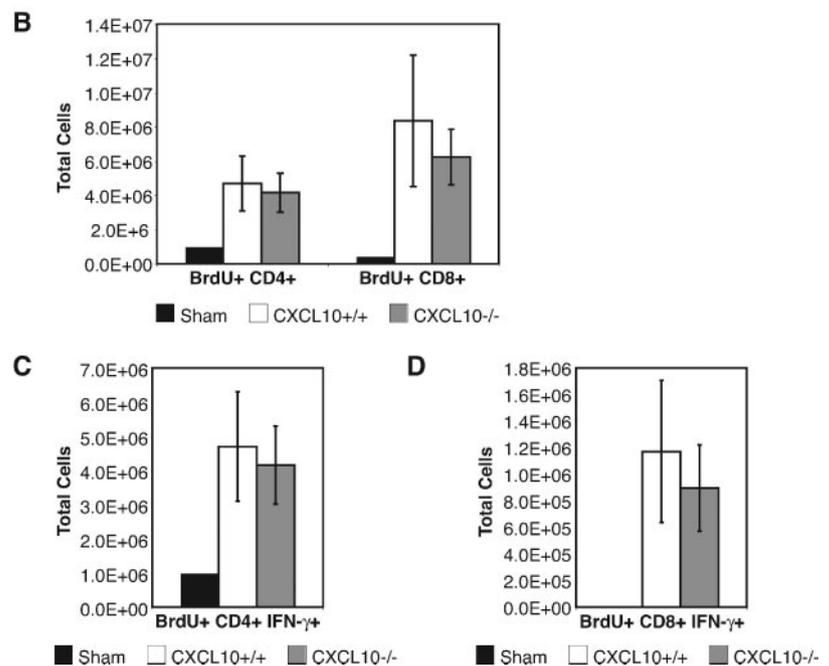


FIGURE 2. T cell proliferation is not affected in CXCL10^{-/-} mice. Splenocytes were harvested at day 7 p.i. from CXCL10^{+/+} and CXCL10^{-/-} mice infected i.p. with MHV and treated with BrdU. There were no differences in the frequency (A) or total numbers (B) of CD4⁺ and CD8⁺ T cells that incorporated BrdU at 7 days p.i. between CXCL10^{+/+} and CXCL10^{-/-} mice. In addition, there were no significant differences in proliferation of Ag-specific T cells, indicated by similar total numbers of virus-specific CD4⁺ T cells (C) or CD8⁺ T cells (D) expressing IFN- γ between CXCL10^{+/+} and CXCL10^{-/-} mice as determined by intracellular cytokine staining following stimulation with the CD4 epitope M_{133–147} or the CD8 epitope S_{510–518}. Representative dot blots are shown in A, and the frequency (average \pm SEM) of dual-positive cells is indicated in the upper right quadrant. Data shown in B, C, and D represent the average \pm SEM. Experiments were performed a minimum of two separate times with at least two to three mice per experiment.



promoting T cell effector functions, suggesting a broader role in the immune response following antigenic challenge beyond influencing T cell trafficking (6, 9, 10, 12, 32, 33). Further support for this possibility is derived from studies that show T cell signaling through CXCR3 also influences certain effector functions including proliferation and secretion of IFN- γ by Ag-specific T cells (11, 34). Therefore, it is possible that blocking CXCL10 after MHV infection results in increased disease severity, not only as a result of deficient trafficking, but also because of impaired T cell effector responses. This study was designed to characterize the contributions of CXCL10 with regards to generating functional antiviral T cells.

Materials and Methods

Virus and mice

MHV strains DM and J2.2 were used for experiments described (35). Age-matched 5- to 7-wk-old CXCL10^{+/+} (C57BL/6, H-2^b background; National Cancer Institute, Bethesda, MD) and CXCL10^{-/-} mice (C57BL/6, H-2^b background, kindly provided by A. Luster, Harvard University, Cambridge, MA) were used for all experiments. Animals were infected by i.p. injection with 2×10^5 PFU of MHV suspended in 200 μ l of sterile saline. Control (sham) animals were injected with 200 μ l of sterile saline alone.

Animals were sacrificed at defined time points, and spleens were removed for analysis. Experiments for all animal studies described have been reviewed and approved by an appropriate institutional review committee.

T cell isolation and flow cytometry

CXCL10^{+/+} and CXCL10^{-/-} mice were infected i.p. with 2×10^5 PFU of MHV and animals were sacrificed at day 7 postinfection (p.i.). Spleens were harvested, RBC lysis was performed, and single-cell suspensions were obtained. Abs used in these studies include allophycocyanin rat anti-mouse CD4 and CD8 and PE-conjugated rat anti-mouse CD44 and CCR5 (BD Pharmingen). Additionally, polyclonal rabbit anti-mouse CXCR3 was used for primary detection of CXCR3, and FITC-conjugated goat anti-rabbit Ab was used as a secondary Ab (Zymed Laboratories). In all cases, isotype-matched conjugated Abs were used as controls. Cells were incubated with Abs for 20–40 min at 4°C, washed, and analyzed using a FACStar flow cytometer (BD Biosciences) and FlowJo software (Tree Star). Frequency data are presented as the percentage of positive cells within the gated population. Total cell numbers were calculated by multiplying these values by the total number of live cells isolated.

Intracellular cytokine staining

Intracellular staining for IFN- γ was performed on splenocytes using a previously described procedure (36, 37). In brief, cells were stimulated with 5 μ M viral peptide corresponding to either the CD4 epitope present in the membrane (M) protein between aa 133 and 147 (M_{133–147}) or the CD8

epitope located within the spike (S) glycoprotein spanning residues 510–518 ($S_{510-518}$) (38, 39). Stimulated cells were incubated for 6 h at 37°C in medium containing GolgiStop (Cytofix/Cytoperm kit; BD Pharmingen), at which point cells were washed and blocked with PBS containing 10% FBS and a 1/200 dilution of CD16/32 (BD Pharmingen). Cells were then stained for surface Ags with allophycocyanin-conjugated CD4 or CD8 Abs and their cognate isotype controls (BD Pharmingen) for 20–40 min at 4°C. Cells were fixed and permeabilized using the Cytofix/Cytoperm kit and stained for intracellular IFN- γ using PE-conjugated anti-IFN- γ (BD Pharmingen) for 20–40 min at 4°C. Cells were analyzed on a FACStar flow cytometer (BD Biosciences) using FlowJo software (Tree Star). Frequency data are presented as the percentage of positive cells within the gated population. Total cell numbers were calculated by multiplying these values by the total number of live cells isolated.

In vivo T cell proliferation assay

CXCL10^{+/+} and CXCL10^{-/-} mice were infected and subsequently treated with 1.0 mg of BrdU (Sigma-Aldrich) suspended in sterile saline on days 3, 4, 5, and 6 p.i. Mice were sacrificed on day 7 p.i., and splenocytes were isolated for flow cytometric analysis using the BrdU Flow Kit (BD Pharmingen).

CTL assay

Spleen-derived CD8⁺ T cells were analyzed for lytic activity at day 7 following i.p. infection of CXCL10^{+/+} and CXCL10^{-/-} mice with 2.5×10^5 PFU of MHV. A CD8⁺ T cell-enriched population of cells was obtained via positive selection through use of a magnetically labeled Ab specific for the CD8 Ag followed by passage over a magnetic column (Miltenyi Biotec) (40). Numbers of $S_{510-518}$ -specific CD8⁺ T cells were determined by tetramer staining, and these cells were used as the effector population. CTL assays were performed with Na⁵¹CrO₄ (New England Nuclear)-labeled RMA-s (H-2^b) target cells preincubated with 5 μ M $S_{510-518}$ peptide or OVA peptide as a control that were combined with immune CD8⁺ T cells at various E:T ratios. ⁵¹Cr release was determined after a 6-h incubation and specific lysis was defined as $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{detergent release} - \text{spontaneous release})]$.

IFN- γ ELISA

Splenocytes were isolated from sham and MHV-infected CXCL10^{+/+} and CXCL10^{-/-} mice at day 7 p.i. and stimulated with 5 μ M concentrations of the CD4 epitope M₁₃₃₋₁₄₇ or CD8 epitopes S₅₁₀₋₅₁₈ or S₅₉₈₋₆₀₅ (Biosynthesis) or OVA peptide (American Peptide Co.) for 48 h at 37°C at 5% CO₂. Supernatants were harvested, and IFN- γ was quantified using the Mouse IFN- γ DuoSet (R&D Systems).

Adoptive transfer and histology

Splenocytes obtained from sham and MHV-infected CXCL10^{+/+} and CXCL10^{-/-} mice (day 7 p.i.) were adoptively transferred (2.5×10^6 cells suspended in 100 μ l of sterile HBSS) via i.v. injection into the retro-orbital sinus of C57BL/6 RAG1^{-/-} mice i.c. infected with 500 PFU of MHV J2.2,3 days before transfer (36). Control animals included MHV-infected (i.c.) RAG1^{-/-} mice receiving i.v. sterile HBSS. Mice were sacrificed 7 days posttransfer (10 days p.i.), and brains were removed. One half of the brains was used for flow analysis, and the remaining half was used to determine viral burden with a plaque assay on the DBT astrocytoma cell line (29, 41). Spinal cords were obtained from experimental groups at day 19 p.i. and fixed by immersion overnight in 10% normal buffered formalin after which portions of tissue were embedded in paraffin or TAAB resin (Electron Microscopy Services). Spinal cords (8- μ m sections) were stained with Luxol fast blue and analyzed by light microscopy. Demyelination was scored as follows: 0, no demyelination; 1, mild inflammation accompanied by loss of myelin integrity; 2, moderate inflammation with increasing myelin damage; 3, numerous inflammatory lesions accompanied by significant increase in myelin stripping; and 4, intense areas of inflammation accompanied by numerous phagocytic cells engulfing myelin debris. Slides containing stained spinal cord sections were blinded and scored. Scores were averaged and presented as average \pm SEM.

Statistical analysis

Statistically significant differences between groups of mice were determined by Student's *t* test, and $p \leq 0.05$ was considered significant.

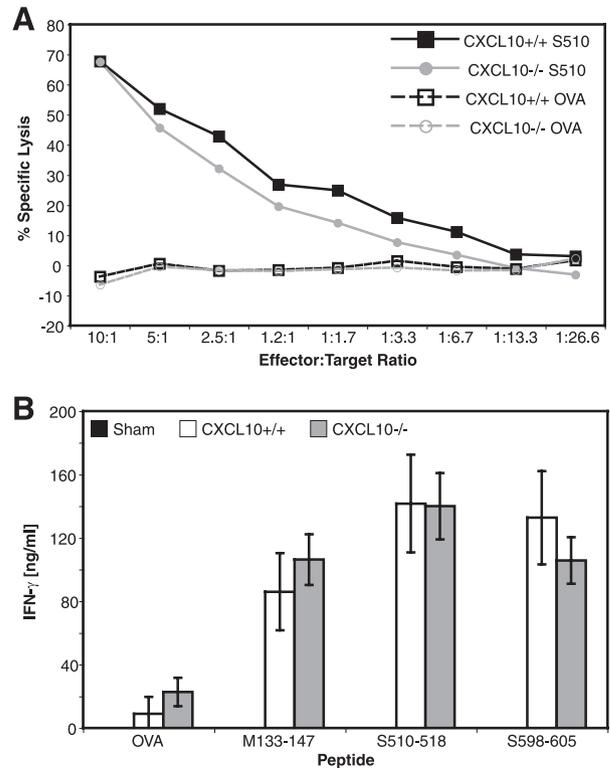


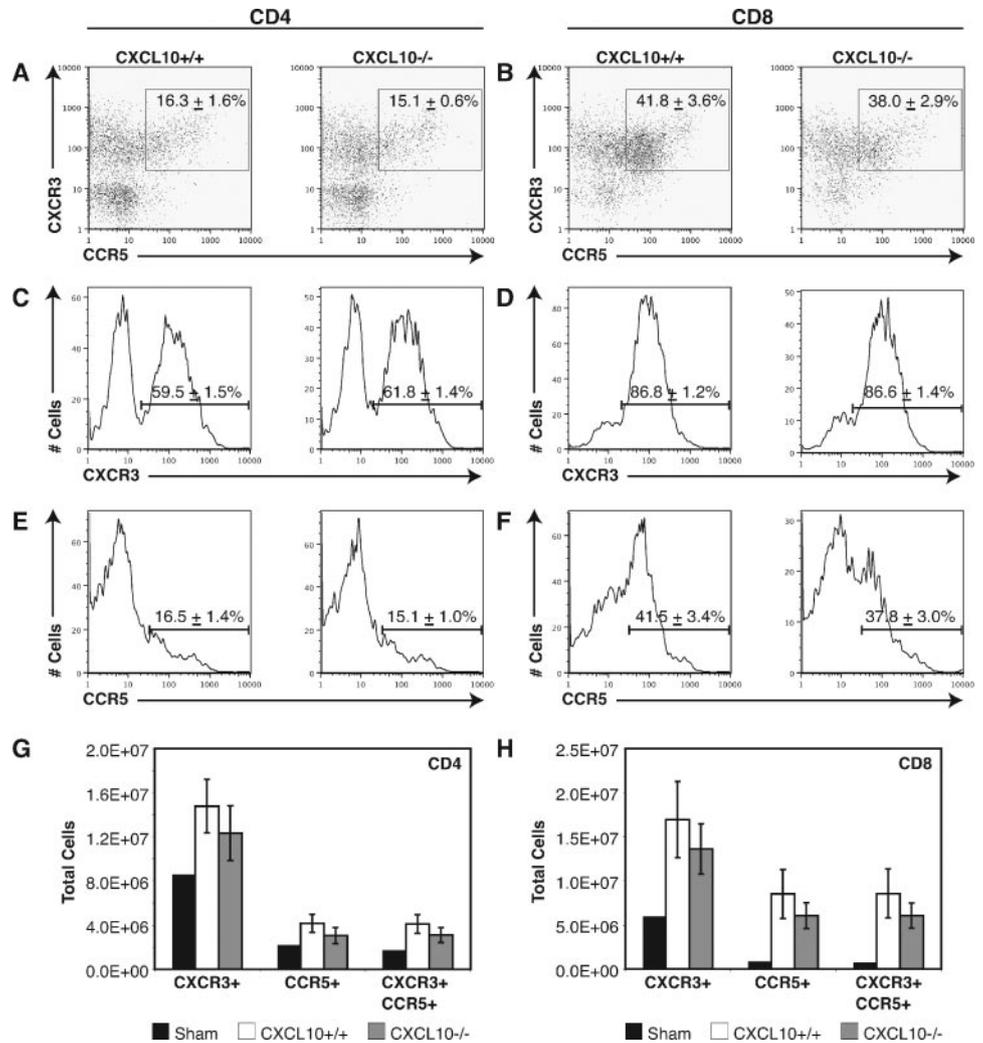
FIGURE 3. CTL activity and IFN- γ secretion are not impaired in CXCL10^{-/-} mice. *A*, CXCL10^{+/+} and CXCL10^{-/-} T cells exhibited comparable CTL activity measured by chromium release assay at all E:T ratios tested. *B*, IFN- γ production was not altered in virus specific CD4⁺ and CD8⁺ T cells between CXCL10^{+/+} and CXCL10^{-/-} mice as shown by ELISA. Data in *B* represent the average \pm SEM. Experiments were performed a minimum of two separate times with at least three mice per experiment.

Results

Generation of virus-specific T cells is not impaired in the absence of CXCL10 following MHV infection

CXCL10^{+/+} and CXCL10^{-/-} mice were infected i.p. with MHV, and T cell responses within the spleens of infected mice were evaluated at day 7 p.i. in which maximal T cell response to virus occurs. The results show that CXCL10 signaling does not influence expression of the T cell activation marker CD44 given that similar frequencies and numbers of CD4⁺ and CD8⁺ T cells obtained from infected CXCL10^{+/+} and CXCL10^{-/-} mice were CD44-positive (Fig. 1, *A* and *B*). Likewise, genetic deletion of CXCL10 did not have an appreciable effect on the development of CD4⁺ T cells specific for immunodominant epitope located within the M protein at residues 133–147 (M₁₃₃₋₁₄₇) as determined by intracellular staining for IFN- γ (Fig. 1, *C* and *D*). Similarly, tetramer staining showed that the frequency and total numbers of CD8⁺ T cells recognizing the S glycoprotein epitope residues 510–518 ($S_{510-518}$) were comparable between CXCL10^{+/+} and CXCL10^{-/-} mice; however, both the frequency and number of virus-specific CD8⁺ T cells measured by intracellular IFN- γ staining were reduced ($p < 0.02$) in CXCL10^{-/-} mice, which is consistent with earlier observations (9) (Fig. 1, *E* and *F*). These data indicate that CXCL10 signaling does not impair T cell activation and/or the generation of virus-specific CD4⁺ T cells or tetramer-positive CD8⁺ T cells. However, IFN- γ production by CD8⁺ T cells, as determined by intracellular staining, is reduced in the absence of CXCL10.

FIGURE 4. Chemokine receptor expression on CD4⁺ and CD8⁺ T cells is not altered in CXCL10^{-/-} mice. Mice were infected i.p. with MHV, splenocytes were harvested at 7 days p.i., and flow cytometry was performed to assess CXCR3 and CCR5 expression on T cells. There were no differences in the frequencies of either CD4⁺ (A) or CD8⁺ (B) T cells expressing both CXCR3⁺ and CCR5⁺. The frequencies of CD4⁺ T cells expressing either CXCR3 (C) or CCR5 (E) from either infected CXCL10^{+/+} or CXCL10^{-/-} mice was comparable. Similarly, there were no differences in the frequency of CD8⁺ T cells expressing CXCR3 (D) or CCR5 (F) from infected CXCL10^{+/+} or CXCL10^{-/-} mice. Representative dot blots and histograms are shown, and the frequency (average \pm SEM) of dual-positive cells in infected mice is indicated. Evaluation of total numbers of cells revealed no differences in numbers of CD4⁺ T cells (G) or CD8⁺ T cells (H) expressing CXCR3, CCR5, or dual-positive cells. Data presented in G and H represent the average \pm SEM. Experiments were performed a minimum of two separate times with at least three mice per experiment.



T cell proliferation is not affected in the absence of CXCL10 signaling

We next tested the contribution of CXCL10 to T cell proliferation in response to MHV infection. CXCL10^{+/+} and CXCL10^{-/-} mice were infected i.p. with MHV and simultaneously injected with BrdU to measure proliferative responses in vivo. As shown in Fig. 2, A and B, the absence of CXCL10 did not affect T cell incorporation of BrdU at day 7 p.i. In addition, there were no differences in M₁₃₃₋₁₄₇-specific CD4⁺ T cell responses between infected CXCL10^{+/+} and CXCL10^{-/-} mice (Fig. 2C), and although proliferation of S₅₁₀₋₅₁₈-specific CXCL10^{-/-} CD8⁺ T cells was reduced compared with CXCL10^{+/+} CD8⁺ T cells, this difference was not significant (Fig. 2D). Collectively, these results specify that T cell proliferation following MHV infection is not dependent on CXCL10.

T cells from CXCL10^{-/-} mice exhibit no defects in CTL activity or IFN- γ production

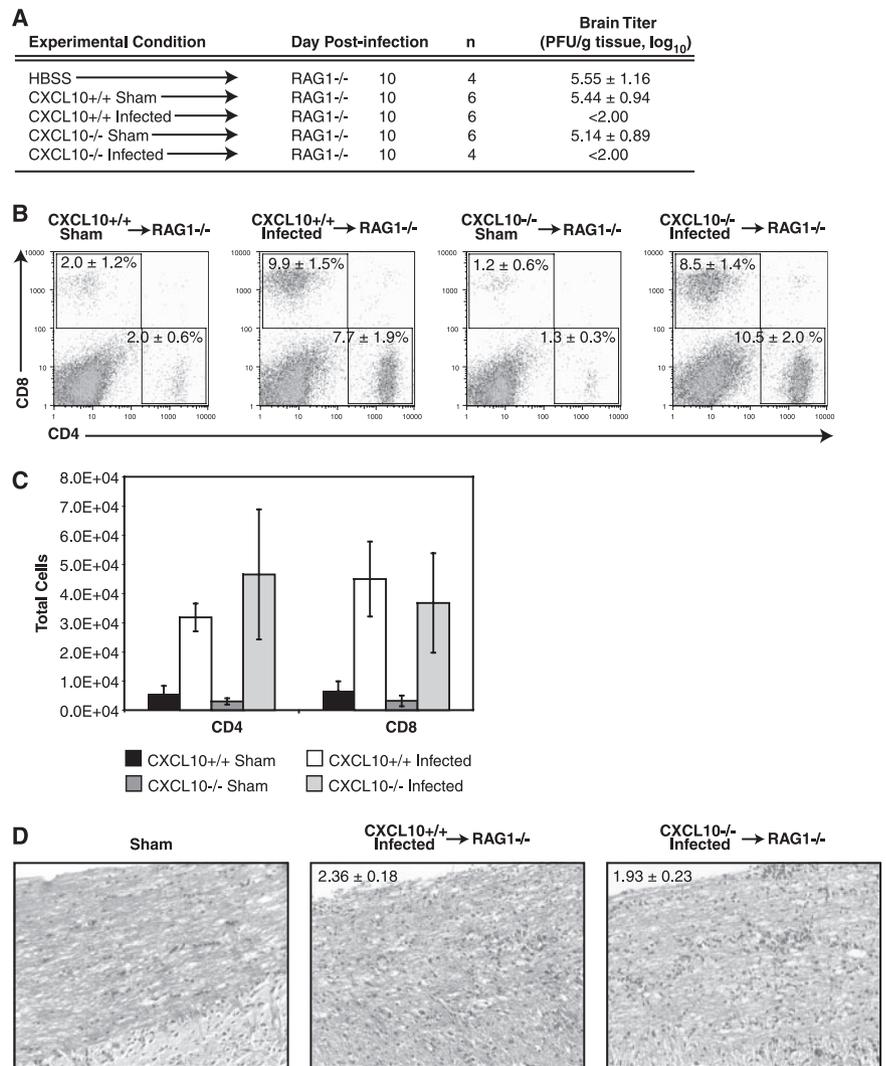
To determine whether CTL activity is influenced by CXCL10 signaling, ex vivo CTL assays were performed. CXCL10^{+/+} and CXCL10^{-/-} mice were infected i.p. with MHV, and spleens were removed at day 7 p.i., at which point CD8⁺ T cells were enriched, and the numbers of S₅₁₀₋₅₁₈-virus specific CD8⁺ T cells were determined by tetramer staining. Consistent with earlier results, similar numbers of tetramer-positive CD8⁺ T cells were detected in both MHV-infected CXCL10^{+/+} and CXCL10^{-/-} mice (data

not shown). As shown in Fig. 3A, no differences in CTL activity were detected at all E:T ratios tested, indicating that CXCL10 is not required for optimal cytolytic activity following MHV infection. In addition to CTL activity, IFN- γ is also important in reducing viral burden within the brain (26, 27). Therefore, IFN- γ production by virus-specific CD4⁺ and CD8⁺ T cells was determined by stimulating immune T cells from MHV-infected mice to defined viral peptides. Such analysis revealed no difference in amounts of IFN- γ secreted between CXCL10^{+/+} and CXCL10^{-/-} cell cultures following incubation with either CD4 or CD8 epitopes (Fig. 3B). Together, these findings indicate that the absence of CXCL10 does not attenuate specific antiviral T cell effector functions used to control MHV replication.

Trafficking of T cells is not affected in the absence of CXCL10

To eliminate the possibility that there is impaired trafficking of T cells derived from CXCL10^{-/-} mice compared with CXCL10^{+/+} mice due to alterations in homing receptor expression, we next evaluated the expression of chemokine receptors on T cell subsets from CXCL10^{+/+} and CXCL10^{-/-} mice infected i.p. with MHV. Previous studies have shown that MHV infection results in increased T cell expression of the chemokine receptors CXCR3 and CCR5 that play a prominent role in T cell trafficking to sites of MHV infection presumably by binding to the ligands CXCL10 and CCL5, respectively, which are expressed within the

FIGURE 5. T cell trafficking is not affected in the absence of CXCL10. CXCL10^{+/+} and CXCL10^{-/-} mice were infected i.p. with MHV, and splenocytes were isolated 7 days p.i. and then transferred i.v. into MHV-infected RAG1^{-/-} mice to determine the effect on viral titers and T cell trafficking into the brain. *A*, T cells from MHV-immunized CXCL10^{-/-} mice transferred to MHV-infected RAG1^{-/-} mice were capable of clearing replicating virus as were T cells from MHV-immunized CXCL10^{+/+} mice. There were no differences in the frequency (*B*) or total numbers (*C*) of CD4⁺ or CD8⁺ T cells within the brains of RAG1^{-/-} mice receiving splenocytes from MHV-immunized CXCL10^{+/+} or CXCL10^{-/-} mice. In addition, representative Luxol fast blue staining of experimental groups of mice reveals similar levels of myelin destruction in MHV-infected RAG1^{-/-} mice receiving donor cells derived from either MHV-infected CXCL10^{+/+} and CXCL10^{-/-} mice (*D*). The severity of demyelination in recipient animals is indicated in the panels and represents the average \pm SEM. Data in *D* are representative of two separate experiments with $n = 7$ for CXCL10^{+/+} and CXCL10^{-/-} recipients. Data presented in *A* and *C* represent the average \pm SEM. The n values for *A* are indicated. Representative dot blots are shown in *B*, and the frequency (average \pm SEM) of dual-positive cells in infected mice is indicated in the upper right quadrant. Experiments were performed a minimum of two separate times with at least three mice per experiment.



CNS (15, 29, 42). CXCL10^{+/+} and CXCL10^{-/-} mice were infected i.p. with MHV, and the expression of CXCR3 and CCR5 on T cells was evaluated. No differences in the frequencies or total numbers of either CD4⁺ or CD8⁺ T cells expressing both CXCR3 and CCR5 were observed between infected CXCL10^{+/+} and CXCL10^{-/-} mice (Fig. 4, *A*, *B*, *G*, and *H*). Regardless of whether T cells were obtained from infected CXCL10^{+/+} or CXCL10^{-/-} mice, there was a marked difference between expression of chemokine receptors on T cell subsets. Although \sim 15% of CD4⁺ T cells were dual-positive for CXCR3 and CCR5, on average 40% of CD8⁺ T cells expressed both receptors (Fig. 4, *A*, *B*, *G*, and *H*). In addition, $>$ 95% of either CD4⁺ or CD8⁺ T cells that were CCR5⁺ also expressed CXCR3. In contrast, the majority of CXCR3⁺ T cells did not express CCR5. Analysis of either CXCR3 or CCR5 expression on gated populations of either CD4⁺ T cells (Fig. 4, *C*, *E*, and *G*) or CD8⁺ T cells (Fig. 4, *D*, *F*, and *H*) revealed no differences in the overall frequency or numbers of cells expressing either receptor between CXCL10^{+/+} or CXCL10^{-/-} mice, indicating that CXCL10 is not required for expression of these receptors. Interestingly, CXCR3 expression was greater on CD8⁺ T cells in terms of both frequency and mean fluorescence intensity when compared with CD4⁺ T cells (Fig. 4, *C* and *D*). More than 85% of CD8⁺ T cells were CXCR3⁺ as compared with \sim 60% of CD4⁺ T cells. This trend was also observed when CCR5 expression was determined. There was a $>$ 2-fold increase in the frequency of CCR5⁺CD8⁺ T cells compared with CCR5⁺CD4⁺ T

cells (Fig. 4, *E* and *F*). Moreover, the overall mean fluorescence intensity for CCR5 expression on either CD4⁺ or CD8⁺ T cells was lower than that for CXCR3 expression (Fig. 4, *C*–*F*). Together, these data demonstrate that expression of CCR5 and/or CXCR3 is not dependent on CXCL10 signaling during expansion of T cells and suggest that migration of T cells is not impaired.

To confirm this hypothesis, splenocytes derived from MHV-immunized CXCL10^{-/-} mice were adoptively transferred into RAG1^{-/-} mice that had been infected i.c. 3 days before transfer. Recipient mice were injected i.v. with 2.5×10^6 total splenocytes and sacrificed 7 days after transfer (10 days p.i.), and brains were collected to determine viral titer and T cell accumulation. RAG1^{-/-} recipients of splenocytes derived from sham-infected mice or vehicle (HBSS) alone were unable to control replicating virus as evidenced by high viral load within the brains (Fig. 5*A*). In contrast, recipients of immune splenocytes from immunized CXCL10^{+/+} or CXCL10^{-/-} mice were both capable of clearing replicating virus below the levels of assay sensitivity ($\leq 2 \log_{10}$ PFU/g) (Fig. 5*A*). In addition, CD4⁺ and CD8⁺ T cell populations within the brains were indistinguishable with regard to both frequency and overall numbers between recipient mice receiving either CXCL10^{+/+} or CXCL10^{-/-} donor cells from MHV-immunized donors (Fig. 5, *B* and *C*). These data demonstrate that CXCL10^{-/-} T cells have trafficking capabilities comparable with those of CXCL10^{+/+} T cells and are able to exert antiviral effects in vivo. Finally, assessment of the severity of neuropathology was

performed on spinal cords obtained from mice at day 10 p.i. As shown in Fig. 5D, similar levels of myelin destruction within the spinal cords were observed between RAG1^{-/-} recipients of either CXCL10^{+/+} (2.4 ± 0.2 , $n = 7$) or CXCL10^{-/-} (1.9 ± 0.2 , $n = 7$) splenocytes. Therefore, the absence of CXCL10 did not mute the pathogenic potential of T cells following infiltration into the CNS.

Discussion

Numerous animal models of inflammatory disease support the role of CXCL10 interactions in T cell trafficking by establishing that inhibition of CXCL10 signaling reduces the accumulation of CXCR3⁺ T cells into sites of disease activity (32, 43–47). Through use of blocking Abs specific for CXCL10 or mice lacking CXCL10 (CXCL10^{-/-}), it has been implicated as a key molecule contributing to a wide variety of pathologies including allograft rejection, tumor biology, and various autoimmune models of disease including diabetes and arthritis (33, 48–51). In addition, CXCL10 is important in defense against microbial pathogens by promoting T cell trafficking to sites of infection. For example, Luster and colleagues (10) demonstrated that Ab neutralization of CXCL10 in mice infected with *Toxoplasma gondii* inhibited T cell influx into the infected tissue, which ultimately resulted in enormous increases in tissue parasite burden and mortality. Furthermore, CXCL10 is valuable in providing optimal defense following infection of the CNS of susceptible mice with neurotropic viruses including MHV, HSV-1, and West Nile virus by allowing T cell access to infected tissue that subsequently reduce viral burden through either cytokine secretion and/or cytolytic activity (13, 45, 52). Similarly, Thomsen and colleagues (53, 54) demonstrated that lack of either CXCL10 or CXCR3 protects mice from death following intracranial infection with lymphocytic choriomeningitis virus (LCMV) by limiting T cell infiltration into the brain. Collectively, these studies illustrate that CXCL10 expression is important in providing directional signals to T cells that enable accumulation within infected tissue.

Along these lines, data presented in this study provide insight into chemokine receptor expression on T cells following MHV infection. A greater percentage of CD8⁺ T cells were CXCR3⁺CCR5⁺ dual-positive when compared with CD4⁺ T cells, highlighting a potentially more important role for CD8⁺ T cells during acute viral infection with regard to controlling viral replication and spread. Expression of both receptors may enhance trafficking to sites of viral infection as well as positional migration within tissue after exit from the vasculature. Interestingly, although the majority of CXCR3⁺ T cells did not express CCR5, almost all of CCR5⁺ were also CXCR3⁺. This observation is consistent with earlier reports examining CXCR3 and CCR5 expression on CD8⁺ T cells following LCMV infection (55). Further, the fact that CXCR3 was more abundant on both CD4⁺ and CD8⁺ T cells compared with CCR5 suggests a more important role for CXCR3 in host defense following MHV infection. However, treatment with anti-CXCR3 neutralizing Ab during acute MHV-induced encephalitis blocks CD4⁺ T cell, but not CD8⁺ T cell recruitment into the CNS suggesting differential roles for CXCR3 in T cell trafficking (15). This is also supported by the demonstration that anti-CXCL10 treatment of MHV-infected mice during chronic disease preferentially reduces CD4⁺ T cell recruitment but has little effect on CD8⁺ T cells (14). Therefore, it is possible that while CXCR3 is a predominant receptor detected on CD8⁺ T cells in response to MHV infection, alternative chemokine receptors can promote directional migration of these cells to sites of infection.

Additional studies have suggested a broader role for CXCL10 in coordinating immune responses and suggest that, beyond leuko-

cyte recruitment, CXCL10 may play a role in the generation and function of effector cells. Characterization of CXCL10^{-/-} mice revealed impaired T cell responses following antigenic challenge, including diminished proliferation and IFN- γ secretion (9). In the autoimmune model of demyelination, experimental autoimmune encephalomyelitis, sensitivity to disease severity is modulated in the absence of either CXCL10 or CXCR3, which did not correlate with deficient trafficking of T cells to the CNS suggesting that the CXCL10-CXCR3 signaling axis helps coordinate T cell responses (6, 11). Tumor protective immunity by IL-12 was also compromised following CXCL10 neutralization in a murine neuroblastoma model. Specifically, treatment with anti-CXCL10 Ab resulted in diminished IFN- γ production by proliferating T cells, reduced tumor cell lysis, and the inhibition of systemic immunity against disseminated metastases (32). Protective immunity was only abrogated if CXCL10 was neutralized in the early immunization phase, but no defects were detected when CXCL10 depletion occurred in the effector phase (32). Impaired CXCL10 signaling has also been suggested to impede polarization of Ag-sensitized T cells to a Th1 profile and may promote a Th2-type response (11, 12, 33). Taken together, the above studies highlight the importance of CXCL10 in the induction of a protective immune response and suggest that it may play a broad role during the pathogenesis of some diseases.

If CXCL10 is involved in T cell effector function in response to MHV infection then there would likely be differences in the T cell response between CXCL10^{+/+} and CXCL10^{-/-} mice at the levels of virus specific T cell generation, proliferation, and function. The data presented here clearly indicate that CXCL10 is not required for generation and/or expansion of virus-specific T cells, demonstrated by the lack of significant deficiencies in either activation profiles (measured by CD44 expression) or numbers of virus-specific CD4⁺ T cells (measured by intracellular IFN- γ secretion in response to M_{133–147} peptide) or CD8⁺ T cells (determined by S_{510–518} tetramer staining) between CXCL10^{+/+} and CXCL10^{-/-} mice. However, there was a significant reduction ($p < 0.02$) in numbers of IFN- γ -positive CXCL10^{-/-} CD8⁺ T cells compared with wild-type mice and this was consistent with our earlier findings (9). Yet there were no differences in the ability of CD8⁺ T cells obtained from MHV-infected CXCL10^{+/+} or CXCL10^{-/-} mice to secrete IFN- γ in response to S_{510–518} peptide as determined by ELISA. Why this differential exists with regard to production of IFN- γ by virus-specific CD8⁺ T cells obtained from infected CXCL10^{-/-} mice is not clear at this time but may reflect differences in assays used. Whereas intracellular IFN- γ production is measured following a relatively brief (6-h) pulse with peptide, IFN- γ secretion is measured by ELISA following 48 h of peptide stimulation. Therefore, there may be a paucity in the synthesis of IFN- γ early after antigenic stimulation of CD8⁺ T cells, but this is eventually overcome. However, we would argue that the collective data indicate that CXCL10 does not appear to play a major role in the acquisition of antiviral T cell effector function measured by CTL activity or IFN- γ secretion following MHV infection.

It was also possible that CXCL10 may influence T cell effector function by modulating chemokine receptor expression. Previously, our laboratory demonstrated an important role for the chemokine receptors CXCR3 and CCR5 during MHV infection, presumably by directing T cell entry into the MHV-infected CNS where CXCL10 and CCL5 are strongly expressed (15, 42). Here we show the absence of CXCL10 does not diminish expression of either CXCR3 or CCR5 on either CD4⁺ or CD8⁺ T cells and that there are no differences in the numbers of T cells expressing both receptors between infected CXCL10^{-/-} and CXCL10^{+/+} mice, suggesting CXCL10 signaling is not required for expression of

homing receptors on activated Ag-specific T cells. Importantly, this study confirms that homing of T cells and the antiviral activity of these cells occurs independently of CXCL10 during the generation of effector cells in response to MHV infection as transfer of immune T cells from CXCL10^{-/-} mice into infected RAG1^{-/-} recipient mice resulted in the accumulation of T cells into the CNS and a reduction in viral titers within the brain.

T cells are important in amplifying the severity of demyelination in mice persistently infected with MHV (21, 56–62). This study also demonstrates that the absence of CXCL10 does not mute the pathogenic activity of T cells following entry into the CNS, demonstrated by similar levels of myelin destruction in recipients of CXCL10^{+/+} or CXCL10^{-/-} T cells. Taken together, these data argue that expression of CXCL10 within the CNS following MHV infection serves to attract T cells and does not influence effector functions. Similarly, Christensen et al. (53) showed that T cell effector responses were not impaired following LCMV infection of CXCL10^{-/-} mice. Further, CXCR3^{-/-} mice infected with *Leishmania major* exhibited defects in CD4⁺ and CD8⁺ T cell trafficking to the site of infection yet could mount a parasite-specific Th1 response (63). In our hands, Ab-mediated neutralization of CXCR3 in MHV-infected mice did not result in muted antiviral activity but did diminish T cell accumulation into the CNS, which is similar to our findings when CXCL10 is blocked (15).

These data differ from previous reports suggesting a requirement for CXCL10 and/or CXCR3 in the development of T cell effector function (9, 10, 12, 32, 33). Blocking CXCL10 following *T. gondii* infection not only impaired T cell trafficking but also muted specific effector functions including proliferation and cytokine secretion (10). Similarly, T cell functions are modulated in mice lacking either CXCL10 or CXCR3 following immunization with myelin epitopes, which correlates with changes in the severity of neuroinflammation and disease (6, 11). We do not feel that these findings necessarily conflict with our data and others demonstrating that CXCL10 has no significant impact on T cell effector function. Rather, these differences are likely due to the unique model systems used and the fact that different stimuli, i.e., Ag(s), costimulatory molecules and/or cytokines, are elicited when administered to induce an immune response. Clearly, numerous interrelated factors converge during the course of Ag recognition by T cells that are critical to the outcome of the T cell response.

Disclosures

The authors have no financial conflict of interest.

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