Chemokine receptors CCR2 and CX3CR1 regulate viral encephalitis-induced hippocampal damage but not seizures

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Viral encephalitis is a major risk factor for the development of seizures, epilepsy, and hippocampal damage with associated cognitive impairment, markedly reducing quality of life in survivors. The mechanisms underlying seizures and hippocampal neurodegeneration developing during and after viral encephalitis are only incompletely understood, hampering the development of preventive treatments. Recent findings suggest that brain invasion of blood-borne monocytes may be critically involved in both seizures and brain damage in response to encephalitis, whereas the relative role of microglia, the brain’s resident immune cells, in these processes is not clear. CCR2 and CX3CR1 are two chemokine receptors that regulate the responses of myeloid cells, such as monocytes and microglia, during inflammation. We used Ccr2-ko and Cx3cr1-ko mice to understand the role of these receptors in viral encephalitis-associated seizures and neurodegeneration, using the Theiler’s virus model of encephalitis in C57BL/6 mice. Our results show that CCR2 as well as CX3CR1 plays a key role in the accumulation of myeloid cells in the CNS and activation of hippocampal myeloid cells upon infection. Furthermore, by using Cx3cr1-creERtdTomatoSow reporter mice, we show that, with regard to CD45 and CD11b expression, some microglia become indistinguishable from monocytes during CNS infection. Interestingly, the lack of CCR2 or CX3CR1 receptors was associated with almost complete prevention of hippocampal damage but did not prevent seizure development after viral CNS infection. These data are compatible with the hypothesis that CNS inflammatory mechanisms other than the infiltrating myeloid cells trigger the development of seizures during viral encephalitis.

Significance

Viral encephalitis is a frequent medical emergency, often resulting in acute seizures and brain damage, which reduce quality of life, promote the development of epilepsy, and can cause death. The relative roles of activation of microglia, the brain-resident innate immune cells, versus invasion of blood-borne immune cells such as monocytes in the acute and chronic consequences of viral encephalitis are only incompletely understood. Here we show that lack of the chemokine receptors CCR2 or CX3CR1, which regulate the responses of myeloid cells such as monocytes and microglia, prevents hippocampal damage but not seizures in a mouse model of viral encephalitis. Treatment strategies aimed at inhibiting peripheral immune cells from entering the brain during encephalitis could reduce brain damage.

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are only poorly understood (3). In a mouse model of viral encephalitis-induced seizures and hippocampal damage, using intracerebral inoculation of Theiler’s murine encephalomyelitis virus (TMEV)) in C57BL/6dJ (B6) WT mice, two groups independently reported that brain-infiltrating inflammatory monocytes damage the hippocampus (12, 13) and are key to the development of acute seizures (14). However, the experimental methods used to analyze and reduce monocyte invasion were not specific, so a role of other immune cells could not be excluded. Using a more selective approach for inhibiting monocyte invasion, i.e., administration of clodronate liposomes, we did not observe any prevention of hippocampal damage in this viral encephalitis model (15). Interestingly, in another mouse strain (SJL), in which infection with TMEV induces severe spinal cord demyelination, the use of Ccr2-KO mice reduced monocyte infiltration, demyelination, and long-term disease severity (16).

The objectives of the present study were threefold. The first aim was to better differentiate brain-resident myeloid cells, including microglia, from invading monocytes in the TMEV encephalitis model of TLE. For this, we compared virus-induced effects in B6 WT vs. B6-based Cx3cr1<sup>-</sup>Cre<sup>ER<sup>-</sup>/tdTomato<sup>S</sup>St/Wt and Ccr2<sup>-</sup>-KO, and Cx3cr1<sup>-</sup>-KO animals were mock infected or infected with TMEV intracranially. Animals were killed at 2 or 7 dpi. After enzymatic digestion, we perfused mice, prepared and homogenized the brain, isolated immune cells following discontinuous Percoll gradient centrifugation, and immunolabeled the specimens for analysis.

### Results

#### Lack of CCR2, but Not of CX3CR1, Leads to Reduced Accumulation of Myeloid Cells in the CNS After Intracerebral TMEV Infection.

Because CCR2 is implicated in the accumulation of infiltrating monocytes in the CNS after infection (7), whereas CX3CR1 has been proposed to be involved in microglia activation and neuron–microglia communication after CNS injury (5), we aimed to address the roles of CCR2 and CX3CR1 in viral encephalitis-induced hippocampal damage and seizure development. To this end we first investigated myeloid cell accumulation in the CNS after TMEV infection of B6 WT mice and Ccr2-KO or Cx3cr1-KO mice. For this, in brief, we perfused mice, processed and homogenized the brain, isolated immune cells following discontinuous Percoll gradient centrifugation, and immunolabeled the cells for analysis.

![Flow cytometry data of immune cells isolated from the brains at 7 dpi.](image)

**Fig. 1.** Decreased accumulation of myeloid cells in the brains of CCR2-deficient but not C3XCR1-deficient mice after intracranial TMEV infection. C57BL/6 WT, Ccr2-KO, and Cx3cr1-KO animals were mock infected or infected with TMEV intracranially. Animals were killed at 2 or 7 dpi. After enzymatic digestion, immune cells were isolated using Percoll gradient and were immunolabeled, and flow cytometry was performed. Myelin debris and dead cells were excluded by FSC/SSC gating, and singlet populations were analyzed. (A) Representative flow cytometry data of immune cells isolated from the brains at 7 dpi. (B) Quantification of CD45<sup>high</sup>CD11b<sup>high</sup> myeloid cells in the brain at 2 dpi (red-marked population in A). Shown are combined data of two independent experiments; n = 7. (C) Quantification of CD45<sup>high</sup>CD11b<sup>high</sup> myeloid cells in the brain at 7 dpi (red-marked population in A). Shown are combined data of two independent experiments; n = 6–14. (D) Quantification of CD45<sup>high</sup>CD11b<sup>high</sup> cells in the brain at 7 dpi (green-marked population in A). Shown are combined data of two independent experiments; n = 6–13. The data in B–D are shown as mean ± SEM (plus individual data). Analysis of data in C by two-way ANOVA indicated a significant effect of infection [F (1, 57) = 13.91; P = 0.0004], genotype [F (2, 57) = 3.98; P = 0.0241], and interaction [F (2, 57) = 3.601; P = 0.0337]. Similar, analysis of data in D indicated a significant effect of infection [F (1, 52) = 36.29; P < 0.0001], genotype [F (2, 52) = 7.034; P = 0.0019], and interaction [F (2, 52) = 7.034; P = 0.002]. Post hoc results in B–D are indicated by asterisks: **P < 0.01; ***P < 0.001); ns, not significant.
cells and analyzed them cytofluometrically by first excluding myelin debris and dead cells by forward scatter versus side scatter (FSC/SSC) gating followed by subsequent gating on singlet populations (Methods). In accordance with previous reports (12–15), infection of B6 mice with TMEV resulted in a significant increase in the number of CD45<sup>high</sup>CD11b<sup>high</sup> myeloid cells in the CNS at 2 and 7 d postinfection (dpi) as indicated by flow cytometry analysis of brains (Fig. 1 A–C). Since we observed particularly high accumulation of myeloid cells in the CNS at 7 dpi, we chose this time point for further analysis.

CCR2 is required for the egress of monocytes from the bone marrow to the blood as well as for migration of blood monocytes into the inflamed tissue (7, 19). TMEV infection of Ccr2<sup>−/−</sup> mice resulted in a significant accumulation of CD45<sup>high</sup>CD11b<sup>high</sup> myeloid cells at 7 dpi in the CNS (Fig. 1 A and C). However, this accumulation of CD45<sup>high</sup>CD11b<sup>high</sup> myeloid cells was significantly less than in B6 WT animals (Fig. 1 A and C). It has been shown previously that lack of CX3CR1 leads to apoptosis of Ly6<sup>−</sup>C<sup>−</sup> and Cx3cr1<sup>−/−</sup> monocytes in the blood (20). TMEV infection of Cx3cr1<sup>−/−</sup> mice also resulted in a significant accumulation of CD45<sup>high</sup>CD11b<sup>high</sup> myeloid cells at 7 dpi, which was comparable with the accumulation in B6 WT animals. Minute accumulation of immune cells was also observed in sham controls, most likely as a result of intracerebral injection of mock solution.

Both Ccr2<sup>−/−</sup> and Cx3cr1<sup>−/−</sup> animals showed significantly reduced accumulation of CD45<sup>+</sup>CD11b<sup>+</sup> cells compared with B6 WT animals, although the difference from infected B6 WT mice was low (Fig. 1 A and D). To exclude the possibility that the minor reduction of lymphocytes in the genetically manipulated hosts led to inefficient virus clearance, we determined TMEV antigen in the whole brain and hippocampus of infected mice at 7 dpi. In B6 WT mice, the virus is cleared within 2 wk after infection, predominantly by CD8<sup+</sup> cytotoxic T lymphocytes (3). As shown in SI Appendix, Fig. S3, there was no indication that genetically manipulated hosts cleared the virus less rapidly than infected WT mice; rather, the opposite was found.

Interestingly, unlike CD45<sup>high</sup>CD11b<sup>high</sup> myeloid cells, CD45<sup>low</sup>CD11b<sup>low</sup> cells (i.e., microglia) were not significantly increased in infected WT mice (SI Appendix, Fig. S1). However, TMEV-infected Ccr2<sup>−/−</sup> and Cx3cr1<sup>−/−</sup> mice had significantly fewer CD45<sup>low</sup>CD11b<sup>low</sup> cells than WT mice.

Since Ccr2<sup>−/−</sup> animals showed reduced recruitment of CD45<sup>high</sup>CD11b<sup>high</sup> myeloid cells in the CNS upon TMEV infection, we investigated the number of monocytes in the blood at 7 dpi. In the WT animals there was normal distribution of CSF-1R<sup>+</sup> monocytes in the blood (SI Appendix, Fig. S2). As reported previously (19, 20), we detected reduced numbers of circulating CSF-1R<sup>+</sup> monocytes in the blood of both Ccr2<sup>−/−</sup> and Cx3cr1<sup>−/−</sup> mice (SI Appendix, Fig. S2). However, CSF-1R<sup>+</sup> monocytes were entirely absent in Ccr2<sup>−/−</sup> animals, whereas in Cx3cr1<sup>−/−</sup> animals there was only a reduction in the number of such monocytes, which accounted for the accumulation of CD45<sup>high</sup>CD11b<sup>high</sup> myeloid cells in the brain. In conclusion, the lack of CSF-1R<sup>+</sup> monocytes in Ccr2<sup>−/−</sup> mice leads to reduced accumulation of myeloid cells in the CNS during TMEV infection, whereas in Cx3cr1<sup>−/−</sup> animals the accumulation of myeloid cells is comparable to that in B6 WT animals.

**CCR2 and CX3CR1 Regulate the Activation and Proliferation of Myeloid Cells in the Hippocampus After TMEV Infection.** During the development of TLE after brain insults, morphological and functional alterations of the hippocampus are thought to contribute critically to the epileptogenesis and cognitive impairment often associated with TLE (1). Hippocampal sclerosis with degeneration

![Image](https://example.com/image.png)

Fig. 2. Genetic deficiency of CCR2 but not CX3CR1 leads to decreased activation of myeloid cells in the hippocampus after intracranial TMEV infection. Animals were treated as described in Fig. 1. On day 7 after perfusion brains were removed, and immunohistology was performed. (A) Representative examples of Iba-1<sup>+</sup> and Mac-3-stained hippocampal sections. Scale bars: 50 μm. (B) Area of the hippocampus from which the sections shown in A were taken. (C) Quantification of hippocampal Iba-1<sup>+</sup> cells shown in A. (D) Semiquantitative data for Mac-3 staining shown in A. The data in C and D are shown as mean ± SEM (plus individual data). Analysis of data in C by two-way ANOVA indicated a significant effect of infection [F (1, 36) = 20.89; P < 0.0001] but not genotype [F (2, 36) = 0.4271; P = 0.6556] or interaction [F (2, 36) = 0.03501; P = 0.9656]. Analysis of data in D indicated a significant effect of infection [F (1, 41) = 38.21; P < 0.0001], genotype [F (2, 41) = 3.088; P ≤ 0.05], and interaction [F (2, 41) = 8.251; P = 0.0010]. Post hoc results are indicated by asterisks: *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.
of neurons in CA1, CA3, and the dentate hilus is the most common pathology of TLE, but the underlying mechanisms of the neuronal degeneration are only poorly understood (1). A similar type of hippocampal damage is also observed in the TMEV model of TLE in B6 mice (14, 15, 21). Since we observed a decreased accumulation of myeloid cells by flow cytometry analysis of whole brains from TMEV-infected Ccr2-KO animals, we next investigated the accumulation of myeloid cells in the hippocampus of infected mice by immunohistochemical analysis of the common myeloid cell marker Iba-1, which is expressed by resting and activated microglia as well as monocytes (22). Similar to the flow cytometry analysis, the histochemical analysis also revealed that TMEV-infected WT animals showed a significant accumulation of Iba-1+ myeloid cells in the hippocampus compared with control animals at 7 dpi (Fig. 2A and C). In addition to counting Iba-1+ cells in the hippocampus, we also examined the shape of Iba-1+ cells. In WT control animals the Iba-1+ cells showed the typical microglial morphology with a small cell body and many long and thin ramifications. Upon TMEV infection of WT animals most of the Iba-1+ cells showed an increased cell body volume with short thick processes, suggesting activation of microglia, whereas some cells had round or elongated shapes suggesting infiltration of monocytes from the periphery (Fig. 2A). Infected Ccr2-KO animals showed similarly increased numbers of Iba-1+ cells in the hippocampus as infected WT animals, suggesting increased accumulation of myeloid cells in the hippocampus in infected Ccr2-KO mice (Fig. 2A and C). The shape of the Iba-1+ cells suggested that activated microglia similar to those observed in infected WT controls were present in several mice (Fig. 2A). Similarly, Cx3cr1-KO animals showed a significant increase in the number of Iba-1+ cells in the hippocampus of TMEV-infected animals compared with noninfected Cx3cr1-KO animals (Fig. 2A and C), and the shape of the Iba-1+ cells suggested that activated microglia were present in several mice (Fig. 2A). Comparison of the accumulation of Iba-1+ myeloid cells in infected WT, Ccr2-KO, and Cx3cr1-KO mice showed that there was no significant difference in the accumulation of myeloid cells within the hippocampus among these three groups of animals upon TMEV infection (Fig. 2C). This, however, did not allow any conclusions about differences in the type of myeloid cells (resting vs. activated vs. proliferating microglia vs. monocytes) across genotypes.

Activated myeloid cells release many proinflammatory mediators which damage neurons and cause their death (21). We analyzed the activation of Iba-1+ myeloid cells by analyzing hippocampal expression of Mac-3, which, like Iba-1, is expressed by activated microglia as well as monocytes (23). This analysis showed that myeloid cells from noninfected WT control animals did not express Mac-3, suggesting that these cells were not activated, whereas TMEV infection of WT animals led to a high expression of Mac-3 in the hippocampus, indicating that the myeloid cells were activated after infection (Fig. 2A and D). In the Ccr2-KO or Cx3cr1-KO animals we observed a low expression of Mac-3 in the hippocampus of control animals, suggesting mild activation of myeloid cells. Mac-3 expression did not increase significantly upon TMEV infection in Ccr2-KO animals (P = 0.1934), but a significant increase was observed in Cx3cr1-KO animals (Fig. 2A and D). However, compared with infected B6 WT mice, the increase in Mac-3–labeled cells in infected Cx3cr1-KO mice was significantly less marked (Fig. 2D).

Response to a variety of CNS insults such as microbial invasion, resting microglia have the capacity to dramatically change their morphology into an activated phenotype (24). However, microglia activation can also be the consequence of the proliferation of microglia, as previously shown in B6 mice infected with TMEV (25). We therefore examined whether microglia proliferation is altered by Ccr2 KO or Cx3cr1 KO, using colabeling for Ki67 and Iba-1. As shown in Fig. 3A and C, in noninfected controls of either genotype almost no Ki67/Iba-1 double-labeled cells were observed, whereas a significant increase in Ki67/Iba-1 double-labeled cells was determined in the hippocampus of infected WT mice. Thirteen to sixty-seven percent (mean 36%) of the Iba-1+ cells were also labeled by Ki67 in infected WT mice (Fig. 3D). In contrast, microglia proliferation was suppressed in both Ccr2-KO and Cx3cr1-KO mice (Fig. 3A, C, and D).

Activated Myeloid Cells Present in the CNS After TMEV Infection

Activated Myeloid Cells Consist of Microglia and Infiltrating Monocytes. Based on flow cytometry analysis of CD45 and CD11b, previous studies have reported the accumulation of infiltrating monocytes in the CNS during TMEV infection (12–15). However, recent studies have shown that during neuroinflammation microglia up-regulate CD45 expression and become indistinguishable from monocytes (22, 26, 27), so the specific function of invading monocytes has been difficult to address (4). To differentiate infiltrating monocytes from CNS-resident myeloid cells such as microglia, we used Cx3cr1-creER<sup>+</sup>/tdTomato<sup>ER</sup> reporter mice carrying a tamoxifen-inducible cre<sup>B6</sup> and a cre-inducible tdTomato reporter. In these mice Cx3CR1<sup>+</sup> myeloid cells, such as microglia and circulating monocytes, are marked by expression of the tdTomato reporter upon tamoxifen injection. Due to repopulation of all recirculating tdTomato-expressing Cx3CR1<sup>+</sup> cells (such as monocytes) by tdTomato<sup>+</sup> cells within 8 wk following the tamoxifen treatment, this approach allows the discrimination of long-lived Cx3CR1<sup>+</sup> cells, such as microglia, from peripheral monocytes (17, 18).

In the first step, by flow cytometry analyses we ensured that the genetic marking of myeloid cells did not alter the accumulation of immune cells in the CNS after TMEV infection. Indeed, upon TMEV infection 8 wk after tamoxifen treatment, we observed myeloid cell accumulation in the brains of these mice that was comparable with that in WT animals (Fig. 4A and D; see Fig. 1 for WT). Next, we used tdTomato expression to distinguish microglia from infiltrating monocytes by flow cytometry. In the CD45<sup>high</sup>/CD11b<sup>high</sup> population most of the cells expressed the tdTomato reporter (Fig. 4B), showing high targeting of microglia as reported previously (17). Since we wanted to investigate whether some of the CD45<sup>low</sup>/CD11b<sup>low</sup> microglia up-regulated CD45 and became CD45<sup>high</sup> upon TMEV infection, we compared the number of tdTomato<sup>+</sup> cells in the CD45<sup>high</sup>/CD11b<sup>high</sup> populations in control and TMEV-infected animals. This analysis showed that there was a significant increase in the number of tdTomato<sup>+</sup> cells within the CD45<sup>high</sup>/CD11b<sup>high</sup> population of mice upon TMEV infection (Fig. 4B). In the Cx3cr1-cre<sup>B6</sup>/tdTomato<sup>ER</sup> mice, tamoxifen treatment also leads to the expression of the tdTomato reporter in CNS vessel-associated myeloid cells such as perivascular macrophages and meningeal macrophages, and these cells are also long-lived (18). These cells express high levels of CD45 as well as the mannose receptor CD206, whereas microglia typically do not express CD206 (28). Therefore, to differentiate between CD45<sup>high</sup> microglia and the vessel-associated macrophages present within the CD45<sup>high</sup>/CD11b<sup>high</sup>tdTomato<sup>+</sup> population from infected mice, we analyzed the expression of CD86 and CD206 on these cells. In noninfected control animals as well as in TMEV-infected animals we detected more than 90% cells within the CD45<sup>high</sup>/CD11b<sup>high</sup>tdTomato<sup>+</sup> population that did not express CD206 and very few cells expressing CD206 (Fig. 4C). Expression of CD86 on these cells confirmed that these cells were activated (Fig. 4C). This analysis showed that upon TMEV infection of the CNS most, if not all, of the CD45<sup>high</sup>/CD11b<sup>high</sup>tdTomato<sup>+</sup> cells were microglia and not CNS vessel-associated macrophages. We further confirmed infiltration of peripheral monocytes in the CNS by the analysis of the number of tdTomato<sup>+</sup> cells within the CD45<sup>high</sup>/CD11b<sup>high</sup> population, which significantly increased
upon infection (Fig. 4 B and F). In conclusion, upon TMEV infection a population of microglia up-regulates CD45 expression and by CD45 and CD11b expression becomes indistinguishable from infiltrating monocytes.

CCR2 and CX3CR1 Are Involved in Hippocampal Neurodegeneration After TMEV Encephalitis. As reported previously (21), TMEV-induced encephalitis in B6 WT mice was predominantly associated with damage in the hippocampus as a result of the virus tropism to this region in this mouse strain. Within the hippocampus, the CA1 and CA2 pyramidal cell layers were severely damaged, as indicated by the decrease in NeuN staining (Fig. 5 A and B). No such damage was observed in NeuN-immunostained sections from infected Ccr2-KO or Cx3cr1-KO mice (Fig. 5 A and B). For further analysis of neurodegeneration in the hippocampus, sections were stained with Fluoro-Jade C (FJC), a sensitive and specific fluorescent marker of dying neurons (29). In mock-infected controls, no FJC-stained neurons were detected, while intense staining of pyramidal cells was observed in the CA1 and CA2 layers of several TMEV-infected WT animals (Fig. 5 C and D). In Ccr2-KO mice, no FJC-labeled cells were observed, whereas a few FJC-labeled cells were detected in Cx3cr1-KO mice (Fig. 5 C and D). In conclusion, CCR2 and CX3CR1 play a key role in hippocampal neurodegeneration during TMEV encephalitis.

Neither Ccr2 nor Cx3cr1 Knockout Prevents Acute Seizures in TMEV-Infected Mice, but Lack of CCR2 Reduces Seizure Severity. Since hippocampal damage is frequently associated with seizure development in animal models and patients with acquired partial epilepsy (1), we hypothesized that decreased hippocampal neurodegeneration could lead to a decrease in TMEV encephalitis-induced seizure development. To address this question, we monitored seizure development in TMEV-infected WT, Ccr2-KO, and Cx3cr1-KO animals. The temporal (bell-shaped) profile of seizure incidence over the 7 d after infection did not differ among groups (Fig. 6A); in all three groups maximum seizure incidence occurred at 2 dpi. Cumulative seizure incidence was 67% in infected WT mice, 54% in infected Ccr2-KO mice, and 69% in infected Cx3cr1-KO mice, without significant intergroup differences (Fig. 6B). Similarly, seizures were observed in 82% of Cx3cr1-creERSt/WttdTomato+/- animals. Mock-infected WT and mock-infected Ccr2-KO, Cx3cr1-KO, or Cx3cr1-creERSt/WttdTomato+/- animals did not exhibit any seizures.

Raw Racine scores (30) recorded for each animal indicated that seizure severity was significantly lower in Ccr2-KO mice than in WT or Cx3cr1-KO mice, due to a change in the number of low (stage 3) Racine events vs. high (stage 4/5) Racine events in the Ccr2-KO mice (Fig. 6C). Similarly, when average seizure severity was calculated per mouse with seizures, seizure severity was significantly lower in Ccr2-KO mice (3.207 ± 0.0955) than in B6 WT mice (3.808 ± 0.1897; P = 0.0403) and Cx3cr1-KO mice (3.887 ± 0.1647; P = 0.0029).

The number of observed seizures (i.e., seizure frequency) did not differ among the groups (Fig. 6D). Furthermore, the cumulative seizure burden was similar in all groups (Fig. 6E). In conclusion, CCR2 and CX3CR1 do not regulate seizure development following TMEV encephalitis, but CCR2 seems to be involved in seizure severity.

Discussion

Recent studies indicated that brain infiltration with inflammatory monocytes plays a major role in the pathological consequences of viral encephalitis in B6 mice, a widely used model of TLE (21). While some neuronal loss during viral encephalitis may result from direct virus-mediated injury, much of the damage is associated with bystander pathology as a result of activation of the innate

Fig. 3. Genetic deficiency of CCR2 and CX3CR1 prevents microglia proliferation in the hippocampus after intracranial TMEV infection. Animals were treated as described in Fig. 1; on day 7 after perfusion, brains were removed, and immunohistochemistry was performed. (A) Representative examples of the wild-type (WT) and CX3CR1 (Cx3cr1) knockout (KO) mice. (B) Knockout prevents acute seizures in TMEV-infected mice. (C) Infection in the hippocampus, sections were stained with Fluoro-Jade C (FJC), a sensitive and specific fluorescent marker of dying neurons (29). In mock-infected controls, no FJC-stained neurons were observed, whereas a few FJC-labeled cells were detected in Cx3cr1-KO mice (Fig. 5 C and D). In conclusion, CCR2 and CX3CR1 play a key role in hippocampal neurodegeneration during TMEV encephalitis.

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Discussion

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immune system (12, 13, 31). The same is true for the insult-associated seizures observed in the first week after infection (32). Inflammatory monocytes can secrete proinflammatory cytokines such as IL-6, IL-1β, and TNF-α, which have been implicated in the development of acute seizures and hippocampal damage in the TMEV model of encephalitis (21). Furthermore, activation of the calcium-dependent cysteine protease calpain in hippocampal neurons seems to be involved in viral encephalitis-induced neurodegeneration (31, 33). However, neuroinflammation is generally a collaborative interaction between brain-resident cells such as microglia and astrocytes and infiltrating cells such as inflammatory monocytes, neutrophils, and other leukocytes. Therefore, it is highly unlikely that encephalitis-induced pathology is a result of monocyte invasion alone. Indeed, a marked activation of microglia has been determined in the hippocampus of TMEV-infected B6 mice (25, 34). Once the innate immune response is triggered, secretion of proinflammatory chemokines and cytokines contributes to the induction of the adaptive immune response by recruiting immune cells, such as T lymphocytes, to the site of infection, resulting in virus clearance (35). However, in contrast to innate immunity, the adaptive immune system seems not to be critically involved in hippocampal damage and early seizures in the TMEV model of TLE (21, 35). In the present study we examined the roles of CCR2 and CX3CR1, two important chemokine receptors expressed by myeloid cells such as microglia and monocytes, in TMEV CNS infection-induced seizure development, using the Daniel’s (DA) strain of TMEV. Further, we used Cx3cr1-creER<sup>T2</sup>"tdTomato<sup>Sl/wt</sup>" mice to differentiate between long-lived CNS-resident myeloid cells such as microglia and infiltrating peripheral monocytes during TMEV CNS infection. Several surprising findings were obtained that significantly change the current understanding of viral encephalitis-induced brain pathology.

In accordance with the previously published literature, our experiments in Cx3cr1-creER<sup>T2</sup>"tdTomato<sup>St/wt</sup>" mice substantiated the massive monocyte infiltration in the brain of TMEV-infected mice. In previous studies the accumulation of myeloid cells in the CNS and hippocampus was shown to be associated with hippocampal neurodegeneration and the development of seizures (12–15, 21). Lack of CCR2 but not CX3CR1 markedly reduced the accumulation of the myeloid cells in the brains of TMEV-infected mice, which could be explained by the lack of circulating monocytes in the blood of Ccr2-KO mice (19). One of the key problems associated with studies using WT mice is the inability to differentiate between different types of myeloid cells in the CNS, particularly under CNS inflammation. It is known that under CNS inflammation microglia can up-regulate the expression of CD45, which is commonly used to distinguish between microglia and other myeloid cells in the CNS during steady-state conditions (22). Previous studies used LysM-GFP reporter mice or bone marrow chimera to address the phenomenon of CD45 up-regulation of microglia during TMEV CNS infection (13, 14). However, because in these studies monocytes and neutrophils were marked by GFP expression, it was not possible to selectively identify microglia, which might have been present in the CD45<sup>high</sup> population. Thus, these studies could only conclude that there was infiltration of monocytes from periphery upon TMEV CNS infection (13, 14), so the possibility of microglia up-regulating CD45 expression has not yet been addressed in this
Using Cx3cr1-creER<sup>+</sup>/tdTomato<sup>St/Wt</sup> mice, we show here that during viral encephalitis a population of microglia upregulates CD45 and thus become indistinguishable from infiltrating monocytes. Therefore, the conclusions from previous studies (12–15, 21), which relied on CD45 and CD11b expression to differentiate microglia from infiltrating CD45<sup>high</sup> monocytes under conditions of viral encephalitis in the TMEV model, need to be reevaluated.

Since inflammatory response is the final effector mechanism of hippocampal neurodegeneration and seizures, we next focused on assessing the impact of CCR2 and CX3CR1 on CNS inflammation. As myeloid cells have been implicated in hippocampal damage and epilepsy (12–15, 21), we decided to assess the activation of myeloid cells present in the hippocampus of TMEV-infected animals. Determination of virus antigen demonstrated that neither Ccr2-KO nor Cx3cr1-KO retarded virus clearance in the brain. Staining of the myeloid cell-activation marker Mac-3 revealed that TMEV-infected WT mice showed high activation of myeloid cells in the hippocampus, whereas knock out of Ccr2 or Cx3cr1 resulted in decreased infection-dependent activation. One of the reasons for the decrease in Mac-3 expression could be the decreased accumulation of myeloid cells in the hippocampus. Although we observed decreased accumulation of myeloid cells in the flow cytometry analysis of whole brains of Ccr2-KO animals upon infection, Iba-1 immunostaining for myeloid cells showed that there was similar accumulation of Iba-1<sup>+</sup> myeloid cells in the hippocampus upon TMEV infection, independent of genotype of the animals, suggesting that the decrease in Mac-3 staining was indeed due to reduced activation of myeloid cells. Double-labeling of proliferating cells with Ki67 and Iba-1 substantiated increased proliferation of myeloid cells. Representative photomicrographs illustrating Iba-1 immunostaining for myeloid cells showed that there was similar accumulation of myeloid cells in the hippocampus of infected WT mice, as previously reported (25), but this was suppressed in both Ccr2-KO and Cx3cr1-KO mice. These data show that both CCR2 and CX3CR1 play a role in the activation and proliferation of myeloid cells in the CNS during viral encephalitis-induced neurodegeneration and epilepsy.

The reduced activation and proliferation of myeloid cells in the hippocampus of infected Ccr2-KO and Cx3cr1-KO mice motivated us to investigate their impact on hippocampal neurons, as hippocampal damage is associated with the development of seizures and epilepsy following different brain insults, including viral encephalitis (1), although neuronal degeneration is not always a prerequisite for generation of seizures (36). NeuN and FJC staining of the hippocampus showed that TMEV infection in WT animals can lead to heavy loss of neurons in the

**Fig. 5.** Genetic deficiency of CCR2 and CX3CR1 leads to decreased neuronal death in the hippocampus after TMEV infection. Animals were treated as described in Fig. 1. Brains were removed on day 7 after perfusion, and histology was performed on sections of the dorsal hippocampus. Section levels (−2.06 ± 0.26 mm from bregma) were similar in all groups. (A) Representative photomicrographs illustrating NeuN<sup>+</sup> neurons in mock-infected and TMEV-infected B6, Ccr2-KO, and Cx3cr1-KO mice. (Scale bars: 200 μm (overview, Top and Center Rows) or 50 μm (magnification, Bottom Row).) (B) Semi-quantitative data on NeuN<sup>+</sup> neurons in the CA1/CA2 regions of the hippocampus. Data are shown as boxplots with whiskers indicating minimum and maximum values; the horizontal line in the boxes represents the median value. In addition, individual data are shown (n = 5–13). Analysis of data by two-way ANOVA indicated a significant effect of infection [F (1, 41) = 5.33; P = 0.0261], genotype [F (2, 41) = 6.348; P = 0.0040], and interaction [F (2, 41) = 7.35; P = 0.0019]. Significant differences from post hoc testing of mock-infected mice are indicated by the hash sign: *P < 0.05; **P < 0.01. Significant differences between infected groups are indicated by asterisks: *P < 0.05; **P < 0.01. (C) The number of FJC<sup>+</sup> neurons in infected mice (n = 6–14). No FJC staining was observed in controls. Analysis of data by one-way ANOVA indicated a significant difference between groups [F (2, 28) = 5.174; P = 0.0122]. Significant differences between infected groups are indicated by asterisks: *P < 0.05; **P < 0.01. (D) Representative photomicrographs illustrating FJC staining in infected mice. (Scale bars: 200 μm (Top Row) or 50 μm (Bottom Row).)
CA1 and CA2 sectors, whereas neuronal loss was prevented in the Ccr2-KO and Cx3cr1-KO animals. These data show that during viral encephalitis-induced brain pathology hippocampal inflammation and neurodegeneration can be regulated by modulating CCR2 and CX3CR1. The observed suppression of microglia proliferation in infected Ccr2-KO and Cx3cr1-KO may be involved in the prevention of hippocampal neurodegeneration.

To assess if CCR2 and CX3CR1 also could be potential targets for therapy upon viral encephalitis-induced seizures and epilepsy, we investigated the impact of CCR2 and CX3CR1 on the development of seizures after TMEV infection. Surprisingly, we did not observe any significant reduction in seizure development upon Ccr2 or Cx3cr1 knock down except for a moderate decrease in seizure severity in the Ccr2-KO mice. Potential caveats to the interpretation of the present seizure data are the lack of continuous video-EEG monitoring and the restriction of seizure analyses to the day cycle, although the incidence of early seizures that we observed in B6-WT mice (67%) was quite similar to the 75% reported with continuous video-EEG monitoring (37).

Overall, the present data indicate that accumulation of myeloid cells in the CNS or hippocampus via infiltration of monocytes from periphery is not required for seizure development. Thus, it seems that, in contrast to several previous publications pointing toward monocytes being responsible for seizure development (14, 15, 21), our data with KO mice targeting monocytes suggest that monocytes alone are not responsible for the development of seizures during viral encephalitis. However, it must be mentioned that knockout of Ccr2 still led to a significant decrease in seizure intensity. This observation suggests that monocytes could be involved in exacerbating the pathology during viral encephalitis-induced seizures. Thus, our data indicate that targeting the inflammation during viral encephalitis-induced brain pathology is a better strategy than targeting only specific cells, such as infiltrating monocytes.

The role of microglia activation in seizure development in the TMEV model is less clear. Previous studies with minocycline seemed to suggest that microglial activation contributes to seizures in the TMEV model (38, 39). However, the tetracycline antibiotic minocycline is not a selective inhibitor of microglial activation but exerts effects on multiple cellular targets involved in neuroinflammation, such as suppression of monocyte/macrophage activation and decreased infiltration of monocytes, T cells, and neutrophils into the CNS (40). In the present experiments, the lack of the fractalkine receptor CX3CR1, which is critical in microglia activation (5), was not associated with any reduction in the incidence or frequency of early (encephalitis-associated) seizures in the TMEV model. However, this does not exclude...
a critical role for microglia in the development of epilepsy in this model.

Neuroinflammation with monocyte infiltration and microglial activation, as observed in rodent models of TLE, is also a frequently reported finding in patients with TLE (41–43). Thus, countering such inflammatory processes is considered a potential strategy for preventing or modifying epilepsy in patients at risk (2, 3, 44–47). For this strategy, it is imperative to better understand the complex crosstalk between microglia and other CNS-resident cells and infiltrating peripheral monocytes. Importantly, the delayed invasion of peripheral monocytes following brain insults provides a window of opportunity for treatment strategies aimed at preventing peripheral immune cells from entering the brain. CCR2 antagonists are efficacious after traumatic brain injury (47) and have entered clinical trials for the treatment of neuropathic pain (48). Fractalkine/CX3CR1 signaling is increased in microglia and modulates GABAergic currents in human epileptic brain (49). CX3CR1 is also involved during neuropathic pain (50), and the first CX3CR1 antagonists have recently been described (51, 52). One of these CX3CR1 antagonists has been reported to attenuate disease in a chronic-relapsing rat model for multiple sclerosis (52). The present data indicate that both CCR2 and CX3CR1 antagonists might be promising candidates for preventing the hippocampal damage after epileptogenic brain insults in response to viral encephalitis.

Materials and Methods

Animals and Tamoxifen Treatment. JAX C57BL/6J (B6) WT mice were purchased from Charles River Laboratories. According to a cooperative agreement between Charles River Laboratories and The Jackson Laboratory, the JAX C57BL/6J mouse strain bred by Charles River Laboratories in Europe is genetically equivalent to that bred by The Jackson Laboratory in the United States. Cx3cr1-KO mice (15, 52) and Cx3cr1-creER mice (53–55) were bred in-house. Cx3cr1-creER;tdTomato mice were bred at TWINCORE, Centre for Experimental and Clinical infection Research, Hannover, Germany. All three mouse lines were generated on a JAX C57BL/6J background. To induce creER activity, animals were treated s.c. after weaning with 4 mg of tamoxifen (Sigma) two times with a 48-h interval between treatments. To eliminate monocyte targeting, experiments were performed 6–8 wk after tamoxifen treatment. All mice were housed in groups under standardized conditions, with a 12-h/12-h day-night cycle, 50–60% humidity, 22–24 °C temperature, ad libitum tap water, and standard rodent diet (Altromin 1324 standard diet; Altromin International). The diet is produced by the vendor under strict quality control and regular analyses, including microbiology. Environmental enrichment was ensured by group houses and nesting material. Mice were randomly assigned to experimental groups (control vs. virus infection and enrichment was ensured by group houses and nesting material. Mice were recorded twice daily for 1 h in the morning (between 9 and 12 AM) and 1 h in the afternoon (between 3 and 4 PM), which was set 3–4 h after the recording periods by randomly assigning animals to the recording sessions. Care was taken to avoid any group differences in recording periods by randomly assigning animals to the recording sessions. Recording was performed by experienced researchers, and the choice of recording periods was based on previous experiments with video-EEG monitoring of acute seizures in this model (for more details see Bröer et al. [56]). All flow cytometric and histological data shown in this study are from animals infected with TMEV, not only from the animals infected with TMEV that presented with seizures.

Perfusion. On day 2 or 7 postinfection animals were deeply anesthetized with chloral hydrate i.p. and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were removed and left in 4% formaldehyde overnight before being cut and embedded in paraffin. Animals that were used for flow cytometric analysis were perfused with 4 °C PBS only. Brains were harvested and stored in 4 °C PBS until being further processed. EDTA-blood for flow cytometry was sampled before the start of perfusion analysis and was stored at 4 °C.

Histology and Immunohistochemistry. To assess inflammation and neurodegeneration, paraffin slices (2 μm) were stained with H&E or immunohistochemistry and analyzed as published recently (15, 25, 57). Histological analyses were focused on the dorsal hippocampus at [−2.06 ± 0.26 mm from bregma (mean ± SD; n = 49); section levels were determined by the stereotaxic mouse brain atlas of Paxinos and Franklin (58)], as this brain structure is mainly damaged due to a hippocampal tropism of TMEV and is also associated with the development of seizures and epilepsy. Section levels did not differ among groups. Neurodegeneration was assessed using NeuN immunostained slides (Merck Millipore) and was scored semiquantitatively [for details see Waltl et al. (15) and Polascheck et al. (59)]. Also, FJC staining was performed to visualize the presence of degenerating neurons (15, 60). Viral antigen labeling for TMEV in brain sections was performed via immunohistochemistry, using a polyclonal rabbit anti-TMEV capsid protein VP1-specific antibody (61).

For characterization of inflammatory processes, slides were stained using H&E and anti–Mac-3 (Bio-Rad) and were analyzed semiquantitatively (51). For quantification of myeloid cells, slides were stained using anti–iiba-1 (Abcam), and cells were counted manually. Cell proliferation was quantified by double staining slides using anti-iiba-1 (Wako) and anti-Ki67 (BD Pharmingen), and cells were counted manually in the ipsilateral hippocampus. All analyses were performed by two experienced researchers blinded to the experimental groups, and data of both researchers were averaged.

Immune Cell Isolation and Flow Cytometry. Immune cells were isolated from whole-brain homogenates as recently described (15). Briefly, brain tissue was processed using the neural tissue dissociation kit (Miltenyi Biotec) in accordance with the manufacturer's specifications. Afterward, cells were separated using a discontinuous Percoll density gradient (30–37–70% Percoll layers), which results in the enrichment of all immune cell types and removes a lot of myelin, which is auto-fluorescent. Following density gradient centrifugation, immune cells were washed and stained for 30 min at 4 °C using the following antibodies: CD11b APC-Cy7 (BD Biosciences), CD45.2 Pacific blue, CD86 PE-Cy5, and CD206 PE-Cy7 (BioLegend). Respective isotype control antibodies or unstained samples were used to determine positive populations. Myelin debris and dead cells were excluded by FSC/SSC gating, and singlet populations were analyzed.

For flow cytometric analyses of blood samples, EDTA-blood was stained at 4 °C for 15 min with the following antibodies: B220 APC-Cy7 (BD Biosciences), CD45.2 Pacific blue, CD45 PE-Cy5, and CD206 PE-Cy7 (BioLegend). Relative isotype control antibodies or unstained samples were used to determine positive populations. Myelin debris and dead cells were excluded by FSC/SSC gating, and singlet populations were analyzed.

Flow cytometry was performed using an LSR II flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Statistics. GraphPad Prism Version 7 was used for all statistical analyses. Depending on the distribution of the data, either parametric or non-parametric statistical tests were applied. For comparisons of two groups, either a Student's t test or a Mann–Whitney U test was used; for groups larger than two an ANOVA F test, followed by a post hoc Dunnett’s test or a Kruskal–Wallis test and a Dunn’s post hoc test were performed. For comparison of different genotypes and treatments (sham vs. infection) and their interactions, the data were analyzed with two-way ANOVA with appropriate post hoc tests. Seizure frequencies were compared using Fisher’s exact test. P ≤ 0.05 was considered significant.

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