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Fulminant Lymphocytic Choriomeningitis Virus-Induced Inflammation of the CNS Involves a Cytokine-Chemokine-Cytokine-Chemokine Cascade[1]

Jeanette E. Christensen,* Stine Simonsen,* Christina Fenger,† Maria R. Sørensen,* Torben Moos,‡ Jan P. Christensen,* Bente Finsen,† and Allan R. Thomsen[2]*

Intracerebral inoculation of immunocompetent mice with lymphocytic choriomeningitis virus (LCMV) normally results in fatal CD8$^+$ T cell mediated meningoencephalitis. However, in CXCL10-deficient mice, the virus-induced CD8$^+$ T cell accumulation in the neural parenchyma is impaired, and only 30–50% of the mice succumb to the infection. Similar results are obtained in mice deficient in the matching chemokine receptor, CXCR3. Together, these findings point to a key role for CXCL10 in regulating the severity of the LCMV-induced inflammatory process. For this reason, we now address the mechanisms regulating the expression of CXCL10 in the CNS of LCMV-infected mice. Using mice deficient in type I IFN receptor, type II IFN receptor, or type II IFN, as well as bone marrow chimeras expressing CXCL10 only in resident cells or only in bone marrow-derived cells, we analyzed the up-stream regulation as well as the cellular source of CXCL10. We found that expression of CXCL10 initially depends on signaling through the type I IFN receptor, while late expression and up-regulation requires type II IFN produced by the recruited CD8$^+$ T cells. Throughout the infection, the producers of CXCL10 are exclusively resident cells of the CNS, and astrocytes are the dominant expressors in the neural parenchyma, not microglial cells or recruited bone marrow-derived cell types. These results are consistent with a model suggesting a bidirectional interplay between resident cells of the CNS and the recruited virus-specific T cells with astrocytes as active participants in the local antiviral host response. *The Journal of Immunology,* **2009, 182: 1079–1087.**

The CNS is classically considered a site of "immune privilege." This concept was originally introduced as a result of the now classical finding that allografts were only slowly rejected when introduced into the CNS (1). However, subsequent observations have greatly modified the original concept, and it is now clear that the CNS is not beyond the reach of normal immunological surveillance, but rather that induced responses are subject to an extremely restrictive regulation (2, 3). Such stringent control has probably developed to minimize the risk of debilitating pathology in an organ with limited regenerative potential. Specific features influencing the immune surveillance of the CNS are the blood-brain and blood-cerebrospinal fluid barriers and a relative lack of lymphatic drainage as well as endogenous APCs (2–4). One result of this stringent regulation is that the migration of T lymphocytes into the CNS is kept at a very low level under normal conditions (5). However, during infections and neuroinflammatory diseases such as multiple sclerosis (MS)[3] and its animal model, experimental autoimmune encephalomyelitis, a distinct leukocyte infiltration of the CNS can be found and may even be responsible for the related symptoms.

Generally, the recruitment of leukocytes to any organ site is a complex, multistep process, and extravasation as well as the final positioning of the infiltrating cells is tightly regulated through stringent control of the local expression of adhesion molecules and chemokines (6, 7). Similarly, in the CNS there is mounting evidence that certain chemokines/chemokine receptors play a critical role in controlling the pattern of T cell infiltration, and in this respect the pair CXCL10 (IFN-γ-inducible protein 10, IP-10)/CXCR3 has attracted particular attention. Thus, the concentration of CXCL10 has been found to be elevated in the CSF of patients with MS and in active MS lesions (8–10). Additionally, T cells in the lesions express the matching receptor, CXCR3 (9–11). Importantly, expression of CXCL10 in the CNS may also be potentially beneficial to the host, e.g., by attracting effector T cells critically involved in controlling viral infections of the CNS (12–14). CD8$^+$ T cells are often important in the response to viral encephalitides and for a number of years, studies performed by our group have been focused on studying the chemokines and chemokine receptors involved in regulating CD8$^+$ T cell migration during viral infection of the CNS (15–17). These studies along with those of other groups have established that expression of CXCL10 leads to a type I cytokine polarized response with the accumulation of primed CD8$^+$ T cells in the CNS. Indeed, in some cases (lymphocytic choriomeningitis virus (LCMV), West Nile virus, dengue virus; Refs. 12, 13, 15, 16) the outcome of viral infection may be

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[3] Abbreviations used in this paper: MS, multiple sclerosis; LCMV, lymphocytic choriomeningitis virus; i.c., intracerebral; 2′-5′ OAS, 2′-5′-oligoadenylate synthetase 1A; p.i., post infection; WT, wild type; Q-PCR, quantitative PCR; AP, alkaline phosphatase; GFAP, glial fibrillary acidic protein; DAPI, 4′,6-diamidino-2-phenylindole; CC, corpus callosum; EAE, experimental autoimmune encephalomyelitis.

decided by the presence or absence of CXCL10-induced CXCR3 dependent $CD8^+$ T cell recruitment to the infected CNS.

For this reason, it was of obvious interest to elucidate key aspects concerning the regulation of the expression of CXCL10 in the virus-infected CNS. Using the LCMV intracerebral (i.c.) infection model and various knock-out mouse strains as our model system, we addressed the question of how the expression of CXCL10 is regulated in the virus-infected CNS.

The LCMV model is ideal for studying the molecular mechanisms underlying virus-induced T cell mediated inflammation of the CNS, because the virus itself is noncytolytic (18), and pathogenesis and death is directly related to the influx of virus-specific $CD8^+$ T cells (19–21). LCMV-induced CNS disease occurs in adult mice inoculated i.c. with the virus, and the infection presents with $CD8^+$ T cell mediated inflammation of the meninges, the choroid plexus, the ependymal lining of the ventricles and the nearby neuroparenchyma (15, 21). Normal adult mice succumb from this $CD8^+$ T cell-mediated meningoencephalitis between 7 and 10 days after virus inoculation (22). However, in CXCL10- or CXCR3-deficient mice, the accumulation of $CD8^+$ T cells is delayed, despite the generation of a normal T cell response, and unlike wild-type (WT) mice, 50–60% of the former mice do not develop lethal T cell-mediated disease (15–17).

Because production of CXCL10 is known to be positively regulated by IFNs, it was our working hypothesis that expression of CXCL10 in the LCMV-infected CNS was controlled at least in part by members of this broad family of cytokines. Based on the finding that initial LCMV-induced up-regulation of CXCL10 expression coincided with up-regulation of several other IFN-regulated genes, we went on to study various gene-targeted and chimeric mice to more directly address the role of type I and type II IFNs in the regulation of LCMV-induced CXCL10 production.

Our results indicate that LCMV initially triggers the production of CXCL10 through a type I IFN-dependent pathway. Upon recruitment of circulating virus-specific T cells to the infected CNS, Ag-driven local production of IFN-γ serves to substantially up-regulate CXCL10 expression, which in turn leads to the recruitment of more effector T cells. Thus, a cytokine-chemokine-cytokine-chemokine cascade is crucially involved in mediating virus-induced inflammation in the LCMV-infected CNS.

Materials and Methods

Mice

CXCL10, IFN-γR, and IFN-γ-deficient mice were bred locally from breeder pairs originally provided by A. D. Luster (Harvard Medical School, Boston, MA) and The Jackson Laboratory, respectively. IFN-$\alpha\beta$R-deficient mice on a C57BL/6 background were the progeny of breeder pairs provided by D. Pinschewer and R. Zinkernagel (Universitätspital, Zürich, Switzerland). C57BL/6 WT and matched *nu/nu* mice were purchased from Taconic Farms, and these mice were always allowed to acclimatize to the local environment for at least a week before entering into experiments; by that time the animals were ~7–9 wk old. Transgenic C57BL/6 mice (TCR-318) expressing a TCR specific for an immunodominant, MHC class I-restricted LCMV epitope (gp33–41) on ~60% of their $CD8^+$ T cells were bred locally from breeder pairs originally provided by H. Pircher and R. Zingernagel (Universitätspital, Zürich, Switzerland) (23). Animals were housed under controlled (specific pathogen-free) conditions as validated by 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 regarding animal experiments.

Virus infection

Mice were infected i.c. with a virus dose of 10^3 LD_{50} (~200 pfu) of LCMV Traub. LCMV is a noncytolytic virus that causes little if any disease in immunodeficient mice (18, 24). However, intracerebral inoculation of LCMV leads to infection of the CNS, and in adult, immunocompetent mice the result is a severe $CD8^+$ T cell-mediated meningoencephalitis from which the animals succumb around day 8–9 post infection (p.i.) (22).

Adoptive transfer of TCR-318 spleen cells

For cell transfers, spleens were removed from naive TCR-318 transgenic mice sacrificed by cervical dislocation. Single cell suspensions were obtained by pressing the organs through a fine steel mesh, and cells were washed and counted. Three $\times$ 10^6 TCR-318 splenocytes were injected i.v. into nontransgenic syngeneic recipients 1 day before virus challenge. With this number of TCR-318 transgenic cells transferred, donor-derived $CD8^+$ T cells will totally dominate the virus-specific $CD8^+$ T cell response (25).

Bone marrow chimeras

Syngeneic and allogeneic bone marrow chimeras were made using $CXCL10^{-/-}$ and WT mice. Mice were lethally irradiated (9 Gy) in the morning and transplanted i.v. with 20 $\times$ 10^6 femur cells from allo- or syngeneic donors in the afternoon. Eight weeks later, the mice were infected with virus as described.

Isolation of total RNA for quantitative PCR

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 RNeasy midi kit (Qiagen).

Detection of mRNA in the brain by quantitative PCR (Q-PCR)

One microgram of mRNA was reverse transcribed to cDNA using RevertAid First strand cDNA synthesis kit (MBT Fermentas). For Q-PCR reaction, a Brilliant SYBR Green QPCR Mastermix was used according to the manufacturer's instructions (Stratagene, AH Diagnostics). In brief, the Q-PCR components included Brilliant QPCR master mix, distilled water, ROX reference dye, reverse transcribed cDNA, and the forward and reverse target gene primers (Table I). IFN-α primers detected a consensus sequence covering IFN-α1, 5, 6, 7, and 12. Target gene expression was normalized against the housekeeping genes GAPDH or porphobilinogen.

The Q-PCR program used in an Mx3000P Real-time QPCR instrument was: denaturation at (95°C/10 min), 40 cycles of denaturation (95°C/30 s), annealing (58°C/60 s), and extension (72°C/30 s). Each reaction was run in duplicates or triplicates plus a control without reverse transcriptase and a control without 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 (26): $R = (E_{target})^{\Delta CP(control\ -\ sample)}/(E_{reference})^{\Delta CP(control\ -\ sample)}$.

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, based on a 10-fold titration of each primer used ($E = 10^{(-1/slope)}$). Thus, E_{target} corresponds to the target gene primers and $E_{reference}$ to the household gene primers (GAPDH or porphobilinogen). In this article, WT brains infected i.c. 3 or 7 day earlier with LCMV were used as standard curve template. ΔCP(control $-$ sample) refers to the difference in threshold cycle (C_t) between day 0 (control) and day 3, 5, or 7 p.i. (sample). C_t reflects the number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background (26).

Quantitative PCR for IFN-regulated genes

To evaluate the expression of IFN-regulated genes we used a RT (2) Profiler PCR array kit from SABiosciences. The applied kit (PAMM-016A) profiles signaling molecules involved in the IFN-α and -β response and IFN-responsive genes. Genes associated with virally induced and intrinsic IFN resistance are included as well. The preparation of cDNA, the running of the assay and the analysis of the results were all performed according to the manufacturer's instructions.

Quantification of chemokine production from Ag-stimulated $CD8^+$ T cells

$CD4^+$ and MHC class II^+ cells were removed from splenocytes by negative selection. Cultures of 2.5 and 5 $\times$ 10^5 of the remaining cells (primarily $CD8^+$ cells) were either stimulated with a dominant LCMV MHC

Table I. *Primer sequences used for Q-PCR*

2′-5′-OAS	Forward primer	5′-CTT TGA TGT CCT GGG TCA TGT-3′
	Reverse primer	5′-CTC CGT GAA GCA GGT AGA G-3′
CXCL10	Forward primer	5′-CGA TGA CGG GCC AGT GAG AATG-3′
	Reverse primer	5′-TCA ACA CGT GGG CAG GAT AGG CT-3′
IFN-α	Forward primer	5′-GCT AGG CTC TGT GCT TTC CTG ATG-3′
	Reverse primer	5′-CTC AGG TAC ACA GTG ATC CTG TGG-3′
IFN-β	Forward primer	5′-AAC AGG TGG ATC CTC CAC GCT GCG-3′
	Reverse primer	5′-GTG GAG AGC AGT TGA GGA CAT CTC C-3′
IFN-γ	Forward primer	5′-AAC GCT ACA CAC TGC ATC TGG G-3′
	Reverse primer	5′-GCC GTG GCA GTA ACA GCC-3′
PBDG	Forward primer	5′-GTG AGT GTG TTG CAC GAT C-3′
	Reverse primer	5′-GGG TCA TCT TCT GGA CCA T-3′
GAPDH	Forward primer	5′-CAA TGT GTC CGT CGT GGA-3′
	Reverse primer	5′-GAT GCC TGC TTC ACC ACC-3′

class I restricted epitope (GP_{33-41}) in vitro for 6 h or left unstimulated. Supernatants were harvested and assayed for CXCL10 and CCL3 using a chemokine 5-plex bead immunoassay (BioSource Cat. No. LMC0005) for Luminex 100 system according to the manufacturer's instructions (Luminexcorp). More than 100 events were acquired per bead set. StarStation ver. 2.0 software (Applied Cytometry Systems) was used for chemokine quantification analysis.

In situ hybridization and immunohistochemistry

Mice were deeply anesthetized with tribromoethanol (Sigma-Aldrich) and decapitated. Brains were rapidly dissected and frozen in CO_2-snow. Subsequently, brains were cut into serial 30-μm cryostat sections, mounted on RNase-free super frost plus glass slides (Hounisen), and stored in sealed boxes at −80°C.

The in situ hybridization technique was performed as described by Lambertsens et al. (27). The CXCL10 mRNA was detected by a mixture of two alkaline phosphatase (AP)-labeled DNA probes (5′GGCAGGATAG GCTCGCAGGGATGATTTC′3 and 5′GGGTGTGTGCGTGGCTTCAC TCCAGTTA′3 (4 pmol/ml)) both complementary to murine CXCL10 mRNA. The probes were designed by use of Oligo-design software 6.0 and purchased from DNA Technology A/S (Aarhus). Hybridization took place overnight at 37°C and sections were then rinsed. The in situ hybridization signal was developed in a Tris-HCl $MgCl_2$ buffer containing the AP substrates, 5-bromo-4-chloro-3-indolyl phosphatate (Sigma-Aldrich) and nitro blue tetrazolium (Sigma-Aldrich) and after 3 days in dark, the staining was arrested in distilled water. The specificity of the hybridizations was documented by showing that 1) sections hybridized with individual CXCL10 probes yielded similar signal to sections hybridized with the probe mixture, 2) the hybridization signal was abolished when hybridizing RNase A (Pharmacia Biotech) digested sections, or when hybridizing sections with a 100-fold excess of the unlabelled probe mixture, and 3) incubation of sections with buffer yielded no signal. Finally, hybridization of parallel sections with an AP-labeled GAPDH probe ensured the overall suitability of the tissue for hybridization.

When in situ hybridization for CXCL10 mRNA and immunohistochemistry for glial fibrillary acidic protein (GFAP) protein were combined (27), sections were subjected to the standard in situ hybridization procedure, except that development was arrested after 1–1/2 day to reduce the amount of chromogenic signal. Sections were then rinsed and incubated with monoclonal Alexa 488-conjugated mouse-anti-GFAP IgG1 (5 μg/ml) (A21294, Invitrogen) or mouse IgG1 isotype control (5 μg/ml) in TBS-buffer for 2 h. After a final rinse in TBS, sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining. Using PhotoShop software, the bright field pictures of the chromogenic in situ hybridization signal was color inverted and the in situ signal was recolored with red before being merged with pictures of the Alexa 488 and DAPI staining.

In the case of double immunohistochemistry for CXCL10 protein and GFAP or CD11b (27), sections were fixed in 4% paraformaldehyde, rinsed in TBS-buffer, and then incubated with purified goat-anti-CXCL10 IgG (0.5 μg/ml) (AF466NA, R&D Systems) alone or with monoclonal rat-anti-CD11b IgG2b (1.7 μg/ml) (MCA711, Serotec) overnight at 4°C. Parallel sections were incubated with goat IgG (0.5 μg/ml) (DakoCytomation) or rat IgG2b isotype control (1.7 μg/ml) (Nordic BioSite). After another rinse, sections were incubated with Alexa 594-conjugated donkey-anti-goat IgG (10 μg/ml) (A11058, Invitrogen) and monoclonal Alexa 488-conjugated mouse-anti-GFAP IgG (5 μg/ml) or Alexa 488-conjugated donkey-anti-rat IgG (10 μg/ml) (A21208, Invitrogen) in TBS buffer plus 10% bovine serum for 1 h. After a final rinse, sections were counterstained with DAPI nuclear staining.

Statistical analysis

Quantitative results were compared using the Mann-Whitney *U* test. A *p*-value of <0.05 was considered as evidence of statistical significance.

Results

Expression of CXCL10 is up-regulated in the CNS during i.c. LCMV infection and correlates with expression of IFN-regulated 2′,5′-oligoadenylate synthetase 1A (OAS)

Our working hypothesis was that the expression of CXCL10 in the brains of mice infected i.c. with LCMV reflects the induction of type I and/or type II IFNs.

As the first approach to test this assumption, we compared the kinetics of CXCL10 expression with that of 2′-5′-OAS, which is a classical IFN-regulated gene (28), important for the antiviral state in IFN-treated cells (29). Expression of this molecule may therefore serve as a convincing surrogate marker for the fact that IFNs have been induced and functionally expressed.

WT mice were infected intracerebrally with LCM virus, and on days 3, 5, and 7 p.i., we determined the mRNA levels for CXCL10 and 2′,5′-OAS in the brain by use of Q-PCR. As previously shown (15, 16, 30) there is a slight, but significant increase in CXCL10 expression in virus-infected mice on day 3 p.i. as compared with mice inoculated with PBS (Fig. 1*A*). With time, CXCL10 expression gradually become more pronounced, and very high expression is found on day 7 p.i. coinciding with maximal $CD8^+$ T cell mediated local inflammation (22). Similar to the expression of CXCL10, there is an early, significant expression of 2′,5′-OAS on day 3 p.i. followed by further increases on days 5 and 7 p.i. Notably, the increase in expression of 2′,5′-OAS over time is not as marked as that regarding expression of CXCL10. However, this may reflect differences in the responsiveness of the two genes to type I vs type II IFNs.

The presence of 2′,5′-OAS strongly indicates that IFNs have been produced, but does not reveal any details regarding amount and type of IFN. Therefore, to address these issues, we determined mRNA levels for IFN-α,-β, and -γ in the same LCMV-infected brains as evaluated above.

Already on day 3 p.i. we observed a small, but consistent, increase in the expression of IFN-β in LCMV-infected mice compared with PBS-injected controls (Fig. 1*C*). Minimal expression of type I IFNs was noted in sham-injected mice, probably as a consequence of the inoculation trauma. By day 5 and 7 p.i., increasing levels of IFN-α and in particular IFN-β was seen (Fig. 1*C*). However, at all time-points studied, the relative increase in expression

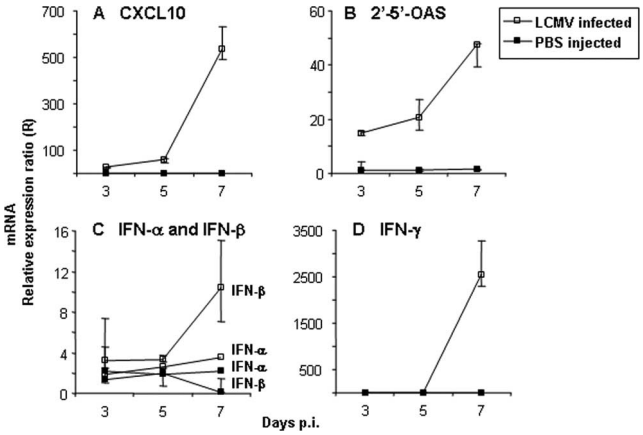

FIGURE 1. The kinetics of expression of CXCL10 (*A*), 2′,5′-OAS (*B*), IFN-α, -β (*C*) and -γ (*D*) in the CNS of C57BL/6 mice infected i.c. with LCMV; controls were mice injected i.c. with PBS. On days 3, 5, and 7 p.i., mRNA levels for CXCL10, 2′,5′-OAS, IFN-α, -β and -γ in the CNS were determined by Q-PCR; the level of expression in the brains of completely unmanipulated mice were used as set point ($R = 1$). Medians and ranges of three mice/group are presented.

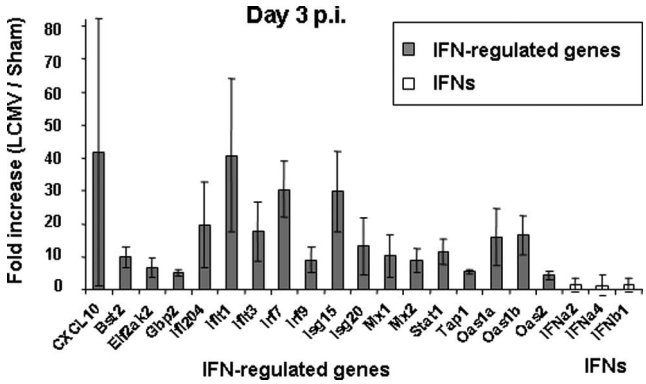

FIGURE 2. Increased expression of multiple IFN-regulated genes in WT mice infected i.c. 3 days earlier. mRNA was extracted from the CNS of WT mice injected with LCMV 3 days earlier; brains from mice injected with PBS served as controls. The expression of the indicated genes was analyzed by Q-PCR. Four infected mice and two sham-inoculated mice were analyzed.

of type I IFNs was limited. This pattern is in contrast to expression of type II IFN, which was not clearly detectable until day 7 p.i. (Fig. 1*D*), at which time, however, very high levels of expression was noted, consistent with earlier analysis of the cerebrospinal fluid on this day (31).

Evidence for general up-regulation of IFN-regulated genes

The fact that we could find substantial expression of 2′,5′-OAS in the brain at day 3 p.i. despite marginal expression of IFNs, at first seemed puzzling. However, simple explanations for such a discrepancy are not difficult to find. First, in the above analysis a burst of type I IFN activity earlier than day 3 (explaining the expression of 2′,5′-OAS on this day) would have been entirely missed. Alternatively, while few cells may actually produce IFNs, many more cells may be involved in the response to this cytokine. In the latter case, it is very likely that up-regulation of down-stream mediators of IFN activity is more easily detected. This would also be the case if the levels of expression of mRNA for IFNs and their regulated products in the individual cell differed markedly.

To test the first possibility, we analyzed the brains of mice infected i.c. 1 or 2 days before sampling of the brain for PCR analysis. However, using IFN-β as target gene, we did not find any evidence indicating that type I IFNs were produced in high amounts before day 3 p.i. (data not shown). In an attempt to evaluate the likelihood of the second possibility, we decided to screen widely on day 3 p.i. for the LCMV-induced expression of additional IFN-regulated genes. Using a Q-PCR-based super array dedicated to analysis of IFN-regulated molecular pathways, we obtained results (Fig. 2) that clearly support the assumption that IFN are being produced and induce widespread cell activation at an early stage of LCMV in the CNS. Thus, in four of four mice we found distinct up-regulation of at least eighteen genes whose expression is known to be influenced by IFNs. In addition, this analysis, which employs primer sets different from those used in our in-house PCRs, confirmed the low expression of both IFN-α (α2 and α4) and β at this time-point.

CXCL10 expression is T cell independent during the early host response

Based on the above results, we wanted to investigate more directly whether the early expression of CXCL10 in the LCMV-infected brain was dependent on induction of type I IFNs, thus directly reflecting the innate host response, or resulted from IFN-γ secreted from the early recruited NK cells and nonspecific memory T cells. First, we compared CXCL10 mRNA levels in LCMV-infected brains from T cell deficient (*nu/nu*) and WT mice on day 3 p.i. As can be seen in Fig. 3*A*, a tendency toward lower CXCL10 levels was observed in mice lacking T cells. However, the difference was not statistically significant, and therefore suggests that early expression of CXCL10 for the most part is T cell independent. A similar expression pattern for 2′,5′-OAS levels was noted, indicating the presence of a significant, early IFN response in the T cell-deficient mice (Fig. 3*B*).

Early CXCL10 expression requires type I IFNs, but not type II IFN

To directly assess whether it is the release of IFNs that drives CXCL10 expression in the brain during the early host response, IFN-$\alpha\beta R^{-/-}$ mice and IFN-$\gamma^{-/-}$ mice were next subjected to LCMV infection, and their brains analyzed for expression of CXCL10 3 days later. As can be seen in Fig. 4*A*, CXCL10 expression was almost completely absent in IFN-$\alpha\beta R^{-/-}$ mice, whereas the expression of CXCL10 in IFN-$\gamma^{-/-}$ mice did not differ significantly from that in WT mice. The complete absence of 2′,5′-OAS expression in IFN-$\alpha\beta R^{-/-}$ mice, but not in IFN-$\gamma^{-/-}$ mice (Fig. 4*B*), confirms that the expression is IFN driven, and that

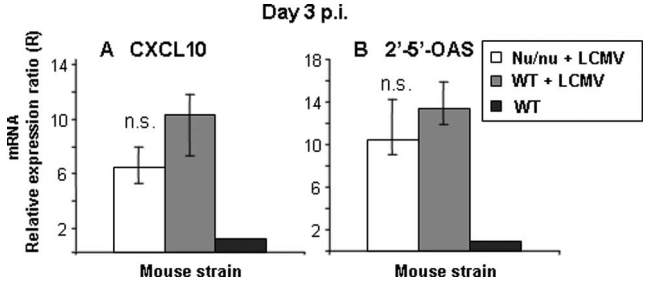

FIGURE 3. Early CXCL10 expression is T cell independent. mRNA was extracted from the CNS of nude (*nu/nu*) and WT mice injected with LCMV 3 days earlier; brains from mice injected with PBS served as controls. The expression of mRNA for CXCL10 (*A*) and 2′,5′-OAS (*B*) was analyzed by Q-PCR. Medians and ranges of four mice/group are presented. Results are representative of two similar experiments. n.s. = not significantly different from WT mice.

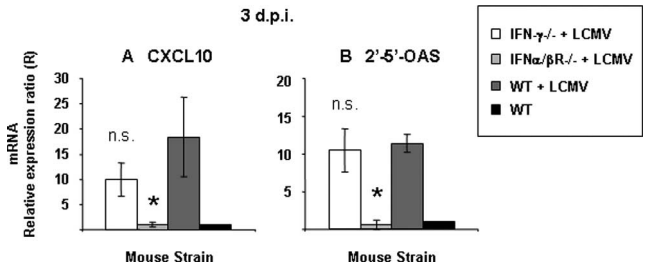

FIGURE 4. Early CXCL10 expression is dependent on production of type I, but not type II IFNs. Levels of mRNA for CXCL10 (*A*) and 2′,5′-OAS (*B*) were determined in the CNS of IFN-$\gamma^{-/-}$, IFN-$\alpha\beta R^{-/-}$, and WT mice infected i.c. with LCMV 3 days earlier. Brains from PBS-injected mice served as controls. Medians and ranges of four mice/group are presented. *, $p < 0.05$ relative to infected WT mice. n.s. = not significantly different from WT mice. Results are representative of several similar experiments.

type I IFNs are the dominating IFN types during the early phase of the host response.

During the acquired immune response, increased expression of CXCL10 are linked to T cell recruitment and production of IFN-γ

In Fig. 1*D*, an exceptionally marked increase in the level of expression of IFN-γ was found to take place between day 5 and 7 p.i., which could imply that IFN-γ produced by the initially recruited virus-specific T cells exerted a critical positive feedback on the expression of CXCL10 during the acquired immune response. To directly investigate this assumption, brains from T cell-deficient, nude mice, and IFN-$\gamma^{-/-}$ mice infected i.c. 7 days earlier were analyzed for CXCL10 mRNA. As can be seen in Fig. 5*A*, neither IFN-$\gamma^{-/-}$ nor nude mice showed any substantial expression of CXCL10 compared with WT, convincingly suggesting that IFN-γ secreted from T cells recruited to the virus-infected brain induce CXCL10 expression. With the exception of some residual expression of 2′,5′-OAS, probably reflecting the activity of type I IFNs (cf. Fig. 1), a similar expression pattern was observed with regard to this gene. This finding underscores that type I IFNs do not suffice for the sustained expression and the late up-regulation of CXCL10 observed in LCMV-infected mice (Fig. 5*B*).

Ag-stimulated CD8$^+$ T cells do not secrete CXCL10 in vitro

The ability of Ag stimulated, LCMV-specific effector CD8$^+$ T cells to produce CXCL10 was next evaluated. Splenocytes from LCMV-infected mice were negatively selected, removing CD4$^+$

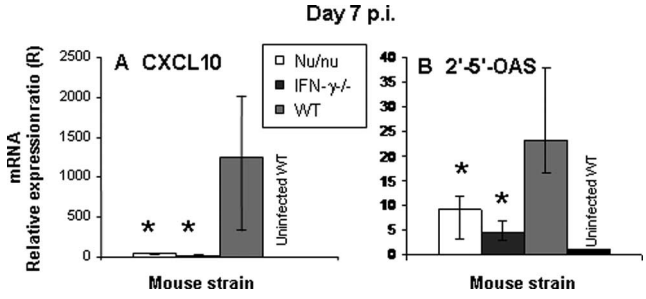

FIGURE 5. Late expression of CXCL10 requires IFN-γ and T cells. Levels of mRNA for CXCL10 (*A*) and 2′,5′-OAS (*B*) were determined in the CNS of nu/nu, IFN-$\gamma^{-/-}$ and WT mice infected i.c. with LCMV 7 days earlier. Brains from PBS-injected mice served as controls. Medians and ranges of 3–4 mice/group are presented. *, $p < 0.05$ relative to infected WT mice. Results are representative of two similar experiments.

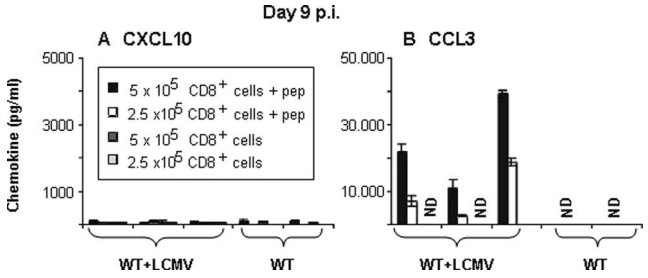

FIGURE 6. Ag-stimulated T cells do not themselves produce CXCL10 protein. CD4$^+$ and MHC II$^+$ cells were removed from splenocytes isolated from WT mice infected i.v. with LCMV 9 days earlier (WT plus LCMV) or uninfected controls (WT). The remaining cells were either stimulated with a dominant LCMV MHC I restricted epitope for 6 h (+pep) or left unstimulated. The supernatants were harvested and the presence of CXCL10 (*A*) and CCL3 (*B*) protein was assayed using a Multiplex Bead Immunoassay. Splenocytes from uninfected WT mice served as controls. Results from duplicate culture of cells from two to three mice/group are presented. N.D. = not detectable.

and MHC II$^+$ cells. The remaining cells were stimulated with a dominant MHC class I restricted LCMV epitope for 6 h, and the supernatants were next analyzed for the presence of CXCL10 protein and, as positive control, CCL3. Fig. 6 shows that activated CD8$^+$ T cells do not themselves secrete significant amounts of CXCL10 despite the fact that they are fully capable of secreting CCL3, confirming earlier results (32). Thus, our in vitro data indicate that CXCL10 is not produced by the T cells themselves.

Restored CXCL10 production in IFN-γ, but not IFN-γR-deficient, mice reconstituted with IFN-γ$^+$/IFN-γR$^+$ T cells

The results presented so far all support the possibility that the recruited T cells via secretion of IFN-γ induced local non-T cells to increase their expression of CXCL10. To investigate this in greater detail, IFN-$\gamma^{-/-}$, IFN-$\gamma R^{-/-}$, and WT mice were given a low number of splenocytes from IFN-$\gamma^{+/+}$, transgenic mice expressing a TCR with specificity toward a dominant LCMV MHC class I restricted epitope. Mice were infected i.c. the next day and, 5 days later, when inflammation is maximal in T cell transplanted WT mice, brains were removed and the level of CXCL10 expression in the CNS was determined. Although the level of CXCL10 expression in LCMV-infected IFN-$\gamma^{-/-}$ mice almost matched that in transplanted WT mice, little or no CXCL10 expression was detected in similarly treated IFN-$\gamma R^{-/-}$ mice (Fig. 7*A*). This observation confirms the assumption that production of type II IFN by the recruited LCMV-specific T cells is responsible for the increased expression of CXCL10 in the LCMV-infected CNS. However, it is equally evident that responding host cells are the major source of CXCL10 in the infected CNS. Matching results were found with regard to 2′,5′-OAS expression, underscoring the parallel regulation of this gene, which is known to reflect the activity of IFNs, and the gene for CXCL10 (Fig. 7*B*).

CXCL10 originates from resident cells of the CNS

To investigate whether CXCL10 mRNA derives from resident cells of the CNS or from hematopoietic, non-T cells recruited during the inflammatory response, syngeneic, and mixed bone marrow chimeras were generated using CXCL10$^{-/-}$ and WT mice. Eight weeks after transplantation, all the inflammatory cells recruited to the infected brain will be of donor origin (T cells and macrophages) whereas the radio resistant cells of the CNS are of recipient type. Chimeras were then infected with LCMV virus, and CXCL10 levels analyzed on day 3 p.i. when an acquired immune

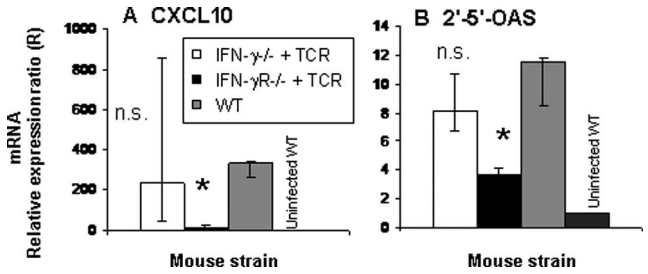

FIGURE 7. CXCL10 production is restored in IFN-γ, but not IFN-γR$^{-/-}$ mice reconstituted with T cells. Three × 10^6 TCR-transgenic splenocytes were transferred into IFN-γ, IFN-γR$^{-/-}$, and WT mice. The next day all mice were infected i.c. with LCMV, and on day 5 p.i. mRNA was isolated from the brains. The expression of mRNA for CXCL10 (*A*) and 2′,5′-OAS (*B*) was determined by Q-PCR. Brains from PBS-injected mice served as controls. Medians and ranges of four mice/group are presented. ∗, $p < 0.05$ relative to infected WT mice. n.s. = not significantly different from WT mice. Results are representative of two similar experiments.

response is not yet induced, and on day 7 p.i. when T cell-dependent inflammation is at its maximum. A similar pattern of CXCL10 expression was found on days 3 and 7 p.i. Thus, irrespective of the genotype of the bone marrow-derived cells, only recipients with radioresistant CNS cells of WT genotype showed substantial CXCL10 expression (Fig. 8), thus strongly indicating that expression of IFNs stimulates resident cells of the brain to produce and secrete CXCL10.

Astrocytes are the main producers of CXCL10 in the CNS proper

To define further the type(s) of cells in the brain that produces CXCL10 in response to LCMV infection, relevant brain sections from LCMV-infected WT mice were hybridized with probes detecting CXCL10 mRNA and Abs detecting CXCL10, the astroglial marker GFAP and the microglial marker CD11b.

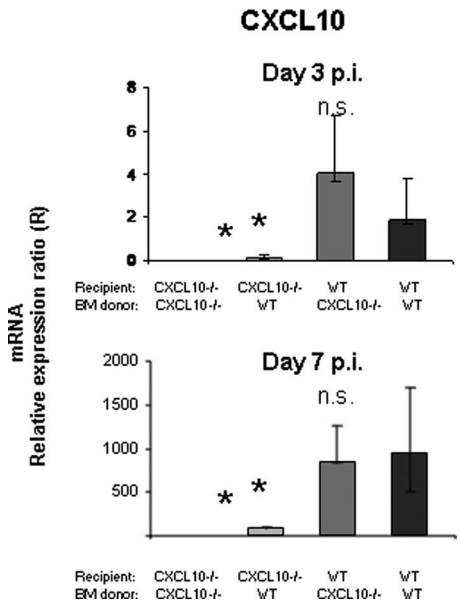

FIGURE 8. CXCL10 is predominant expressed in resident cells of the CNS. Syngeneic and allogeneic bone marrow chimeras were generated using CXCL10$^{-/-}$ and WT mice. Eight weeks after bone marrow reconstitution, chimeric mice were infected i.c. with LCMV virus. On days 3 and 7 p.i. brains were removed, mRNA extracted, and the level of mRNA for CXCL10 was determined by Q-PCR. Medians and ranges of 3–4 mice/group are represented. ∗, $p < 0.05$ relative to reconstituted WT mice. Similar results were obtained in mice infected 15 wk after reconstitution.

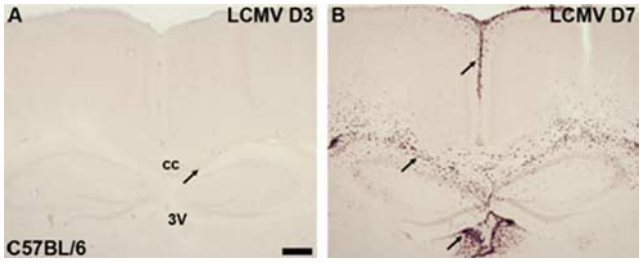

FIGURE 9. Localization of CXCL10 producing cells in the CNS of WT mice as a function of time. Brain sections from C57BL/6 mice infected with LCMV 3 (D3) or 7 (D7) days earlier were analyzed by in situ hybridization using CXCL10 probes. Scale bar (*A* and *B*), 100 μm. Results are representative of four mice/group.

As can be seen in Fig. 9*A*, very few CXCL10 mRNA expressing cells were present in the meningeal membranes and in the neural parenchyma as shown for the corpus callosum (CC) (arrow) on day 3 p.i. This is in contrast to the situation on day 7 p.i., at which time multiple intensely labeled cells were detected both in perimeningeal region (arrow), the periventricular zones (arrow), the choroid plexus and CC (arrow) (Fig. 9*B*). CXCL10 mRNA expressing cells were present at the site of injection both on day 3 and 7 p.i. (data not shown). As expected, in situ hybridization or immunohistochemistry of sections from WT mice and CXCL10$^{-/-}$ mice injected with PBS 3 and 7 days earlier yielded no signal of CXCL10 outside the injection-region (data not shown).

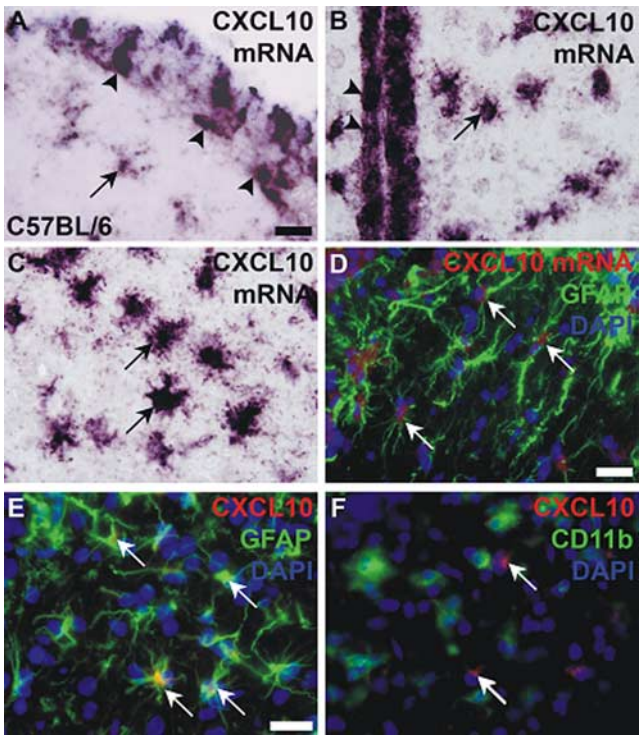

FIGURE 10. Astrocytes and meningeal and ependymal cells are the major CXCL10 producers in the neural parenchyma during LCMV infection of CNS. Brains from WT mice infected with LCMV 7 days earlier were analyzed by in situ hybridization and immunohistochemistry. *A–C*, In situ hybridization detecting CXCL10 mRNA. *D*, Double staining by in situ hybridization for CXCL10 mRNA and immunohistochemistry for GFAP protein. *E* and *F*, Double immunohistochemistry for CXCL10 protein and either GFAP protein (*E*) or CD11b protein (*F*). Scale bar (*A* and *B*), 20 μm; *C–F*, 50 μm. Results are representative of four mice/group.

Further analyses evaluating which cells of the CNS that express CXCL10 mRNA on day 7 p.i., showed that CXCL10 is expressed by the cells forming the meningeal membranes (arrow heads), ependymal cells (arrow heads, here shown around the 3 ventricle (3V)), and glial-like cells (arrows) as can be seen in Fig. 10, *A–C*, respectively.

To determine the identity of the involved glial-like cells, double staining with in situ hybridization for CXCL10 mRNA and immunohistochemistry for GFAP protein was performed. This approach revealed that CXCL10 mRNA (arrows) is expressed by GFAP-immunoreactive astrocytes (Fig. 10*D*). The LCMV-induced up-regulation of CXCL10 mRNA demonstrated by in situ hybridization was translated into increased protein expression by immunohistochemistry for CXCL10 protein. The immunohistochemical staining resulted in a weak, however specific, staining of glial-like cells (arrows) in the periventricular zones on day 7 after LCMV infection (Fig. 10, *E* and *F*). Double staining for CXCL10 protein and either GFAP or CD11b protein supported the suggestion that CXCL10 is expressed by astrocytes (arrows) (Fig. 10*E*) and not by microglial cells (Fig. 10*F*). Thus, CXCL10 is expressed by meningeal cells, ependymal cells, and by astrocytes in CC, the perimeningeal, and periventricular zones even at a distance from the injection site.

Discussion

CXCL10 appears to represent a key chemokine in relation to many viral infections. Thus, CXCL10 is one of the first proinflammatory cytokines to be produced at sites of viral infection (33, 34), and the expression of this chemokine appears to be a common denominator in the mammalian host response to most viral infections (7, 35). On these grounds there is ample reason to assume that CXCL10 comprises a pivotal component in the innate host defense toward a viral challenge. In support of this hypothesis, the induced absence of CXCL10 as observed in either gene-targeted or Ab-treated mice has been found to markedly affect the outcome of several viral infections (12, 13, 16). For this reason it seems pertinent to understand better the mechanisms regulating the expression of CXCL10 in sites of viral infection. In previous studies (15–17), we have clearly documented that CXCR3/CXCL10 interactions play a key role in the $CD8^+$ T cell dependent, inflammatory process underlying fatal LCMV-induced CNS disease, establishing this as a relevant model to study the regulation of CXCL10 expression during viral infection of the CNS. For this reason, we wanted to define the events leading to and regulating the expression of CXCL10 in the context of i.c. infection with LCMV.

Our results confirm that the expression of CXCL10 increases very substantially as a function of time after infection. However, this does not merely reflect a straight-forward relationship to the increased viral load in the CNS, as the latter is maximal already around day 5 p.i (36). Moreover, the use of gene-targeted mice reveals two distinct phases of regulation. During the first few days, expression of CXCL10 is independent of T cells and type II IFN. Although this is not too surprising, the finding that early expression of CXCL10 is almost totally abolished in type I IFN receptor-deficient mice is remarkable. Thus, triggering of both TLRs and other viral sensors such as the RNA helicases may directly induce the expression of CXCL10 (37), and it has previously been suggested that LCMV directly activate CXCL10 expression locally in the brain (35). Moreover, recent results obtained in studies on experimental autoimmune encephalomyelitis (EAE) strongly indicate that type I IFNs may actually serve as an anti-inflammatory signal down-regulating CXCL10 expression in the CNS (38, 39). However, based on the results in the present report it is evident that early CXCL10 expression is markedly reduced in the CNS of LCMV-infected mice in the absence of signaling through the type I IFN receptor. Whether this reflects a direct role for type I IFNs in inducing CXCL10 expression or type I IFNs are simply required to increase the sensitivity of the relevant sensor systems in the virus-infected CNS cells (34, 40, 41) is not clear at the moment. Indeed, these explanations are not mutually exclusive.

One may wonder why type I IFNs seemingly contribute to the inflammatory response in one situation (the LCMV infection), while acting as an anti-inflammatory modulator in another (EAE). Clearly the two situations are quite different. Thus, we evaluate the effect very early after a viral challenge, before the induction of an adaptive T cell response. Moreover, in our case the target cells for the action of type I IFNs are resident cells of the CNS. This is in contrast to the situation in the EAE model, where type I IFNs seemingly act on the recruited myeloid cells to down-modulate the local T cell-induced effector response (38). From this comparison, it is tempting to propose that the temporal and cellular context plays a critical role in determining the direction of the effects induced by type I IFNs in the CNS. Thus, acute expression of these cytokines as part of the early, innate response involving the resident cells including CXCL10 producing astrocytes may act to initiate the inflammatory response, while sustained expression during ongoing inflammation may reduce the production of inflammatory mediators (including CXCL10) by the recruited cells. Whether this suggestion is correct or not, with the appearance of a strong adaptive $CD8^+$ T cell response, type I IFNs no longer suffice as positive regulators of CXCL10 in the LCMV-infected CNS, and instead type II IFN becomes the key inducer of CXCL10 expression. Whether type I IFNs actually contribute to reduce inflammation at this stage, unfortunately cannot easily be addressed experimentally in this model, as the course of the LCMV infection is fundamentally altered in IFN-$\alpha\beta R^{-/-}$ mice.

Our results in vitro and in vivo clearly document that the T cells are not themselves important producers of CXCL10. This is in contrast to the local production of CCL3, which is predominantly produced by Ag-stimulated $CD8^+$ T cell as demonstrated both in vitro and in vivo (32). However, the local secretion of type II IFN from activated $CD8^+$ T cells seems to induce resident cells of the CNS to express high amounts of CXCL10. This conclusion is supported by our analysis of mixed bone marrow chimeras, which revealed that the genotype of radioresistant, non-bone marrow-derived cells within the CNS determines whether CXCL10 is expressed in the CNS or not. Moreover, detailed histochemical analysis discloses that the predominant producers of CXCL10 within the neural parenchyma express the phenotypic marker of astrocytes, but not of microglial cells. These observations confirm and extend an earlier study by Asenio et al. (42), which, however, failed to identify the pivotal role of type I IFNs in inducing early CXCL10 expression (35). Moreover, although an association between type II IFN and sustained expression of CXCL10 was noted in the former study (42), the precise cellular relationship has not previously been worked out.

As the expression of CXCL10 in certain areas of the neural parenchyma (Ref. 42 and present report) closely matches the regions in which $CD8^+$ T cells are prominent in lethally affected WT mice, but absent in partly resistant CXCL10- or CXCR3-deficient mice (15, 16), our results support the following hypothesis regarding the events leading to fatal LCMV-induced CNS diseases. In the early stages of i.c. LCMV infection local production of type I IFN is required for limited expression of CXCL10 in meningeal cells and a few astrocytes. This expression suffices for recruitment of some of the first few

virus-specific $CD8^+$ T cells released into the circulation following their clonal expansion and differentiation in the secondary lymphoid organs, particularly the spleen (21, 43). These initially recruited $CD8^+$ T cells then infiltrate the most easily accessible parts of the CNS and interact with local APCs to release large amounts of type II IFN, which in turn recruits even more astrocytes to produce CXCL10. As a result, more virus-specific $CD8^+$ T cells are recruited to the CNS including certain critical areas of the neural parenchyma, and eventually fatal cell damage is induced. It is still not absolutely clear at the molecular level how the $CD8^+$ T cells induce fatal disease in mice infected i.c. with LCMV, but perforin-dependent lysis certainly contributes to organ damage as perforin-deficient mice live significantly longer than matched WT mice (44). However, even perforin-deficient mice eventually die from what appears to be classical LCMV disease (45), indicating that other molecular mediators may suffice for the induction of fatal cell damage, albeit less efficiently than perforin.

In conclusion, although our results may not be extrapolated to all viral infection of the CNS, our findings underscore that the inflammatory response within the CNS is an intricately regulated process involving several cell types and steps of potential control and down-regulation. It is also becoming increasingly evident that the resident cells of the CNS are not merely passive bystanders during the local inflammatory process, but may actively participate in the host response to certain viral infections of the CNS (46, 47). Thus, in the case of i.c. LCMV infection, there can be no doubt that astrocytes are central in the marked up-regulation of CXCL10, which is important for the subsequent recruitment of the bulk of the infiltrating $CD8^+$ T cells to critical areas of the neural parenchyma (16). Whether astrocytes themselves can act as sentinels for LCMV infection of the CNS and produce type I IFNs, is the subject of ongoing studies. In vitro studies of astrocyte cultures suggest that this may be the case (48), but results obtained in vitro are not always to be trusted when it comes to extrapolating to whole organs with a complex cellular composition.

Disclosures

The authors have no financial conflict of interest.

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