Monocytes Regulate T Cell Migration Through the Glia Limitans During Acute Viral Encephalitis

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Leukocyte access into the central nervous system (CNS) parenchyma is tightly regulated by the blood brain barrier (BBB). Leukocyte migration through the endothelial cell wall into the perivascular space is well characterized; however, mechanisms regulating their penetration through the glia limitans into the parenchyma are less well studied and the role of monocytes relative to neutrophils is poorly defined. Acute viral encephalitis was thus induced in CCL2 deficient (CCL2\(^{-/-}\)) mice to specifically abrogate monocyte recruitment. Impaired monocyte recruitment prolonged T cell retention in the perivascular space although no difference in overall CNS accumulation of CD4 or CD8 T cells was detected by flow cytometry. Delayed penetration to the CNS parenchyma was not associated with reduced or altered expression of either matrix metalloproteinases (MMP) or the T cell chemoattractants CXCL10 and CCL5. Nevertheless, decreased parenchymal leukocyte infiltration delayed T cell mediated control of virus replication as well as clinical disease. These data are the first to demonstrate that the rapid monocyte recruitment into the CNS during viral encephalitis is dispensable for T cell migration across the blood vessel endothelium. However, monocytes facilitate penetration through the glia limitans. Thus the rapid monocyte response to viral encephalitis constitutes an indirect antiviral pathway by aiding access of effector T cells to the site of viral infection.
Introduction

The blood brain barrier (BBB) is a key feature contributing to the immune specialized environment of the central nervous system (CNS); others are the paucity of dendritic cells, low major histocompatibility complex (MHC) expression, and relative lack of lymphatic drainage (10, 15). The complex composition of the BBB tightly regulates CNS leukocyte entry under physiological conditions (4). However, disruption of the BBB induced by infection, trauma or autoimmunity is critical in initiating parenchymal inflammation. While leukocyte entry into the CNS parenchyma is beneficial in controlling microbial infections, dysregulated recruitment is associated with chronic neuroinflammatory diseases such as HIV-associated neurological disorders and multiple sclerosis (MS) (13, 51). Several distinct physical barriers must be breached during leukocyte migration into the parenchyma. At post-capillary venules of the BBB, where leukocyte extravasate from blood into the CNS (4, 10, 34), cell migration is regulated at two stages (34). First, activated leukocytes enter the perivascular space by migrating across the vessel wall composed of endothelial cells connected by tight junctions and associated with a basement membrane (29). This process involves tethering/rolling, activation, adhesion and diapedesis and is regulated by adhesion molecules, chemokines and chemokine receptors (10, 29). Once in the perivascular space, inflammatory cells must further penetrate the glia limitans to enter the CNS parenchyma. This barrier is composed of astrocyte foot processes associated with a distinct basement membrane (24, 41). In contrast to the well-defined mechanisms regulating migration across the endothelial cell layer, factors governing migration through the glia limitans are less well described. As leukocyte access to the CNS parenchyma is associated with clinical symptoms during inflammatory disorders (46, 48), but is also necessary for antimicrobial control, understanding the components regulating parenchymal leukocyte entry may lead to more refined therapeutic strategies controlling this process.

A role for monocytes in facilitating transmigration across the glia limitans was noted by prevention of clinical disease in the absence of monocytes in the experimental autoimmune encephalitis
(EAE) model of MS due to leukocyte accumulation in the perivascular space (48). By contrast, monocyte depletion does not alter parenchymal T cell infiltration after trauma-induced brain inflammation (14). These opposing data suggest monocyte-dependent migration into the CNS parenchyma depends upon the nature of the CNS insult. Monocytes are a component of viral encephalitis in humans and animal models, including HIV, simian immunodeficiency virus (9, 11, 27) and West Nile virus encephalitis (17). In vitro data further suggest that the chemokine CCL2 (MCP-1), essential for monocyte recruitment (26), enhances the ability of peripheral lymphocytes from HIV infected patients to cross the BBB (9, 11, 27). However, a specific role of monocytes in glia limitans disruption during viral encephalitis has not been addressed.

A well-characterized model of viral encephalitis was chosen to better define the role of monocytes in facilitating lymphocyte access to the CNS parenchyma. Mice infected with the non fatal neurotropic JHM strain of mouse hepatitis virus (JHMV) develop an acute encephalitis associated with immune mediated primary demyelination (6). Neutrophils and monocytes are the first cells to infiltrate the CNS (6), consistent with early upregulation of CCL2 and the neutrophil chemoattractants CXCL1 (KC) and CXCL2 (MIP-2α) (25). Depletion of neutrophils and inflammatory monocytes during acute JHMV infection decreased BBB permeability and CNS leukocyte infiltration (54). However, the relative contribution of neutrophils and monocytes in CNS access remains unresolved. A specific role for monocytes in BBB disruption was analyzed following JHMV infection of CCL2 deficient (CCL2−/−) mice. The absence of CCL2 specifically disrupts recruitment of blood-derived monocytes into parenchymal tissues (20, 26), while neutrophil recruitment remains intact. Importantly, as circulating monocytes and lymphoid tissue macrophages are not affected, priming of antiviral T cell responses in cervical lymph nodes is not expected to be impaired. Altered T cell access to the CNS parenchyma can thus be directly attributed to monocyte-dependent functions.
Impaired parenchymal T cell access in infected CCL2−/− mice correlated with enhanced confinement within the perivascular space and a delay in both onset of clinical symptoms and control of virus replication. Furthermore, monocyte mediated disruption of the glia limitans could not be attributed to enhanced or altered activity of matrix metalloproteinases (MMPs). These data are the first to demonstrate a critical contribution of monocytes in aiding lymphocyte access across the glia limitans into the CNS parenchyma during acute viral encephalitis. Furthermore, while monocyte-facilitated migration of activated lymphocytes through the glia limitans is beneficial in early viral intervention this process contributes to clinical disease. Overall the results support a common role of monocytes in disrupting the BBB via alterations in the glia limitans independent of the distinct stimuli initiating and propagating leukocyte CNS recruitment during autoimmune mediated and virus induced inflammation.
Materials and Methods

**Mice.** C57BL/6 mice were obtained from National Cancer Institute (Frederick, MD, USA). Homozygous CCL2 deficient (CCL2<sup>−/−</sup>) mice were originally obtained from Dr. B.J. Rollins (Dana-Farber Cancer Institute, Boston MA) (26) and were backcrossed for eight generations to C57BL/6 mice (22). All mice were used at 6 to 7 weeks of age. All procedures were performed in compliance with the Cleveland Clinic Institutional Animal Care and Use Committee approved protocols.

**Virus.** The glia tropic JHMV-neutralizing monoclonal antibody (mAb)-derived 2.2v-1 variant was used for all infections (12). Virus was propagated in the presence of mAb J2.2 to prevent reversion to the highly lethal parental virus and plaque assayed on monolayers of DBT cells (43). Mice were infected in the left hemisphere with 250 PFU of JHMV diluted in endotoxin-free Dulbecco’s phosphate-buffered saline (PBS) in a final volume of 30 µl. Clinical disease severity was graded daily as previously described (12): 0, healthy; 1, hunched back; 2, partial hind limb paralysis or inability to maintain the upright position; 3, complete hind limb paralysis; 4, moribund or dead. Virus titers in the CNS were determined as described previously (43). Briefly, brains were homogenized individually in Dulbecco’s PBS using Tenbroeck tissue homogenizers. Homogenates were clarified by centrifugation at 400 x g for 7 min at 4°C. Supernatants were stored at -70°C until assayed for infectious virus by plaque assays.

**Neutrophil depletion.** Neutrophils were depleted by intraperitoneal administration of 250 µg of anti-Ly6G (clone 1A8) mAb at day -1, at the time of infection, and every other day post infection. Control animals received the same amount of a rat IgG2a isotype control mAb.

**Isolation of CNS mononuclear cells.** After perfusion with PBS, brains were homogenized as described above. Cell pellets were resuspended in RPMI containing 25 mM HEPES, pH 7.2 and adjusted to 30% Percoll (Pharmacia, Uppsala, Sweden). A 1 ml underlay of 70% Percoll was added before centrifugation.
at 800 x g for 30 min at 4°C. Cells were recovered from the 30%-70% interface (5) and washed with RPMI before analysis.

**Flow cytometry.** CNS cell suspensions were blocked with anti-mouse CD16/CD32 (clone 2.4G2, BD Pharmingen) mAb on ice for 15 min before staining. Cells were stained with FITC-, PE-, PerCP- or APC-conjugated mAb for 30 min on ice in PBS containing 0.1% bovine serum albumin. Expression of surface markers was characterized with mAb (all from BD Pharmingen except when indicated) specific for CD45 (clone Ly-5), CD4 (clone GK1.5), CD8 (clone 53-6.7), CD11b (clone M1/70), F4/80 (Serotec, Raleigh, NC), Ly-6G (clone 1A8), NK1.1 (clone PK136) and I-A/I-E (clone 2G9). Virus specific CD8 T cells were identified using H-2D<sup>b</sup>/S510 MHC Class I tetramers as described (5). Samples were analyzed using a FACS Calibur flow cytometer and CellQuest Software (BD Biosciences, Mountain View, CA).

**Cell sorting.** CNS cells isolated from infected mice at day 3 p.i. were stained as described above with FITC-Ly6G, PE-F4/80, PerCP-CD11b and APC-CD45. Neutrophils (Ly-6G<sup>+</sup>, F4/80<sup>−</sup>, CD11b<sup>+</sup>) and macrophages (Ly-6G<sup>−</sup>, F4/80<sup>+</sup>, CD11b<sup>−</sup>) were purified using a FACS Aria (BD Biosciences). 88,000 neutrophils and 424,000 macrophages were isolated from 6 mice with 99.6% and 98.7% purity respectively.

**Histopathological analysis.** After PBS perfusion, brains and spinal cords were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin as described (35). For analysis of CD45 and CD4 distribution, mice were perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA). Brains and spinal cords were dissected, fixed for 1h in 4% PFA at 4°C, and then incubated with sucrose gradients as followed: 30 min with 15% sucrose at room temperature, 30 min with 20% sucrose at 4°C, overnight with 30% sucrose at 4°C. Tissues were then stored in cryoprotection solution until preparation of 30-µm microtome sections. For analysis of CD8
distribution, mice were perfused with ice-cold PBS. Brains and spinal cords were then quickly frozen in liquid nitrogen in OCT and kept at -80°C until 10 μm sections were prepared. Sections were fixed with methanol for 5 min for CD8 staining and treated with 1% Triton X-100 for 30 min, blocking solution for 30 min, and then stained with rabbit anti-mouse Laminin (Cedarlane Laboratories, Ontario, Canada), rat anti-mouse CD45 (Serotec), CD4 (BD Pharmingen) or CD8 mAb overnight at 4°C. Alexa Fluor 594 goat anti-rabbit (Invitrogen, Carlsbad, CA) and biotinylated rat anti-mouse (Vector Laboratories) were added for 1h, followed by streptavidin-Alexa 488 (BD Pharmingen). Sections were mounted with Vectashield mounting medium with 4’-6-Diamidino-2-phenylindole (DAPI) (Vector Laboratories) and analyzed using a Leica DM4000B fluorescent microscope. For quantification, 10 pictures per animal were analyzed in areas of inflammation at each time point.

Zymography. Zymography was performed as described (54). Briefly, cells purified from the CNS were resuspended in lysis buffer (1% Triton X-100, 300 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.4) and lysates from 2.5x10^5 cells separated on 10% acrylamide gels containing 1% gelatin (Bio-Rad, Hercules, CA). Following electrophoresis, gels were consecutively placed in 1X renaturing buffer (Bio-Rad) for 30 min at room temperature, 1X developing buffer (Bio-Rad) for 20 min at room temperature, and then overnight at 37°C. Gels were then stained in 0.25% Coomassie brilliant blue R-250 (Bio-Rad) and destained with the destain solution (Bio-Rad).

Gene expression analysis. RNA was prepared from individual brains of 3 mice per group by extraction with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. First-strand cDNA synthesis used SuperScript II Reverse Transcriptase (Invitrogen) with oligo(dT)_{12-18} primers (Invitrogen). Semi-quantitative RNA expression was assessed using a LightCycler and SYBR Green kit (Roche, Basel, Switzerland) and the following primer sets: CXCL10: F: 5’-GACGGTCCGCTGCAACTG-3’, R: 5’-GCTTCCTATGGCCCTCATT-3’; CCL5: F: 5’-
Linearity of each primer pair was confirmed to have a correlation coefficient of >0.98 by measuring 5-fold dilutions of cDNA samples. Levels of mRNA expression were normalized to ubiquitin mRNA and converted to a linearized value using the following formula: $[1.8e(C_{ubiquitin}-C_{gene \times})] \times 10^5$ as previously described (53).
Results

Inflammatory monocytes dominate CNS inflammatory cells early in infection

Neutrophils and monocytes are the first cells to infiltrate the CNS following infection with the sublethal glia tropic JHMV (6). Whereas neutrophils, characterized as CD45$^{hi}$Ly6C$^{lo}$F4/80$^{-}$Ly6G$^{hi}$ (6, 45), represented a small percentage of CNS infiltrating leukocytes following infection (Figure 1A; ~6 % at day 3 p.i.), monocytes exhibiting a CD45$^{hi}$Ly6C$^{hi}$ phenotype comprised ~65% of the total inflammatory population at days 3 and 5 p.i. (Figure 1A-C). This high frequency subsequently declined to ~20% by day 10 p.i. as T cells were recruited into the infected CNS. A minor proportion of Ly6C$^{hi}$ F4/80$^{-}$ monocytes expressed the MHC class II at day 3 p.i. (Figure 1B and C), which nevertheless increased during the course of infection (Figure 1C). These data confirmed that Ly6C$^{hi}$ monocytes, defined as inflammatory cells (44, 45), migrate into the CNS where they represent the major population at early times p.i.

CCL2 is specifically required for CNS monocyte recruitment

The contribution of monocytes in regulating leukocyte accumulation in the CNS following virus infection was assessed by flow cytometry. Recruitment of CD45$^{hi}$ bone marrow-derived leukocytes into the CNS of CCL2$^{-/-}$ infected mice was significantly reduced relative to wild type (WT) mice at days 3 and 5 p.i. (Figure 2). However, CNS infiltration was similar in both groups by day 7 p.i. and subsequently declined with similar kinetics. As monocytes comprised the major population of infiltrating cells early in WT mice, decreased CNS leukocyte recruitment in the absence of CCL2 was most likely attributed to a deficit in monocytes. However, in addition to monocytes, CCL2 also attracts natural killer (NK) cells and T lymphocytes (18) by binding to CCR2. To verify that CCL2 deficiency predominantly affects monocytes, CNS accumulation of monocytes (F4/80$^{-}$), neutrophils (Ly6G$^{hi}$F4/80$^{-}$), NK, CD4 and CD8 T cells were compared in infected CCL2$^{-/-}$ and control mice. In WT mice, monocytes were rapidly recruited, remained stable until day 7 p.i., and decreased to ~20% of the
infiltrating cells by day 10 p.i. (Figure 2). By contrast, monocyte infiltration into the CNS of infected CCL2<sup>−/−</sup> mice was reduced by ~80% at all time points p.i.

Although Ly-6G<sup>+</sup> neutrophils were ~10-fold fewer relative to monocytes within the CNS of WT mice, comparable numbers were present at day 3 p.i. in both WT and CCL2<sup>−/−</sup> mice (Figure 2). However, while neutrophils declined to nearly the limit of detection by day 7 p.i. in WT mice, they persisted in the CNS of CCL2<sup>−/−</sup> mice as evidenced by a 2.5 fold increase, potentially compensating for the absence of monocytes (Figure 2). Similar NK cell numbers in both groups supported a redundant role of CCL2 in NK cell trafficking (data not shown). CD4 and CD8 T cells began to accumulate within the CNS at day 5 p.i. and peaked between days 7 and 10 p.i. in both WT and CCL2<sup>−/−</sup> mice (Figure 2). Similar CD4 and CD8 T cell infiltration in both groups, including CD8 T cells specific for the viral spike epitope (S510), demonstrated that T cell recruitment into the CNS is neither affected by the absence of CCL2 nor other chemokines potentially secreted by monocytes. There was also no indication of reduced Db/S510 tetramer reactivity on CD8 T cells in cervical lymph nodes or spleen in CCL2<sup>−/−</sup> mice (data not shown) supporting unimpaired peripheral virus-specific CD8 T cell activation and expansion. CCL2 thus regulates early CNS infiltration of bone marrow derived cells during viral encephalitis mainly by influencing monocyte recruitment. The resulting decreased overall infiltration is nevertheless overcome as T cells accumulate to high frequencies.

**Impaired monocyte recruitment delays disease onset and virus clearance**

Monocytes have been implicated in exacerbating encephalitis by numerous functions including release of MMPs, tumor necrosis factor and inducible nitric oxide synthase (28, 50). The onset of encephalitic symptoms, characterized by hunched posture and ruffled fur, was clearly different between the two groups (Figure 3A). Whereas WT mice began to exhibit encephalitis at day 6 p.i. (Figure 3A), CCL2<sup>−/−</sup> mice did not develop clinical symptoms until day 7 p.i., indicating a short, yet consistent delay in disease onset (Figure 3A). Nevertheless, both groups displayed identical signs of paralysis at day 9.
and subsequent times p.i. (Figure 3A). Mortality rates (∼10%) did not differ between the two groups (data not shown).

Clinical signs of encephalitis correlate with CNS T cell infiltration and T cell mediated antiviral function in infected WT mice (6). Despite initial containment by interferon (IFN)-α/β mediated mechanisms (23), CD8 T cells play a major role in reducing viral replication via perforin and IFN-γ mediated mechanisms (6). Despite overall similar T cell accumulation in WT and CCL2<sup>−/−</sup> mice, delayed disease onset suggested altered antiviral function. CCL2<sup>−/−</sup> mice harbored similar CNS virus loads as WT mice at days 3 and 5 p.i., confirming no differences in initial viral replication and innate immune control (Figure 3B). However, as infectious virus in the CNS of WT mice began to decrease at day 7 p.i., consistent with exertion of T cell effector function, replication remained uncontrolled in CCL2<sup>−/−</sup> mice. Nevertheless, this impaired viral control was subsequently overcome (Figure 3B). The delay in virus control supported lagging T cell effector function within the CNS in the absence of CCL2, despite comparable virus specific T cell activation and CNS recruitment. Furthermore, the delay in onset of clinical disease was also transient and overcome 3 days later, when both groups displayed similar viral loads and disease symptoms (Figure 3).

**Leukocytes are retained in the perivascular space of CCL2<sup>−/−</sup> mice**

Delayed disease onset and virus clearance in the absence of monocytes suggested that effector T cells are impaired in accessing the parenchyma through the glia limitans. Potential T cell retention in the perivascular space was supported by the absence of clinical symptoms when monocyte recruitment is abrogated during EAE (46, 48). Indeed, perivascular leukocyte accumulation was profound in the CNS of CCL2<sup>−/−</sup> compared to WT mice at day 5 p.i. (Figure 4A). To quantify relative leukocyte distribution, laminin within the basal membranes was visualized to define the borders imposed by endothelial cells on one side and the glia limitans on the other (Figure 4B). Lymphocytes were too sparse at day 3 p.i. for consistent quantitative assessment. However, at day 5 p.i. a higher proportion of leukocytes accumulated...
within the perivascular space in CCL2^{-/-} relative to WT mice, correlating with a significant relative leukocyte reduction in the parenchyma (Figure 4C). However, these differences resolved after 5 days p.i., (Figure 4C), as evidenced by equalized leukocyte distribution in both groups at day 7 and enhanced accumulation within the parenchyma by 10 p.i., concomitant with decreased cellularity within the perivascular space (Figure 4C). These data imply that monocytes do not regulate leukocyte access at the endothelial barrier, but rather play a vital role in promoting migration from the perivascular space, through the glia limitans, into parenchymal sites. The sparse monocyte CNS infiltration in CCL2^{-/-} mice predicted that T cells were the major population accumulating in the perivascular space at day 5 p.i. A correlation between impaired parenchymal T cell access and delayed disease onset and virus control was thus assessed by directly studying the distribution of T cells. Despite their sparse numbers at day 5 p.i., retention of CD8 T cells in the perivascular space was increased in CCL2^{-/-} mice compared to controls (Figure 4D). No difference was observed at later time points p.i., consistent with the lack of retention by the entire inflammatory infiltrate. In both WT and CCL2^{-/-} mice, the majority of CD8 T cells reached the CNS parenchyma by day 10 p.i. (Figure 4D). CD4 T cells also exhibited impaired parenchymal infiltration in CCL2^{-/-} mice. However, in contrast to CD8 T cells, CD4 T cells preferentially accumulated in the perivascular space until day 7 p.i. in CCL2^{-/-} mice (Figure 4E), possibly due to expression of tissue inhibitor of MMP (TIMP)-1 (53). Although CD4 T cells eventually migrated to the parenchyma by day 10 p.i. in CCL2^{-/-} mice, the percentage of parenchymal infiltration remained lower compared to WT mice, in which ~ 80 % of CD4 T cells were localized in the parenchyma at this time. These data supported delayed CD4 T cell trafficking to parenchyma compared to CD8 T cells (42). Retention of CD8 and CD4 T cells in the perivascular space of CCL2^{-/-} mice thus directly correlated with the impaired ability of monocytes to promote T cell migration across the glia limitans.
Monocyte mediated disruption of the glia limitans is MMP-independent

Retention of T cells in the perivascular space of CCL2−/− mice implicated monocytes in promoting migration through the glia limitans via chemokine secretion and/or proteolytic enzyme activity. However, neither transcription of CXCL10 (IP-10) or CCL5 (RANTES) mRNA, encoding two major T cell chemoattractants early during JHMV infection (25) were reduced in infected CCL2−/− compared to WT mice (Figure 5). CCL3 (MIP-1α) mRNA, encoding a chemoattractant for both T cells and macrophages, was also similarly expressed early after infection in both groups (Figure 5). Furthermore, CXCL12 (SDF-1) implicated in retaining inflammatory cells within the perivascular space during EAE and West Nile virus encephalitis (30, 31), decreased with similar kinetics in both WT and CCL2−/− mice during the course of infection (Figure 5). Thus, impaired chemokine production did not account for delayed leukocyte infiltration into the CNS parenchyma in the absence of monocytes, suggesting a direct effect of monocytes on glia limitans integrity (33, 48).

Monocyte/macrophages are a source of MMPs during HIV CNS infections (50) and autoimmune mediated inflammation (2, 47, 49). Several reports support a direct role of MMPs in mediating glia limitans permeability (1, 46). A correlation between leukocyte accumulation in the perivascular space and reduction of MMP expression and/or activity in CCL2−/− mice was thus tested. Distinct from other neuroinflammatory disorders (19, 40), JHMV infection is associated with a more limited pattern of MMPs expression, restricted to MMP-9, -3 and -12 (53). Neutrophil/monocyte mediated loss of BBB integrity implicated neutrophils as the specific source of MMP9 (54). To test whether monocytes constitute an alternate source of MMP9, neutrophils and macrophages were purified from the CNS of infected WT mice. MMP9 activity was only associated with neutrophils, not monocytes (Figure 6A). Furthermore, MMP9 activity was not reduced in the CNS of CCL2−/− mice compared to controls at 3 and 5 days p.i. (data not shown), supporting the finding that monocytes recruited into the CNS during viral encephalitis do not express this enzyme at significant levels. Dysregulation of MMP3, expressed primarily by astrocytes, and MMP12, expressed by CNS resident cells as well as infiltrating leukocytes
following JHMV infection (53), was also tested. However, the absence of monocytes did not alter the kinetics or levels of MMP12. MMP3 mRNA increased in CCL2−/− mice at days 3 and 5 p.i. (Figure 6B) suggesting a compensatory effect. To account for potential upregulation of other compensatory MMPs in the absence of monocytes, MMP2, MMP7 and MMP14 were analyzed based on their upregulation during many CNS inflammatory disorders (19, 40). However, MMP2, constitutively expressed in the CNS, was not increased (Figure 6B) nor was expression of MMP7 and MMP14 mRNA induced in CCL2−/− mice at any time point (data not shown), consistent with previous data in WT mice (53). Expression of the MMP inhibitors, TIMP-1, -2 and -3 was also examined. TIMP-1, mainly expressed by CD4 T cells infiltrating the JHMV infected CNS (53), peaked at day 7 p.i. in both groups and its expression did not significantly differ between the two groups at any time p.i. (Figure 6B). TIMP-2 and -3 mRNA expression was also not upregulated during JHMV infection in either WT or CCL2−/− mice consistent with previous results (53). These data indicate that monocytes are not a major source of MMPs or TIMPs during JMHV infection and that their role in glia limitans disruption is MMP-independent.

**Neutrophils do not compensate for the absence of CNS infiltrating monocytes**

Depletion of both neutrophils and monocytes prior to a lethal JHMV infection resulted in a drastic decrease of both BBB permeability and CNS inflammation (54). Retention of neutrophils at day 7 p.i. in the CNS of sublethally infected CCL2−/− mice thus suggested a potential compensatory mechanism facilitating parenchymal T cell access in absence of monocytes. Neutrophils were therefore depleted from infected CCL2−/− mice to assess their contribution to CNS myeloid and T cell recruitment. Although flow cytometric analysis of CNS derived cells confirmed neutrophil depletion in CCL2−/− mice (Figure 7A), their absence did not alter CNS cellular inflammation compared to neutrophil sufficient CCL2−/− mice at days 3 and 5 p.i.; an overall reduction of CNS inflammation was observed at later times p.i. (Figure 7B). Similarly, neutrophil depletion in WT mice reduced CNS leukocyte recruitment mainly
between days 7 and 14 p.i. (Figure 7B). The absence of neutrophils thus affected CNS leukocyte migration at the peak of inflammation (day 7-10 p.i.), but not at early time points. Importantly, neutrophils did not compensate for the absence of monocytes in CCL2⁺⁻ mice at early time points. Moreover, specific neutrophil depletion from WT mice demonstrated no difference in disease severity. Similarly, neutrophil depletion from CCL2⁺⁻ mice did not alter the delayed disease onset observed in the neutrophil sufficient control group (data not shown). However, no difference were observed at later time points as both neutrophil depleted and control CCL2⁺⁻ showed similar signs of hind limb paralysis as WT mice. Moreover, treated and untreated CCL2⁺⁻ mice both controlled virus replication to below the limit of detection by day 14 p.i. (data not shown).

Taken together these results also implicate monocytes, not neutrophils, in facilitating early access of inflammatory cells into the parenchyma to control virus replication, albeit at the cost of enhancing clinical disease.
Discussion

The BBB restrains parenchymal leukocyte entry and significantly alters the character of CNS inflammation (4). Leukocyte recruitment through the BBB is however crucial to protect the host during CNS infections. Nevertheless, uncontrolled CNS inflammation during neuro-inflammatory disorders such as MS can mediate tissue damage. Thus, defining mechanisms that regulate migration into the CNS parenchyma is of particular interest to promote protective immune responses against CNS infection, while restraining damaging immune responses leading to neuro-inflammatory disorders.

Extensive studies of migration across the blood vessel wall into the perivascular space have identified potential therapeutic targets to modulate leukocyte trafficking into the CNS. For example, treatment of relapsing forms of MS with anti-alpha4-integrin reduced CNS leukocyte entry (1, 39). However, development of progressive multifocal leukoencephalopathy demonstrated how interference with CNS leukocyte trafficking can perturb the delicate immune balance controlling endogenous pathogens. While modulation of chemokine signaling represents another therapeutic target for neurological diseases (7, 32), the complexity of the chemokine system makes this a challenging approach. For example, although CCR2 is the only receptor for CCL2, several other chemokines signal to CCR2. Our data clearly supports the notion that targeting the ligand has a different impact than targeting the chemokine receptor. Thus CCL2 deficiency selectively impaired only monocyte recruitment into the CNS during viral induced encephalitis. By contrast, CCR2−/− mice showed a drastic reduction of monocyte as well as T cells in the CNS during JHMV infection, resulting in uncontrolled virus replication and rapid mortality (8).

Few studies have focused on understanding the molecular and cellular mechanisms regulating leukocyte penetration through the glia limitans (34), which constitutes an alternative target for therapeutic strategies to ameliorate autoimmune mediated disease (48). The present data are the first to demonstrate a critical role of monocytes in specifically breaching the glia limitans during acute viral encephalitis. During EAE, monocyte facilitated parenchymal leukocyte access has been attributed to
chemotactic signaling induced by monocyte derived tumor necrosis factor (48). However, the absence of monocytes in JHMV infected CCL2−/− mice did not correlate with decreased chemokine expression compared to controls, indicating a more direct role of monocytes in disrupting the glia limitans. In addition, no virus-infected cells, detected with the anti-JHMV mAb J3.3 specific for the carboxyl terminus of the virus nucleocapsid protein, could be found in the perivascular space of either controls or CCL2−/− mice (data not shown). These observations indicated that delayed T cell migration across the glia limitans of CCL2−/− mice was thus not due to a defect in T cell restimulation by antigen-presenting phagocytes in the perivascular space as previously described during EAE (3). Prime candidates in monocyte-mediated migration through the glia limitans are MMPs (46). However, no evidence for decreased or altered MMP mRNA expression in the absence of monocytes indicated that monocytes are not a primary source of MMPs during JHMV infection. Several hypotheses may thus explain leukocyte retention in the perivascular space of infected CCL2−/− mice. First, monocytes may secrete extracellular proteases other than MMPs, which may promote migration through the glia limitans. For example, the cysteine protease Cathepsins (K, S and L) can be mobilized extracellularly by macrophages (16, 37) and degrade collagen, a component of the glia limitans and extracellular matrix. Monocytes may also provide an indirect activation signal by inducing the release of tissue plasminogen activator. Thus, conversion of plasminogen into active plasmin by tissue plasminogen activator can lead to the degradation of other components of the glia limitans such as laminin or fibronectin (16, 38). In addition, monocytes are a major source of reactive oxygen species, which can trigger MMP activation (36).

Although our results indicated no difference in MMP mRNA expression in CCL2−/− mice compared to controls, we cannot rule out impaired cleavage of pro-MMP into activated MMP in absence of monocytes. The identification of the molecule(s) released by monocyte to promote migration through the glia limitans is thus critical to develop more specifically targeted therapies for CNS disorders.

Neutrophil depletion prior to virus infection of the CCL2−/− mice did not alter viral pathogenesis compared to CCL2−/− control mice. Consistent with neutrophil-depleted WT mice, depletion of
neutrophils in CCL2\textsuperscript{-/-} mice did not affect early CNS inflammation. Neutrophils did thus not compensate for the absence of monocytes and were not required to mediate sufficient leukocyte recruitment for anti viral control. These observations appear to contradict previous data showing a drastic reduction of CNS inflammation and BBB permeability, yet early mortality after treatment with anti-Gr1 antibody (54). Nevertheless, the previous depletion studies were performed in mice infected with a more virulent JHMV variant causing lethal encephalitis, in which neutrophils are more abundant CNS inflammatory cells (~20\% of total CD45\textsuperscript{hi} cells). By contrast, they only represent ~5\% of total infiltrating cells during the sublethal JHMV infection used in the present study. Lastly, the anti-Gr-1 antibody not only depletes neutrophils and monocytes, but also activated CD8 T cells, preemting direct comparison of these studies. Nevertheless, a recent report using direct CXCR2 ablation to impair neutrophil recruitment also demonstrates enhanced mortality associated with impaired viral clearance in the sublethal infection model (21), supporting previous studies (54) and contradicting the present results. However, anti CXCR2 treatment also affected monocyte recruitment, making it difficult to discern respective effects of neutrophils versus monocytes on diminished T cell recruitment. Despite the apparent redundancy of neutrophils in enhancing parenchymal T cell access and viral clearance even in the absence of monocytes in our model, they clearly affected leukocyte accumulation at the peak of inflammation. In this context, it is of interest to note that MMP9 expression was specific for neutrophils and not detected in monocytes. The precise role of MMP9 in enhancing leukocyte recruitment thus remains to be elucidated.

The transient nature of delayed disease onset and control of virus replication in infected CCL2\textsuperscript{-/-} mice constitutes a further enigma; disease severity and virus clearance were similar to infected WT mice at later time points, indicating that impaired T cell access to the parenchyma is overcome fairly rapidly. MMPs expression by T cells (52) suggests that a critical number of activated CD4 and/or CD8 T cells may directly promote migration through the glia limitans in the absence of either monocytes or neutrophils.
In summary, these data are the first to demonstrate an important direct role of monocytes in promoting T cell migration across the glia limitans into the parenchyma during acute viral encephalitis. In absence of monocytes, T cells accumulated transiently in the perivascular space, leading to delayed disease onset, but also delayed virus control. Contrasting the EAE model, no overt defects in chemotactic signaling during virus infection in CCL2−/− mice indicated a direct effect of monocytes on glia limitans disruption. While dysregulation of MMP expression in the absence of monocytes was ruled out at the transcriptional level, direct effects on MMP proteolytic activity or involvement of proteases other than MMPs remain to be determined. Identification of mechanisms by which monocytes promote glia limitans disruption will be valuable to design less vigorous, more targeted therapeutic approaches to enhance T cell access to the CNS parenchyma or impair entry of potentially infected monocytes themselves in cases of infection, in addition to limiting this access during neuroinflammatory disorders.
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References


Figure 1. Monocytes represent the major population of CNS infiltrated leukocytes early after infection.

A. Representative density plot of CNS derived cells at 3 day p.i. stained with anti-CD45 and Ly-6C mAb. CNS infiltrating leukocytes (CD45hi) contain a prominent population of Ly6C^hi monocytes (R8; 68.13% of CD45^hi) and a minor proportion of Ly6C^int neutrophils (R7; 5.25% of CD45^hi). B. F4/80 and MHC class II expression was analyzed within the CD45^hiLy6C^int and the CD45^hiLy6C^hi myeloid population at days 3 and 5 p.i. C. Kinetics of MHC class II expression by CD45^hiLy6C^hiF4/80^+ monocytes during the course of infection. Data represent means of 3 mice at each time point.
Figure 2. CCL2 is required for CNS monocyte recruitment.
CNS inflammation in infected CCL2−/− and wt mice analyzed by flow cytometry at the indicated times p.i. Number of total inflammatory leukocytes (CD45hi), macrophages (F4/80+), neutrophils (Ly-6G+), CD4+ T cells, CD8+ T cells and Db/S510 tetramer+ virus specific CD8 T cells per brain (white bars= WT, grey bars CCL2−/−). Data represent means (±SEM) of 3 separate experiments with 3 pooled mice per time point per experiment (n=9 per group). *p<0.05.
Figure 3. Impaired monocyte recruitment delays disease onset and virus clearance.
A. Clinical symptoms were monitored daily according to the following grades: 0, healthy; 1, hunched back; 2, partial hind limb paralysis or inability to maintain the upright position; 3, complete hind limb paralysis; 4, moribund or dead. Data represent means (±SEM) of 60 WT mice and 54 CCL2−/− mice from 3 separate experiments. ***p<0.001. B. Virus replication in brains of WT and CCL2−/− mice analyzed by plaque assay. Data represent the average (±SD) of 3 mice per time point per experiment from 2 separate experiments. (n=6 per group) **p<0.01
Figure 4. Leukocyte retention in the perivascular space of CCL2−/− mice.
A. Brain sections of WT and CCL2−/− mice at day 5 p.i. were stained with Hematoxylin & Eosin (H&E). Perivascular inflammation was prominent in CCL2−/− compared to WT mice. Pictures are representative of 3 mice from each group. Scale bar 300 µm B. Leukocyte localization was analyzed using anti-CD45 (green) and anti-Laminin antibody (red). Scale bar 25 µm. CD45+ cells (C), CD8 (D) and CD4 (E) T cells in perivascular space versus parenchyma quantified in CCL2−/− and WT mice at days 5, 7 and 10 p.i. For quantification, 10 pictures per animal were analyzed in areas of inflammation at each time points. Data are representative of 3 mice from each group (mean ±SEM). *p<0.05.
Figure 5. Monocytes do not affect mRNA CNS chemokine expression. Expression of CXCL10, CCL5, CCL3 and CXCL12 chemokine relative to ubiquitin mRNA in brains of naive (n=4), WT and CCL2-/- mice at day 3, 5, 7 and 10 p.i. (n=3 per time point) was measured by real time PCR. No differences in CXCL10, CCL5 or CCL3 upregulation, or CXCL12 downregulation, were observed between WT and CCL2-/- mice during the course of infection.
Figure 6. MMP-independent disruption of glia limitans.
A. MMP9 activity was analyzed by zymography from purified populations of monocytes and neutrophils isolated from WT mice at 3 days p.i. B. Relative mRNA expression of MMP12, MMP3, MMP2 and TIMP1 were analyzed by quantitative real time PCR. Total RNA was extracted from brains of naive mice (n=4), WT and CCL2-/- mice at day 3, 5, 7 and 10 p.i. (n=3 for each time point).
Figure 7. Neutrophils do not compensate for the absence of monocytes.
A. CNS inflammation of neutrophil-depleted CCL2-/- mice and controls (WT mice treated with isotype control antibody) analyzed by flow cytometry. Graphs represent the number of neutrophils (Ly-6G⁺) and macrophages (F4/80⁺) in the infiltrating CD45high population. Data represent means of 3 mice per group at each time point. B. Percentage of maximal inflammation relative to WT mice was compared between CCL2⁺/⁺ and CCL2⁻/⁻ or WT neutrophil-depleted mice. Data represent the average of 3 mice at each time point.