Prolonged Microglial Cell Activation and Lymphocyte Infiltration following Experimental Herpes Encephalitis

Cristina P. Marques, Maxim C-J. Cheeran, Joseph M. Palmquist, Shuxian Hu, Stina L. Urban and James R. Lokensgard

*J Immunol* 2008; 181:6417-6426; doi: 10.4049/jimmunol.181.9.6417
http://www.jimmunol.org/content/181/9/6417

### References
This article *cites 51 articles*, 24 of which you can access for free at:
http://www.jimmunol.org/content/181/9/6417.full#ref-list-1

### Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

### Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

### Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Prolonged Microglial Cell Activation and Lymphocyte Infiltration following Experimental Herpes Encephalitis

Cristina P. Marques,* Maxim C-J. Cheeran,2*† Joseph M. Palmquist,* Shuxian Hu,* Stina L. Urban,* and James R. Lokensgard*

Experimental murine herpes simplex virus (HSV)-1 brain infection stimulates microglial cell-driven proinflammatory chemokine production which precedes the presence of brain-infiltrating systemic immune cells. In the present study, we investigated the phenotypes and infiltration kinetics of leukocyte trafficking into HSV-infected murine brains. Using real-time bioluminescence imaging, the infiltration of luciferase-positive splenocytes, transferred via tail vein injection into the brains of HSV-infected animals, was followed over an 18-day time course. Flow cytometric analysis of brain-infiltrating leukocytes at 5, 8, 14, and 30 days postinfection (d.p.i.), was performed to assess their phenotype. A predominantly macrophage (CD45<sup>high</sup>CD11b<sup>+</sup>Ly6<sup>Chigh</sup>) and neutrophil (CD45<sup>high</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup>) infiltration was seen early during infection, with elevated levels of TNF-α mRNA expression. By 14 d.p.i., the phenotypic profile shifted to a predominantly lymphocytic (CD45<sup>high</sup>CD3<sup>+</sup>) infiltrate. This lymphocyte infiltrate was detected until 30 d.p.i., when infectious virus could not be recovered, with CD8<sup>+</sup> and CD4<sup>+</sup> T cells present at a 3:1 ratio, respectively. This T lymphocyte infiltration paralleled increased IFN-γ mRNA expression in the brain. Activation of resident microglia (CD45<sup>int</sup>CD11b<sup>+</sup>) was also detected until 30 d.p.i., as assessed by MHC class II expression. Activated microglial cells were further identified as the predominant source of IL-1β. In addition, infected mice given primed immunocytes at 4 d.p.i. showed a significant increase in mortality. Taken together, these results demonstrate that intranasal infection results in early macrophage and neutrophil infiltration into the brain followed by prolonged microglial activation and T lymphocyte retention. Similar prolonged neuroimmune activation may contribute to the neuropathological sequelae observed in herpes encephalitis patients. *The Journal of Immunology, 2008, 181: 6417–6426.

Herpes simplex virus (HSV)<sup>1</sup>-1 infection of the brain produces a focal, necrotizing encephalitis characterized by severe neuroinflammation and prolonged neuroimmune activation (1). Despite antiviral therapy, 70% of herpes encephalitis patients suffer significant long-term neurological deficits (2, 3), suggesting that targeting viral replication alone may not be sufficient to prevent infection-induced brain damage. Previous studies from our laboratory using a murine model support the notion that vigorous neuroimmune responses against HSV-1 may not be sufficient to protect against fatal disease (4). It is possible that overactive neuroimmune responses may contribute to the long-term neuropathological sequelae associated with HSV-1 brain infection.

1 Herpes simplex keratitis has been demonstrated to be largely an immunopathologic condition. Athymic nude mice, which do not develop herpes simplex keratitis, develop disease following adoptive transfer of HSV-1-specific T lymphocytes (5, 6). CD4<sup>+</sup> T cells, along with corneal Langerhans cells, have been identified as critical mediators of corneal pathology (7–10). In addition, IL-2 and IFN-γ have been reported to regulate inflammation by mediating extravasation of immune cells from blood vessels into the cornea (11). Both the protective and immunopathologic roles of leukocyte trafficking into the brain during herpes encephalitis remain to be elucidated.

2 Previous in vitro studies from our laboratory have identified microglial cells (the resident brain macrophages) as a source of proinflammatory cytokines and chemokines in response to HSV-1 (12, 13). We have also shown that this chemokine production precedes peripheral immune cell infiltration into the brain (4). Given that this robust neuroimmune response fails to protect susceptible BALB/c mice, the present study was undertaken to assess the cellular profile, kinetics, function, and persistence of peripheral immune cells which traffic into the brain during herpes encephalitis.

3 Abbreviations used in this paper: HSV, herpes simplex virus; d.p.i., days p.i.

Materials and Methods

**Virus and infection**

HSV-1 strain 17syn<sup>+</sup>, a neurovirulent strain of HSV, provided by L. T. Feldman (University of California, Los Angeles, CA) was used in all experiments. The virus was propagated in rabbit skin fibroblasts (CCL68; American Type Culture Collection), sucrose purified, and titrated using standard plaque assay. Eight to 10-wk-old female BALB/c or FVB/N mice (Charles River Laboratories) were infected intranasally with 2.0 × 10<sup>3</sup> PFU/mouse.
Adoptive transfer

Spleen and lymph nodes (lumbar and inguinal) from HSV-1 primed (1 × 10^7 PFU/mouse, i.p. injection) or unprimed donor animals were collected aseptically at 7 days postpriming. Single cell suspensions of immunocytes were depleted of RBC by treatment with 0.87% ammonium chloride, washed twice, and cell viability was confirmed using trypan blue. Immune cells were harvested 7 d.p.i. Adoptive transfer of 1 × 10^5 primed or unprimed immunocytes via tail vein injection into MHC-matched, HSV-infected animals at 4 d.p.i. (6 × 10^5 PFU of strain 17syn−) or uninfected recipients was performed. Dorsal bioluminescence images of recipient mice are shown at 5 d.p.i./1 day posttransfer (p.t.), 7 d.p.i./3 d.p.t., 11 d.p.i./7 d.p.t., and 13 d.p.i./9 d.p.t. B, Signal intensity of the luciferase expression, indicative of the number of immune cells present, was quantified in the brain as photons/sec/cm² at each time point. Data are reported as mean intensities (± SEM) from at least three animals plotted over the 18-day time course. C, Extracorporeal images of the dorsal (left panel) and ventral (right panel) aspects of HSV-1-infected brains collected at 10 d.p.i./6 d.p.t. demonstrating bioluminescence signal in both the cortices and brain stems.

Bioluminescence imaging

Imaging of brightly luciferase expression in live animals was performed using an IVIS50 (Xenogen) equipped with a charge-coupled camera device, as previously described with minor modifications (14). In brief, 150 μg of D-luciferin (Gold Biotechnology) was administered to anesthetized mice by i.p. injection. Animals were imaged 5 min after D-luciferin administration and data were acquired using a 5-min exposure window. Bioluminescence imaging studies were conducted with age-matched 8- to 10-wk-old female FVB/N mice as recipients for all experiments. Isolation of brain leukocytes and FACS sorting were performed using this technique. Four treatment groups were set up for these experiments: 1) HSV-infected mice that received virus-primed immunocytes, 2) HSV-infected mice that received naive cells, 3) uninfected mice given HSV-primed immunocytes, and 4) uninfected mice given naive cells.

Isolation of brain leukocytes and FACS

Leukocytes were isolated from HSV-1-infected murine brains using a previously described procedure with minor modifications (15–18). In brief, brain tissues harvested from four to six animals were minced finely in RPMI 1640 (2 g/L D-glucose and 10 mM HEPES) and digested in 0.0625% trypsin (in Ca/Mg-free HBSS) at room temperature for 20 min. Single cell preparations from infected brains were resuspended in 30% Percoll and banded on a 70% Percoll cushion at 900 g for 10 min. Brain leukocytes obtained from the 30–70% Percoll interface were treated with FcBlock (anti-CD3/CD16 in the form of 2.4G2 hybridoma culture supernatant with 2% normal rat and 2% normal mouse serum) to inhibit non-specific Ab binding and were stained with anti-mouse immune cell surface markers for 45 min at 4°C (anti-CD45-allophycocyanin (eBioscience), anti-CD11b-FITC or anti-CD11b-APC-CY7, anti-CD4-FITC, anti-Ly6G-FITC, anti-Ly6C-FITC, anti-MHC class II-PE, anti-CD8-PE, and anti-CD3-PE-Cy7 (BD Biosciences)) and analyzed by flow cytometry. Control isotype Abs were used for all isotype and fluorochrome combinations to assess nonspecific Ab binding. Live leukocytes were gated using forward scatter and side scatter parameters on a BD FACScanto flow cytometer (BD Biosciences). Data was analyzed using FlowJo software (TreeStar). For sorting brain leukocytes, nonoverlapping populations of cells stained with anti-mouse CD45-Allophycocyanin (eBioscience), anti-mouse CD11b-FITC, and CD3e-Cy7-PE (BD Biosciences) were separated using a FACs (BD FACSaria, BD Biosciences). Total RNA isolated from sorted cell populations was analyzed by quantitative real-time RT-PCR for IFN-γ, IL-1β, and TNF-α expression.

Real-time PCR

Total RNA and DNA were extracted from brain tissue homogenates using the TRIzol reagent (Invitrogen Life Technologies). cDNA was synthesized using 1 μg of total RNA, SuperScript II reverse transcriptase (Invitrogen Life Technologies) and oligo dT₁₈ primers (Sigma-Aldrich). Real-time PCR was performed using the FullVelocity SYBR Green QPCR master mix (Stratagene) following the manufacturer’s specifications. The 25 μl final reaction volume consisted of premade reaction mix (SYBR Green I dye, reaction buffer, TaqDNA polymerase, and dNTPs), 0.3 mM each primer, and 0.5 ng cDNA in water. Reaction conditions for the Mx3000P QPCR System (Stratagene) were as follows: polymerase activation at 95°C for 5 min, 40 denaturation cycles of 95°C for 10 s, and annealing/elongation at 60°C for 30 s. Primer sequences used in the amplification of cytokines have been described previously (19). For real-time viral DNA PCR, total DNA was eluted in water and stored at −80°C until quantification using real-time PCR. Primers for HSV-1 were designed from the gene

FIGURE 1. Real-time bioluminescence imaging of luciferase-positive immune cells trafficking into HSV-1-infected brains. A, Splenocytes and lymph node cells from β-actin-luciferase transgenic FVB mice that were injected i.p. with 1 × 10^6 PFU of HSV-1 strain 17syn− (primed) or uninfected (unprimed) were harvested 7 d.p.i. Adoptive transfer of 1 × 10^6 primed or unprimed immunocytes via tail vein injection into MHC-matched, HSV-infected animals at 4 d.p.i. (6 × 10^5 PFU of strain 17syn−) or uninfected recipients was performed. Dorsal bioluminescence images of recipient mice are shown at 5 d.p.i./1 day posttransfer (p.t.), 7 d.p.i./3 d.p.t., 11 d.p.i./7 d.p.t., and 13 d.p.i./9 d.p.t. B, Signal intensity of the luciferase expression, indicative of the number of immune cells present, was quantified in the brain as photons/sec/cm² at each time point. Data are reported as mean intensities (± SEM) from at least three animals plotted over the 18-day time course. C, Extracorporeal images of the dorsal (left panel) and ventral (right panel) aspects of HSV-1-infected brains collected at 10 d.p.i./6 d.p.t. demonstrating bioluminescence signal in both the cortices and brain stems.
FIGURE 2. Macrophage and neutrophil infiltration into the brain during herpes encephalitis. Age-matched BALB/c mice were intranasally infected with $2 \times 10^5$ PFU of HSV-1 strain 17syn+. Single cell suspensions of brain tissue obtained from HSV-infected mice (3–6 animals) per time point were banded on a 70% Percoll cushion. Brain leukocytes collected at 30–70% Percoll interface were labeled with Abs specific for mouse CD45, CD11b, Ly6G, and Ly6C and analyzed using flow cytometry (Canto, BD Biosciences), with FlowJo software (Tree-Star). Representative dot plots from three to five experiments at 5 d.p.i. (A) showing distinguishable populations of cells delineated by their CD45 and CD11b expression patterns. Histograms showing differential expression of Ly6C and Ly6G among the CD45$^+$ cells were used to distinguish between macrophages (clear) and neutrophils (black). Gray plot on the histogram denotes isotype Ab binding. Representative dot plots of CD45$^+$CD11b expression at 8 (B), 14 (C), and 30 (D) d.p.i. are also presented. Pooled data indicating ratios of the various cell populations, based on a leukocyte gate, are expressed as mean ± SEM.

Results

Kinetics of peripheral leukocyte migration into the brain during herpes encephalitis

Using bioluminescent imaging of live animals, we examined the kinetics of leukocyte infiltration into the brain longitudinally in both HSV-1-infected and uninfected mice. Within 24 h of adoptive transfer, luciferase-positive cells were detected in the spleen, cervical lymph nodes, and inguinal lymph nodes of all animals regardless of their infection status. Movement of leukocytes into the brain was observed within 24 h after transfer, but was detected only in HSV-1-infected animals, receiving either primed or unprimed immune cells (Fig. 1A). Intensity of the luciferase signal in the brain was highest among HSV-1-infected mice that received immunocytes from primed animals. This signal peaked between 7 and 11 d.p.i., and remained noticeably elevated over the other groups until 13 d.p.i. (Fig. 1C). By 9 days post transfer, the overall signal generated by the luciferase-positive splenocytes markedly decreased (Fig. 1B). Extracorporeal analysis of bioluminescence signal from infected mouse brains showed highest luminescence signals, indicative of leukocyte infiltration into the brain, in the rostral/ventral cortical region and in the brain stem (Fig. 1C).

Early macrophage and neutrophil infiltration into the brains of HSV-infected mice

To identify cell types involved in the neuroimmune response to HSV-1 brain infection, leukocytes were isolated from the brains of infected BALB/c mice at 5, 8, 14, and 30 d.p.i. These isolated cells were subsequently immunostained using markers that distinguished microglia, T lymphocytes, macrophages, and neutrophils (CD45, CD11b, CD3, Ly6C, MHC class II, and Ly6G). At 5 d.p.i., ~65% of the total brain leukocytes gated were identified as resident microglia expressing a CD45$^+$CD11b$^+$ phenotype, and 20% were found to be CD45$^+$, indicative of peripheral myeloid cells (vs resident brain myeloid cells, Fig. 2A). Among the CD45$^+$ population, a larger proportion of the leukocytes expressed CD11b$^+$ (14.1 ± 2.5%). This CD11b$^+$ cell population increased up to 8 d.p.i. (36.8 ± 7.2%, Fig. 2B), but waned among the surviving animals at 14 and 30 d.p.i. (8.5 ± 0.5% and 1.9 ± 0.1%, respectively; Fig. 2, C and D). At 5 d.p.i., approximately half (48.6 ± 2.5%) of the CD45$^+$ cells expressed a macrophage phenotype (CD45$^+$CD11b$^+$Ly6C$^+$), and 15.2 ± 2.4% were identified as neutrophils (CD45$^+$CD11b$^+$Ly6G$^+$MHCII$^+$, Fig. 2A and 3D). In addition, at 5 d.p.i., some of the CD45$^+$Ly6C$^+$ cells (1.1 ± 0.3% of gated cells) also expressed the NK cell marker, CD49b.
Influx of CD45\textsuperscript{high}CD11b\textsuperscript{+} \textit{brains}

Persistent T lymphocyte infiltration into HSV-infected murine brains represented the bulk of CD45\textsuperscript{high} infiltrates at 14 and 30 d.p.i. (Fig. 3). CD3\textsuperscript{+} T lymphocytes, peaked at 14 d.p.i. and, although reduced in numbers, neutrophils (5.7 ± 1.6\%) were also present at this time point (Fig. 3B). By 14 d.p.i. less than 10\% of the gated cells (8.5 ± 0.6\%) were CD45\textsuperscript{high}CD11b\textsuperscript{−} and this cell population reduced considerably (1.9 ± 0.7\%) by 30 d.p.i. (Fig. 2, C and D).

**Persistent T lymphocyte infiltration into HSV-infected murine brains**

An influx of CD45\textsuperscript{high}CD11b\textsuperscript{−} cells was also detected during the course of herpes encephalitis. Interestingly, these CD11b\textsuperscript{−} cells, largely composed of CD3\textsuperscript{+} lymphocytes, peaked at 14 d.p.i. and represented the bulk of CD45\textsuperscript{high} infiltrates at 14 and 30 d.p.i. (Fig. 2, C and D, and 3B). The infiltration of CD3\textsuperscript{+} cells increased in HSV-1-infected brains from 3.2 ± 0.2\% of total gated cells at 5 d.p.i. to 13.7 ± 1.5\%, 53.5 ± 1.5\%, and 18.8 ± 1.0\% at 8, 14, and 30 d.p.i., respectively (Fig. 3A). CD3\textsuperscript{+} lymphocytes constituted 14.9 ± 2.8\%, 19.7 ± 1.0\%, 62.6 ± 2.4\%, and 72.9 ± 0.7\% of the CD45\textsuperscript{high} infiltrating brain leukocyte population at 5, 8, 14, and 30 d.p.i. (Fig. 3B).

The absolute numbers of brain leukocytes isolated from HSV-1-infected brains also increased over time, with peak numbers observed at 14 d.p.i. At 14 and 30 d.p.i. a significant proportion of the brain leukocytes were T cells (Fig. 3C). Further analysis of the T lymphocyte population at 14 and 30 d.p.i. indicated that CD8\textsuperscript{+} lymphocytes outnumbered CD4\textsuperscript{+} cells. At 14 d.p.i. 49.9 ± 1.8\% of the CD3\textsuperscript{+} cells were of the CD8 phenotype, whereas at 30 d.p.i., CD8 cells outnumbered CD4\textsuperscript{+} cells three to one, with CD8 cells forming 73.8 ± 2.0% of CD3\textsuperscript{+} cells (Fig. 3D). The presence of T lymphocytes in the cortex, subcortex, and cerebellum of HSV-1-infected brains was also examined by assessing TCR \(\beta\)-chain mRNA expression using real-time RT-PCR. TCR \(\beta\)-chain mRNA was present in the highest amount in the cerebellum where it peaked at 7 d.p.i. and remained elevated through 30 d.p.i. (Fig. 3E). By 56 d.p.i., the levels of TCR expression levels in virus-infected brains returned to those seen in uninfected animals.

The sustained presence of T lymphocytes in the brains of HSV-infected animals was observed at time points where neither infectious virus nor viral gene expression products were detectable. Infectious virus was detected by plaque assay in various brain regions as early as 3 d.p.i., titers peaked by 9 d.p.i., and infectious virus was undetectable by 16 d.p.i. (Fig. 3D).
4A). The expression of viral glycoprotein B, analyzed by real-time RT-PCR, followed a similar pattern and was undetectable at 14 or 30 d.p.i., a time point when T lymphocytes were present in the brain (Fig. 4B).

**Lymphocytes are the source of IFN-γ in HSV-infected brains**

Given the persistence of T lymphocytes in the CNS of HSV-infected animals, we went on to investigate the function of these cells by assessing IFN-γ mRNA expression. To determine the cellular source of IFN-γ production, brain leukocytes isolated at 7 d.p.i. were sorted into four distinct populations (A-D) using FACS with anti-mouse CD45 and CD11b Abs (Fig. 5A). Post-sort analysis indicated that the separated populations were >93% positive for their associated surface markers. The sorted populations were then individually assayed for IFN-γ mRNA expression using quantitative real-time RT-PCR. In these experiments, elevated levels of IFN-γ mRNA expression were detected specifically in the CD45highCD11b− cell population, a population previously identified as T lymphocytes (Fig. 5B). To further evaluate IFN-γ expression at the protein level, macrophages were separated from the other brain leukocytes based on their ability to adhere to gelatin-coated plates. Adherent and nonadherent cells were analyzed by ELISPOT assay to enumerate IFN-γ production in the brain. Confirming the data obtained by real-time PCR from FACS sorted populations, 5-fold greater numbers of IFN-γ producing cells were counted in the nonadherent cell population. Of the 5.5 × 10⁵ cells isolated from each brain at 7 d.p.i., 115 IFN-γ producing spots were counted for every 10⁴ nonadherent cells (vs 21 per 10⁴ adherent cells). In comparison, 6.5 IFN-γ producing spots were counted per 10⁴ nonadherent cervical lymph node cells obtained from HSV-1-infected animals (Fig. 5C). IFN-γ spots were not observed in the naive cervical lymph node cells. In subsequent time-course experiments, total RNA extracted from the cortex, subcortex, and cerebellum of HSV-infected mice was analyzed for IFN-γ expression by real-time RT-PCR. IFN-γ levels in all three brain regions peaked at 7 d.p.i., with levels reaching 50–150-fold greater than uninfected controls in the subcortex and cerebellum. These expression levels remained elevated until 28 d.p.i. (1.5-fold) with levels ranging between 1.5–10-fold over those observed in uninfected brain (Fig. 5D).

**Persistent microglial cell activation in HSV-1-infected brains**

Previous in vitro studies from our laboratory suggest that resident microglial cells are key mediators of the neuroimmune response to HSV (12, 13). To determine whether microglial cells are activated during herpes encephalitis in vivo, the kinetics of MHC class II expression, as an indicator of activation, during the course of HSV-1 infection was assessed using flow cytometry. In these experiments, MHC class II expression on CD45intCD11b− cells was evaluated at 5, 8, 14, and 30 d.p.i. (Fig. 6). Although resting microglia (CD45highCD11b−) in the uninfected brain, or at 5 d.p.i., expressed very little MHC class II, this molecule was induced by 8 d.p.i., and was present on 79.2 ± 3.6% of the cells. MHC class II expression on microglial cells remained highly elevated at 14 and 30 d.p.i. (Fig. 6).

To further understand the functional significance of this microglial cell activation, leukocytes isolated from HSV-infected murine brains (8 d.p.i.) were sorted into four distinct populations (A-D) by FACS, as described above (Fig. 5A). Expression of TNF-α and IL-1β mRNA among the different sorted cell populations was analyzed by quantitative real-time RT-PCR. Although, TNF-α mRNA was detected in both the CD45highCD11b− and CD45highCD11b− cell populations, the highest expression was found in the CD45intCD11b− population, which was previously shown to be predominantly macrophages (Fig. 7A). In contrast, IL-1β mRNA expression was highest in the CD45intCD11b− microglial cell population (Fig. 7B). TNF-α protein production by adherent brain leukocytes was 5-fold greater (average of 1025 spots per 10⁴ cells) than from the nonadherent cell population (201 spots per 10⁴ cells; Fig. 7C). TNF-α was also higher among the adherent cell population from spleen and cervical lymph node of

---

**FIGURE 4.** Kinetics of viral replication and gD expression during herpes encephalitis. A, Titers of infectious virus in cell-free homogenates of cortex, subcortex, and cerebellum obtained at the indicated times p.i. (two animals/time point) were determined using plaque assay. Data are expressed as PFU/gram of tissue. B, Total RNA extracted from cortex, subcortex, and cerebellum of three to five mice/time point was reverse transcribed and examined for the expression of gD mRNA. Mean RNA transcript levels normalized to HPRT expression from three to five animals per time point are presented.

**FIGURE 5.** A, FACS analysis for CD45 and CD11b expression from sorted populations of HSV-1-infected brain at 7 d.p.i., 115 IFN-γ producing spots were counted per 10⁴ nonadherent cells (vs 21 per 10⁴ adherent cells). In contrast, every 10⁴ nonadherent cells were analyzed by ELISPOT assay to enumerate IFN-γ production in the brain. Confirming the data obtained by real-time PCR from FACS sorted populations, 5-fold greater numbers of IFN-γ producing cells were counted in the nonadherent cell population. Of the 5.5 × 10⁵ cells isolated from each brain at 7 d.p.i., 115 IFN-γ producing spots were counted for every 10⁴ nonadherent cells (vs 21 per 10⁴ adherent cells). In comparison, 6.5 IFN-γ producing spots were counted per 10⁴ nonadherent cervical lymph node cells obtained from HSV-1-infected animals (Fig. 5C). IFN-γ spots were not observed in the naive cervical lymph node cells. In subsequent time-course experiments, total RNA extracted from the cortex, subcortex, and cerebellum of HSV-infected mice was analyzed for IFN-γ expression by real-time RT-PCR. IFN-γ levels in all three brain regions peaked at 7 d.p.i., with levels reaching 50–150-fold greater than uninfected controls in the subcortex and cerebellum. These expression levels remained elevated until 28 d.p.i. (1.5-fold) with levels ranging between 1.5–10-fold over those observed in uninfected brain (Fig. 5D).

**FIGURE 6.** Persistent microglial cell activation in HSV-1-infected brains. Experimental design (A) and flow cytometric plots (B) showing the kinetics of MHC class II expression on microglial cells in vivo. In these experiments, MHC class II expression on CD45intCD11b− cells was evaluated at 5, 8, 14, and 30 d.p.i. (Fig. 6). Although resting microglia (CD45highCD11b−) in the uninfected brain, or at 5 d.p.i., expressed very little MHC class II, this molecule was induced by 8 d.p.i., and was present on 79.2 ± 3.6% of the cells. MHC class II expression on microglial cells remained highly elevated at 14 and 30 d.p.i. (Fig. 6).

**FIGURE 7.** Expression of TNF-α and IL-1β mRNA among the different sorted cell populations was analyzed by quantitative real-time RT-PCR. Although, TNF-α mRNA was detected in both the CD45highCD11b− and CD45highCD11b− cell populations, the highest expression was found in the CD45intCD11b− population, which was previously shown to be predominantly macrophages (Fig. 7A). In contrast, IL-1β mRNA expression was highest in the CD45intCD11b− microglial cell population (Fig. 7B). TNF-α protein production by adherent brain leukocytes was 5-fold greater (average of 1025 spots per 10⁴ cells) than from the nonadherent cell population (201 spots per 10⁴ cells; Fig. 7C). TNF-α was also higher among the adherent cell population from spleen and cervical lymph node of...
HSV-1-infected animals (45 and 48.5 spots per 10^4 cells) compared with uninfected animals (12 and 12.5 spots per 10^4 cells, respectively).

Increased mortality following adoptive transfer of activated immune cells

In the course of performing the bioluminescence imaging experiments described above (Fig. 1), we observed that mortality among HSV-infected mice that had received primed immune cells via tail vein injection at 4 d.p.i. was markedly higher than those receiving unprimed (naive) immunocytes (data not shown) or no cells. To further investigate this unexpected mortality in animals receiving primed immune cells, the experiment was repeated using groups of BALB/C mice. In this study, HSV-1 primed, MHC-matched immune cells (1 x 10^7/animal) given to infected BALB/C mice at 4 d.p.i. increased the mortality rate from 42% in control mice (without splenocytes, n = 12) to 90% in animals receiving HSV-primed immunocytes.

**FIGURE 5.** Expression of IFN-γ mRNA by brain-infiltrating CD45^hi^CD11b^−^ leukocytes during herpes encephalitis. A. Single cell suspension of brain tissue obtained from HSV-infected mice (five animals, 7 d.p.i.) were banded on a 70% Percoll cushion. Brain leukocytes banded at the 30–70% Percoll interface were collected, labeled with mouse Abs specific for CD45 and CD11b, and sorted into four populations using FACS. A, CD45^−^CD11b^−^ non-myeloid cells; B, CD45^hi^CD11b^−^ predominantly lymphocytes; C, CD45^hi^CD11b^+^ are infiltrating macrophages or neutrophils; and D, CD45^int^CD11b^+^ the resident brain macrophage, microglial cells. B. Total RNA extracted from each of the separated populations (A through D) was analyzed by real-time RT-PCR for expression of IFN-γ. Relative transcript levels in cells isolated from brain tissues of five animals normalized to HPRT expression are presented. C. IFN-γ production by leukocytes isolated from the cervical lymph nodes (LN) and brain (BMNC) of HSV-1-infected mice was analyzed by ELISPOT assay (R&D Systems). Isolated leukocytes, separated into adherent and nonadherent cells based on their property to adhere to gelatin-coated plastic, were assayed overnight for IFN-γ production. Data presented are average spots counted from two pooled samples using two animals each at 7 d.p.i. D. Relative IFN-γ mRNA expression levels in the brains of HSV-1-infected animals were analyzed by real-time RT-PCR at the indicated time points. IFN-γ expression levels, in the cortex, subcortex, and cerebellum of infected mice, normalized to the housekeeping gene HPRT-1 were compared with those of sham-infected controls. Average fold induction values calculated from three to seven mice per time point, are presented.
The immune cells transferred into recipient mice were composed of 40% T lymphocytes (CD3), with CD4 and CD8 cells comprising 60 and 35%, respectively, and 4% CD11b cells. To exclude the immune cell preparation as a source of additional infectious virus, splenocyte preparations used in the adoptive transfer experiments were analyzed by plaque assay and quantitative real-time quantitative PCR for infectious virus and viral gene expression or viral DNA, respectively. These splenocyte preparations were found to be negative for infectious virus, gD mRNA, and viral DNA.

\[ n = 10, p = 0.00928 \text{ log rank test; Fig. 8.} \]

The immune cells transferred into recipient mice were composed of 40% T lymphocytes (CD3), with CD4 and CD8 cells comprising ~60 and ~35%, respectively, and ~4% CD11b cells. To exclude the immune cell preparation as a source of additional infectious virus, splenocyte preparations used in the adoptive transfer experiments were analyzed by plaque assay and quantitative real-time quantitative PCR for infectious virus and viral gene expression or viral DNA, respectively. These splenocyte preparations were found to be negative for infectious virus, gD mRNA, and viral DNA.

\[ n = 10, p = 0.00928 \text{ log rank test; Fig. 8.} \]
Discussion

The present study clearly demonstrates that HSV-1 brain infection induces neuroimmune responses which persist in the absence of detectable virus replication. Early during infection, immune responses in the brain are dominated by the influx of macrophages and neutrophils, which remain the prominent component of the cellular infiltrate until 8 d.p.i. In addition to macrophages and neutrophils, the infiltrating cell profile at 8 d.p.i. included T lymphocytes, which become the predominant leukocyte infiltrate at 14 d.p.i. This lymphocytic infiltration into the brain was largely composed of CD8+ T cells, and persisted in the brains of surviving mice until 30 d.p.i., at a time when neither infectious virus nor viral replication products could be detected. The presence of lymphocytosis was corroborated by elevated levels of TCR β-chain mRNA transcription in the cerebellum and correlated with expression of IFN-γ. Furthermore, evidence for long-term activation of resident microglia was demonstrated by increased MHC class II expression on CD45+CD11b− cells.

Early neutrophil and macrophage responses play a critical role in the pathogenesis of HSV-1 infection. It has previously been shown that macrophages and neutrophils are confined to infectious foci at the trigeminal nerve roots and the olfactory bulbs, which are sites of viral entry into the brain (21). In contrast, reactive microglial cells show a more widespread distribution and remain activated for several weeks p.i. (4, 21). In the present study, neutrophil infiltration was the highest at 5 d.p.i. and decreased as the infection progressed. A biphasic neutrophil infiltration response has been reported during HSV-1 infection of the cornea, which peaks quickly after infection, wanes, and then re-emerges later during the infection (22). This neutrophil response is known to both inhibit viral replication and induce corneal pathology (22, 23). Similar results have been reported with macropage responses to ocular HSV-1 infection. Depleting macrophages early in the infection had a profound effect on viral replication and Ag presentation (24), but depleting them later in infection reduced the pathology without effecting viral clearance (25). It is clear that neutrophils and macrophages provide the first leukocyte response to HSV-1 brain infection, which is supported by subsequent infiltration of T lymphocytes.

In the present study, peak lymphocyte infiltration was seen at 14 d.p.i. About half of the infiltrating CD3+ T cells at 14 d.p.i. were CD8+ lymphocytes, whereas >70% of the lymphocytes were CD8+ at 30 d.p.i. It has been shown that CD4+ T cells are necessary to control virus replication at mucosal and nonneural sites and CD8+ T cells are essential for preventing a lethal infection within the CNS (26, 27). The HSV-1 corneal infection model provides evidence implicating a role for T lymphocytes in herpes keratitis (7–10, 28). The continued detection of lymphocytes, primarily CD8+ cells in the brain is suggestive of an ongoing neuroimmune response as well. Both lytic and IFN-mediated mechanisms have been proposed to explain CD8 T cell function in the HSV-1-infected brain (29, 30). It has been postulated that the ability of CD8 cells to produce IFN and lyse infected cells are both important mechanisms of defense early during the infection. Delay in the infiltration of CD8 cells may result in increased mortality (29). The implications for the persistence of CD8 lymphocytes in the brain are under investigation.

The lymphocyte-containing subpopulation of infiltrating cells was the major source of IFN-γ in the brain. The kinetics of IFN-γ mRNA expression are intriguing given reports that IFN-γ production by virus-specific CD8+ lymphocytes is induced by Ag exposure. Lymphocytes rapidly produce IFN-γ upon Ag stimulation and also rapidly cease production when contact with Ag is disrupted (31–33). In this study, IFN-γ mRNA expression analysis revealed an increase in expression in the cerebellum at 6 d.p.i., followed by a decrease by 14 d.p.i. This pattern correlates with data that peak infectious virus titers are seen between 7 and 9 d.p.i. and the virus can no longer be detected at 16 d.p.i. It has been shown that IFN-γ is critical in keeping the virus latent in the ganglion (34). Although the issues of viral latency in the brain parenchyma are still speculative, these data provide precedent for a persistent lymphocyte response in the absence of infectious virus.

In addition to persistent lymphocyte infiltration, our study also demonstrated long-term activation of resident microglia. Up-regulated MHC class II expression on microglial cells was observed until 30 d.p.i. It is well known that IFN-γ induces MHC class II expression, but it is important to note that the turnover of these molecules on resident microglia may be slow and may persist in the absence of IFN-γ (35). Our laboratory has identified HSV-infected microglia as key mediators of neuroimmune responses via production of proinflammatory cytokines and chemokines (12). In this study, we show that while the infiltrating macrophage population is the major source of TNF-α, microglial cells express dramatically increased levels of IL-1β during the acute phase of infection. A synergistic effect of TNF-α and IFN-γ on the up-regulation of endothelial cell adhesion molecules has been reported (36). The dramatic increase in both TNF-α and IFN-γ mRNA expression, from macrophages and T lymphocytes, holds implications for effects on the blood-brain barrier which may also exacerbate brain inflammation. Recent investigations into the role of TNF-α and IL-1β in the neuropathogenesis of HSV-1 infection demonstrate that although wild-type C57BL/6 mice are resistant to HSV infection, IL-1β and/or TNF-α knock out mice are highly vulnerable to fatal brain disease (37, 38).

Although the immune system serves to both protect and defend the host from invading pathogens, uncontrolled inflammatory responses can be deleterious. The synergistic effect of TNF-α and IFN-γ is also known to exacerbate NO-induced neurodegeneration and demyelination in murine brains (39). Studies exploring the role of macrophages in the pathogenesis of HSV hepatitis suggest that overproduction of free radicals in combination with cytokines, such as TNF-α, IL-6, and IFN-γ may result in hepatic cell apoptosis (40). The levels of inflammatory cytokines in the cerebrospinal fluid, but not viral load in the CNS, are good correlates for clinical severity of HSV-1 encephalitis (41, 42). A recent study in a mouse model of HSV-1 encephalitis reports that the early inflammatory response, composed largely of macrophages and neutrophils, induced widespread damage in the brainstem and contributed to HSV-1-induced mortality (43). Reports of HSV-1 encephalitis recurrence, after acyclovir treatment, and in the absence of detectable viral DNA provide more supporting evidence for the role of the immune response in HSV-1 brain disease (44–48). Regardless, about two-thirds of the patients with herpes encephalitis given antiviral treatment suffer significant long-term neurological impairment. Studies examining the effects of glucocorticoids on the progression of HSV-1 encephalitis has shown that glucocorticoid treatment 3 days after intranasal infection significantly increases the survival rate of HSV-infected mice, whereas those treated with the same agent concomitant with viral infection had severe neuronal damage leading to increased mortality (49). This notion is further supported by evidence from a recent clinical case report where a short course of high-dose steroid therapy resulted in a remarkable improvement.
of severe herpes encephalitis-associated neurologic deterioration despite appropriate antiviral therapy and a decreasing viral load in cerebrospinal fluid (50). Furthermore, recent studies investigating the role of CXCR3 (CXCR3 ligands that are known potent chemok�ractants for activated T cells) in the progression of herpes brain infection, showed greatly reduced mortality in CXCR3−/− mice is associated with decreased CNS inflammation, compared with wild-type animals (51). Further studies to evaluate the extent and effects of prolonged neuroimmune activation are necessary to determine its contribution to the long-term neurological sequelae following HSV-1 encephalitis.

While it remains unknown how immune responses contribute to tissue damage in HSV-induced neuropathies, this report clearly demonstrates that inflammation persists long after viral clearance. Other animal models of HSV infection have indicated that activated immune cells and cytokine production persist for a prolonged period of time in the trigeminal ganglion, which suggests similar long-term immune activation, presumably to control viral replication and reactivation events (24). The dramatically increased mortality of mice given primed splenocytes suggests that the persistent neuroimmune activation identified in the present study could be detrimental as well as beneficial.

Acknowledgments

We thank Rajesh Nair for sharing technical expertise in flow cytometry and Paul Marker for assistance in sorting brain leukocyte populations.

Disclosures

The authors have no financial conflict of interest.

References


Downloaded from http://www.jimmunol.org by guest on July 27, 2017


