Opposing Effects of CXCR3 and CCR5 Deficiency on CD8+ T Cell-Mediated Inflammation in the Central Nervous System of Virus-Infected Mice

Carina de Lemos, Jeanette Erbo Christensen, Anneline Nansen, Torben Moos, Bao Lu, Craig Gerard, Jan Pravsgaard Christensen and Allan Randrup Thomsen

*J Immunol* 2005; 175:1767-1775; doi: 10.4049/jimmunol.175.3.1767
http://www.jimmunol.org/content/175/3/1767

---

**References**
This article cites 46 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/175/3/1767.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
Opposing Effects of CXCR3 and CCR5 Deficiency on CD8+ T Cell-Mediated Inflammation in the Central Nervous System of Virus-Infected Mice

Carina de Lemos,* Jeanette Erbo Christensen,* Anneline Nansen,* Torben Moos,† Bao Lu,‡ Craig Gerard,‡ Jan Pravsgaard Christensen,* and Allan Randrup Thomsen2*

T cells play a key role in the control of viral infection in the CNS but may also contribute to immune-mediated cell damage. To study the redundancy of the chemokine receptors CXCR3 and CCR5 in regulating virus-induced CD8+ T cell-mediated inflammation in the brain, CXCR3/CCR5 double-deficient mice were generated and infected intracerebrally with noncytolytic lymphocytic choriomeningitis virus. Because these chemokine receptors are mostly expressed by overlapping subsets of activated CD8+ T cells, it was expected that absence of both receptors would synergistically impair effector T cell invasion and therefore protect mice against the otherwise fatal CD8+ T cell-mediated immune attack. Contrary to expectations, the accumulation of mononuclear cells in cerebrospinal fluid was only slightly delayed compared with mice with normal expression of both receptors. Even more surprising, CXCR3/CCR5 double-deficient mice were more susceptible to intracerebral infection than CXCR3-deficient mice. Analysis of effector T cell generation revealed an accelerated antiviral CD8+ T cell response in CXCR3/CCR5 double-deficient mice. Furthermore, while the accumulation of CD8+ T cells in the neural parenchyma was significantly delayed in both CXCR3- and CXCR3/CCR5-deficient mice, more CD8+ T cells were found in the parenchyma of double-deficient mice when these were analyzed around the time when the difference in clinical outcome becomes manifest. Taken together, these results indicate that while CXCR3 plays an important role in controlling CNS inflammation, other receptors but not CCR5 also contribute significantly. Additionally, our results suggest that CCR5 primarily functions as a negative regulator of the antiviral CD8+ T cell response. The Journal of Immunology, 2005, 175: 1767–1775.
expressed, i.e., concurrent inhibition of both chemokine receptors would lead to a severely impaired inflammatory response. Whether in fact a synergistic inhibitory effect on T cell-mediated inflammation will be obtained by combined blockade of CXCR3 and CCR5 is very important from a pharmacotherapeutic point of view considering that this receptor combination seems to characterize the majority of tissue-infiltrating lymphocytes (16, 24–27). Therefore, to test our hypothesis, transgenic mice deficient in both CXCR3 and CCR5 were generated, and the outcome of LCMV infection in these mice was studied. Much to our surprise, we found that double-deficient mice are more susceptible to LCMV-induced T cell-mediated disease.

Materials and Methods

Mice

The generation of CXCR3-deficient mice has been described before (28). The animals used in these experiments were the progeny of breeder pairs kept at the Panum Institute, University of Copenhagen. CCR5-deficient mice (B6.129P-CmKbr5tm/Kn2) were bred locally from breeder pairs obtained from The Jackson Laboratory. Mice deficient in CXCR3/CCR5 were produced in the following way: CCR5-deficient mice were mated with CXCR3-deficient mice to generate an F1 generation. These mice were then backcrossed to CCR5-deficient mice, and offspring heterozygous at both loci (+/−;−/−) were then selected and interbred. From the subsequent, offspring homozygous double-deficient mice were selected for further in-breeding. Assessment of genotypes was performed by PCR. Wild-type (WT) C57BL/6 mice were purchased from Taconic M&B. Mice from outside sources were always allowed to rest for at least a week before entering into experiments; by that time, the animals were −7−9 wk old. Animals were housed under controlled (specific pathogen free) conditions, and experiments were conducted according to national guidelines.

Virus infection

Mice were infected i.c. with a virus dose of 3 × 10^6 LD_{50} of LCMV Traub in a volume of 0.03 ml. Intracerebral infection induces a fatal CD8^+ T cell-mediated meningitis from which immunocompetent mice succumb on days 7–10 p.i. (29). In a few cases the same dose of virus was injected i.v.; in this case no disease is induced.

Survival study

Mortality was used to evaluate the clinical severity of acute LCMV-induced meningitis. 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 organs, these were first homogenized in PBS to yield 10% (v/v) organ suspensions, and serial 10-fold dilutions were prepared. Each dilution was then plated in duplicates on MC57G cells. Forty-eight hours after infection, infected cell clusters were detected using monoclonal anti-LCMV (VL–4) Ab, peroxidase-labeled goat anti-rat Ab, and α-phenylenediamine (substrate) (30). The numbers of PFU were counted, and results were expressed as PFU/g tissue.

Cell preparations

Single-cell suspensions of spleen cells were obtained by pressing the organs through a fine steel mesh and, when required, erythrocytes were lysed by 0.83% NH4Cl treatment.

CSF cell count

CSF 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 hemocytometer, and phenotypic analysis was conducted by flow cytometry (see Flow cytometric analysis).

Abs for flow cytometry

The following mAbs were purchased from BD Pharmingen as rat anti-mouse Ab: PE- and CyChrome-conjugated anti-CD8, allophycocyanin-conjugated anti-CD4, FITC-conjugated anti-VLA-4 (CD49d), FITC-conjugated anti-CD44, FITC-conjugated anti-Mac-1 (CD11b), FITC-conjugated anti-L-selectin (CD62L), PE-conjugated anti-B220 (CD45R), PE-conjugated anti-CCR5, PE-conjugated anti-IFN-γ, and PE-conjugated IgGl isotype standard. Rabbit anti-CXCR3 Ab and biotin–SP-conjugated goat anti-rabbit Ab were purchased from Zymed Laboratories. Streptavidin–Tricolor was purchased from Caltag Laboratories.

Flow cytometric analysis

Staining of cells for flow cytometry was performed according to standard protocols (31, 32). For enumeration of LCMV-specific CD8^+ T cells, splenocytes were incubated in vitro for 5 h at 37°C in 5% CO2 with gp33–41 peptide (0.1 μg/ml) in the presence of monensin (3 μM; Sigma-Aldrich) and murine rIL-2 (10 U/well; R&D Systems Europe). After incubation cells were surface stained, washed, and permeabilized using 0.5% saponin. Cells were then stained with anti-IFN-γ or IgG1 isotype control for 20 min at 4°C. Samples were analyzed using a BD Biosciences FACSCalibur, and at least 10^4 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 (BD Biosciences).

Detection of mRNA in the brain

Brains from mice deeply anesthetized and exsanguinated were immediately removed, snap frozen in liquid nitrogen, and stored in a liquid nitrogen freezer. Total RNA was extracted from homogenized brains by use of a RNeasy Midi kit (Qiagen). Transcription levels were studied using the RiboQuant multiprobe RNase protection assay (RPA) system (BD Pharmingen) (21, 33). The following templates sets (from BD Pharmingen) were used: T cell marker mRNA (CD3ε, CD4, CD11b, CD8β, and F4/80); cytokine marker mRNA (TNF-β, LTβ, TNF-α, IL-6, IFN-γ, IFN-β, TGF-β1–3, and MIF) chemokine receptor marker mRNA (CCR1, CCR3, CCR4, CCR5, CCR2, CCR7, CCR8, CXCR3, CXCR2, CXCR4, and CCR5X), and chemokine marker mRNA (XCL1 (lymphotactin), CCL5 (RANTES), CCL11 (eotaxin), CCL4 (MIP-1α), CCL3 (MIP-1β), CXCL1–2, CXCL10 (interferon protein-10), CCL2 (MCP-1), and CCL1 (TCA-3)). All sets of probes included templates for the housekeeping genes L32 and GADPH 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. Single-stranded RNA was digested with an RNase/T1 mixture, and the hybrids were analyzed on a denaturing urea-polyacrylamide gel. For qualitative and quantitative results, gels were subjected to PhosphorImager analysis (Amersham Biosciences), and the data were subsequently analyzed using ImageMaster TotalLab software (Amersham Biosciences).

Immunohistochemistry

Mice were deeply anesthetized with tribromoethanol (Sigma-Aldrich) i.p. and decapitated. Brains were then processed for immunohistochemistry (19). Ten-micrometer cryostat sections fixed in acetone were washed three times for 5 min in 0.1 M PBS (pH 7.4), followed by incubation with 5% normal swine serum (X0901; DakoCytomation) 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) diluted 1/50 in BSA/TX100, or monoclonal anti-rat LCMV Ab (VL–4, kindly provided by R. M. Zinkernagel, Zurich, Switzerland) (30, 34) diluted 1/100 was used to detect viral protein in the brain. Specific binding of the primary Abs was verified using HRP-streptavidin–biotin complex followed by diaminobenzidine.

Quantitative morphological analysis

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 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 CCR5-deficient mice on day 7 p.i. and CXCR3-deficient and CXCR3/CCR5 double-deficient mice on days 7 and 9 p.i. Data were examined by ANOVA, and means were tested with the Student-Newman-Keuls test for differences between individual means. Evidence of statistically significant difference between mean values was considered to be p < 0.05.
Statistical analysis

For survival curves comparisons were performed using the Mantel-Cox test; quantitative results were compared using the Mann-Whitney U test. A value of \( p < 0.05 \) was considered as evidence of statistical significance.

Results

**CXCR3 and CCR5 expression on primed CD8\(^+\) T cells**

We initially asked whether CXCR3 and CCR5 are coexpressed on CD8\(^+\) T cells during acute viral infection. To address this question, mice were infected i.c. with LCMV, and on day 7, p.i. splenocytes were isolated from WT as well as single and double knockout mice, and chemokine receptor expression on CD8\(^+\) T cells was evaluated using flow cytometry. From an earlier study, we know that a subset of the CD8\(^+\) T cells from naive mice express CXCR3. The majority of these cells are CD44\(^{high}\), indicating that they represent primed (effector/memory) cells (19). Extending this observation, we now find that \( \sim 15–20\% \) of the CD8\(^+\) T cells in young naive mice express CXCR3, whereas none are CCR5\(^{high}\) (Fig. 1 (numbers seen in brackets)). In mice infected 7 days earlier, 50–80\% of splenic CD8\(^+\) T cells have an activated (CD44\(^{high}\)) phenotype (19), and more than half of the CD8\(^+\) T cells express CXCR3 (Ref. 19; Fig. 1). Furthermore, about one-fifth of splenic CD8\(^+\) T cells from recently infected mice express CCR5, and these cells all coexpress CXCR3. These finding are consistent with the hypothesis that CXCR3 might compensate for the lack of CCR5 and that CXCR3 should be more important in effector T cell homing. Notably, the results in Fig. 1 also confirm the phenotypes of the different knockout strains used in this study.

**Leukocyte recruitment to CSF in i.c. infected CXCR3/CCR5-deficient mice**

To evaluate the redundancy of CXCR3 and CCR5 in regulating the migration of leukocytes to the infected CNS, WT and single and double knockout mice were inoculated i.c. with \( 3 \times 10^3 \) LD\(_{50}\) of LCMV, and the number of leukocytes present in the CSF of mice infected 7 days earlier was quantified (Fig. 2A). Confirming earlier reports (19, 21), lack of CXCR3 or CCR5 individually did not significantly delay the accumulation of leukocytes in this CNS compartment. The average cell infiltrate in double knockout mice matched that in CXCR3-deficient mice. However, a lesser interindividual variation was observed, which could indicate that meningeal cell accumulation might be more consistently delayed in these mice. Therefore, to study in greater detail the kinetics of cell accumulation in the CSF of double knockout mice, these mice and WT mice were analyzed in parallel at several different time points after i.c. infection. As seen in Fig. 2B, we observed no difference in the number of cells in the CSF between WT and double knockout mice on day 6 p.i. In contrast, on day 7 p.i., we could detect a

![FIGURE 1](image1.png)  
**FIGURE 1.** CXCR3 and CCR5 expression on virus-activated CD8\(^+\) T cells. Splenocytes were harvested from WT-, CXCR3-, CCR5-, and CXCR3/CCR5-deficient mice that were infected i.c. with \( 3 \times 10^3 \) LD\(_{50}\) LCMV 7 days earlier; uninfected WT mice served as controls (percentages in parenthesis). All cells were stained with anti-CD8, anti-CXCR3, and anti-CCR5. Gates were set for CD8\(^+\) cells; results are representative of two independent experiments with three mice per group in each case.

![FIGURE 2](image2.png)  
**FIGURE 2.** CXCR3 and CCR5 are not pivotal in LCMV-induced meningoitis. A, WT-, CXCR3-, CCR5-, and CXCR3/CCR5-deficient mice were infected i.c. with \( 3 \times 10^3 \) LD\(_{50}\), and 7 days later, CSF was harvested, and cells in the CSF were counted (\( n = 3–7 \) mice/group). B, WT and CXCR3/CCR5 were similarly infected, and on the indicated days, cell numbers in CSF were determined (\( n = 4–9 \) mice/group). C, On day 7 p.i., cells from CSF were stained with anti-CD8, anti-CD4, anti-Mac-1, and anti-B220 (B cell marker), and the composition of the cellular infiltrate was analyzed in a flow cytometer (\( n = 10 \) group). Averages ± SDs are depicted; statistical evaluation of the difference between CXCR3/CCR5-deficient and WT mice was performed using the Mann-Whitney U test (\( *, p < 0.05 \)).
small but statistically significant reduction in the number of infiltrating mononuclear cells in CXCR3/CCR5-deficient mice compared with WT mice ($p < 0.05$). At this time point, the composition of the inflammatory exudate was also investigated (Fig. 2C), and in both mouse strains, CD8$^+$ T cells and macrophages made up the majority of cells in the CSF, whereas few CD4$^+$ T cells and B cells were present. Most importantly, no significant differences between the genotypes with regard to composition of the infiltrate were observed. On day 9 p.i., all WT mice had succumbed to LCMV disease, and in the double knockout mice, the number of inflammatory cells reached its maximum, which together with the results from day 7 could point to a slight delay in the accumulation of cells in the CSF of these mice. Nonetheless, it is evident that CXCR3 and CCR5 neither individually nor together are pivotal for the accumulation of mononuclear cells in the CSF.

**Susceptibility to LCMV-induced T cell-mediated meningitis**

To further study the importance of CXCR3 and CCR5 in the recruitment of effector T cells to the virus-infected CNS, WT and single and double knockout mice were again infected i.c. with $3 \times 10^5$ LD$_{50}$ of LCMV, and for the next 14 days, their mortality was registered (Fig. 3). WT mice succumb to LCMV-induced CD8$^+$ T cell-mediated meningitis around days 7–10 p.i., and a similar pattern is observed in CCR5-deficient mice confirming earlier results by our group (21). As recently reported (19), CXCR3-deficient mice are less susceptible to i.c. infection with LCMV, and ~60% of these mice survive i.c. infection. Surprisingly, mice lacking both chemokine receptors present a disease phenotype intermediate to that of CCR5 and CXCR3 single-deficient mice, and only ~30% of i.c. infected double knockout mice survived the infection. This reduction in the survival rate, relative to CCR5-deficient mice, is small but statistically significant ($p < 0.05$; Mantel-Cox). Analysis of the viral load in the brain of i.c. infected mice on days 4 and 7 p.i. did not reveal any consistent genotype-related differences, indicating that the differences in survival rate do not reflect differences in the extent of viral replication in the brain (see also Fig. 8 for immunohistochemical analysis). Thus, our results indicate that CCR5 is not redundant, but instead of the expected positive influence on survival in absence of CXCR3 expression, the clinical evaluation reveals that the two receptors exert opposite effects on the severity of LCMV-induced disease.

**Generation of virus-activated CD8$^+$ T cells is accelerated in i.c. infected CXCR3/CCR5-deficient mice**

Because the clinical outcome of i.c. LCMV infection directly reflects cerebral CD8$^+$ T cell-mediated immunopathology and we have previously obtained results suggesting an augmented T cell response in LCMV-infected CCR5-deficient mice (21), a simple explanation for the increased mortality of i.c. infected double knockout mice compared with CCR5-deficient mice could be the generation of a stronger antiviral CD8$^+$ T cell response in the former mice. To test this possibility, we compared the virus-specific CD8$^+$ T cell response in CXCR3- and CXCR3/CCR5-deficient mice, including WT- and CCR5-deficient mice for further comparison. On days 7 and 9 p.i., cells were isolated from the spleen, which is the predominant site of effector T cell generation (35), and CD8$^+$ T cells specific for one of the immunodominant epitopes were measured as the fraction of CD8$^+$ T cells, which respond to stimulation with the MHC class I-restricted LCMV peptide (gp31-44) by production of IFN-γ. As WT- and CCR5-deficient mice would all have succumbed by day 9 after i.c. infection, i.v. infection was used for these mice. This approach is valid because in i.c. infected mice ~90% of the inoculum in fact goes i.v. due to a combination of the pressure and volume applied.

Seven days after infection, similar virus-specific CD8$^+$ T cell responses were measured in the spleens of CXCR3-deficient and WT mice (Fig. 4). In contrast, more virus-specific CD8$^+$ T cells were found in the spleens of CCR5- and CXCR3/CCR5-deficient mice. Additional experiments (data not shown) confirmed that these differences were reproducible and statistically significant. Two days later (day 9 p.i.), the virus-specific CD8$^+$ T cell response was further increased in all groups, and although the absolute differences were less pronounced at this time point, a pattern similar to that seen on day 7 p.i. was observed: significantly higher

![FIGURE 3. Increased susceptibility to fatal LCMV-induced meningitis in mice deficient for both CXCR3 and CCR5. WT-, CXCR3-, CCR5-, and CXCR3/CCR5-deficient mice were infected i.c. with $3 \times 10^5$ LD$_{50}$ of LCMV, and mortality was registered ($n = 19–29$/group). Statistical evaluation of the difference between CXCR3/CCR5- and CXCR3-deficient mice was performed using the Mantel-Cox test ($p < 0.05$). Brain virus titers of days 4- and 7-infected WT and gene-targeted mice were determined and expressed as mean PFU/g tissue ± SD of three to four mice per group.](image-url)

![FIGURE 4. Accelerated CD8$^+$ T cell expansion in LCMV-infected CXCR3/CCR5-deficient mice. WT-, CCR5-, CXCR3-, and CXCR3/ CCR5-deficient mice were infected with $3 \times 10^5$ LD$_{50}$ LCMV, and on days 7 and 9 p.i., splenocytes were isolated. All mice were infected i.c., except for WT- and CCR5-deficient mice analyzed on day 9 p.i., which were infected i.v. To determine the frequency of Ag-specific CD8$^+$ T cells, cells were stained for intracellular IFN-γ following in vitro stimulation with LCMV gp33-41 peptide for 5 h.](image-url)
responses were detected in CCR5- and CXCR3/CCR5-deficient mice compared with WT- and CXCR3-deficient mice.

To see whether early differences in the viral load in the spleen was the cause of the genotype-related differences in the kinetics of the antiviral T cell response, spleen virus titers were determined in mice infected 4 days earlier, and this roughly marks the last day before onset of the T cell response; in all cases, the virus titer was $\sim 0.5-1.0 \times 10^6$ PFU/g organ, indicating that innate defenses were not significantly influenced by the absence of CCR5.

In addition to cell numbers, the quality of the virus-specific CD8$^+$ T cells was also compared. Using intracellular cytokine staining, we did not find any difference in the capacity of the cells to produce IFN-γ following standard peptide stimulation (Fig. 5). However, interestingly, unstimulated CD8$^+$ T cells from virus-infected double-deficient mice “spontaneously” produced low amounts of IFN-γ. Thus, cytokine production in absence of overt stimulation was consistently higher in mice lacking CCR5 expression. However, because the virus infection in the spleen is not completely eliminated at this time, these results may simply indicate that CD8$^+$ T cells from CCR5-deficient mice are more easily triggered by the remaining infected cells (notably, we have no evidence indicating delayed virus clearance in the spleen as a result of CCR5 deficiency (21)). Consistent with this interpretation, neither naive cells nor virus-primed cells from CCR5-deficient mice taken $\geq 14$ days p.i. produced IFN-γ unless stimulated (data not shown). If CD8$^+$ T cells from CCR5-deficient mice were more responsive in vivo, this would further augment the inflammatory reaction in these mice.

**Comparison of cerebral mRNA expression in i.c. infected chemokine receptor-deficient and WT mice**

To evaluate the local immune response in the brain, we quantified transcripts for cell subset markers and proinflammatory cytokines by RPs. mRNA was isolated from the brains of WT and single and double knockout mice 7 days after i.c. infection and from matched control mice inoculated i.c. with PBS. The level of CD8β and CD3ε mRNA is reduced in the CXCR3- and CXCR3/CCR5-deficient mice compared with WT- and the CCR5-deficient mice (Fig. 6A). The lower expression of transcripts for T cell markers probably reflects delayed accumulation of leukocytes in the CNS of CXCR3- and CXCR3/CCR5-deficient mice. Furthermore, we find that the level of TNF-α-specific transcripts is similarly increased in all infected mice, whereas the level of transcripts for IFN-γ is lower in double knockout and CXCR3-deficient mice (Fig. 6B), which may be explained by the reduced level of CD8$^+$ T cells. Other proinflammatory cytokines were either not detected (TNF-β and LTβ) or only expressed at a very low level (IL-6) (data not shown).

To determine whether the existence of an inflammatory response in double knockout mice could be explained by up-regulation of other chemokines/receptors, mRNA from the brain was further analyzed for transcripts for relevant chemokines and receptors. As can be seen in Figs. 6C and 7, no change in the chemokine and receptor profile readily explains the increased susceptibility to i.c. LCMV infection noted in double knockout mice.

**Absence of CXCR3 expression delays but does not prevent CD8$^+$ T cell accumulation in neural parenchyma**

To study T cell accumulation in CNS more directly, the localization of CD8$^+$ T cells was evaluated through immunohistological analysis. WT-, CXCR3-, CCR5-, and CXCR3/CCR5-deficient mice were infected i.c. with LCMV, and brain sections were analyzed 7 and 9 days after infection. As previously reported, the meninges of i.c. infected mice exhibited prominent signs of inflammation, including multiple CD8$^+$ immunoreactive cells. WT- and CCR5-deficient mice also harbored CD8$^+$ cells in many gray and white matter regions in close vicinity of the surfaces of the ventricular system (Fig. 8A and Table I). By contrast, this finding was not recapitulated in brains of CXCR3- and CXCR3/CCR5-deficient mice (Fig. 8B and Table I). Hence, a careful examination of the corpus callosum revealed very few CD8$^+$ cells in this area in CXCR3- and CXCR3/CCR5-deficient mice infected 7 days earlier (Table I). When examined 2 days later, around the time when part of these mice succumbs to LCMV-induced disease, more cells were found in the corpus callosum of these strains. Furthermore, consistent with the higher mortality of double-deficient mice, the number of CD8$^+$ T cells was also significantly higher in these mice (Table I).

Finally, to determine whether the difference in the kinetics of the accumulation of CD8$^+$ T cells reflected a difference in the localization of virus-infected cells, brain sections were also analyzed with regard to the distribution of virus-infected cells. Irrespective of genotype, viral protein was detected in choroid plexus epithelial

**FIGURE 5.** Spontaneous production of IFN-γ by CD8$^+$ T cells from CCR5-deficient mice. CXCR3- and CXCR3/CCR5-deficient mice were infected i.c. with $3 \times 10^4$ LD$_{50}$ LCMV, and CCR5-deficient and WT mice were infected i.v. with $3 \times 10^3$ LD$_{50}$ LCMV. Splenocytes were isolated on day 9 p.i., and cells were stained for intracellular IFN-γ following in vitro stimulation with or without LCMV gp33-41 peptide for 5 h. The cutoff was set by use of isotypic control Ab (data not shown). The depicted plots represent the medians of each group; values are the averages $\pm$ SD of five animals. Statistical evaluation was performed using the Mann-Whitney U test: *, $p < 0.05$; CXCR3/CCR5- vs CXCR3-deficient mice and CCR5-deficient vs WT mice.
cells, ependymal cells, cells of the meninges, and cells with a morphology corresponding to microglial cells and monocytes (Fig. 8, D–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.

**Discussion**

In the past, the CNS was classified as an immune-privileged site. However, it is now evident that lymphocytes do patrol the CNS, and during i.c. infection, large numbers of effector T cells are seen to accumulate in areas of microbial replication. Three routes of leukocyte entry into the CNS can be defined (3). One route follows the formation of the CSF, with the leukocytes crossing the blood-CSF barrier. Following the second route, leukocytes extravasate across postcapillary venules at the level of the pial surface, accessing the subarachnoid and the Virchow-Robin perivascular spaces, where they may meet hemopoietic cells competent for Ag presentation. Finally, leukocytes may cross the blood-brain barrier extravasating through postcapillary venules inside the neural parenchyma.

Chemokines are thought to be important gatekeepers in leukocyte extravasation and migration into the different compartments of the brain, and based on studies of MS patients and related animal models, CXCR3 and CCR5 are believed to be key receptors in the accumulation of Th1 cells at sites of inflammation inside the CNS (e.g., Refs. 7, 10, 22). The same chemokine receptors are expressed by Tc1 cells, and we have demonstrated recently that CXCR3 expression is essential for optimal Tc1 surveillance of the neural parenchyma (19). In contrast, expression of CCR5 does not seem to be essential in the recruitment of Tc1 cells in the virus-infected CNS (21, 36). One reason why CCR5 might be redundant is that other chemokine/receptor pairs exert a similar function in effector cell recruitment. Because CCR5 is often expressed on a subset of activated T cells, which also express CXCR3, we hypothesized that lack of both these receptors might result in a nearly complete block in CD8$^+$ T cell migration to the infected CNS.

**FIGURE 6.** Cerebral expression of inflammation-related transcripts in mice infected i.c. with LCMV. WT-, CXCR3-, CCR5-, and CXCR3/CCR5-deficient mice were infected i.c. with $3 \times 10^3$ LD$_{50}$ LCMV or injected with PBS (control). Seven days later, mice where sacrificed, and transcript levels in the brain were assayed by RPAs; the intensity of the different bands have been normalized against the housekeeping gene L32. A, Cell subset markers; B, cytokines, and C, chemokines. Averages ± SDs of three mice per group are depicted.

**FIGURE 7.** Analysis of cerebral chemokine receptor profile in mice infected i.c. with LCMV. WT-, CXCR3-, CCR5-, and CXCR3/CCR5-deficient mice were infected i.c. with $3 \times 10^3$ LD$_{50}$ LCMV or injected with PBS (control). Seven days later, mice where sacrificed, and chemokine receptor transcripts in the brain were visualized using RPAs. Each lane represents one animal. Cerebral mRNA was also analyzed for transcript for CXCR2, CXCR4, and CXCR5, but none of these were found to be up-regulated as a result of infection (data not shown). Except for weak CCR5 and CXCR4 bands, control mice did not have detectable transcripts for any of the chemokine receptors evaluated here (data not shown).
Therefore, to unravel the biological functions and possible interplay of CXCR3 and CCR5, double knockout mice were generated. For studying the mechanism of CD8\(^{+}\) T cell migration into the virus-infected CNS, LCMV infection is an ideal model system because the virus itself is noncytolytic, and a fatal outcome is therefore directly related to the invasion of the CNS by virus-specific cytotoxic CD8\(^{+}\) T cells (17, 18). Using this model, it has been demonstrated previously that ligands of both CXCR3 and CCR5 act as chemokine receptors for relevant effector cells (CD8\(^{+}\) T cells and monocytes/macrophages) in vitro (37–39).

To evaluate the in vivo consequences of lacking expression of both CXCR3 and CCR5, the ability of the inflammatory cells to accumulate in the virus-infected CNS and cause lethal disease was analyzed. Following invasion by two of the routes through which leukocytes may access the CNS, the infiltrating cells end up in the CSF (3). Therefore, accumulation of mononuclear cells in the CSF was evaluated both quantitatively and qualitatively. Except for a slight delay, no difference between WT and double knockout mice was observed. Thus, contrary to expectations (16), CD8\(^{+}\) T cells deficient in both CXCR3 and CCR5 are capable of crossing the blood-CSF barrier, albeit with a minor delay. A delay in the influx of CD8\(^{+}\) T cells is also reflected in a reduced level of transcripts for CD8\(^{+}\) T cell markers and the proinflammatory cytokine IFN-\(\gamma\), which is produced by the infiltrating CD8\(^{+}\) T cells (40, 41).

Surprisingly, mice lacking both CXCR3 and CCR5 were less resistant to the CD8\(^{+}\) T cell-mediated meningoencephalitis than CXCR3-deficient mice (Fig. 3). This increase in the susceptibility toward the LCMV infection was not readily explained by up-regulation of new chemokine receptors or chemokines (Fig. 6). Evidently other chemokine receptors suffice to allow the infection could explain the increased mortality in double-deficient mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Days after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
</tr>
<tr>
<td>WT</td>
<td>16.6 ± 2.08</td>
</tr>
<tr>
<td>CCR5</td>
<td>13.4 ± 3.10</td>
</tr>
<tr>
<td>CXCR3</td>
<td>2.5 ± 1.73</td>
</tr>
<tr>
<td>CCR5/CXCR3</td>
<td>1.9 ± 1.24</td>
</tr>
</tbody>
</table>

Table 1. CD8\(^{+}\) T cell accumulation in corpus callosum of i.e. LCMV-infected mice as a function of mouse strain and time

*No significant difference between WT and CCR5 knockout mice.
No significant difference between CXCR3-deficient mice and two-thirds of double knockout mice 9 days after infection (p < 0.05).
No significant difference between CXCR3-deficient mice on days 7 and 9 after infection (p < 0.05).
Significant difference between CXCR3-deficient mice and CCR5-deficient mice on days 7 and 9 after infection (p < 0.05).
Significant difference between CXCR3/CXCR5-deficient mice on days 7 and 9 after infection (p < 0.05).
Significantly different from WT mice on day 7 after infection (p < 0.05).
admixiture of 129-derived genes should influence the immune response to LCMV (unpublished observation). Altered tissue distribution could also play a role; delayed accumulation in infected, nonlymphoid tissues could result in a build-up of effector cells in the spleen. However, we have preliminary data showing that CD8+ T cell homing to the virus-infected liver is not impaired in double-deficient mice (P. Holst et al., manuscript in preparation), and changes in the localization to other organs are very unlikely to have a substantial impact on T cell numbers in the spleen. Finally, we did not observe critical differences in the early viral load in the spleen, indicating that the augmented response in CCR5-deficient mice does not relate to increased Ag presentation. Therefore, the most likely explanation is that absence of CCR5 somehow augments the generation of effector CD8+ T cells. This is in agreement with a number of recent reports suggesting a negative regulatory function for CCR5 (21, 36, 42, 43) and indicates that this chemokine receptor serves a negative regulator of effector CD8+ T cell generation. Using the mouse hepatitis virus model, Glass and Lane (36) have previously found that CCR5 signaling imparts a downstream effect on the effector capacity of the individual effector CD8+ T cell. Our studies have not confirmed this effect (Ref. 21 and data not shown). However, we did find that primary effector T cells generated in absence of CCR5 produce more INF-γ in the absence of overt stimulation. Because this phenomenon was only observed during the period when virus-infected cells were still present in the spleen, this could indicate that CD8+ T cells from CCR5-deficient mice are more easily triggered by the low number of virus-infected cells present at this time. If this is the case also in vivo, it could add further to the observed numerical difference, creating the basis for more intense inflammation in double-deficient mice.

In conclusion, the principle finding of this study is that inhibition of CCR5/ligand interaction fails to further suppress T cell-mediated inflammation even in a situation where CXCX3/ligand interaction is already inhibited. Hence, the redundancy of CCR5 (21) does not reflect a functional overlap with CXCR3. This information is highly pertinent from a pharmacotherapeutic perspective because these receptors tend to characterize most tissue-infiltrating T cells, e.g., during allograft rejection (27) and autoimmune disease (7, 25).

Additionally, based on the above results in combination with our prior analysis of mice lacking the individual chemokine receptors (19, 21), we are inclined to suggest that CXCR3 and CCR5 predominantly influences different stages in the immune response to viral invasion of CNS. Thus, while CXCR3 critically influences local T cell accumulation, particularly within the neural parenchyma, CCR5 seems primarily to influence the generation of effector T cells. Notably, the present data also reveal that while CXCR3 is clearly involved in promoting tissue-accumulation, the protective impact of blocking this receptor may be reduced, if the availability of effector T lymphocytes is increased. Together with the histological analysis, this underscores that T cell surface receptors other than CXCR3 and CCR5 play a role in controlling CNS invasion. Molecules that could play a role are the chemokine receptors CCR2 (33) and CXCR6 (44), as well as receptors for leukotrienes; leukotriene B4 has been identified recently as a potent nonchemokine chemoattractant of cytototoxic effector T cells (45, 46).

Acknowledgments

We thank Grethe Thotner Andersen, Lone Malte, Grazyna Hahn, and Susan Peters for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

References


