Impact of deficiency in CCR2 and CX3CR1 receptors on monocytes trafficking in herpes simplex virus encephalitis

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The role played by resident microglia and by the infiltration of peripheral monocytes/macrophages in the innate immune response during herpes simplex virus type 1 (HSV-1) encephalitis was evaluated in mice deficient for the CCR2 and CX3CR1 receptors. CCR2−/−, CX3CR1−/− and C57BL/6 wild-type (WT) male mice were infected intranasally with 7×105 p.f.u. of an HSV-1 clinical strain and monitored for signs of encephalitis and survival. In addition, brain viral DNA load and cytokine levels were evaluated by RT-PCR and magnetic bead-based immunoassay, respectively. The cellular response was assessed by fluorescence-activated cell sorting of blood and brain leukocytes. Infected CX3CR1−/− mice had a significantly lower mean life expectancy than WT mice (P < 0.05, log-rank test) and demonstrated an increased infiltration of Ly-6Chigh ‘inflammatory’ macrophages in the brain (P < 0.05). Infected CCR2−/− mice had fewer monocytes (P < 0.05), with a lower proportion of Ly-6Chigh ‘inflammatory’ monocytes in the blood than the other groups (P < 0.05). Brain viral DNA loads were only slightly higher in knockout mice than in WT mice (P-value not significant). These data suggest that CCR2 and especially CX3CR1 receptors are necessary to initiate a proper immune response during HSV encephalitis. More precisely, CCR2 is crucial for the emigration of monocytes from the bone marrow to the blood, whereas CX3CR1 is mostly implicated in the regulation of infiltrating cells from the blood to the site of infection and in the control of the immune homeostasis of the brain.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a common human pathogen, most frequently associated with orolabial, genital and ocular infections. In addition, HSV-1 is the most frequent cause of sporadic and potentially fatal viral encephalitis in Western countries (Tyler, 2004; Whitley & Kimberlin, 2005). Despite the use of intravenous acyclovir aimed at blocking virus replication, the mortality rate associated with HSV encephalitis (HSE) is still high (20–30 %), with the majority of surviving individuals developing neurological sequelae. The neuronal damage and mortality attributable to HSV-1 could involve both virus and immune-related mechanisms.

It has previously been demonstrated that microglial cells are required to initiate a vigorous but yet non-protective immune response during HSV-1 infection in BALB/c mice (Marques et al., 2006). Indeed, during a non-productive infection with HSV-1, human microglia induce a robust in vitro expression of tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, RANTES and C-X-C motif ligand 10 (CXCL10) chemokine (Lokensgard et al., 2001). On the other hand, after HSV-1 intranasal infection of mice, an early predominant infiltration of macrophages and neutrophils occurs in the brain, followed by prolonged microglial activation and T-lymphocyte retention (Marques et al., 2008). Macrophages and cytokines play an important role in the early defence against HSV, but they need to be controlled to avoid excessive harm to the host (Ellermann-Eriksen, 2005). However, it is still under debate whether the cerebral immune response and the inflammatory process occurring during HSE result from the activation of resident microglia and/or from the infiltration of peripheral monocytes/macrophages into the central nervous system (CNS).

Recently, two subsets of peripheral blood monocytes have been described, namely the ‘inflammatory’ and ‘circulating’ monocytes (Auffray et al., 2009). These two cell types can be distinguished from each other on the basis of their surface receptors, as inflammatory and circulating monocytes are CD45high/CD115+/Ly-6Chigh/CCR2+/CX3CR1low and CD45high/CD115+/Ly-6Clow/CCR2−/CX3CR1high, respectively. Monocytes expressing CCR2 (i.e. inflammatory
monocytes) are able to emigrate from the bone marrow, infiltrate injured tissues (where they become macrophages) and produce high levels of TNF-α and IL-1, whereas monocytes expressing high levels of CX3CR1 (i.e. circulating monocytes) are thought to be involved in monitoring organic insult and replenishing tissue macrophages during homeostatic conditions (Auffray et al., 2007; Getts et al., 2008; Prinz & Priller, 2010; Serbina & Pamer, 2006). Therefore, these two monocyte subsets may play distinct roles in the immune response following HSE.

To further investigate the role of infiltrating inflammatory and circulating monocyte subsets as well as resident microglia, we used chimeric mice with bone marrow (BM)-derived cells expressing GFP and knockout mice for the CCR2 and CX3CR1 receptors. The kinetics of inflammatory and circulating monocytes/macrophages, neutrophils and resident microglia were analysed at different time points following intranasal infection with HSV-1 in the blood and brain of mice by fluorescence-activated cell sorting (FACS) using appropriate cell-surface markers.

RESULTS

Immunohistochemistry of brain tissues of chimeric mice

The infiltration of peripheral monocytes/macrophages into brain tissues following HSE was evaluated in chimeric mice with BM-derived cells expressing GFP. In all chimeric mice, infected or not, we observed a basal level of infiltrating GFP-positive cells in the brain at all time points (Fig. 1b), which probably resulted from the irradiation process. These cells had a ramified shape, suggesting that they were macrophages that were already differentiated into resident ‘sensing’ microglia (Davoust et al., 2008). Interestingly enough, a strong infiltration of GFP-positive cells (Fig. 1c), which co-localized with iba1 (ionized calcium-binding adaptor molecule-1, specifically expressed by macrophages/microglia and upregulated during their activation) (Fig. 1d), was observed at day 6 post-infection in infected chimeric mice, but not in uninfected mice. In contrast to the ramified GFP-expressing cells observed at basal level in all chimeric mice, these macrophages had a round, amoeboid shape and tended to surround the virus in brain tissues (Fig. 1e). The amoeboid shape of these macrophages suggests that they had recently infiltrated the brain, in contrast to the ramified cells, which were already differentiated into resident microglia. Moreover, some GFP-expressing cells did not co-localize with iba1, suggesting that other cell types may also infiltrate the brain due to alterations of the blood–brain barrier caused by the infection. No GFP-positive cells were detected in brain tissues of infected non-chimeric C57BL/6 mice (data not shown).

Survival rates and brain viral DNA loads

In order to evaluate the role played by the CCR2 and CX3CR1 signalling pathways on monocytes/macrophages trafficking during HSE, we first compared the survival rates of C57BL/6 WT mice with those of CCR2 and CX3CR1 knockout mice after intranasal infection with HSV-1. Because of the relative resistance of C57BL/6 mice to HSV, we used a high intranasal inoculum of 7×10⁵ p.f.u. Following the infection, mice started to demonstrate sickness signs from days 4–6 post-infection. CX3CR1⁻/⁻ mice exhibited more symptoms than the CCR2⁻/⁻ and WT groups and had a mean ±SD life expectancy of 8.67 ± 0.43 days compared with 9.33 ± 0.41 and 9.77 ± 0.22 days, respectively (censored at 10 days; P<0.05 between WT and CX3CR1-knockout mice). Survival rates were higher in the WT (92.3 %) than in the CCR2⁻/⁻ (75.0 %) and CX3CR1⁻/⁻ (50.0 %) groups (Fig. 2). However, there was only a modest, non-significant increase in the brain viral DNA loads of CX3CR1⁻/⁻ and CCR2⁻/⁻ mice compared with WT at day 5 post-infection (205, 172 and 164 copies ng⁻¹, respectively).

Cytokine levels in the brain

There was a significantly higher level of TNF-α in the brain of CX3CR1⁻/⁻ mice than in those of CCR2⁻/⁻ mice at day 5 post-infection (P<0.05) (Fig. 3). There was also a non-significant increase of IL-1β in the CX3CR1⁻/⁻ group, whereas CCL2 was non-significantly increased in the CCR2⁻/⁻ group of mice compared with WT.

Monocyte and neutrophil populations in the blood

The kinetics of different populations of blood monocytes and neutrophils in C57BL/6, CCR2⁻/⁻ and CX3CR1⁻/⁻ mice were analysed prior to and at different time points following intranasal infection with HSV-1 using flow cytometry (Fig. 4). Prior to the infection, we observed that the proportion of blood monocytes expressing CD11b and CD115 (a receptor for macrophage colony-stimulating factor) from all leukocytes (CD45+), which were significantly lower at days 2, 4 and 6 post-infection compared with those measured prior to the infection (P<0.05). Following the infection, CD45⁺/CD11b⁺/CD115⁺ monocytes increased until day 4 in CX3CR1⁻/⁻ mice, whereas the peak level was already reached on day 2 for WT mice (Fig. 4a). In CX3CR1⁻/⁻ mice, the amounts of CD45⁺/CD11b⁻/CD115⁺ monocytes were significantly higher at days 2, 4 and 6 post-infection compared with those measured prior to the infection (P<0.05). In contrast, no significant increase in the amounts of CD45⁺/CD11b⁻/CD115⁺ monocytes was detected in the blood of CCR2⁻/⁻ mice following the infection. Their mean amounts remained at 0.6–1.0 % at all time points and were significantly lower (P<0.05) than those of the CX3CR1⁻/⁻ and WT groups.

The CD45⁺/CD11b⁺/CD115⁺ monocytes were then differentiated according to their level of expression of Ly-6C into Ly-6C⁹⁰ high ‘inflammatory’ monocytes and Ly-6C⁶⁰ ‘circulating’ monocytes. Prior to the infection, the proportion of Ly-6C⁹⁰ inflammatory monocytes was
significantly lower in the blood of mice lacking CCR2 compared with the CX3CR1−/− and WT groups (P<0.05) and it remained unchanged after infection (Fig. 4b). The proportion of this monocyte subset increased up to day 4 post-infection in CX3CR1−/− mice, whereas it already reached its peak by day 2 in the WT group; the difference at day 4 between the two groups was just above the level of significance (P=0.06).

Similarly, a lower proportion of Ly-6Clow circulating monocytes was observed in CCR2−/− mice compared with the WT (P<0.05) prior to the infection. The proportion of this monocyte subset increased significantly until day 4 post-infection in all groups (P<0.05). However, this proportion remained significantly lower in the CCR2−/− group than in CX3CR1−/− and WT mice at all time points following the infection (Fig. 4c). These observations suggest that the CCR2, but not the CX3CR1, signalling pathway may have an impact on the egress of circulating monocytes from the bone marrow to the blood.

Finally, a slight increase of neutrophils was observed in CX3CR1−/− mice at days 2, 4 and 6 (P<0.05) compared with pre-infection levels (Fig. 4d). In contrast to monocytes, an important increase of neutrophils was observed in CCR2−/− mice although, due to high variation between mice, these levels were not significantly different from those in the two other groups.

**Resident microglia populations in the brain**

Brains from C57BL/6 and CCR2- and CX3CR1-deficient mice were processed and analysed by flow cytometry to evaluate the kinetics of resident microglial cells prior to...
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and at day 5 after HSV infection. We selected this time point because most mice started to demonstrate signs of encephalitis by that time, but were not succumbing to the infection. In our studies, the level of CD45 expression was first used to distinguish CD45low/CD11b+ and CD45high/Ly-6C- and low/Ly-6G− from CD45high/CD11b+ cells, which were attributed to microglia and infiltrating cells, respectively. Infiltrating cells (CD45high/CD11b+) were further separated into macrophage-like cells (Ly-6G−) and neutrophils (Ly-6C+/Ly-6G+). The level of expression of Ly-6C was also used to differentiate blood-derived macrophage subtypes into Ly-6Chigh and Ly-6Clow, which were attributed to inflammatory and circulating macrophages, respectively. Prior to the infection, microglial cells represented 85.5, 88.0 and 90.9% of all leukocytes (CD45+) in the brain of CCR2−/−, CX3CR1−/− and WT groups, respectively. On day 5 post-infection, these proportions decreased to 67.5, 43.1 and 83.8% (P<0.05 compared with pre-infection) in the same respective groups (Fig. 5a).

Infiltration of macrophages and neutrophils in the brain

The kinetics of infiltration of monocytes/macrophages and neutrophils were also analysed in brains of C57BL/6, CCR2−/− and CX3CR1−/− mice processed for flow cytometry analyses prior to and at day 5 after HSV infection. Prior to the infection, 5.3% of leukocytes found in the brain of WT mice expressed CD11b and high levels of CD45 (i.e. CD11b+/CD45high) and were attributed to peripheral myeloid cells. This cell population represented 8.5 and 5.7% in CCR2−/− and CX3CR1−/− mice, respectively. Following the infection, the infiltration of peripheral leukocytes increased by 2.4-fold (P<0.05), 6.8-fold (P<0.05) and 1.9-fold (P>0.05) in CCR2−/−, CX3CR1−/− and WT mice, respectively (Fig. 5b). In addition, peripheral myeloid cells were further differentiated according to the presence or absence of expression of Ly-6G and were attributed to neutrophils and macrophages, respectively. Macrophages represented 3.9, 2.9 and 3.4% of all leukocytes (CD45+) at baseline, followed by a 4.8-fold (P<0.05), 13.1-fold (P<0.01) and 2.7-fold (P<0.05) increase after infection in CCR2−/−, CX3CR1−/− and WT mice, respectively (Fig. 5c). The levels of macrophages after infection were significantly higher in CX3CR1−/− mice than in the CCR2−/− and WT groups (P<0.05) but were below the level of significance in CCR2−/− mice compared with the WT group (P=0.08). In contrast, the proportion of neutrophils decreased after infection from 4.5 to 1.3% in CCR2−/− (P=0.01), from 2.8 to 1.2% in CX3CR1−/− and from 1.9 to 0.7% in WT groups (Fig. 5d).

Finally, as done previously for blood-cell populations, macrophages were also differentiated according to their level of expression of Ly-6C into Ly-6Chigh inflammatory macrophages and Ly-6Clow circulating macrophages. Both subsets increased following infection in all groups (P<0.05) (Fig. 5e). However, the highest levels of both Ly-6Chigh (21.4%) and Ly-6Clow (7.6%) macrophages after infection were found in the brain of CX3CR1−/− mice [P<0.05 vs C57BL/6 WT (4.1 and 2.1%) and CCR2−/− (6.8 and 4.4%) mice].

**DISCUSSION**

In the present study, we have demonstrated that infiltration of peripheral monocytes/macrophages into the CNS plays a key role in the cerebral innate immune response during HSE. By using chimeric mice, we first showed that
macrophages infiltrate the brain and migrate to the infected area to surround the virus. However, the model of chimeric mice had some weaknesses (Davoust et al., 2008). First, GFP-expressing cells represented both newly ‘infiltrating’ macrophages as well as those already differentiated into ‘resting’ microglia, some of which could have been activated following the infection. Indeed, a basal level of GFP-positive cells with a ramified shape, which is characteristic of ‘resting’ microglia, was observed in the brain of both uninfected and infected chimeric mice and probably resulted from the irradiation process. Secondly, the use of chimeric mice could not allow the distinction between ‘inflammatory’ and ‘circulating’ macrophages, which may play distinct roles in the immune process. C57BL/6 WT mice are naturally resistant to intranasal infection with HSV-1 (Sergerie et al., 2007). In contrast, mice deficient for the CCR2 and CX3CR1 receptors (especially the latter), which are involved in monocytes/macrophages trafficking (Boring et al., 1997; Jung et al., 2000) from the bone marrow to the blood and from the blood to the infected tissue, respectively, were more vulnerable to this infection and had higher mortality rates. As there was no significant difference in brain viral DNA load between the three groups of mice, we believe that the higher mortality rate in CX3CR1−/− mice is mostly the result of an excessive inflammatory response due to an uncontrolled infiltration of monocytes. However, as virus titres have not been measured in those mice, we cannot rule out the possibility that the difference in survival rates was related to amounts of infectious virus present in the CNS tissue.

By using mice knocked out for the expression of TNF-α and IL-1β, we have already demonstrated that an inflammatory immune response is necessary for the control of virus replication (Sergerie et al., 2007). However, an exaggerated inflammation caused by a prolonged activation of microglia and by an overproduction of pro-inflammatory cytokines such as TNF-α (Lokensgard et al., 2001; Marques et al., 2008), which may itself induce blood–brain barrier breakdown (Candelario-Jalil et al., 2007), is thought to result in higher mortality rates in a mouse model of HSE. In addition, an uncontrolled infiltration of monocytes/macrophages from the blood to the CNS, which may result in higher levels of TNF-α (Marques et al., 2008), may also contribute to this excessive inflammatory response. In this respect, the enhanced infiltration of CD45high peripheral immune cells in the brain of CX3CR1-deficient mice compared with the CCR2−/− and WT groups following HSV infection was associated with increased levels of TNF-α and a higher mortality rate. As there was no significant difference in brain viral DNA load between the three groups of mice, we believe that the higher mortality rate in CX3CR1−/− mice is mostly the result of an excessive inflammatory response due to an uncontrolled infiltration of monocytes. However, as virus titres have not been measured in those mice, we cannot rule out the possibility that the difference in survival rates was related to amounts of infectious virus present in the CNS tissue.

The signalling pathways responsible for immune-cell trafficking linked to CCR2 and CX3CR1 play respectively a key role in the egress of monocytes/macrophages from the bone marrow to the blood and their infiltration from the blood to the site of infection. Previous studies have suggested that CCR2 is needed for the egress of Ly-6Chigh inflammatory monocytes from the bone marrow, as this monocyte subtype is reduced in the peripheral blood of mice lacking this receptor (Mildner et al., 2007; Serbina & Pamer, 2006; Tsou et al., 2007). Such reduction in monocytes, as seen in CCR2−/− mice, enhances susceptibility to pathogens such as Listeria monocytogenes (Jia et al., 2008; Serbina & Pamer, 2006), Cryptococcus neoformans

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**Fig. 3.** TNF-α (a), IL-1β (b) and CCL2 (c) levels in brain homogenates of CCR2−/− (filled bars), CX3CR1−/− (shaded bars) and C57BL/6 (empty bars) mice infected intranasally with HSV-1. Entire brains (four mice per group) were removed rapidly on day 5 after infection and homogenized. Cytokine levels were determined in brain homogenates by magnetic bead-based immunoassay. Bars show means±SEM. *Significantly different (P<0.05, Student’s t-test).
Fig. 4. Kinetics of different populations of monocytes and neutrophils in the blood of CCR2−/− (●), CX3CR1−/− (■) and C57BL/6 (▲) mice prior to and at varying time points following intranasal infection with HSV-1. (a) Percentage of CD11b+ /CD115+ monocytes compared with all leukocytes. (b) Percentage of CD11b+/CD115+ monocytes expressing Ly-6C high, which corresponds to ‘inflammatory’ monocytes, compared with all leukocytes (CD45+). (c) Percentage of CD11b+/CD115+ monocytes expressing Ly-6C low, which corresponds to ‘circulating’ monocytes, compared with all leukocytes (CD45+). (d) Percentage of neutrophils (CD45+/Ly-6C+/Ly-6G+) compared with all leukocytes. (e, f) Representative flow plots from one infected C57BL/6 WT mouse. Blood was taken from the submandibular vein of three to five mice per group at each time point and processed for flow-cytometry analyses. Bars show means ± SEM. *Significantly different from the proportion observed prior to the infection; †significantly lower or higher in CCR2−/− mice compared with CX3CR1−/− and C57BL/6 mice (P<0.05, Student’s t-test).
Fig. 5. Kinetics of different populations of resident microglial cells (CD45<sup>low</sup>), infiltrating macrophages and neutrophils (CD45<sup>high</sup>) in the brain of CCR2<sup>−/−</sup>, CX3CR1<sup>−/−</sup> and C57BL/6 mice prior to (filled bars) and at day 5 following (empty bars) intranasal infection with HSV-1. (a) Percentage of both CD45<sup>low</sup>/CD11b<sup>+</sup>/Ly-6C<sup>−</sup> and low/Ly-6G<sup>−</sup> resting and activated microglial cells compared with all leukocytes. (b) Percentage of infiltrating cells (CD45<sup>high</sup>/CD11b<sup>+</sup>) compared with all leukocytes (CD45<sup>+</sup>). (c) Percentage of CD45<sup>high</sup>/CD11b<sup>+</sup>/Ly-6C<sup>+/−</sup>/Ly-6G<sup>−</sup> macrophages compared with all leukocytes (CD45<sup>+</sup>). (d) Percentage of CD45<sup>high</sup>/CD11b<sup>+</sup>/Ly-6C<sup>+/−</sup>/Ly-6G<sup>−</sup> neutrophils compared with all leukocytes (CD45<sup>+</sup>). (e) Percentage of infiltrating monocytes differentiated according to their expression level of Ly-6C into Ly-6C<sup>high</sup> 'inflammatory' (CD45<sup>high</sup>/CD11b<sup>+</sup>/Ly-6C<sup>high</sup>/Ly-6G<sup>−</sup>) and Ly-6C<sup>low</sup> 'circulating' monocytes (CD45<sup>high</sup>/CD11b<sup>+</sup>/Ly-6C<sup>low</sup>/Ly-6G<sup>−</sup>) compared with all leukocytes (CD45<sup>+</sup>). (f, g) Representative flow plots from one infected CX3CR1<sup>−/−</sup> mouse, depicting an important infiltration of CD45<sup>high</sup> and Ly-6C<sup>high</sup> 'inflammatory' macrophages. Brains were taken from four mice per group prior to and at day 5 post-infection and processed for flow-cytometry analyses. Bars show means±SEM. *Significantly different (P<0.05, Student's t-test).

(Osterholzer et al., 2009), Mycobacterium tuberculosis (Peters et al., 2001; Scott & Flynn, 2002), West Nile virus (WNV) (Lim et al., 2011) and HSV-2 (Iijima et al., 2011), whereas it causes a delay in disease onset and decreased clinical severity during experimental autoimmune encephalomyelitis (Mildner et al., 2009). The role of the CCL2–CCR2 signalling pathway in the migration of Ly-6C<sup>high</sup> inflammatory monocytes into the CNS is not clearly defined, as it resulted in different outcomes in non-lethal and lethal mouse models of WNV encephalitis (Getts et al., 2008; Lim et al., 2011). In our experiments, the proportion of Ly-6C<sup>high</sup> inflammatory monocytes measured prior to and following the infection was lower in the blood of CCR2-deficient mice than in the CX3CR1<sup>−/−</sup> and WT groups. However, following the infection with HSV-1, some Ly-6C<sup>high</sup> inflammatory monocytes were recruited to the brain of CCR2<sup>−/−</sup> mice, suggesting that their infiltration into the CNS is not completely dependent on the CCR2–CCL2 signalling pathway. In this respect, studies performed with mouse models of WNV encephalitis have highlighted the role of the chemokine receptor CCR5, in addition to CCR2, in the migration of monocytes, T-cells and NK cells from the blood to the CNS (Glass et al., 2005). Besides CCR5, other chemokine receptors such as CXCR3 may also be involved in the infiltration of monocytes into the CNS. In a C57BL/6 mouse model of HSV-1 corneal infection, CXCR3 deficiency resulted in exacerbated corneal eye disease and periocular skin disease. In this model, the CXCR3 pathway contributed to regulation of the innate response that ensures virus elimination (Lundberg et al., 2007).

More interestingly, the deficiency in the CX3CR1–CX3CL1 signalling pathway was responsible for a higher recruitment of Ly-6C<sup>high</sup> inflammatory monocytes in the brain after infection with HSV-1 than in CCR2<sup>−/−</sup> and WT mice. These data suggest that the immune response induced by a higher infiltration of Ly-6C<sup>high</sup> inflammatory monocytes can produce more deleterious effects in the CNS, which resulted in increased mortality rates. The CX3CR1–CX3CL1 pathway needs to be investigated further, as leukocyte infiltration in CX3CR1<sup>−/−</sup> mice was not affected in a model of septic peritonitis (Ishida et al., 2008), but was associated with an increase number of macrophages in hepatic fibrosis (Karlmark et al., 2010).

The Ly-6C<sup>low</sup> circulating monocytes also seem to be involved in the innate immune response during HSE. Indeed, they infiltrated the CNS of both WT and knockout mice, especially the CX3CR1<sup>−/−</sup> ones, at higher rates following HSV infection, but to a lesser extent compared with Ly-6C<sup>high</sup> inflammatory monocytes. These cells patrol on the resting endothelium and are dependent on the integrin LFA-1 and on CX3CR1 for rapid tissue invasion at the site of infection, where they initiate an early immune response (Auffray et al., 2007). Because the CX3CR1 receptor is responsible for regulating the trafficking and recruitment of immune cells from the blood to the CNS, its absence may disturb the immune homeostasis in the brain (Huang et al., 2006). Therefore, mice deficient for this receptor could develop an exaggerated inflammatory response after infection with HSV-1, which may result from an increased infiltration of both Ly-6C<sup>low</sup> circulating and Ly-6C<sup>high</sup> inflammatory monocytes in the brain and may contribute to aggravating the disease.

We also found a slightly higher percentage of neutrophils in the blood of CCR2<sup>−/−</sup> mice before and after the infection. However, the increase of neutrophils in the blood at days 4 and 6 after infection was not reflected in the brain at day 5. Thus, in contrast to monocytes/macrophages, neutrophils seem to be less involved in the innate immune response in our model of HSE at day 5, but their effect may be more important at an earlier time post-infection.

Many studies on the role of CX3CR1 and its ligand have also demonstrated additional effects for this signalling pathway. CX3CR1 deficiency has been shown to disturb microglial responses, resulting in neurotoxicity (Cardona et al., 2006; Harrison et al., 1998). In a model of Parkinson’s disease, CX3CR1-deficient mice showed enhanced neurodegeneration and inflammation compared with WT littermates (Cardona et al., 2006). In addition, increased microglial activation was also noted in CX3CR1-deficient mice challenged with intraperitoneal injections of lipopolysaccharide (Corona et al., 2010). The communication between neurons and microglial cells is altered with the deletion of CX3CR1 and results in severe sickness in various models of neurodegenerative diseases (Cardona et al., 2006; Huang et al., 2006). In contrast, studies with
mouse models of Alzheimer’s disease have demonstrated that signalling through CX3CR1 alters the activation of microglia and decreases their phagocytic capability, resulting in less severe disease (Lee et al., 2010). Thus, microglial activation differs depending on the nature and the chronicity of the challenging stimuli. Therefore, we may also suggest that, in our model of HSE, the CX3CR1 signalling pathway plays a key role in the control of HSV-1 infection by regulating the inflammatory state of the brain.

To our knowledge, this is the first report describing the impact of CCR2 and CX3CR1 deficiency in a mouse model of HSE. One study has evaluated monocyte infiltration following intravaginal challenge with HSV-2 in CCR2−/− mice (Iijima et al., 2011). This study, like ours, reported that HSV infection was associated with increased mortality rates in CCR2-deficient mice compared with WT mice. However, after intravaginal challenge, the number of monocytes was reduced at the site of infection whereas, in our model of HSE, we observed a slight increase of monocytes in the blood and some infiltration of Ly-6Chi inflammatory and Ly-6Clo circulating macrophages in the brain of CCR2−/− mice, as seen after L. monocytogenes infection (Serbina & Pamer, 2006). This discrepancy could be due to the different sites of infection and/or to the virus inoculum, as well as to the use of Depo-Provera to increase susceptibility to infection in the intravaginal model.

It would have been also interesting to look at the kinetics of NK cells and lymphocytes, as these cells could express one or both receptors (CCR2 and CX3CR1) (Campbell et al., 2001). However, the contribution of NK cells in HSE is still controversial (Kastrukoff et al., 2010), while lymphocytes are mostly involved after the first week post-infection (Marques et al., 2008). Thus, we have focused our analysis on monocytes/macrophages and neutrophils because these cells are highly important in the early immune response during HSE and clearly affect mortality (Marques et al., 2008). Also, we did not analyse the infiltration of monocytes/macrophages and neutrophils in the brain at different time points after infection, which is the main limitation of our study.

In conclusion, our data derived from chimeric mice demonstrate that the infiltration of peripheral monocytes/macrophages into the CNS plays a key role in the innate immune response and inflammatory process occurring during HSE in mice. Our results with knockout mice also highlight the role of CCR2 and especially CX3CR1 signalling pathways in the innate immune response to HSV replication in the brain. More specifically, the CCR2–CCL2 pathway is important for the emigration of monocytes from the bone marrow to the blood, whereas the CX3CR1–CX3CL1 pathway controls the infiltration of macrophages in the brain. Moreover, the increased mortality observed in CX3CR1-deficient mice reinforces the notion that immune-related mechanisms aggravate the pathogenesis of HSE in addition to virus replication in the brain.

**METHODS**

**Animals and experimental procedures.** C57BL/6 WT male mice were purchased from Charles River Canada. Hemizygous transgenic male mice expressing GFP under control of the chicken β-actin promoter and the cytomegalovirus enhancer [B6-Tg (CAG-EGFP) 10bs/J], CCR2−/− (B6.129S4-Ccr2tm1Ifc/J) and CX3CR1−/− (B6.129P-Cx3cr1tm1Litt/J) male mice, which are all maintained in a C57BL/6 background, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). CCR2−/− mice were generated by inserting a neomycin-resistance gene to disrupt the entire CCR2 coding region except for the first 39 nt and the 5’ UTR (Boring et al., 1997). CX3CR1−/− mice were generated by inserting the GFP gene into the Cx3cr1 gene and all peripheral blood monocytes express GFP (Jung et al., 2000). Mice were housed three to five per cage and acclimated to standard laboratory conditions.

In the first experiment, we generated chimeric mice by irradiation of C57BL/6 mice followed by transplantation of BM cells expressing GFP. Briefly, C57BL/6 WT mice (n = 30) were exposed to 10 Gy total-body irradiation using a 60Co source (Theratron-780 model; MDS Nordion). A few hours after irradiation, animals were injected via the tail vein with approximately 15 × 106 BM cells freshly collected from hemizygous transgenic mice expressing GFP, as described previously (Simard & Rivest, 2004). Irradiated mice transplanted with this suspension were housed in autoclaved cages and treated with antibiotics [0.2 mg trimethoprim and 1 mg sulfamethoxazole (ml drinking water)−1 ad libitum, started 1 week before and maintained for 2 weeks after irradiation]. Seven weeks after transplantation, chimeric mice were infected intranasally with 7 × 106 p.f.u. of HSV-1 clinical strain H25 (grown and passaged five times in Vero cells) in 20 μl minimal essential medium as described elsewhere (Sergerie et al., 2007). Infected C57BL/6 WT mice (n = 7), as well as infected (n = 20) and uninfected (n = 10) chimeric mice, were deeply anaesthetized with ketamine and rapidly perfused transcardially on days 1, 3, 6, 10 and 20 following the infection. The brain and spinal cord were removed, post-fixed and cut as described previously (Boivin et al., 2002).

In the second experiment, C57BL/6 WT (n = 17), CCR2−/− (n = 16) and CX3CR1−/− (n = 16) mice were infected intranasally at the age of 8–10 weeks with HSV-1 (7 × 106 p.f.u. H25) (Sergerie et al., 2007); HSE-related symptoms (namely ruffled fur, ocular swelling and shacking movements), as well as weight loss and mortality, were monitored for 25 days. Animals were sacrificed when a ≥ 20% weight loss or a combination of two obvious sickness signs were observed. On day 5 following the infection, four mice per group were sacrificed by intracardiac perfusion with saline for both viral DNA load and protein quantification in brain homogenates.

In the third experiment, C57BL/6 WT (n = 18), CCR2−/− (n = 17) and CX3CR1−/− (n = 18) mice were infected intranasally with HSV-1 as described above to perform flow-cytometry analysis of blood and brain leukocytes. Blood was collected from the submandibular vein of three to five mice per group before and on days 2, 4, 6 and 10 following infection. Brains of four mice per group were collected before and on day 5 following the infection.

All experimental procedures were approved by the Animal Care Ethics Committee of Laval University.

**Immunohistochemistry on brain tissue of chimeric mice.** Brain sections (25 μm thick) were washed three times for 15 min each in KPBS (potassium PBS), and incubated for 20 min in KPBS containing 0.4% Triton X-100, 4% chicken serum and 1% BSA to block non-specific sites. The sections were then incubated for 2 h with the primary antibody [polyclonal rabbit anti-HSV-1/2, 1:1000 or polyclonal goat anti-GFP, 1:1000 (both from Abd Serotec) or polyclonal rabbit anti-iba1, 1:1500 (Wako Chemicals)] at room
temperature. After three consecutive washes of 15 min with KPBS, sections were incubated in the dark for 2 h with the fluorochrome-conjugated chicken secondary antibody [anti-rabbit Alexa 594 or anti-goat Alexa 488 (Invitrogen)]. Sections were then rinsed three times for 15 min each in KPBS, mounted onto SuperFrost slides (Fisher Scientific) and coverslips were applied with Fluoromount-G (Electron Microscopy Sciences). All images were captured using a Nikon Eclipse 80i microscope equipped with a digital camera (QImaging).

**Viral DNA load and cytokine measurements.** For viral DNA load quantification, mouse brains were homogenized in PBS with a homogenizer (BioSpec Products, Inc.). DNA was extracted with a Magnapure LC total nucleic acid isolation kit (Roche Molecular Systems) and eluted in 100 μl elution buffer. Real-time PCR was performed using 5 μl extracted DNA and an internal control as described previously (Boivin et al., 2006).

For cytokine quantification, aliquots of 500 μl brain homogenate were mixed with protease inhibitor cocktail (Sigma-Aldrich) and 200 μl KPO4 containing 0.4 % CHAPS [3-[3-cholamidopropyl]-dimethylammonio]-1-1-propanesulfonate]. Samples were centrifuged at 13,800 g for 10 min at 4 °C and the supernatants were stored at −20 °C until use. Quantification of CCL2, TNF-α and IL-1β was performed by magnetic bead-based immunoassay using a Milliplex mouse cytokine 3-plex kit (Millipore). Results were analysed with the Bio-Plex system equipped with Bio-Plex Manager 5.0 software (Bio-Rad).

**Flow-cytometry analysis of blood leukocytes.** Blood aliquots (100 μl) were collected into EDTA-containing tubes. An equal volume of PBS containing 4 % FBS was added and the mixture was incubated for 30 min at 4 °C. After centrifugation and resuspension in 100 μl PBS containing 2 % FBS, 4 μl CD16/CD32 antibody (BD Biosciences) was added to block non-specific binding of IgGs to Fc receptors. Cell suspensions were then labelled with several immune cell-surface markers [anti-CD45-FITC, anti-Ly-6C–V450, anti-Ly-6G–PE–Cy5, anti-CD11b–PE–Cy7, anti-Ly-6C–V450 and anti-Ly-6G–PE] for 40 min at 4 °C. After centrifugation and resuspension for 10 min at 4 °C, red blood cells were lysed by the addition of 40 μl Immuno-lyse from a Whole Blood Lysing Reagent kit (Beckman-Coulter). The 30–70 % Percoll interface. Cells were harvested in 15 ml Falcon tubes filled with 5 ml DPBS (Sigma) containing 2 % FBS and the 30–70 % Percoll interface. Cells were harvested in 15 ml Falcon tubes filled with 5 ml DPBS (Sigma) containing 2 % FBS and centrifuged for 10 min at 300 g. Cell preparations were treated with 4 μl anti-CD32/CD16 to inhibit non-specific antibody binding and then labelled with anti-mouse immune cell-surface markers (anti-CD45–PE–Cy5, anti-CD11b–PE–Cy7, anti-Ly-6C–V450 and anti-Ly-6G–PE) for 40 min at 4 °C. Final processing of the samples and analyses were performed as described above for blood leukocytes.

**Statistical analysis.** Life expectancies between groups of mice were estimated by the Kaplan–Meier method and were compared using a log-rank test. Brain viral DNA loads, cytokine levels and cell populations were analysed using Student’s t-test for independent samples. Statistical analyses were carried out with the spss software 11.0 (SPSS Inc.).

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