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Microglia and a Functional Type I IFN Pathway Are Required To Counter HSV-1–Driven Brain Lateral Ventricle Enlargement and Encephalitis

Christopher D. Conrady,* Min Zheng,[†] Nico van Rooijen,[‡] Douglas A. Drevets,[§] Derek Royer,* Anthony Alleman,^{§,¶} and Daniel J. J. Carr*[†]

HSV-1 is the leading cause of sporadic viral encephalitis, with mortality rates approaching 30% despite treatment with the antiviral drug of choice, acyclovir. Permanent neurologic deficits are common in patients that survive, but the mechanism leading to this pathology is poorly understood, impeding clinical advancements in treatment to reduce CNS morbidity. Using magnetic resonance imaging and type I IFN receptor–deficient mouse chimeras, we demonstrate HSV-1 gains access to the murine brain stem and subsequently brain ependymal cells, leading to enlargement of the cerebral lateral ventricle and infection of the brain parenchyma. A similar enlargement in the lateral ventricles is found in a subpopulation of herpes simplex encephalitic patients. Associated with encephalitis is an increase in CXCL1 and CXCL10 levels in the cerebral spinal fluid, TNF- α expression in the ependymal region, and the influx of neutrophils of encephalitic mouse brains. Reduction in lateral ventricle enlargement using anti-secretory factor peptide 16 reduces mortality significantly in HSV-1–infected mice without any effect on expression of inflammatory mediators, infiltration of leukocytes, or changes in viral titer. Microglial cells but not infiltrating leukocytes or other resident glial cells or neurons are the principal source of resistance in the CNS during the first 5 d postinfection through a Toll/IL-1R domain-containing adapter inducing IFN- β –dependent, type I IFN pathway. Our results implicate lateral ventricle enlargement as a major cause of mortality in mice and speculate such an event transpires in a subpopulation of human HSV encephalitic patients. *The Journal of Immunology*, 2013, 190: 2807–2817.

Herpes simplex virus-1 is the leading cause of sporadic viral encephalitis, a rapidly progressive and severely debilitating disease with mortality rates approaching 70% in the untreated human patient (1). In those patients who do survive, permanent neurologic deficits are not uncommon (1). During herpes simplex encephalitis (HSE), patients suffer from acute psychosis, temporal lobe hemorrhaging, and cingulate gyrus involvement implicating the lateral ventricles as a critical site in the pathogenic process (2, 3). Moreover, enlargement of the lateral ventricles leading to compression of cortical tissue has been

shown to correlate with decreased cognitive function, suggesting a possible role in the development of acute neurologic dysfunction during HSE (4, 5). However, acute ventricle enlargement has not been reported other than in neonatal HSE cases in which a physical obstruction of drainage or overproduction of cerebrospinal fluid (CSF) results in hydrocephalus (6–8).

In the human patient, cytokines including IL-6 and IFN- γ and additional soluble factors including soluble Fas within the CSF are observed in patients diagnosed with HSE (9, 10). Furthermore, enhanced susceptibility to HSE has been described in preadolescent populations with genetic mutations in TLR-3 or TLR-3 signaling (11–13) and in a subpopulation of rheumatoid arthritic patients treated with TNF- α inhibitors (14). Such findings have begun to identify pathways and molecules ascribed to susceptibility and neuropathogenesis. However, the fundamental series of events that ultimately lead to neuropathology and morbidity associated with HSE in the human patient remain elusive.

Experimental models of HSE have been established in mice with results that suggest inflammation-induced and direct viral-induced pathways as the cause of encephalitis and death following acute infection. For example, CD4⁺ and CD8⁺ T cells including $\gamma\delta$ ⁺ T cells contribute to resistance to HSE in that in their absence, mice succumb to infection with greater frequency (15–17). Cytokines secreted by T cells including TNF- α and IFN- γ are protective and reduce the incidence of encephalitis as reported in mice administered exogenous cytokines or in mice deficient in the respective cytokine or cognate cytokine receptor (18–22). Even though the host innate and adaptive immune responses are critical in viral resistance, a fine balance to limit inflammation, on the one hand, yet suppress virus replication and cell death on the other is absolutely necessary in sensitive tissue such as the brain (23, 24). To further explore the relationship among viral insult, predicted

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Abbreviations used in this article: AF-16, anti-secretory factor 16; BM, bone marrow; BS, brain stem; CD118^{−/−}, type I IFN receptor α -chain–deficient; CSF, cerebral spinal fluid; CT, computed tomography; HSE, herpes simplex encephalitis; MMP, matrix metalloproteinase; MRI, magnetic resonance imaging; MyD88^{−/−}, MyD88–deficient; p.i., postinfection; TK, thymidine kinase; TRIF^{−/−}, TRIF, deficient; WT, wild-type.

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anatomical changes within the CNS, and the inflammatory immune response with the development of encephalitis, we chose to incorporate a mouse model of HSE using wild-type (WT), type I IFN receptor α -chain-deficient (CD118^{-/-}), and CD118^{-/-} mouse chimeras infected with a highly virulent, neurotropic strain of HSV-1, strain McKrae.

In the current study, we find a pathologic enlargement of the lateral ventricles in mice associated with ependymal cell susceptibility to HSV-1 infection. CD118^{-/-} mouse chimeras demonstrated greater susceptibility to HSV-1. The increased susceptibility correlates with lateral ventricle enlargement, which occurs in the host exclusively when the majority of the microglial cell population does not possess a functional type I IFN pathway. Such results indicate a functional type I IFN pathway by resident cells is required to maintain resistance to HSV-1 replication and lateral ventricle pathology during acute infection. Finally, the therapeutic application of a peptide previously reported to alleviate intracranial pressure due to HSV-1 infection (25) suppressed viral-induced lateral ventricle dilation and improved survival of mice. The efficacy was not reflected in changes in viral titer or local CNS inflammation in terms of leukocyte infiltration or cytokine expression. Collectively, we interpret the findings to suggest HSE-induced mortality is the direct result of lateral ventricle enlargement. As such, the treatment of HSE patients with acyclovir along with compounds that lessen ventricular dilation may improve the long-term outlook and diminish the incidence of protracted neurologic deficits.

Materials and Methods

Human subject data collection

The study was approved by the Institutional Review Board at the University of Oklahoma Health Sciences Center (protocol 15984). All data analyzed were anonymized. Patients were identified by retrospective screening of the hospital database for confirmed cases of herpetic encephalitis by an International Classification of Diseases, Ninth Revision code (054.3). To identify age- and sex-matched controls, a query of the picture archiving and communication system was performed to identify subjects with reports containing the phrases "normal ventricular size," "normal ventricles," and/or "no ventricular enlargement" to be used as "healthy controls." Controls include both inpatient and outpatient exams. Once records were retrieved, a neuroradiologist examined the magnetic resonance images (MRIs) in a masked fashion on two separate occasions to evaluate neuroinflammation proximal to the lateral ventricles and ventricular enlargement. Values were then calculated to obtain a computed tomography (CT) Evan's Index, and those above a value of 0.30 were considered pathologic as previously described (26, 27).

$$\text{CT Evan's Index} = \frac{a}{b} = \frac{\text{Maximum axial width of anterior horns of lateral ventricle}}{\text{Maximum intracranial (inner table to inner table)}}$$

Mice and virus

C57BL/6J WT [CD45.1 and CD45.2] mice were purchased from The Jackson Laboratory and Charles River and housed alongside CD118^{-/-} and MyD88-deficient (MyD88^{-/-}) mice (28, 29) in Dean A. McGee Eye Institute's animal facility. All mice were age- and sex-matched. Animal treatment was consistent with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals. All experimental procedures were approved by the University of Oklahoma Health Sciences Center and Dean A. McGee Eye Institutes' Institutional Animal and Care Use Committees. HSV-1 McKrae, HSV-1 GFP (McKrae background), and HSV-1 Tomato Red (KOS background) were propagated Vero cells and maintained at a stock concentration of 10⁸ PFU/ml. Anesthetized mice were infected with HSV-1 by applying 3 μ l PBS containing 1,000–10,000 PFU virus as indicated onto the scarified mouse cornea. For plaque assays, at the indicated time postinfection (p.i.), tissue and cells were isolated, suspended in 500 μ l RPMI 1640 media, and homogenized with a tissue miser for ~20–30 s. Media was clarified of cellular debris by centrifugation for 60 s at 10,000 \times g. Infectious content within the clarified supernatant was then evaluated as previously described (30).

Magnetic resonance imaging

WT, CD118^{-/-}, and mouse chimeras were subjected to gas anesthesia (1.5–2.5% isoflurane, 0.8 l/min O₂). At the indicated time prior to or p.i., mice were imaged using a Bruker Biospec 5.0 imaging system to capture T2-weighted images. Volumes of tissue were calculated by drawing regions of interest around consecutive 1-mm slices of T2-weighted images that were then added together for total volume using Paravision software. Four to six mice were analyzed per group.

Cell culture

A human ependymoma cell line, BXD-1425EPN (31), was maintained in DMEM supplemented with 10% FBS. Once confluency had been reached in tissue-culture wells, cells were infected with a multiplicity of infection of 0.1 or 0.01. HSV-1 was allowed to adhere for 1 h at 37°C. After 1 h, media was changed, and 24 h p.i., the medium was evaluated for infectious content by plaque assay or stained for HSV-1 Ag expression.

Immunohistochemical staining and confocal imaging

WT and CD118^{-/-} mice were anesthetized with ketamine and xylazine and transcardially perfused with 10 ml cold 4% paraformaldehyde. Brains and livers were removed and fixed for another 48 h at room temperature. Samples were then embedded in paraffin blocks, and 10- μ m sections were cut and mounted on slides. Sections were then subjected to H&E staining or deparaffinized and blocked overnight at 4°C with donkey serum. Samples to be viewed by confocal microscopy were then subjected to anti-HSV-1 Ab (DakoCytomation) overnight at 4°C. Following three washes, secondary Ab (Jackson ImmunoResearch Laboratories) was added overnight at 4°C. Slides were then washed three more times and incubated with DAPI overnight. The sections were then imaged with an Olympus IX81-FV500 epifluorescent/confocal laser-scanning microscope (Olympus) as previously described (30).

RT-PCR

At the indicated time p.i., the indicated tissues were harvested and resuspended in 500 μ l to 1.0 ml TRIzol (Invitrogen). Total RNA was then isolated per the manufacturer's guidelines (Invitrogen), and semiquantitative real-time PCR was performed with an iCycler (Bio-Rad) as previously described (32). Primer sequences were as follows: β -actin forward, 5'-CTTCTACAATGAGCTGCGTGTG-3' and reverse, 5'-TTGAAGGTC-TCAAACATGATCTGG-3'; IFN- α forward, 5'-CTCATCTCTGCAATGACCTCCA-3' and reverse, 5'-CCTGATGGTCTTGGTGGTG-3'; IFN- β forward, 5'-CAAGAGGAAAGATTGACGTGG-3' and reverse, 5'-TAA-GGTACCTTTGCACCCTCC-3'; Oas1a forward, 5'-CTTTGATGTCTTGGTCATGT-3' and reverse, 5'-GCTCCGTGAAGCAGGTAGAG-3'; thymidine kinase (TK) forward, 5'-ATACCGACGATCTGCGACCT-3' and reverse, 5'-TTATTGCCGTCATAGCGCG-3'; and TNF- α forward, 5'-CATCTTCTCAAATTCGAGTGACAA-3' and reverse, 5'-TGGGAG-TAGACAAGGTACAACCC-3'.

Bioplex and ELISA assays

CSF was accessed through the cisterna magna and collected from uninfected and infected WT and CD118^{-/-} mice at the indicated time p.i. Samples were then analyzed by suspension array (Millipore) per the manufacturer's protocol for CXCL1, CCL2, CXCL9, CXCL10, IL-1 α , and IL-1 β . Matrix metalloproteinase (MMP)-9 levels in the CNS were determined by ELISA (R&D Systems).

Flow cytometry

At the indicated time p.i., brain stem (BS), brain cortex, and ependymal tissue were surgically removed, and single-cell suspensions were generated using a Wheatley Dounce homogenizer (Fisher Scientific) followed by filtration through a sterile 70- μ m cell strainer (BD Biosciences). Cells were phenotypically characterized following preincubation with Fc Block anti-mouse CD16/32 (BD Biosciences) using combinations of fluorochrome-conjugated Abs to CD3, CD4, CD8, CD11b, CD11c, Pan-leukocyte CD45, CD45.1, CD45.2, NK1.1, and Gr-1 from BD Biosciences or F4/80 obtained from Serotec using a Coulter Epics XL flow cytometer as previously described (Beckman Coulter) (32). In the case of microglia, cells were gated on CD45^{medium} and stained with CD11b. The contribution of resident versus hematopoietic cells in the microglia population in the mouse chimeras was further delineated using anti-CD45.1 or anti-CD45.2 Ab. The percentage of the total microglia population for resident and donor sources is reported.

Brain-slice culture

WT and CD118^{-/-} mice were transcardially perfused with 10 ml 1× PBS. Whole brains were then removed, placed in slice media (DMEM, 5% FBS), and sliced into 400-μm transverse sections on a Leica vibratome (Leica Microsystems). Two to three slices were then moved to Millipore organotypic membranes (Millipore) and supplied with 1.2 ml neural basal media supplemented with B-27, 10% FBS, HEPES, N2, glutamine, and glutamax as described (33). Tissue was then inoculated with 1,000–10,000 PFU/slice HSV-1 in 3 μl neural basal media. After 24 h, the media was replaced with 1.2 ml fresh neural basal media without FBS and changed every other day thereafter as described (33). In the indicated experiments, clodronate liposomes or PBS liposome controls were suspended in media at a concentration of 1000 μM and changed every other day with neural basal media. For clodronate experiments, on the second day, slices were infected with 10,000 PFU/slice diluted in 3 μl neural basal media. To confirm, slices were viable over the time of experimental manipulation, Presto Blue (Invitrogen) was topically applied to slices and incubated for 30 min. The slices were then imaged for a color change from blue to red, which indicates tissue viability.

Stereotaxic inoculation of reporter virus or Evan's blue dye in mice

To directly inoculate HSV-1-expressing tomato red or Evan's blue dye into the lateral ventricles, mice were anesthetized using ketamine and xylazine as previously described. Hair and skin overlying the skullcap were resected and a pen-size hole drilled 0.2 mm caudal and 1.2 mm lateral of the bregma. One thousand PFU virus or 5 μl 1% Evan's blue dye was then injected into the lateral ventricles of WT or CD118^{-/-} mice at a depth of 1.0 mm. Uninfected mice served as controls. For HSV infection, skin was then sutured closed, and mice were treated with antibiotic-supplemented

(trimethoprim and sulfamethazole) water. Seventy-two hours later, mice were sacrificed and brains cut on the vibratome as previously described. For Evan's blue dye experiments, dye was injected into the lateral ventricle, and the clearance of the dye (time to the 4th ventricle) was recorded by direct visualization of the dye draining into the cisterna magna with the aid of a dissecting microscope. Mice were then perfused and tissue was then fixed in 4% paraformaldehyde, cut at a thickness of 600 μm, and imaged.

Western blots

WT organotypic brain slices were cut as previously described at 400 μm. The slices were then subjected to clodronate or PBS liposomes as previously described. Twenty-four hours p.i., media and liposomes were changed. Two days later, tissue and isolated cells were suspended in Radio-Immunoprecipitation buffer containing protease inhibitors (Santa Cruz Biotechnology) and briefly homogenized with a tissue miser. Protein and nuclear extracts were then isolated, electrophoresed, and transferred onto polyvinylidene fluoride membranes as previously described (30). The membranes were then washed and subjected to primary Ab (GFAP, Invitrogen; NeuroD, Abcam) overnight at 4°C. Following three additional washes, samples were incubated with secondary anti-rabbit Ab (Abcam; 1:5000 dilution) for 1 h at room temperature. The membranes were then washed, and signal intensity was evaluated (Pierce) using a Kodak imager (Kodak). Relative protein expression was then calculated using β-actin or TATA-binding protein as the housekeeping control.

Anti-secretory factor 16 peptide treatment

Anti-secretory factor 16 (AF-16) peptide (25) with the sequence VCHSKTRSNPENNVGL was synthesized by EZBiolab. Immediately prior

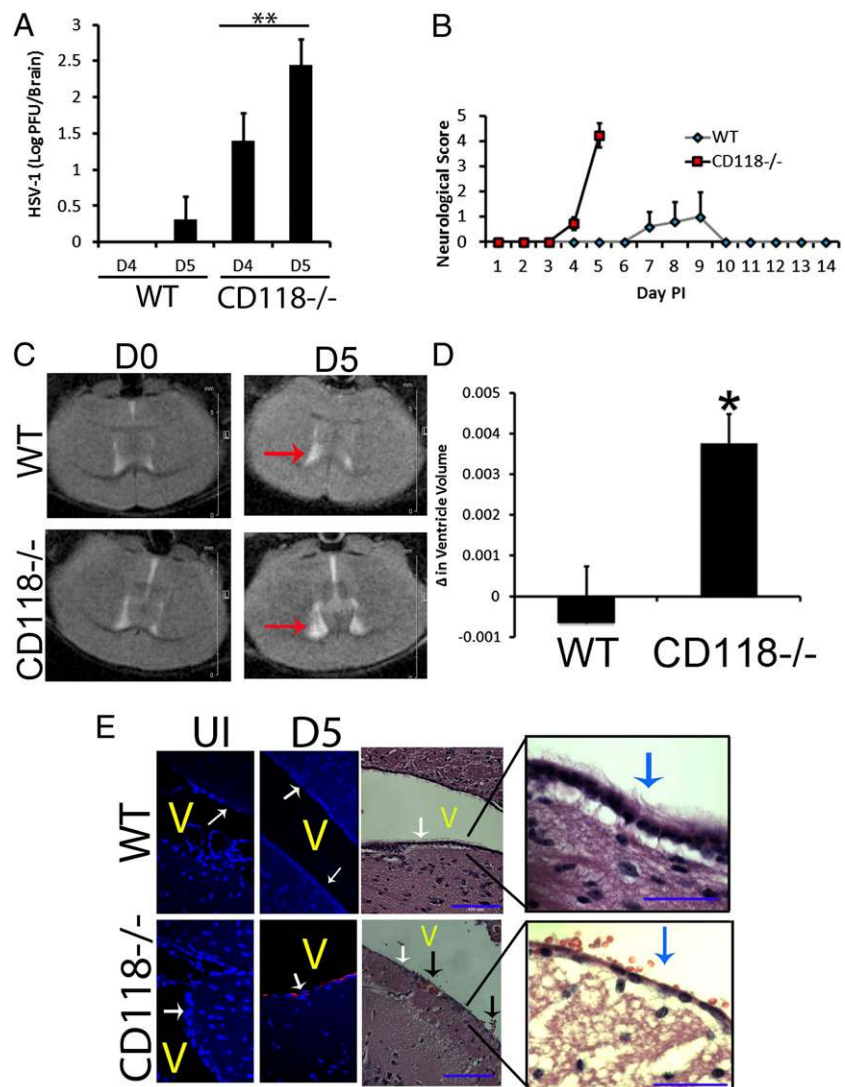


FIGURE 1. CD118^{-/-} mice suffer from lateral ventricle involvement. WT and CD118^{-/-} mice were evaluated for viral content (**A**), overt encephalitic symptoms (**B**), and in vivo pathology (**C**, **D**) following ocular HSV-1 infection (1000 PFU/eye). Values are the mean ± SEM. *n* = 4 to 5 mice/group. Red arrows, lateral ventricles. (**E**) To identify the precise location of HSV Ag (red) and surrounding pathology, immunostaining and H&E images were captured. White arrows in fluorescent images, ependymal layer; white/blue arrows in H&E, cilia; black arrows, subependymal hemorrhaging (RBCs in CSF fluid). *n* = 4 to 5 mice/group. Scale bars, 105 μm. **p* < 0.05, ***p* < 0.01. V, Ventricle.

to use, the peptide was dissolved in PBS at a concentration of 1.0 mg/ml. CD118^{-/-} and WT mice were infected with either 1,000 or 10,000 PFU/eye HSV-1, respectively. Starting on day 3, mice were anesthetized and treated twice a day with an intranasal injection of 20 μ l AF-16 peptide (1 μ g/ μ l) or PBS through the duration of the experiment up to day 12 p.i. for WT mice.

Statistical analysis

Statistical analysis was performed using the GBSTAT one-way ANOVA followed by an ad hoc Tukey's *t* test in experiments comparing more than two groups, whereas a Student *t* test was used to compare two variables. Significance was defined as a *p* value <0.05 throughout the paper.

Results

MRI of the brain reveals lateral ventricle involvement in mice and man

We have previously observed CD118^{-/-} mice are highly susceptible to ocular HSV-1 infection with signs consistent with HSE including the absence of grooming, food intake, and neurologic signs (motor dysfunction, including gait, posture, and spontaneous movement) (Ref. 34 and C.D. Conrady and D.J.J. Carr, unpublished observations). Consistent with the appearance of HSE, the viral burden in the cerebral cortex of CD118^{-/-} mice was significantly greater than in WT animals 4 d p.i. (Fig. 1A) with observable features of encephalitis (35) (Fig. 1B). This trend continued to day 5 p.i. (Fig. 1A), resulting in severely swollen heads and death of ~100% of CD118^{-/-} mice (Fig. 1B). WT mice were not refractory to disease and exhibited similar symptoms as early as 7 d p.i. but with only an ~20% mortality rate (Fig. 1B). As a means to further evaluate global pathology within the CNS, mice were imaged using MRI, a useful and non-invasive modality to monitor HSE (36). CD118^{-/-} but not WT mice displayed ventricular enlargement by 5 d p.i., which mirrored that of human disease (Fig. 1C, 1D). This finding was restricted to the lateral ventricles, as no overt pathology or enlargement was identified in the third ventricle (Fig. 1C).

Diagnosis of HSE patients typically present with uni- or bilateral cerebrocortical lesions in the temporal lobe but may also include parietal or frontal lobe involvement (37, 38). Because acute ventricle enlargement has previously been reported in neonatal cases of HSE (6–8) and was observed in susceptible CD118^{-/-} mice, we hypothesized this phenomena may occur in a subpopulation of HSE adults. In fact, MRI scans of 19 patients with HSE analyzed proximal to tissue within millimeters of the boundary of the Foramen of Monroe revealed a gross dilatation of the lateral ventricles in 7 patients compared with 0 out of 19 in controls (Fig. 2A, 2B). Although these results were not found to be significant (Fig. 2C), they do suggest that lateral ventricle enlargement may be a feature found in a subpopulation of HSE patients certainly worth further investigation.

HSV-1 targets ependymal cells

Resident cells of the CNS and regions adjacent to lateral ventricles can be infected by HSV-1 in vitro and ex vivo (39–42). In our in vivo encephalitic model, HSV-1 Ag expression was limited to the ependymal cells within the CNS of CD118^{-/-} mice shortly before or during encephalitis following ocular infection (Fig. 1E). Along with viral tropism to the ependymal region, a loss of ciliated ependymal cells and detection of erythrocytes within the ventricle were also routinely observed in encephalitic CD118^{-/-} mice (Fig. 1E). In a similar fashion, the human ependymoma cell line BXD-1425EPN (31) was found to be highly susceptible to HSV-1 evident by expression of HSV-1 Ag (Fig. 2D) and recovery of progeny virus following infection (Fig. 2E). These results are consistent with the epithelial origin of ependymal cells (43) and high susceptibility of epithelial cells including ependymal cells (44) to HSV-1 infection.

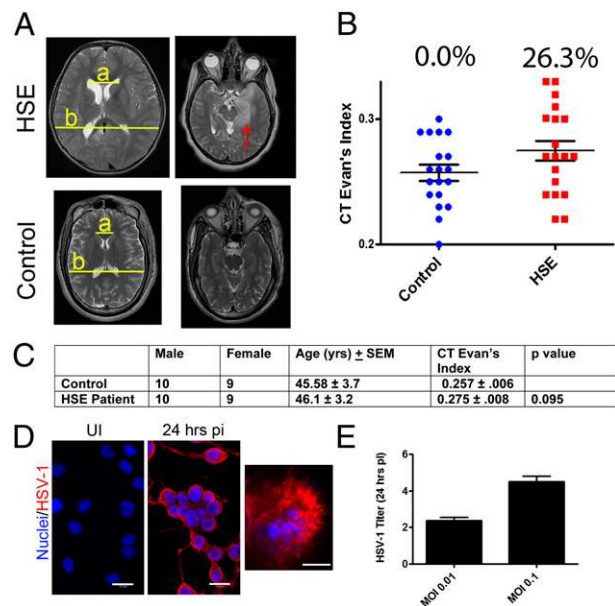


FIGURE 2. Lateral ventricle enlargement in human HSE. (A and B) *Left panel*, Ventricle size was compared between HSE patients and controls at the Foramen of Monroe and is presented as the mean CT Evan's Index \pm SEM. CT Evan's index was calculated as the ratio of a/b. Pathologic ventricle enlargement was defined as an index >0.30. *Right panel*, Axial images of the temporal lobe demonstrate mesial temporal lobe edema (red arrow) characteristic of HSE. (C) Patient demographics. Human ependymoma cells were infected with HSV-1 at an multiplicity of infection of either 0.1 or 0.01 and expressed Ag (in red, D) and reproduced infectious virus 24 h p.i. (E). Images are representative of two independent experiments. Results are presented as the mean log PFU \pm SEM. Scale bars, 20 μ m. a, Maximum width of the lateral ventricles; b, maximal intracranial width same image; MOI, multiplicity of infection; UI, uninfected.

HSV-1 tropism for the ependyma was further substantiated in mice both after direct viral inoculation into the lateral ventricles in vivo and ex vivo infection of brain slices at sites proximal to the ventricles (Fig. 3). Specifically, stereotaxic placement of tomato red-expressing HSV-1 into the ventricle of WT mice localized selectively to the ependymal region detected within 72 h post-administration (Fig. 3A). In organotypic brain-slice cultures, inoculation of ventricle (red asterisk) but not cerebral cortex (blue asterisk) led to the detection of GFP expressing reporter virus that localized to the ependymal region (Fig. 3B–D). To identify the selective mode of transit of HSV-1 into the CNS of WT and CD118^{-/-} mice, two routes of entry were considered. HSV may undergo viremia as seen with HIV (45) and West Nile virus (46) and/or follow neuronal retrograde axonal transport from the periphery such as rabies and West Nile viruses (47, 48). Systemic HSV dissemination has been reported in adults and children (49, 50) and was a plausible route of CNS entry modeled by the rapid spread of virus in CD118^{-/-} mice (34). To test this idea, highly sensitive real-time RT-PCR of viral TK was performed on sections of the CNS including the ependyma, cortex, and BS (Fig. 4A) as well as blood samples. TK mRNA expression was detected as early as 3 d p.i. in the BS and ependymal-rich tissue sections of highly susceptible CD118^{-/-} mice, but was not detected in blood samples until 5 d p.i. (Fig. 4B, 4C). In addition, TK expression was detected earlier (day 3 p.i.) in the ependyma compared with the cortex (day 4 p.i.) of the CD118^{-/-} mice (Fig. 4B). We interpret the results to suggest HSV-1 enters the CNS through a neural route and rapidly but selectively progresses from the BS to the ependymal cells and then into the cortex.

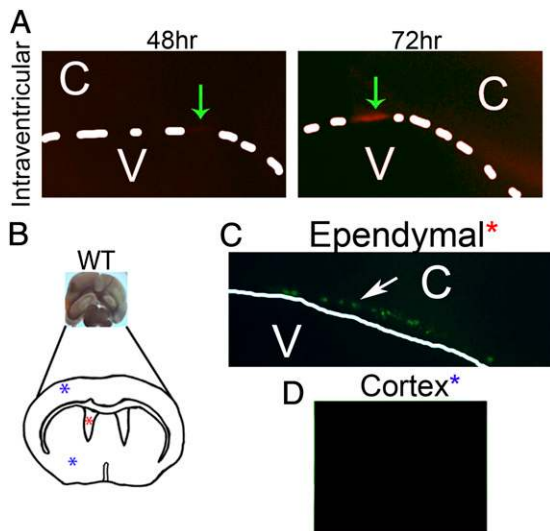


FIGURE 3. HSV infection results in ependymal inoculation. (A) To confirm infection of ependymal cells, tomato red-expressing HSV-1 was directly inoculated into the lateral ventricles. Two days p.i., WT brains were isolated, sectioned on a vibratome, and imaged for consecutive days for tomato red expression. Green arrows, area of tomato red expression. To substantiate infection of ependymal cells, WT brain slices (B) were infected with GFP-expressing HSV in either the cortex (D) or ependymal area (C). Slices were then imaged 2 d p.i. for GFP expression. Images are representative of two to three independent experiments. Dotted white line, ependymal cell layer; white arrow, infected ependymal cells. C, Cortex; V, ventricle.

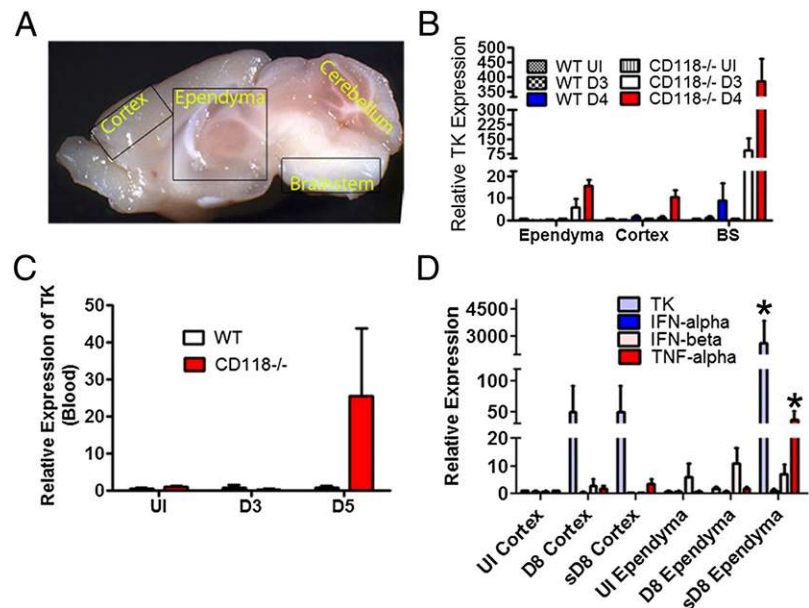
By comparison, WT mice exhibiting severe symptoms of encephalitis (HSE) were compared with those mice with no overt signs (gait, posture, and spontaneous movement), which routinely occurred at day 8 p.i. Viral replication was more pronounced in the ependymal-rich areas of the brain of HSE WT mice for which there was ~60-fold more TK transcript relative expression compared with the surrounding cortex (Fig. 4D). Furthermore, there was no difference in the expression of TK in the brain cortex of HSE and non-HSE WT mice at day 8 p.i., whereas there was ~600-fold more TK transcript expressed in the ependymal region of HSE versus non-HSE WT mice (Fig. 4D). Taken together, the results

suggest viral levels as measured by TK expression within the ependyma but not cortex of the brain are clearly aligned with encephalitis. This notion is further supported by the expression of TNF- α , which was significantly elevated in the ependymal region but not cortex of the brain of HSE or non-HSE WT mice (Fig. 4D). Ironically, there was no significant difference in the expression of IFN- α or IFN- β in the ependyma or cortex comparing encephalitic to nonencephalitic WT mice (Fig. 4D). Similar to what was found in CD118^{-/-} mice, HSV-1 TK expression was not detected in the blood of WT mice (data not shown) at the time when some WT mice display signs of encephalitis (day 8 p.i.), suggesting HSV-1 is distributed to the CNS via neuronal routes.

Inflammatory response profile in the CNS of encephalitic CD118^{-/-} and WT mice

Because changes were observed in the expression of the proinflammatory cytokine TNF- α comparing HSE to non-HSE WT mice, additional experiments were performed to further identify factors and cell infiltrates associated with HSE. The CSF of HSV-1-infected, HSE CD118^{-/-} mice had detectable levels of IL-1 α , CCL2, CXCL1, CXCL9, and CXCL10 compared with uninfected controls at day 5 p.i., of which CXCL1 and CXCL10 were prominently expressed (Fig. 5A). Likewise, HSE WT mice had detectable levels of IL-1 α , CCL2, CXCL1, CXCL9, and CXCL10, with CXCL1 and CXCL10 expression significantly above that recovered in the CSF of non-HSE WT mice day 8 p.i. (Fig. 5B). Upon analysis of infiltrating cells within the CNS of HSE CD118^{-/-} mice, the ependymal region contained more neutrophils (F4/80⁺Gr-1⁺) and total CD45^{hi} leukocytes but not NK cells (NK1.1⁺CD3⁻), inflammatory monocytes (F4/80⁺Gr-1⁺), or macrophages (F4/80⁺Gr-1⁻) compared with brain cortex day 5 p.i. (Fig. 5C). In comparing HSE to non-HSE WT mice, neutrophils, inflammatory monocytes, macrophages, and total leukocyte numbers were all elevated in the HSE WT mice in the brain cortex and ependymal region compared with the non-HSE WT mice (Fig. 5D). Because neutrophils are highly inflammatory cells and the levels were elevated in the ependymal region of both the HSE CD118^{-/-} and HSE WT mice, we hypothesized neutrophils may be the principal driver of neuropathogenesis and mortality in HSE mice. Therefore, by targeting the neutrophil population, the result may alleviate the host of much of the inflammatory process within

FIGURE 4. HSV-1 targets the ependyma via neural projections rather than by a hematogenous route. (A) Anatomical location of the ependyma relative to cortex, cerebellum, and BS of mouse. (B) Viral TK mRNA expression detected in the ependyma, cortex, and BS of WT and CD118^{-/-} mice comparing uninfected (UI) mice to mice infected with 1000 PFU/cornea and assessed at day 3 (D3) or day 4 (D4) p.i. Values are the mean \pm SEM. $n = 4$ –6 mice/group. (C) Viral TK mRNA expression detected in the blood of HSV-1-infected mice at the indicated time p.i. Values are the mean \pm SEM. $n = 4$ –6 mice/group. (D) WT mice were infected with 1000 PFU/cornea HSV-1. Eight days p.i., relative expression of TK, IFN- α , IFN- β , and TNF- α in the cortex or ependymal region of extremely sick (encephalitic) mice ("s") and those not exhibiting signs of encephalitis were evaluated and normalized to the housekeeping gene β -actin. Uninfected (UI) mice served as controls. The results are expressed as the mean \pm SEM of three independent experiments of one to four mice per group per experiment. * $p < 0.05$ comparing sD8 ependyma group to UI and D8 ependyma groups.



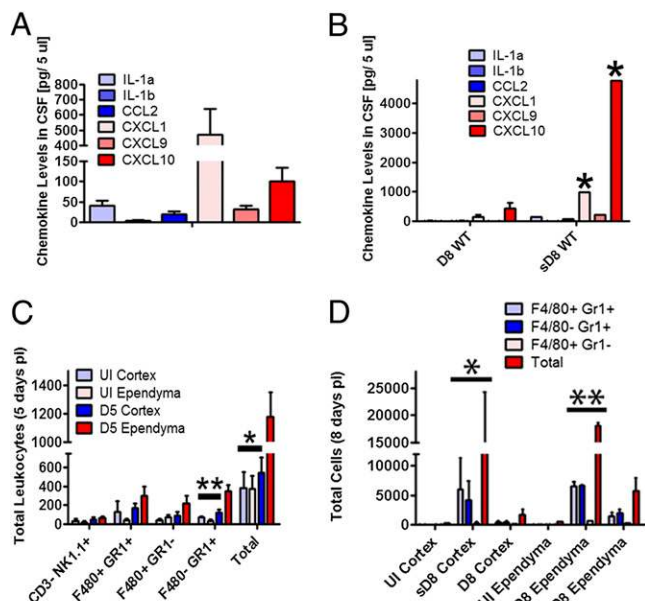


FIGURE 5. Inflammatory profile in the CNS of HSE CD118^{-/-} and WT mice to non-HSE WT mice. In all cases, mice were infected with 1000 PFU/cornea HSV-1. **(A)** Uninfected and infected CD118^{-/-} mouse CSF ($n = 5$ /group) was isolated and evaluated for select proinflammatory cytokines and chemokines by suspension array. Uninfected CSF levels for each analyte measured were as follows (expressed as the mean in pg/5 μ l CSF): IL-1 α , 19.4; IL-1 β , 0.0; CCL2, 12.1; CXCL1, 169; CXCL9, 4.0; and CXCL10, 38. Uninfected levels served as background levels and were subtracted from the levels detected in the infected CD118^{-/-} mice. Levels are expressed as mean \pm SEM. With the exception of IL-1 β , all analyte levels in the infected CD118^{-/-} were significantly above those found in uninfected CD118^{-/-} mice. **(B)** Same as (A) except comparing HSE(s) to non-HSE WT mice in which CSF was extracted from the mice day 8 (D8) p.i. Levels are expressed as mean \pm SEM. * $p < 0.05$ comparing HSE to non-HSE CSF samples for CXCL1 and CXCL10. **(C)** Leukocyte infiltration was evaluated in serial dissections of ependymal-enriched regions and cortex of uninfected (UI) and infected CD118^{-/-} mice euthanized at day 5 p.i. (D5) by flow cytometry. The data are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ comparing the day 5 p.i. ependymal-enriched group to the day 5 p.i. cortex and UI ependymal-enriched and cortex groups. **(D)** Leukocyte infiltration was evaluated as in (C) in WT mice ($n = 4$ –12/group summarized from three experiments) with overt symptoms of HSE (abbreviated “s”) compared with those with no HSE clinical presentation within the cortex and ependymal-enriched region by flow cytometry day 8 (D8) p.i. The data are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ comparing the HSE ependymal-enriched and cortex groups to the non-HSE and UI ependymal-enriched and cortex groups.

the CNS and, as a consequence, reduce death. However, anti-Ly6G Ab depletion of neutrophils within the CNS of HSV-1-infected CD118^{-/-} and WT mice did not attenuate the disease course (data not shown), suggesting another pathogenic mechanism.

Lateral ventricle enlargement is detrimental to survival

Increased intracranial pressure is a typical feature of viral encephalitis and has been hypothesized to cause enlargement of the lateral ventricles (51, 52). Thus, we hypothesized that the loss of cilia on ependymal cells of the CD118^{-/-} mice (Fig. 1E) would result in reduced clearance of CSF, resulting in the pathogenic expansion of the lateral ventricles seen in both mouse and man that has been previously attributed to parenchymal swelling (51) (Fig. 2A). In fact, we found infected CD118^{-/-} mice had a significantly reduced capacity to move dye from the lateral ventricles to the fourth ventricle in comparison with uninfected controls (Fig. 6A, 6B).

CSF distribution, circulation, and turnover along with intracranial tissue mass influence the intracranial pressure (53). Furthermore, an increase in intracranial pressure can lead to brain herniation and death consistent with outcomes in cases of human HSE (37, 54). To this end, the therapeutic intranasal delivery of a peptide previously reported to reduce intracranial pressure, AF-16 (25), was found to slightly prolong survival of sensitive CD118^{-/-} mice following HSV-1 infection (Fig. 7A, 7B) but significantly reduce mortality in HSV-1-infected WT mice compared with vehicle-treated controls (Fig. 7C, 7D). The dramatic improvement in cumulative survival in peptide-treated WT mice correlated with restoration in lateral ventricle volume as indicated in the MRI profile (Fig. 7E, 7F) and improved neurologic signs (data not shown).

AF-16 peptide efficacy does not dampen brain inflammation

As a result of the therapeutic efficacy of the AF-16 peptide applied to HSV-1-infected mice, we next sought to determine the mechanism behind the efficacy. We previously found an elevation in TNF- α , CXCL1, and CXCL10 expressed in the ependyma or CSF as well as an increase in leukocyte infiltrates into the brain of HSE CD118^{-/-} or WT mice compared with nonencephalitic animals (Figs. 4, 5). However, analysis of brain cortex and ependyma for cytokine and chemokine (TNF- α and CXCL10) expression by suspension array or leukocyte infiltrate (including neutrophils, macrophages, inflammatory monocytes, and NK cells) by flow cytometry revealed no significant difference in levels or cell numbers comparing peptide- to vehicle-treated mice day 7 to 8 p.i. (data not shown). Furthermore, there was no difference in viral titers recovered from the brain of peptide- to vehicle-treated groups day 7 to 8 p.i. (data not shown). These results confirm and extend those previously published using an encephalitic rat model (25).

MMPs including MMP-9 but not MMP-2 are associated with an increase in neuropathology during acute HSE (55). Upon comparison of MMP-9 levels in the brain ependyma of peptide- to vehicle-treated, HSV-1-infected mice, we found no difference in total MMP-9 levels, which ranged from 50–150 pg/mg tissue. Attempts were made to recover CSF from AF-16 peptide-treated mice to compare levels of inflammatory cytokines. Whereas 3–5 μ l of CSF could be recovered in vehicle-treated mice, CSF could not be obtained in peptide-treated mice day 7 to 8 p.i., and therefore, no such comparison was possible. As such, the peptide experiment reinforces the detrimental lateral ventricle enlargement as the major pathologic predictor of mouse mortality, as evident in the similarities in the inflammatory response and viral

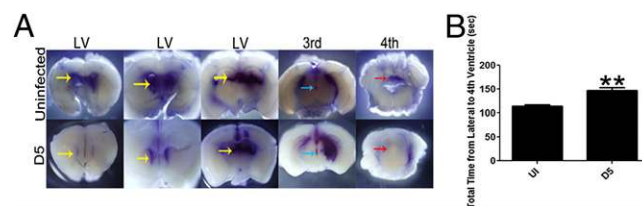


FIGURE 6. CSF is blocked from draining in the ventricle of HSE CD118^{-/-} mice. CD118^{-/-} mice were infected with 1000 PFU/eye HSV-1. Five days p.i., the stereotactic placement of Evan's blue dye was administered into the lateral ventricles. **(A)** Tissue was then fixed and imaged for Evan's blue dye. **(B)** The time it took the dye to travel from the lateral ventricle to the fourth ventricle was recorded and compared with uninfected (UI) controls. Results and images are representative of two independent experiments of three to four mice per group. Yellow arrows, lateral ventricle; blue arrow, third ventricle; red arrow, fourth ventricle. ** $p < 0.01$ comparing the encephalitic to uninfected animals. D5, Day 5.

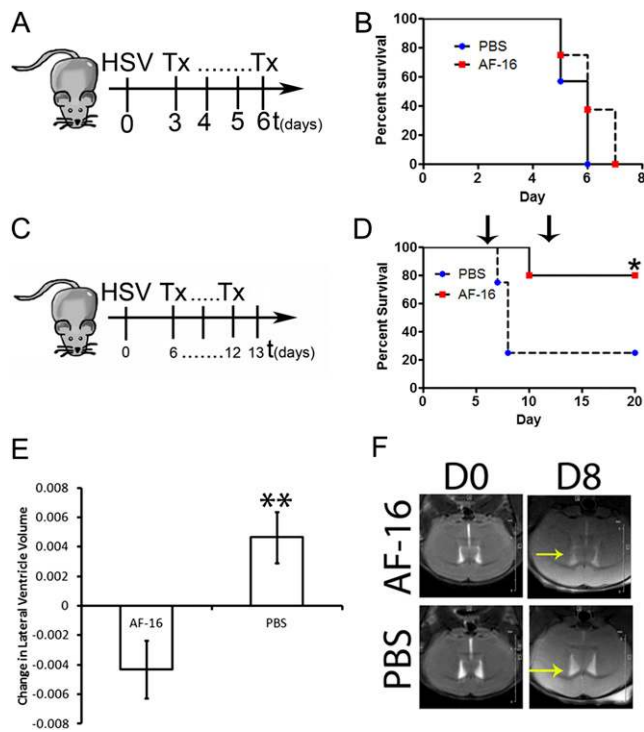


FIGURE 7. A reduction in lateral ventricle enlargement attenuates disease course in $CD118^{-/-}$ mice and significantly improves survival in WT mice infected with HSV-1. **(A)** To evaluate the effect increased CSF pressure had on mouse survival, highly susceptible $CD118^{-/-}$ mice were infected and then treated 3 d p.i. with an intranasal dose of PBS or AF-16 twice daily. **(B)** $CD118^{-/-}$ mice treated with AF-16 on average lived longer than PBS-treated controls. Results are representative of two independent experiments of a total of seven to eight mice per group. **(C–F)** WT mice were infected with 10,000 PFU/eye HSV-1 and then treated twice per day with AF-16 or PBS starting on day 6 p.i. until day 12 p.i. (indicated by black arrows). Survival **(D)** and lateral ventricle volume at day 8 **(E)** were then assessed. The graph represents two experiments of two to three mice per group per experiment. **(F)** Representative MRI scans at day 0 and day 8 p.i. expressed as mean change in volume \pm SEM (yellow arrows, lateral ventricles). $*p < 0.05$, $**p < 0.01$ comparing peptide- to vehicle-treated groups.

loads of AF-16- and PBS-treated groups despite significant differences in survival.

Surveillance of HSV-1 in the brain is due to microglia

Once the virus has infected the tissue lining the lateral ventricles in the CNS, the identification of cells that contribute in resistance against further viral progression remained elusive in WT mice. To first dissect the contribution of resident cells from infiltrating leukocytes, a mouse chimera model was employed. Five days p.i., mice with a $CD118^{-/-}$ recipient background regardless of the bone marrow (BM) source had significantly more virus in the CNS, enlarged lateral ventricles, and swollen heads compared with WT mouse recipients of $CD118^{-/-}$ or WT BM (Fig. 8A–C). We interpret these results to suggest a resident cell population such as microglia, astrocytes, and/or neurons afforded protection from HSV-1 infection within the CNS of WT mice.

The microglial cell is thought to be the innate immune cell of the CNS due to its constant surveillance of the tissue (56). In the $CD118^{-/-}$ /WT mouse chimera model, a majority of the microglia in the BS and cortex were derived from the original resident population during an uninfected or infected state (Fig. 9A–E). Specifically, prior to infection, 80–100% of microglia residing in the BS (Fig. 9B) or brain (Fig. 9C) were WT-derived in WT

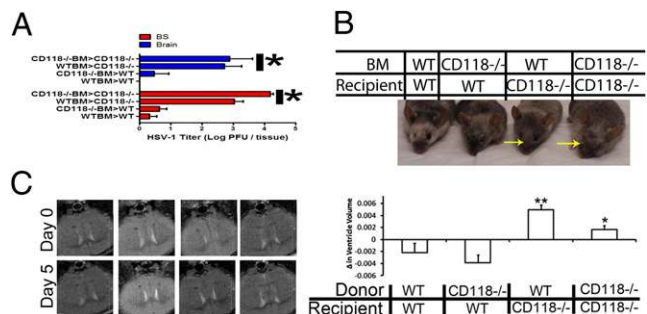


FIGURE 8. Loss of resident protection results in ventricular enlargement. To identify the cell responsible for viral containment in the CNS, a chimeric mouse model using WT and $CD118^{-/-}$ mice was developed. Mice were infected with 1000 PFU/cornea of HSV-1 and euthanized 5 d p.i. **(A)** Tissue obtained from the BS and brain cortex (brain) were collected and processed for viral titer by plaque assay. Results are presented as the mean log PFU HSV-1/ tissue \pm SEM; $n = 5$ –12/group from two independent experiments. It should be noted the resident population afforded CNS protection from HSV-1 with >2 log reduction in HSV-1 recovered regardless of the source of BM cells. **(B)** Irradiated WT mice given WT or $CD118^{-/-}$ BM were resistant to head swelling observed in irradiated $CD118^{-/-}$ mice given WT or $CD118^{-/-}$ BM. Yellow arrows indicate significant head swelling. **(C)** $CD118^{-/-}$ and WT mouse chimeras were imaged by T2-weighted imaging for ventricle enlargement following infection. By day 5 p.i., $CD118^{-/-}$ mouse recipients of WT or $CD118^{-/-}$ BM had significantly larger lateral ventricles compared with WT recipient chimeras. Results represent three independent experiments of one mouse per group per experiment. $*p < 0.05$, $**p < 0.01$ comparing WT recipient to $CD118^{-/-}$ recipient mouse chimeras.

recipients of WT or $CD118^{-/-}$ BM. By comparison, 60–100% of microglia populating the BS or brain of uninfected $CD118^{-/-}$ recipients of WT or $CD118^{-/-}$ BM were $CD118^{-/-}$ derived (Fig. 9B, 9C). Five days following infection, $>60\%$ of the microglia in the BS or brain of WT recipients of WT or $CD118^{-/-}$ BM retained the WT phenotype, whereas $>75\%$ of the microglia in the BS or brain of $CD118^{-/-}$ recipients of WT or $CD118^{-/-}$ BM were of the $CD118^{-/-}$ background (Figure 9D, 9E). Taken together with the data showing less HSV-1 recovered in the BS or brain of WT recipients of $CD118^{-/-}$ BM in comparison with $CD118^{-/-}$ recipients of WT BM (Fig. 8A), we conclude a resident CNS cell population controls HSV-1 replication within the first 5 d p.i.

To exclusively characterize the contribution of resident CNS cells (microglia, astrocytes, or neurons) in viral surveillance in a relatively native state in the absence of a leukocyte contribution, an organotypic brain-slice culture system was engineered as previously described (33) (Fig. 10A). Organotypic brain-slice cultures were established from naive WT and $CD118^{-/-}$ mice. Upon infection, the $CD118^{-/-}$ mouse organotypic cultures had significantly more virus than WT cultures, confirming a role for IFN-responsive resident cells in the CNS as well as verification that these cultures behaved similar to that seen in vivo (Fig. 10B). Specific depletion (50% loss) of microglia by clodronate liposomes in WT brain-slice cultures (Fig. 10C) resulted in a significant increase in viral titer compared with tissue slices treated with control liposomes (Fig. 10D). Clodronate liposome-mediated depletion of microglia had no apparent effect on astrocytes or neurons as measured by GFAP or NeuroD expression, respectively, suggesting microglia were the primary cell population depleted by clodronate liposome treatment and therefore responsible for the innate resistance in the brain to HSV-1 infection (Fig. 10E).

Previous reports indicated TLR-3 signaling deficiencies led to an increased susceptibility to HSE in children due to a loss of IFN

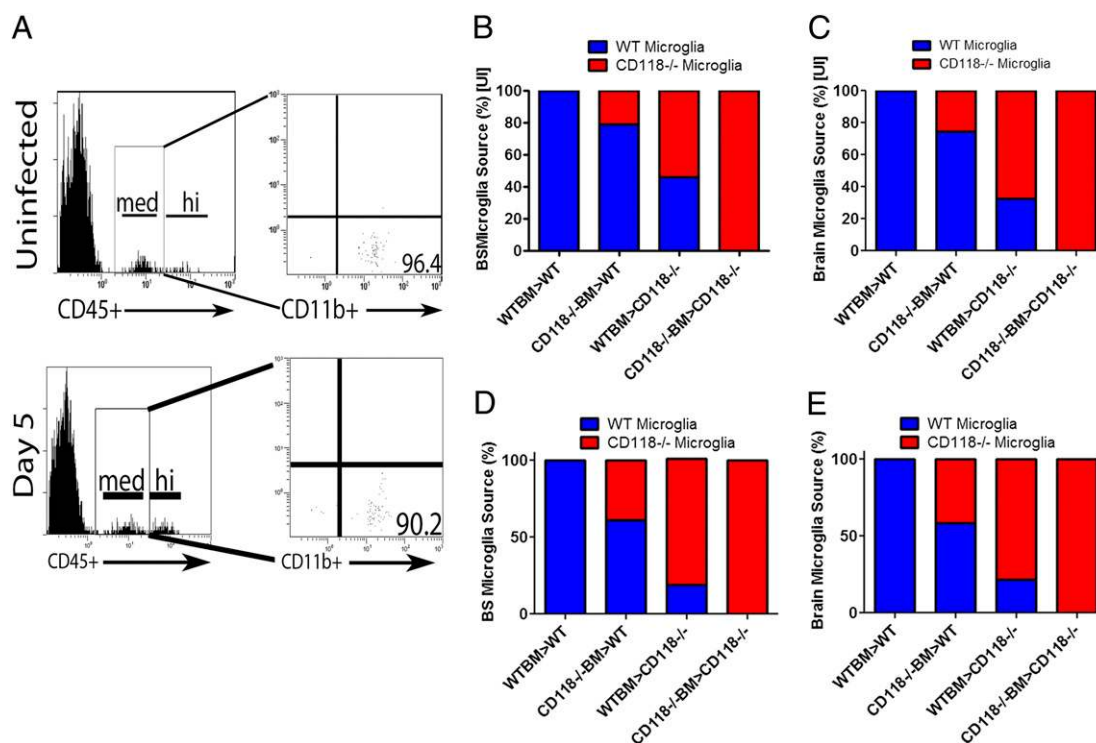


FIGURE 9. The irradiated mouse chimera is the major source of microglia in the CNS. CD118^{-/-} and WT mouse chimeras were established, and CD45^{med}-gated cells (A) obtained from single-cell suspensions of mouse brain were stained for CD11b and analyzed by flow cytometry. Greater than 90% from infected and 95% from uninfected mice of gated cells stained positive for CD11b, confirming a microglia cell population. The source of microglia was linked to the resident population in uninfected (B, C) and infected (D, E) mouse chimeras in the BS (B, D) and brain (C, E) using CD45.1 and CD45.2 gating. Histograms are representative of three independent experiments. $n = 2/\text{group/experiment}$.

production (11–13). It has also been reported a loss of this innate sensor renders astrocytes permissive to HSV infection (57). To further investigate this possibility in our model, we evaluated brain-slice cultures from TRIF-deficient (TRIF^{-/-}), MyD88^{-/-}, and WT mice for resistance to primary HSV-1 infection. HSV-1 titers were significantly elevated in the organotypic brain-slice cultures from TRIF^{-/-} mice in comparison with the WT and MyD88^{-/-} samples, whereas there was no difference comparing WT to MyD88^{-/-} groups (Fig. 10F). Furthermore, microglial cell depletion of TRIF^{-/-} mouse brain-slice cultures did not enhance susceptibility to HSV infection, suggesting the loss of microglia had no further effect on HSV containment in the absence of TRIF (Fig. 10G). Thus, TRIF-dependent pathways within microglia were critical to contain HSV-1.

Discussion

In the present article, we found TRIF-activated microglia control viral replication in the CNS of WT mice. In pediatric patients with TLR-3 signaling deficiencies (11–13), the microglial-driven, IFN- β antiviral response is likely compromised, and astrocytes are now permissive to viral infection (57). These two findings may explain the susceptibility of these children to recurrent HSV infections of the CNS. In our mouse model of HSE, we found for the first time, to our knowledge, that a loss of cilia results in a reduced capacity to move CSF through the ventricular network, leading to a functional obstruction of fluid movement. This process may be an additional mechanism for acute ventricle enlargement in diseases such as traumatic brain injury and hydrocephalus as the result of previous bacterial meningitis (7, 58). Specifically, reports have shown *Streptococcus pneumoniae*-induced meningitis results in damaged ciliated ependymal cells, reduced cilia cell beat frequency, and is a risk factor for the development of hydrocephalus

(59–61). Moreover, traumatic brain injury results in damage to ependymal cells and may result in the development of hydrocephalus (58, 62). The accumulation of CSF and subsequent acute enlargement of the lateral ventricles during HSE has never been reported in adults. We propose such an occurrence may predispose the brain and BS to catastrophic herniation, as seen with more typical cases of hydrocephalus (63). Additionally, herniations have been reported during HSE and other viral encephalitis, further suggesting viral infections of the CNS increase the probability of brain herniations due to enlargement of the lateral ventricles (64, 65). Although CNS herniations of infected mice were not readily appreciable, the rapid decline of CD118^{-/-} mice would suggest this possibility.

Anti-secretory factor, originally described as a protein in the CNS and gut (66) but also found in macrophages (67), was previously found to block cholera toxin-induced intestinal hypersecretion (66). A peptide representing the first 16 aa of the anti-secretory factor AF-16, has previously been reported to enhance HSV-1-infected rat survival, which was correlative with a drop in intracranial pressure (25). Therefore, we evaluated the potential use of this peptide in our mouse model of HSE. Consistent with the previous findings, the intranasal application of AF-16 during the acute phase of HSV-1 infection preserved survival and prevented lateral ventricle dilatation. As there is a gross CNS inflammatory response to HSV-1 infection in the brain (68) and soluble factors generated as a result of the infection including MMP-9 and TNF- α are thought to be pathologic (69) or protective (21), respectively, to the host, we investigated levels of these and other cytokines as a possible means to further explain the efficacy of the peptide. However, the obvious possible mediators that might influence the outcome of the infection were unaltered in the peptide-treated mice. Furthermore, we found no

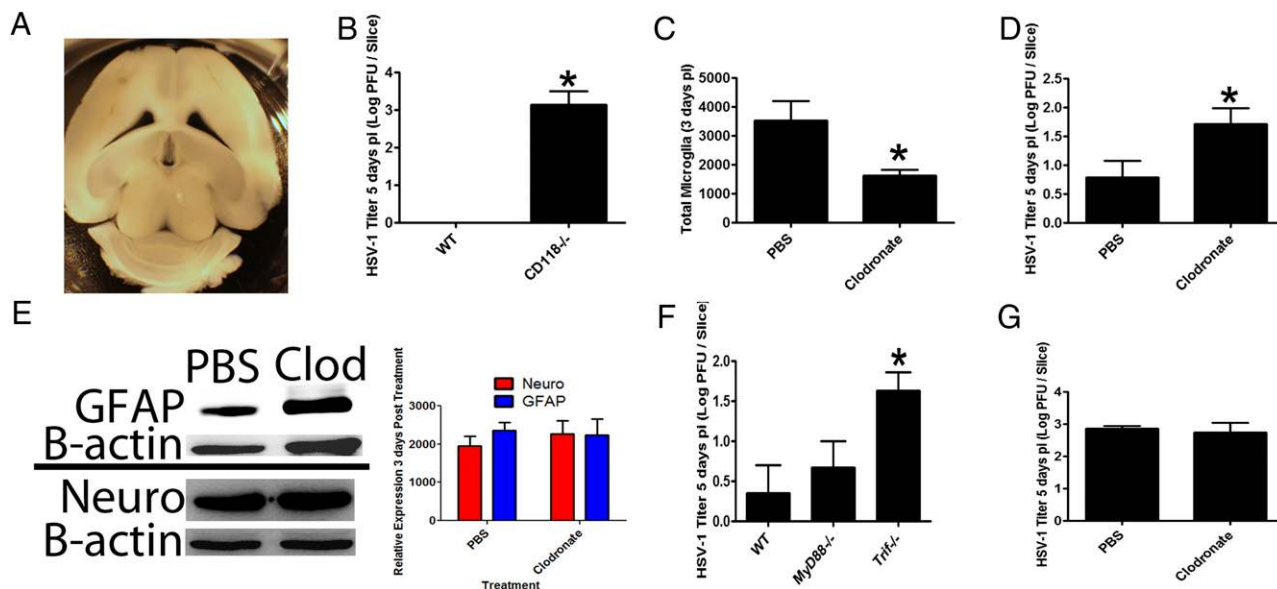


FIGURE 10. TLR-3-driven microglia responses prohibit HSV spread. **(A)** A brain-slice organotypic culture system was developed, and tissue slices were viable for at least 7 d as evident by conversion of Presto blue substrate to red (data not shown). Images are representative of two independent experiments. **(B)** WT and CD118^{-/-} brain slices were infected with 1000 PFU/slice to confirm susceptibility of CD118^{-/-} tissue. Results are a summary of two independent experiments of three to four slices per group. **p* < 0.05 comparing CD118^{-/-} to WT group. **(C)** WT tissue was subjected to specific depletion of microglia with clodronate liposomes resulted in a significant reduction in CD45^{med}CD11b⁺ cells. **p* < 0.05 comparing PBS-liposome- to clodronate-liposome-treated organotypic cultures (*n* = 6–8 slices/group). **(D)** The infection of microglia-depleted organotypic brain-slice cultures with 10,000 PFU/slice resulted in significantly (**p* < 0.05) higher viral titers (*n* = 6–8 slices/group). **(E)** Clodronate liposome and PBS liposome-treated cultures were assessed for astrocyte (GFAP) and neurons (Neuro) by Western blot analysis. A representative blot (taken from two experiments) is shown in the left panel with the summary densitometric analysis shown in the right panel. **(F)** To identify the critical innate sensor within the CNS, WT, MyD88^{-/-}, and Trif^{-/-} organotypic brain-slice cultures were infected with 10,000 PFU/slice and viral content evaluated 5 d p.i. Results are a summary of two independent experiments (*n* = 2 to 3/group/experiment). **p* < 0.05 comparing the TRIF^{-/-} to WT and MyD88^{-/-} brain slice groups. **(G)** Clodronate or PBS liposome-treated brain-slice cultures from TRIF^{-/-} mice were infected as in (F) and viral content evaluated 5 d p.i. Results are a summary of two independent experiments (*n* = 2 to 3/group/experiment) and expressed as mean log PFU HSV-1/slice.

change in leukocyte infiltration or in infectious virus content in the brain, re-emphasizing the death of the animals was unrelated to viral load or gross inflammation. Rather, it would appear survival is related to maintaining equilibrium between fluid accumulation and clearance evident by lateral ventricle enlargement in this study and intracranial pressure in previously published work (25). The results also emphasize the importance of cilia in bulk flow of CSF (70), and the loss of this fluid movement, we hypothesize, allows localized augmentation in titers of virus to occur. We propose the high viral load is able to overcome the local innate (microglia) immune response, allowing HSV access to the surrounding cortex to induce the rapidly progressive disease known as HSE.

Although gross inflammation is not, in and of itself, the driving force behind host survival, we propose CXCL10 as a potential biomarker to delineate magnitude of disease during viral encephalitis. Production of CXCL10 is a well-established finding during viral infections of the CNS (71). We found that the amount of CXCL10 detected in the CSF directly correlated with severity of infection in that WT and CD118^{-/-} HSE mouse CSF contained copious amounts of the chemokine. The fact that CSF CXCL10 levels were less in the HSE CD118^{-/-} mice compared with the HSE WT mice may be due to the time in which the samples were obtained (day 8 p.i. for WT versus day 5 p.i. for CD118^{-/-} mice) or the relationship between a functional type I IFN pathway and HSV-1-induced CXCL10 (34). We speculate CXCL10 may serve as an easily accessible predictor of inflammation severity in patients with no previous history of neuroinflammatory disease.

In conclusion, we have found in mice deficient in a functional type I IFN pathway, ocular HSV-1 infection results in the rapid

spread of the virus to the BS from the cornea. There, the virus traffics to the ependyma, a ciliated, single-cell layer lining the lateral ventricles. It is here the virus rapidly replicates, destroying the ependymal cells and, as a result, increasing fluid accumulation in the lateral ventricles and intracranial pressure that ultimately leads to irreversible brain damage and death of the host. In contrast to CD118^{-/-} mice, WT mice are capable of controlling virus replication and spread to a greater extent, although a subpopulation of animals is still prone to develop encephalitis and die. It is clear from the data presented in this study that viral levels in the ependyma and not the brain cortex correlate with severity of the disease state and that microglia are the principal resident cell source that is required for viral surveillance in a TRIF-dependent manner. Whereas it is likely TRIF activation is mediated via the TLR3 sensor, we have not formally excluded TLR4, which can also signal through TRIF (72). Likewise, TLR-independent sensors including DNA-dependent activator of IFN regulator factor and IFI16/p204 that respond to HSV-1 may also contribute to viral surveillance in the CNS and, specifically, in the ependymal region (30, 73). Ultimately, these pathways converge in the generation of type I IFNs that counter HSV-1 replication and thus would reduce tissue pathology and preserve the ependymal cell lining of the lateral ventricles. We presume the lack of a difference in type I IFN expression in the ependyma region of encephalitic compared with nonencephalitic WT mice was due to the time analyzed (day 8 p.i.) due to the clinical presentation of the animal. Earlier time points could not be predicted based on the phenotype of individual WT mice. Future work is required to more fully address this unproven hypothesis and potentially apply such findings to therapy in the human condition.

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Disclosures

The authors have no financial conflicts of interest.

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