CXCL10 Is the Key Ligand for CXCR3 on CD8+ Effector T Cells Involved in Immune Surveillance of the Lymphocytic Choriomeningitis Virus-Infected Central Nervous System

Jeanette Erbo Christensen, Carina de Lemos, Torben Moos, Jan Pravsgaard Christensen and Allan Randrup Thomsen

J Immunol 2006; 176:4235-4243; doi: 10.4049/jimmunol.176.7.4235
http://www.jimmunol.org/content/176/7/4235

References
This article cites 64 articles, 36 of which you can access for free at: http://www.jimmunol.org/content/176/7/4235.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
CXCL10 Is the Key Ligand for CXCR3 on CD8⁺ Effector T Cells Involved in Immune Surveillance of the Lymphocytic Choriomeningitis Virus-Infected Central Nervous System

Jeanette Erbo Christensen,* Carina de Lemos,* Torben Moos,† Jan Pravsgaard Christensen,* and Allan Randrup Thomsen²*

IFN-γ-inducible protein 10/CXCL10 is a chemokine associated with type 1 T cell responses, regulating the migration of activated T cells through binding to the CXCR3 receptor. Expression of both CXCL10 and CXCR3 are observed during immunopathological diseases of the CNS, and this receptor/ligand pair is thought to play a central role in regulating T cell-mediated inflammation in this organ site. In this report, we investigated the role of CXCL10 in regulating CD8⁺ T cell-mediated inflammation in the virus-infected brain. This was done through analysis of CXCL10-deficient mice infected intracerebrally with lymphocytic choriomeningitis virus, which in normal immunocompetent mice induces a fatal CD8⁺ T cell-mediated meningoencephalitis. We found that a normal antiviral CD8⁺ T cell response was generated in CXCL10-deficient mice, and that lack of CXCL10 had no influence on the accumulation of mononuclear cells in the cerebrospinal fluid. However, analysis of the susceptibility of CXCL10-deficient mice to lymphocytic choriomeningitis virus-induced meningitis revealed that these mice just like CXCR3-deficient mice were partially resistant to this disease, whereas wild-type mice invariably died. Furthermore, despite marked up-regulation of the two remaining CXCR3 ligands: CXCL9 and 11, we found a reduced accumulation of CD8⁺ T cells in the brain parenchyma around the time point when wild-type mice succumb as a result of CD8⁺ T cell-mediated inflammation. Thus, taken together these results indicate a central role for CXCL10 in regulating the accumulation of effector T cells at sites of CNS inflammation, with no apparent compensatory effect of other CXCR3 ligands. The Journal of Immunology, 2006, 176: 4235–4243.

To maintain a constant immune surveillance of all host organs, systemic recirculation of T lymphocytes is essential.

In this context, the chemokine system has been recognized as a crucial component in regulating the trafficking of the involved T lymphocyte subsets. As T lymphocytes mature and undergo activation and differentiation, they develop new patterns of chemokine receptor expression which are associated with changes in their route of migration. In this manner, naïve T cells are directed through the secondary lymphoid organs, whereas effector T cells patrol the nonlymphoid organs and accumulate at inflammatory sites (1–4). However, inflammatory infiltration is not always desirable. Thus, the recruitment of lymphocytes into the CNS can lead to severe pathology as in case of several infectious and autoimmune neuroinflammatory diseases. Because of this, chemokine/receptor interactions have become an important subject to study, when trying to establish the foundation for more efficient and specific treatment of neuroinflammatory diseases.

Abbreviations used in this paper: IP-10, IFN-γ-inducible protein 10; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MHV, murine hepatitis virus; LCMV, lymphocytic choriomeningitis virus; i., intracerebral; WT, wild type; RPA, RNase protection assay; Q-PCR, quantitative PCR; Ct, cycle threshold; DC, dendritic cell; Tc, cytotoxic T cell.
However, the suggested role of CXCL10 in CNS inflammation is not unequivocal (28, 29), and in some situations elimination of CXCL10 may result in augmented pathology (30–32). Therefore, identifying CXCL10 as a therapeutic target is a controversial issue. This is particularly relevant during infection, because CXCL10 has been ascribed a role in the development of Ag-driven type 1 phenotype T cell responses in the secondary lymphoid organs (23, 30, 33), why inhibition of CXCL10 function might lead to redirection of T cell polarization toward a type 2 immune response. Therefore, immunopathology might be prevented, but in most cases, the anti-infective potential of the T cell response will be reduced in parallel (34, 35).

Finally, while some studies have suggested that CXCL9 and CXCL11 have a compensatory effect in the absence of CXCL10, other have suggested that the three ligands are expressed in temporally and site-specific manners. Thus, CXCL9 might be more dedicated to generation of effector lymphocytes in secondary lymphoid organs, whereas CXCL11 regulate migration and/or localization of lymphocytes in the inflamed CNS. Additionally, it has been argued that CXCL10 have a regulatory effect on CXCL9 and CXCL11 expression, consequently resulting in dysregulation of the two other ligands in the absence of CXCL10 (29, 31, 36). To address some of the above issues, we have studied the outcome of intracerebral (i.c.) infection with lymphoproliferative choriomeningitis virus (LCMV) in CXCL10-deficient mice. In wild-type (WT) mice, this infection induces a fatal T cell-mediated meningitis, the outcome of which directly reflects CD8+ T cell-induced cell damage within the CNS. Using this model, our group has previously found that both CXCL10 and CXCR3 are up-regulated during T cell-mediated inflammation. Furthermore, failure of the effector T cells to express CXCR3 delays CD8+ T cell accumulation in critical areas of the brain parenchyma and reduces LCMV-associated mortality (24).

Materials and Methods

Mice

CXCL10-deficient mice (backcrossed on a C57BL/6 (B6) background) were bred locally from breeder pairs originally provided by A. D. Luster (Massachusetts General Hospital, Boston, MA). The generation of CXCR3-deficient B6 mice has been described before (42); the animals used in these experiments were the progeny of breeder pairs kept at the Panum Institute (University of Copenhagen). WT B6 mice were purchased from Taconic Farms, and they were always allowed to rest for at least a week before entering into experiments; by that time, the animals were 7–9 wk old. All animals were housed under controlled (specific pathogen-free) conditions that included the testing of sentinels for unwanted infections according to Federation of European Laboratory Animal Science Association standards; no such infections were detected. Female mice were used in most experiments, but when both sexes were used, no gender effect was observed. Experiments were conducted according to national guidelines.

Virus infection

Mice were infected i.c. with a virus dose of $3 \times 10^3$ LD$_{50}$ (600 PFU) of LCMV Traub in a volume of 0.03 ml. LCMV is a noncytolytic virus that causes little if any disease in immunodeficient mice. However, i.e. inoculation of LCMV leads to infection of the meninges and choroid plexus, and in adult, immunocompetent mice the result is a severe CD8+ T cell-mediated meninitis from which animals succumb within 8–10 days p.i (43).

Survival study

Mortality was used to evaluate the clinical severity of acute LCMV-induced meninitis. Mice were checked twice daily for a minimum of 2 wk after i.c. inoculation; deaths occurring $<$5 days after infection were excluded from analysis.

Organ virus titers

To determine virus titers in the organs, these were first homogenized in PBS to yield 10% (v/w) organ suspensions, and serial 10-fold dilutions were prepared. Each dilution was then plated in duplicate on MC57G cells. Forty-eight hours after infection, infected cell clusters were detected using monoclonal rat anti-LCMV (VL-4) Ab, peroxidase-labeled goat anti-rat Ab, and o-phenylenediamine (substrate) (44). The numbers of PFU were counted, and results were expressed as PFU per gram of tissue.

Cytotoxic assay

LCMV-specific cytotoxic T cell (Tc) activity was assayed in a $^{51}$Cr-release assay using histocompatible EL-4 cells (H-2$b$) pulsed for 1 h with LCMV gp33–41 peptide as targets; unpulsed EL-4 cells served as control targets. Assay time was 5 h, and percent specific release was calculated according to standard procedure (45, 46).

Cerebrospinal fluid cell count

Cerebrospinal fluid was obtained from the fourth ventricle of mice deeply anesthetized and exsanguinated. The total number of inflammatory cells (background level in uninfected mice is <100 cells/μl) was determined by counting in a hemocytometer, and phenotypic analysis was conducted by flow cytometry (see below).

Abs for flow cytometry

The following mAbs were purchased from BD Pharmingen as rat anti-mouse Abs: FITC- and CyChrome-conjugated anti-CD8, FITC- and PE-conjugated anti-CD44, FITC-conjugated anti-Mac-1 (CD11b), and allophycocyanin-conjugated anti-IFN-γ. PE-conjugated anti-CXCR3 were purchased from R&D Systems and finally, allophycocyanin-conjugated H-2D$b$-gp33–41 on a dextramer backbone were obtained from Dako.

Flow cytometric analysis

Single-cell suspensions of spleen cells were obtained by pressing the organs through a fine steel mesh. Staining of cells for flow cytometry was performed according to standard laboratory procedure (47, 48). Briefly, for enumeration of LCMV-specific CD8+ T cells, cells were either incubated for 30 min with H-2D$b$/gp33–41 dextramer before cells were stained with other relevant Abs, washed, and analyzed or cells were incubated in vitro for 5 h at 37°C in 5% CO$_2$ with or without gp33–41 peptide (0.1 μg/ml) in the presence of monoclonin (3 μM; Sigma-Aldrich) and murine rIL-2 (10 U/well; R&D Systems). After incubation, cells were surface-stained with relevant Abs, washed, and permeabilized using 0.5% saponin. Cells were then stained with anti-IFN-γ for 20 min at 4°C. Samples were analyzed using a FACS-Caliber (BD Biosciences), and at least 10,000 mononuclear cells were gated using a combination of low angle and side scatter to exclude dead cells and debris. Data analysis was conducted using CellQuest Pro software (BD Biosciences).

Isolation of total RNA for RPA or quantitative PCR (Q-PCR)

Brains from mice deeply anesthetized and exsanguinated were immediately removed, snap-frozen in liquid nitrogen, and stored in a liquid nitrogen
Detection of mRNA in the brain by RNase protection assay (RPA)

Transcription levels were studied using the RiboQuant multiprobe RPA system (BD Pharmingen) (49, 50). The following template sets (from BD Pharmingen) were used: T cell marker mRNA (CD3ε, CD4, CD11b, CD8β, F4/80), cytokine marker mRNA (TNF-β, LTβ, TNF-α, IL-6, IFN-γ, IFN-β, TGFβ1–3, macrophage migratory inhibitory factor) chemokine-receptor marker mRNA (CCR1, CCR3, CCR4, CCR5, CCR2, CCR7, CCR8, CXCR3, CXCR5), and chemokine marker mRNA (XCL1 (lymphotactin), CCL5 (RANTES), CCL11 (eotaxin), CCL4 (MIP-1β), CCL3 (MIP-1α), CXCL1–2 (MIP-2), CXCL10 (IP-10), CCL2 (MCP-1), and CCL1 (TCA-3)). All sets of probes included templates for the housekeeping genes L-32 and GAPDH to serve as loading controls. The RPA was performed according to the manufacturer’s instructions. Briefly, α-32P-UTP-labeled antisense RNA transcript was generated from the template sets using T7 RNA polymerase. RNA from each sample was allowed to hybridize to the labeled probe for 16–20 h at 56°C. ssRNA was digested with an RNase/T1 mixture, and the hybrids were analyzed on a denaturing urea-polyacrylamide gel. Protected fragments were visualized by autoradiography by placing dried gels in cassettes with intensifying screens (Bio-max MS; Kodak). For qualitative and quantitative results, gels were subjected to phosphor imager analysis (Amersham Biosciences), and the data were subsequently analyzed using ImageMaster TotalLab software (Amersham Biosciences).

Detection of mRNA in the brain by real-time RT-PCR (Q-PCR)

One microgram of mRNA was reverse-transcribed to cDNA using the RevertAid First strand cDNA synthesis kit (MBT Fermentas). For real-time PCRs (Q-PCR), a Brilliant SYBR Green QPCR Mastermix was used according to the manufacturer’s instructions (Stratagene/AH Diagnostics). Briefly, the Q-PCR components included Brilliant QPCR Master mix, dis- tilled water, ROX reference dye, reverse-transcribed cDNA, and the forward and reverse target gene primers (Table I). Target gene expression was normalized against the housekeeping gene GAPDH.

The Q-PCR program used in a Mx3000P Real-time QPCR instrument was: denaturation at (95°C for 10 min), 40 cycles of (denaturation (95°C/30 s), annealing (58°C/60 s), extension (72°C/30 s). Each reaction runs in duplicates or triplicates including a control whiteout reverse transcriptase and a control whiteout template.

The results were analyzed using Mx3000P system software. The relative expression ratio (R) in each sample is calculated by a mathematical model based on the amplification efficiency (49–51): 

$$ R = \frac{(E_{\text{target}} CP(\text{control} - \text{sample}))}{(E_{\text{target}} CP(\text{control} - \text{sample}))}$$

An amplification efficiency (E) of 100% corresponds to a doubling of the PCR product per cycle. E is calculated from the slope of a standard curve, made based on a 10-fold titration of each primer used (E = 10(−1/slope)). Thus, $$E_{\text{target}}$$ corresponds to CXCL9–11 and $$E_{\text{reference}}$$ to GAPDH. In this study, WT brains infected i.e. 7 days earlier with 3 × 104 LCMV were used as standard curve template. ΔCP (control – sample) refers to the difference in threshold cycle (Ct) between day 0 (control) and day 3, 5, or 7 p.i. (sample). CRef reflects the number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background (51).

Analysis of chemokine levels in cerebrospinal fluid

Cerebrospinal fluid was obtained as described above; to obtain sufficient volumes for analysis test, samples consisted of cerebrospinal fluid pooled from two mice. CXCL9 and CXCL10 were assayed using sandwich ELISA kits (R&D Systems Europe). All assays were run according to manufacturer’s instructions, and concentrations were calculated from standard curves generated by use of recombinant chemokines. Detection limits of the assays were ~30 pg/ml, but due to limitations in the volumes of cerebrospinal fluid that can be obtained, predilution of test samples was required increasing the detection limit to 300–400 pg/ml.

Immunohistochemistry

Mice were deeply anesthetized with tribromoethanol (Sigma-Aldrich) i.p. and decapitated. Brains were then processed for immunohistochemistry as described previously (24). For immunohistochemistry, 10 aceton-fixed micron cryostat sections were washed in 0.1 M PBS (pH 7.4) followed by incubation with 5% normal swine serum (X0901; Dakopatts) diluted in 1% BSA in PBS/0.3% Triton X-100 (BSA/TX100) to block nonspecific binding by the Abs. Next, sections were incubated overnight at 4°C with monoclonal rat anti-mouse CD8α (550281; BD Biosciences), or alternatively with monoclonal rat anti-mouse LCMV Ab to detect viral protein in the brain (VL1-4; provided by R. M. Zinkernagel, University Hospital, Zurich, Switzerland) (44, 52). Specific binding of the primary Ab was verified using biotinylated rabbit anti-rat IgG (BA-4001; Vector Laboratories) and HRP-streptavidin-biotin complex (Vectastain; Vector Laboratories). Finally, the sections were developed in diaminobenzidine and 0.01% H2O2 in 0.05 M Tris buffer (pH 7.6). To evaluate the extent of nonspecific binding of the primary Ab in the immunohistochemical studies, the preincubation agent (swine serum and BSA) was substituted for the primary Ab step described above, and results were considered only if this control was negative (24).
Counts of CD8+ T cells were performed in sections using a standardized protocol for estimating cell density, which involved counting the number of CD8+ T cells in a 10 × 10-mm frame overlaying the part of the corpus callosum situated above the body of the lateral ventricle at 250 times magnification equivalent to an area of 10,000 μm². Three sections with an individual distance of ~300 μm were examined in brains of WT and CXCL10-deficient mice on day 7 p.i.

Statistical analysis

Histological data were examined by ANOVA, and means were tested with the Student-Newman-Keuls test for differences between individual means. For survival curves, comparisons were performed using the Mantel-Cox test; quantitative results were compared using the Mann-Whitney U test. A p value of <0.05 was considered as evidence of statistical significance.

Results

Generation of a normal CD8+ T cell response in the absence of CXCL10

It has been suggested that CXCL10 expressed in secondary lymphoid organs influence the activation and differentiation of T cells. The reason for this is, that dendritic cell (DC)-T cell interactions are considered essential to initiate effector T cell generation in lymphoid organs (53, 54), and DC-derived CXCL10 has been reported to stabilize DC-Th1 cell clusters and mediate retention of T cells in lymph nodes (30). Moreover, CXCL10 has been demonstrated to enhance expansion of Ag-specific T cells (23) and induce Th1 polarization of CD4+ T cells (30, 33). Therefore, to determine whether lack of CXCL10 would impair the expansion and differentiation of LCMV-activated Tc1 cells in secondary lymphoid tissues, we investigated the generation of effector cells in terms of total numbers of splenic CD8+ T cells with 1) an activated phenotype (CD44high) and 2) known specificity for an immunodominant LCMV epitope (gp33–41). The T cell response in the spleen was chosen for analysis because during i.c. infection with LCMV, the majority of effector T cells are generated in this organ (55). WT mice and CXCL10-deficient mice were infected i.c. with LCMV, and on days 6 and 7 p.i., splenocytes were analyzed by flow cytometry. As can be seen in Fig. 1, A and B, lack of CXCL10 did not impair the LCMV-induced CD8+ T cell expansion. Moreover, by evaluating the capacity of gp33–41-specific CD8+ T cells to produce IFN-γ (Fig. 1C) and to lyse peptide-loaded target cells (Fig. 1D), we could show that the generated T cells were functionally active Tc1 cells.

We further evaluated CXCR3 expression on the virus-specific T cells in the absence of CXCL10 as it could be argued that CXCR3 binding of its ligand could affect the expression level of CXCR3 due to recycling, as previously documented for CCR5 (56, 57). Chemokine receptor expression on CD8+ splenocytes, from WT and CXCL10-deficient mice infected i.c. with LCMV 7 days earlier were analyzed using flow cytometry. Similar up-regulation of CXCR3 expression was observed in CXCL10-deficient and WT mice, and this included cells with known (H-2Dβ/gp33–41+) specificity for the virus (Fig. 2). Thus, under our experimental conditions, the generation and differentiation of inflammatory Tc1 cells in the spleen is not affected by the absence of CXCL10.

CXCL10 have no impact on leukocyte recruitment to cerebrospinal fluid

CXCL10 expression is up-regulated in the LCMV-infected CNS (49, 58). Therefore, having established that lack of CXCL10 does not impair the generation of virus-specific effector T cells in the spleen, we next studied the requirement for CXCL10 in directing mononuclear leukocytes to the virus-infected CNS. First, we compared the accumulation of mononuclear cells in the cerebrospinal fluid of WT and CXCL10-deficient mice. Mice were infected with 3 × 103 LD50 of LCMV, and on the days leading up to fatal inflammatory disease in WT mice (days 6 and 7 p.i.), the number of leukocytes present in the cerebrospinal fluid was quantified. As can be seen in Fig. 3A, no significant differences in the accumulation of leukocytes were revealed between the mouse strains. On day 7 p.i., we further evaluated the composition of the inflammatory exudate with regard to CD8+ T cells and Mac-1+ cells, as a characteristic of the inflammatory reaction at this time point is the accumulation of mononuclear cells belonging to these two cell lineages (24, 59–61). We did not observe any significant difference in the composition of the cell infiltrates between the two mouse strains (Fig. 3B), leading us to conclude that CXCL10 is not pivotal for the accumulation of mononuclear leukocytes in the cerebrospinal fluid.

Similar to CXCR3-deficient mice, CXCL10-deficient mice are less susceptible to LCMV-induced meningoencephalitis than WT mice

To further evaluate the requirement for CXCL10 in the accumulation of activated CD8+ effector T cells within the virus-infected CNS, WT, CXCL10, and CXCR3-deficient mice were infected with 3 × 103 LD50 of LCMV, and for the next 14 days, their mortality was registered (Fig. 4). We recently showed (24) that CXCR3-deficient mice are less susceptible to this CD8+ T cell-mediated disease compared with WT, which invariably succumb from i.c. infection. It was therefore of interest to see whether CXCL10-deficient mice would be as resistant as CXCR3-deficient
mice, or if the other ligands of CXCR3 would suffice in the absence of CXCL10.

As expected, all infected WT mice died with symptoms of LCMV-induced meningitis around day 8–9 p.i. In contrast, CXCL10-deficient mice tended to die later, and nearly 40% of these mice survived the infection (Fig. 4). This result closely matched that for CXCR3-deficient mice infected in parallel, suggesting that other CXCR3 ligands do not compensate for the lack of CXCL10. The different survival rates in the three mouse strains do not reflect any major differences in peak viral loads, as similar brain virus titers were measured 7 days after infection regardless of genotype (Fig. 4). Furthermore, like in surviving CXCR3-deficient mice, some of the CXCL10-deficient mice had controlled the CNS infection by day 21 after virus inoculation, whereas still harbored substantial amounts of virus in the brain (data not shown).

Thus, from a clinical perspective, absence of CXCL10 weakens the immune attack to the same degree as previously observed in CXCR3-deficient mice.

Comparison of relevant cerebral mRNA expression in CXCL10-deficient and WT mice with fatal meningitis

To directly evaluate the immune response in the brain, we first quantified transcripts specific for cell subset markers and proinflammatory cytokines by RPA. mRNA were isolated from the brains of WT and CXCL10-deficient mice 7 days after i.c. LCMV infection and from matched control mice inoculated the same way with PBS. As can be seen in Fig. 5A, the levels of CD8 and CD3 mRNA tended to be reduced in CXCL10-deficient mice similar to what has previously been observed in CXCR3-deficient mice (24, 61). Furthermore, we found the expression of TNF-α to be similarly increased in CXCL10-deficient and WT mice. In contrast, IFN-γ levels were lower in the absence of CXCL10 (Fig. 5B), probably reflecting the reduced CD8+ T cell accumulation in the CNS. Other proinflammatory cytokines were either not detected (TNF-β) or only expressed at a very low level (IL-6, lymphotoxin β) (data not shown).

In the same mice described above, we also evaluated the cerebral expression pattern of relevant chemokines/chemokine receptors to determine whether lack of CXCL10 would result in a different expression profile of chemokines or chemokine receptors. As can be seen from Fig. 5, C and D, no qualitative change in the composition of the evaluated chemokine/chemokine receptor expression was observed in CXCL10-deficient mice except for the expected lack of CXCL10 expression in CXCL10-deficient mice.

Absence of CXCL10 expression delays CD8+ T cell accumulation in the neural parenchyma.

To study CD8+ T cell accumulation in the CNS more directly, the localization of CD8+ T cells was evaluated through immunohistological analysis. WT and CXCL10-deficient mice were infected i.c. with LCMV, and brain sections were analyzed 7 days after infection, the day before WT mice normally die from this infection (Fig. 6). As previously reported, the meninges of i.c.-infected mice exhibited prominent signs of inflammation, including multiple CD8+ immunoreactive cells. WT mice also harbored CD8+ cells in many gray and white matter regions in close vicinity of the surfaces of the ventricular system (Fig. 6B). This was not the case in brains of CXCL10-deficient mice (Fig. 6E), the histology of which recapitulated the pattern previously observed in CXCR3-deficient mice (24). Thus, a careful examination of the corpus callosum revealed very few CD8+ cells in this area in CXCL10-deficient mice infected 7 days earlier (Table II).

To further determine whether the difference in the accumulation of CD8+ T cells reflected a difference in the localization of
virus-infected cells, brain sections were analyzed with regard to the distribution of virus-infected cells. Irrespective of genotype, viral protein was detected in choroid plexus epithelial cells, ependymal cells, cells of the meninges, and cells with a morphology corresponding to microglial cells and monocytes (Fig. 6, C and F). The labeled inflammatory cells were observed diffusely around the surfaces of the ventricular system and in major white matter tracts such as the corpus callosum, fimbria, internal and external capsules, and the pyramidal tract. In conclusion, a delay in accumulation of fatal CD8\(^+\)/H-11001 T cells was observed in CXCL10-deficient mice, rendering them less susceptible to fatal meningitis.

Cerebral expression of CXCL9 and 11 in CXCL10-deficient mice

Although the above results indicated that CXCL10 is involved in regulating the accumulation of CD8\(^+\) T cells in the virus-infected CNS, it was pertinent to evaluate the behavior of the two related ligands of CXCR3, CXCL9 and 11, in the absence of CXCL10. Besides the role of IFN-\(\gamma\) in regulating the expression of all three ligands, it has been suggested that the level of CXCL10 production may regulate the further expression of CXCL9 and CXCL11 indirectly, through its effect on the balance of type 1 and type 2 cytokines (29).

First, to determine whether CXCL9 and CXCL11 are induced during fatal LCMV-induced meningitis, we studied the kinetics of mRNA expression for these ligands in the brain of LCMV-infected WT mice. For comparison, we also determined the levels of expression of CXCR3 and CXCL10 mRNA, which are already known to be up-regulated in CNS during LCMV infection (24, 49).

As can be seen in Fig. 7, B and D, the kinetics of both CXCL9 and CXCL11 mRNA expression are similar to that of CXCL10 (Fig. 7C), and the expression of mRNA for CXCR3 follows the same pattern (Fig. 7A).

Next, to evaluate whether lack of CXCL10 would influence the expression of the other two ligands, we compared the levels of expression of mRNA for CXCL9–11 from CXCL10-deficient and WT mice infected with LCMV i.c. 7 days earlier (Fig. 8A). Interestingly, we found a higher expression of CXCL11 in CXCL10-deficient mice

Table II. Number of CD8\(^+\) T cells in corpus callosum of WT and CXCL10-deficient mice (CXCL10\(^{-/-}\)) infected with LCMV virus 7 days earlier

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>CXCL10(^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells</td>
<td>13.8 ± 3.56</td>
<td>5.25 ± 0.96(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Number of CD8\(^+\) T cells per 10,000 \(\mu\)m\(^2\) in corpus callosum of mice infected with 3 \(\times\) 10\(^3\) LD\(_{50}\) of LCMV. Each value is the mean ± SD, \(n = 4–5\) group.

\(^b\) Significant difference between WT and CXCL10\(^{-/-}\) mice (\(p < 0.001\)).
FIGURE 8. Comparison of CXCL9–11 levels in CXCL10-deficient and WT mice. WT and CXCL10-deficient mice were either infected i.c. with $3 \times 10^3$ LD$_{50}$ of LCMV or injected with PBS (CP control). A, Seven days later, mRNA, isolated from the brain, was subjected to real-time RT-PCR. Medians and ranges of three mice per group are represented; one of two similar experiments is presented. B, Seven days later, cerebrospinal fluid (CSF) was tapped and chemokine levels were assayed using sandwich ELISAs. Medians and ranges of three pools (each from two mice per group) are presented; levels in uninfected mice were below the detection limit (<400 pg/ml); N.D., not detectable.

Discussion

Today, a wide range of studies debate the possibility of targeting CXCL10 to prevent immunopathology during various infectious and autoimmune neuroinflammatory diseases. Thus, local production of CXCL10 has been associated with a variety of neurodegenerative diseases including MS (13–17), EAE, an animal model of MS (18–20), and viral encephalitides (21–23). Moreover, accumulating evidence point to a central role for CXCL10 in regulating the recruitment of pathological T cells to sites of CNS inflammation. For example, chronic expression of CXCL10 in mice infected intracerebrally with MHV results in T cell accumulation during LCMV infection, but the involved ligand(s) had not been identified.

Our present data showed that CXCL10 is not essential for the expansion and differentiation of an optimal virus-specific Tc1 response in the spleen, despite the fact that other studies have indicated a role for CXCL10 in both optimizing T cell activation and inducing type 1 polarization (23, 30, 33). Although it could be argued that CXCL9 might exert a compensatory effect in the absence of CXCL10 (39), this does not seem to be the explanation in our case, as T cell activation and differentiation is also unimpaired in CXCR3-deficient mice (24). Consequently, it is more likely that differences in the model systems provide the explanation for this discrepancy. In this context, it may be relevant that in our infection model the main part of the inoculum immediately accesses the blood stream due to the abrupt increase in intracranial pressure following i.c. inoculation. In this manner, most vascularized organs, including lymph nodes and spleen, become actively infected, and perhaps this overrules the necessity of CXCL10 signaling to enhance effector cell generation.

Evaluating the requirement for CXCL10 in controlling the accumulation of pathogenic T cells in the virus-infected CNS, we first noted that CXCL10 was not required for CD8$^+$ T cells to extravasate and cross the blood-cerebrospinal fluid barrier, because similar accumulation of CD8$^+$ T cells were detected in the cerebrospinal fluid of CXCL10-deficient and WT mice. Consequently, it is not simply the lack of infiltrating CD8$^+$ T cells which limits the immunopathology in i.c.-infected CXCL10-deficient mice. This conclusion is not surprising, given that we have recently
shown a similar redundant role of CXCR3 in the recruitment of leukocytes to the cerebrospinal fluid of LCMV-infected mice (24). Studies by Callahan et al. (62) may further explain this redundancy mechanistically. Thus, analyzing the transmigration of CXCR3+ memory T cells across an endothelial cell layer with properties resembling the blood-brain barrier, they found that lack of CXCR3 had no effect on the trans migratory skills of the T cells, suggesting that engagement of CXCR3 contribute to retention of activated T cells within the inflamed CNS rather than playing a role in diapedesis.

In contrast with the cerebrospinal fluid results, analysis of transcript levels in the brain directly related to the influx of CD8+ T cells provided the first indication that T cell accumulation in the CNS was impaired. Thus, we found reduced levels of CD8+ T cell markers and IFN-γ around the time of maximal disease intensity in WT mice. Even more convincing was the immunohistological analysis which revealed that CD8+ T cells did not accumulate efficiently in the neural parenchyma of CXCL10-deficient mice, at least not as rapidly as in WT mice. Notably, this was despite a similar level and localization of LCMV-infected cells. Overall, these findings conform to what we have previously observed in CXCR3-deficient mice, in which animals the accumulation of CD8+ T cells inside the neural parenchyma is significantly delayed (24). Taken together, these results indicate that CXCL10/ CXCR3 interaction plays a key role in facilitating the accumulation of activated cells within certain critical areas of the LCMV-infected CNS. However, this may not be the only possible mechanism by which CXCL10 contributes to neuroinflammatory pathologies. It could be argued that CXCL10 expression has a critical role in retaining activated T cells around the cerebral endothelial cells, and that local secretion of inflammatory cytokines (e.g., IFN-γ) and chemokines resulting from this interaction could contribute to the immunopathology (63). This might be another explanation of why WT mice are more susceptible to the fatal meningitis than CXCL10 and CXCR3-deficient mice. Hence, even though it is the impression that similar numbers of CD8+ T cells in CXCL10-deficient mice may not establish sufficient stable interactions to induce real damage. Supporting this possibility, So-rensen et al. (15, 17) have shown that in MS patients, a convincing relationship exists between 1) CXCL10 expression by astrocytes near vessels and 2) CXCR3-expressing perivascular lymphocytes. Another subject of debate is whether CXCL9 and CXCL11 have a compensatory role or are dysregulated in the absence of CXCL10 (31, 37, 38, 41). In our model, we noted a reduced expression and production of CXCL9 in mice lacking CXCL10. However, because CXCL10 is the quantitatively dominating CXCR3 ligand, and CXCL10-deficient mice also tend to express less IFN-γ, the lower level of CXCL9 is probably the result of an impaired inflammatory response rather than the cause of it. In contrast, increased expression of CXCL11 was found in the inflamed CNS of CXCL10-deficient mice. This finding is of interest for two reasons.

First, it indicates that the regulation of the expression of CXCL9 and 11 is partly dissociated in the CNS of CXCL10-deficient mice. Second, Cole et al. (6) have shown that with regard to chemotraction, CXCL11 is the most potent ligand of CXCR3 and thus would be expected to dominate as chemoattractant of CXCR3+ cells in vivo. However, because the survival rate of CXCL10-deficient mice closely matched that of CXCR3-deficient mice, CXCL11 expression did not seem to be biologically relevant in the virus-infected CNS. A recent study examining the expression of CXCL11 in rats subjected to EAE showed that in this case CXCL11 was not expressed until at least 24 h after the appearance of both CXCL10 and its receptor CXCR3 in the CNS (38). A similar delay in the expression of CXCL11 relative to CXCL10 in LCMV-infected mice might explain why we did not observe any compensatory effect of CXCL11. Interestingly, a recent study showed that besides the fact that CXCL10 and CXCL11 are all-totopic agonists for CXCR3, with CXCL10 having a single-binding affinity and CXCL11 having multiple-binding affinities, they also bind to the CXCR3 receptor when it is in different activation states (64). Thus, the two ligands might bind to cells in different activation states and thus mediate different signals to CXCR3-expressing cells, instead of complementing each other.

In conclusion, we have evaluated the role of CXCL10 in regulating CD8+ T cell-mediated inflammation in the virus-infected brain. This was done by use of CXCL10-deficient mice infected i.c. with LCMV, which in WT mice invariably induces a fatal CD8+ T cell-mediated meningitis. In summary, our results dem-onstrate a central role for CXCL10 in regulating the accumulation of CD8+ effector T cells in critical areas of the CNS during this viral infection, with no apparent compensatory function of other CXCR3 ligands.

Acknowledgments
We thank Grethe Thørner Andersen, Lone Malte, and Susan Peters for excellent technical assistance. We also thank Christina Jespersgaard and Jørgen Schöller (Dako) for generously providing the MHC/peptide dextramers used in this study. IP-10-deficient mice were provided by A. D. Luster.

Disclosures
The authors have no financial conflict of interest.

References


