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Role of Specific Innate Immune Responses in Herpes Simplex Virus Infection of the Central Nervous System


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Herpes simplex virus 1 (HSV-1) causes a spectrum of disease, including herpes labialis, herpes keratitis, and herpes encephalitis, which can be lethal. Viral recognition by pattern recognition receptors plays a central role in cytokine production and in the generation of antiviral immunity. The relative contributions of different Toll-like receptors (TLRs) in the innate immune response during central nervous system infection with HSV-1 have not been fully characterized. In this study, we investigate the roles of TLR2, TLR9, UNC93B1, and the type I interferon (IFN) receptor in a murine model of HSV-1 encephalitis. TLR2 is responsible for detrimental inflammatory cytokine production following intracranial infection with HSV-1, and the absence of TLR2 expression leads to increased survival in mice. We prove that inflammatory cytokine production by microglial cells, astrocytes, neutrophils, and monocytes is mediated predominantly by TLR2. We also demonstrate that type I IFNs are absolutely required for survival following intracranial HSV-1 infection, as mice lacking the type I IFN receptor succumb rapidly following infection and have high levels of HSV in the brain. However, the absence of TLR9 does not impact survival, type I IFN levels, or viral replication in the brain following infection. The absence of UNC93B1 leads to a survival disadvantage but does not impact viral replication or type I IFN levels in the brain in HSV-1-infected mice. These results illustrate the complex but important roles that innate immune receptors play in host responses to HSV-1 during infection of the central nervous system.

The earliest interactions between viruses and host cells are critical to determining the outcome of infections. These interactions can lead to the production of type I interferon (IFN) proteins, including IFN-α and IFN-β, which control virus replication. Viruses also trigger host cells to produce cytokines and chemokines that are essential for the development of T cells and antibodies that provide long-term defense against the invaders. At the same time, however, cytokines and chemokines can drive inflammation that may lead to localized tissue damage and/or systemic circulatory collapse and death. Recent work in our laboratories has focused on the role of innate immune pattern recognition receptors in herpes simplex virus (HSV) pathogenesis and immunity (13, 14, 24). Mammalian cells sense DNA viruses via several distinct classes of pattern recognition receptors. These include the Toll-like receptors (TLRs); the RNA helicases RIG-I and MDA5 (also known as the RIG-I-like receptors [RLRs]); NOD-like receptors (NLRs), particularly NLRP3 (also known as cryopyrin or NALP3); the AIM2 inflammasome; and the pyrin and HIN200 domain-containing (PYHIN) protein IFI16 (22). Several distinct pattern recognition receptors have been implicated in HSV-induced cytokine production (12–14, 17, 20, 21, 24, 28). However, the cells and cognate receptors responsible for cytokine responses in HSV-1 infection in animals are not fully defined. It is also unclear which cells and receptor pathways contribute to protective versus destructive responses in HSV encephalitis.

We and others have demonstrated that HSV-1 can trigger TLRs to induce cytokine production (1, 14, 24, 25). TLR2-dependent signals play a role in disease following HSV-1 infection and the development of lethal encephalitis following intraperitoneal injection of virus (14). Recognition of HSV genomic DNA has also been well described, as HSV-1 and HSV-2 stimulate cytokine production in plasmacytoid dendritic cells (DC) in a TLR9-dependent manner (12, 17) and HSV-1 induces cytokines, including IFN-β, via the PYHIN protein IFI16 (28). TLR9 has been reported to be critical for early type I IFN responses following HSV infection in vivo (17). HSV-1 replication also leads to production of double-stranded RNA (dsRNA) intermediates (10), and a role for RIG-I-like helicases in type I IFN production during HSV-1 infection has been investigated previously (20). UNC93B1 is an endoplasmic reticulum (ER)-resident membrane protein that is required for signaling via TLR3, TLR7, and TLR9 in that it permits these TLRs to translocate from the ER to the endolysosomal compartment (4, 11). Deficiency of UNC93B1 is associated with sporadic herpes encephalitis in humans, which appears to be related to impairment of type I and type III IFN cellular responses following viral infection (6).

Because the published data with regard to innate immune recognition of HSV-1 are complex, we sought to clarify the mechanisms of protective and destructive host responses, first in vivo by inoculating virus directly into the brain and then in vitro by using individual types of cells. We present evidence that TLR2 and the type I IFN receptor both play significant roles in survival following central nervous system (CNS) infection with HSV-1. Tabeta et al. identified a mutant mouse strain, called the triple deficient or “3d” mouse, with altered function of UNC93B1 (27). We find that 3d mice demonstrate a mild but statistically significant survival dis-
advantage compared to wild-type mice infected with HSV-1; however, 3d mice have viral titers and type I IFN levels similar to those in wild-type mice. TLR2 knockout, TLR9 knockout, and TLR2/ TLR9 double knockout mice all have intact type I IFN signaling and can control virus replication in the brain. Macrophages, astrocytes, microglial cells, and neutrophils produce inflammatory cytokines in a predominantly TLR2-dependent manner, rather than a TLR9-dependent manner, following infection with HSV-1. Our studies indicate the importance of non-TLR-driven type I IFN signaling in the brain and highlight the importance of TLR2 for driving inflammation in the brain.

MATERIALS AND METHODS

Mice. TLR2 and TLR9 knockout mice were originally obtained from S. Akira, Osaka University (Osaka, Japan), and backcrossed to C57BL/6 mice. TLR2/TLR9 double knockout mice were subsequently bred at the University of Massachusetts Medical School. IFN-α/IFN-β receptor (IFN-α/IFN-βR) knockout mice backcrossed to C57BL/6 mice were provided by J. Sprent (Scripps Research Institute, La Jolla, CA). UNC93B1 mutant (3d) mice were generated by B. Beutler (Scripps Research Institute, La Jolla, CA). Age-matched, wild-type control C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were bred and housed in the animal facility at the University of Massachusetts Medical School. Experimental protocols involving animals were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Inoculation of mice. HSV-1 strain 7134R, a derivative of HSV-1 KOS, was used for inoculations of mice. 7134R is the rescued virus derived from 7134 by introduction of the wild-type ICp0 gene. Mice 4 to 6 weeks old were inoculated intracranially (i.c.) at the confluence of sinuses with 30 μl of virus (3 × 10⁴ PFU diluted in sterile, endotoxin-free saline). Mice were monitored daily for the development of encephalitis (seizures and hunched posture), as well as for symptoms of generalized illness (ruffled fur and hunched posture).

Cell preparation. Peritoneal exudate cells (PEC) were isolated as described previously (14). Primary brain mixed glial cultures were prepared as described previously (31). Murine peripheral blood leukocytes were prepared from heparinized blood treated with Gentra red blood cell (RBC) lysis solution (Qiagen, Valencia, CA) at a ratio of 3:1 by volume for 10 min at room temperature. Cells were washed twice and then seeded at 2 × 10⁶ cells/well in a 96-well plate in Dulbecco modified Eagle medium (DMEM)—10% fetal calf serum (FCS) (HyClone, Logan, UT) with granulocyte colony-stimulating factor (G-CSF; PeproTech, Rocky Hill, NJ) to maintain viability and with granulocyte-macrophage colony-stimulating factor (GM-CSF; ebioScience, San Diego, CA) for priming.

Cytokine measurement. Murine monocytic chemotactrant protein 1 (MCP-1) and interleukin-6 (IL-6) from cell culture supernatants were measured using the OptEIA enzyme-linked immunosorbent assay (ELISA) set (BD Biosciences, San Jose, CA). Murine IFN-α and IFN-β were measured using the VeriKine mouse ELISA kit (PBL, Picataway, NJ). Murine blood cells were stimulated with either virus or TLR ligands and then 10 min later were treated with GolgiPlug, a protein transport inhibitor containing brefeldin A (BD Biosciences, San Diego, CA), and incubated for an additional 18 h. Murine leukocytes were stained using Alexa-Fluor 647-conjugated rat anti-mouse 7/4 monoclonal antibody (Serotec, Raleigh, NC) and F4/80-fluorescein isothiocyanate (F4/80-FITC; ebioScience, San Diego, CA). After treatment with Cytofix/Cytoper (BD Biosciences), phycocyanin-conjugated rat anti-mouse tumor necrosis factor (TNF) monoclonal antibody (BD Biosciences) was used for intracellular staining. Murine astrocytes and microglial cells were stimulated with either virus or TLR ligands and then 1 h later treated with GolgiPlug and incubated for an additional 18 h. Cells were stained using FITC-conjugated CD11b and then were treated with Cytofix/Cytoper, and phycoerythrin-conjugated anti-mouse TNF monoclonal antibody or allophycocyanin-conjugated anti-mouse MCP-1 (BD Biosciences) was used for intracellular staining.

Flow cytometry. Cells were acquired using BD FACSDiva software on a BDLSRII flow cytometer. The 7/4-positive, F4/80-positive cells or CD11b-positive cells were gated, and the percentage positive for cytokine was calculated using FlowJo v8.1 software (TreeStar, Inc.).

Virus plaque assay. For quantification of virus in infected animals, brains were weighed and then homogenized in phosphate-buffered saline (PBS). Homogenates were pelleted by centrifugation, and the supernatants were used for a plaque assay on monolayers of Vero cells seeded in 24-well tissue culture plates. The cells were adhered overnight and then infected for 1 h at 37°C with serial dilutions of organ suspensions. The cells were washed and overlaid with medium containing 1% methylcellulose, and the plates were incubated for 2 days. Following incubation, the plates were stained with 0.5% crystal violet to allow for quantification of plaques. Results were expressed as the number of PFU per gram of tissue.

Stimulants and antiviral compounds. Repurposed lipopolysaccharide (LPS) was prepared as described previously (9). CpG was purchased from Coley Pharmaceuticals (Kanata, Canada). Pam3CSK4 was obtained from EMC Microcollections (Tubingen, Germany). Loxoribine and poly(IC) were purchased from Alexis Biochemicals (Lausen, Switzerland).

Statistical analysis. All analyses were performed using GraphPad Prism software. Survival data were analyzed by comparing Kaplan-Meier survival curves with a log-rank ( Mantel-Cox) test. P values from survival analyses are reported as numerical values. Brain type I IFN levels and viral titers were analyzed by one-way analysis of variance (ANOVA) with Dunnett’s multiple-comparison test. P values of <0.05 were considered significant.

RESULTS

To establish a lethal dose of HSV-1 in mice, we assessed the survival of wild-type C57BL/6 mice following injection of 3 × 10⁴ PFU, 3 × 10⁵ PFU, or 3 × 10⁶ PFU of HSV-1 7134R directly into the brain (Fig. 1A). At the highest dose, 100% of mice died within 5 days, with a median survival time of 3 days. The lowest dose caused only 38.5% lethality over 14 days. The intermediate dose of 3 × 10⁵ PFU resulted in 75% lethality with a median survival time of 5.5 days, which was suitable for comparing different knockout strains of mice. We subsequently challenged age-matched mice between 4 and 6 weeks old with 3 × 10⁵ PFU of HSV-1 delivered directly into the brain by intracranial injection (all preparations were from the same virus stock). In survival studies with this dose (Fig. 1B to F), wild-type mice had an overall median survival time of 6 days. The combined lethality was 78% (n = 110), and lethality ranged between 60 and 87% for individual experiments.

To assess the roles of different innate immune signaling pathways in lethal HSV disease, we first applied the intracranial model of HSV-1 infection to mice with targeted deficiencies in the type I IFN receptor (IFN-α/IFN-βR knockout mice). We found that IFN-α/IFN-βR knockout mice had significantly increased mortality compared to wild-type mice when infected intracranially with HSV-1 and had a median survival time of 3 days (Fig. 1B). This increase in mortality, which was consistent with others’ findings for IFN-α/IFN-βR knockout mice infected with either HSV-1 (5) or HSV-2 (21), validated the model for intracranial delivery of virus.

We demonstrated previously that TLR2 plays a critical role in the development of encephalitis following intraperitoneal systemic infection (14). Others have established that TLR9 is an important receptor for type I IFN production in response to HSV (12, 17, 21, 26). We wanted to determine the roles of TLR2, TLR9, and combined TLR2/TLR9 deficiency in the brain itself. We found

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that TLR2 knockout animals had a significantly increased survival rate compared to wild-type mice following intracranial inoculation with HSV-1 (Fig. 1C), which was consistent with our findings for TLR2 knockout mice infected with HSV-1 using a systemic route (14). On the other hand, TLR9 mice were similar to wild-type animals with regard to their survival following intracranial HSV-1 injection; each group had a median survival time of 5 days (Fig. 1D). While TLR2 knockout mice had a survival advantage compared to wild-type mice, TLR2/TLR9 double knockout mice did not exhibit any difference in survival from their wild-type counterparts, as each group had a median survival time of 6 days (Fig. 1E). Finally, the role of UNC93B1 in murine HSV-1 encephalitis was examined by infecting 3d mice. The 3d mice had a mild but significant survival disadvantage compared to wild-type mice in this infection model ($P = 0.0016$). Wild-type mice had a median survival time of 6 days while 3d mice had a median survival time of 5 days following HSV-1 intracranial injection (Fig. 1F).

Mice deficient in the type I IFN receptor have increased viral titers and high levels of IFN-β following intracranial HSV-1 infection. We next selected a time postinfection for assessing potential differences in viral replication in the brains of different mouse strains. We measured the quantity of HSV-1 in the brains of wild-type mice inoculated i.c. with $3 \times 10^4$ PFU and found that the titers rose between days 2 and 3 (Fig. 2A). Because all IFN-α/IFN-βR knockout mice typically die by day 3 and wild-type mice begin dying at day 4 (Fig. 1), we did not measure titers beyond day 3. We then directly compared brain virus titers from age-matched HSV-1-infected wild-type mice, TLR2, TLR9, TLR2/TLR9, and IFN-α/IFN-βR knockout mice, and 3d mice at day 3 postinfection. Figures 2B and C show the combined results of two experiments with similar results. HSV-1 titers were more than 200 times higher in IFN-α/IFN-βR knockout brains than in wild-type brains (Fig. 2B), which provides evidence that activation of the type I IFN pathway strongly influences viral replication in CNS-mediated disease following HSV-1 infection. However, no differences were seen for any of the other strains tested in comparison to wild-type mice. In particular, HSV-1 titers were similar in wild-type and 3d mice, suggesting that the survival disadvantage imparted by lack of functional UNC93B1 does not relate to increases in viral replication. We also quantified type I IFN in the same tissue samples. IFN-β levels were low but detectable in infected versus mock-infected wild-type mouse brains (Fig. 2C). IFN-β was significantly elevated only in the brains of infected IFN-α/IFN-βR knockout mice in comparison to infected wild-type mice.

![Figure 1](https://jvi.asm.org/)

**FIG 1** Survival of different knockout (KO) strains following intracranial infection with HSV-1 was assessed. (A) Survival of age-matched wild-type (WT) C57BL/6 mice was established over 14 days following i.c. injection with $3 \times 10^6$ PFU, $3 \times 10^5$ PFU, or $3 \times 10^4$ PFU of HSV-1. (B) Age-matched wild-type and IFN-α/IFN-βR knockout mice were inoculated i.c. with $3 \times 10^4$ PFU of HSV-1 and monitored for survival. $P < 0.0001$. (C) Age-matched wild-type and TLR2 knockout mice were inoculated i.c. with $3 \times 10^6$ PFU of HSV-1 and monitored for survival. $P = 0.03$. (D) Age-matched wild-type and TLR9 knockout mice were inoculated i.c. with $3 \times 10^6$ PFU of HSV-1 and monitored for survival. Data are combined from two independent experiments with similar results. (E) Age-matched wild-type and TLR2/TLR9 double knockout (DKO) mice were inoculated i.c. with $3 \times 10^4$ PFU of HSV-1 and monitored for survival. Data are combined from two independent experiments with similar results. (F) Age-matched wild-type and 3d mice were inoculated i.c. with $3 \times 10^5$ PFU of HSV-1 and monitored for survival. Data are combined from two independent experiments with similar results. $P = 0.0016$. 

Mice deficient in the type I IFN receptor have increased viral titers and high levels of IFN-β following intracranial HSV-1 infection. We next selected a time postinfection for assessing potential differences in viral replication in the brains of different mouse strains. We measured the quantity of HSV-1 in the brains of wild-type mice inoculated i.c. with $3 \times 10^6$ PFU and found that the titers rose between days 2 and 3 (Fig. 2A). Because all IFN-α/IFN-βR knockout mice typically die by day 3 and wild-type mice begin dying at day 4 (Fig. 1), we did not measure titers beyond day 3. We then directly compared brain virus titers from age-matched HSV-1-infected wild-type mice, TLR2, TLR9, TLR2/TLR9, and IFN-α/IFN-βR knockout mice, and 3d mice at day 3 postinfection. Figures 2B and C show the combined results of two experiments with similar results. HSV-1 titers were more than 200 times higher in IFN-α/IFN-βR knockout brains than in wild-type brains (Fig. 2B), which provides evidence that activation of the type I IFN pathway strongly influences viral replication in CNS-mediated disease following HSV-1 infection. However, no differences were seen for any of the other strains tested in comparison to wild-type mice. In particular, HSV-1 titers were similar in wild-type and 3d mice, suggesting that the survival disadvantage imparted by lack of functional UNC93B1 does not relate to increases in viral replication. We also quantified type I IFN in the same tissue samples. IFN-β levels were low but detectable in infected versus mock-infected wild-type mouse brains (Fig. 2C). IFN-β was significantly elevated only in the brains of infected IFN-α/IFN-βR knockout mice in comparison to infected wild-type mice.
in addition, some wild-type mice were mock infected i.c. with saline (2276). To investigate TLR-mediated responses to HSV-1 in macrophages and neutrophils, we utilized murine PEC and found that IL-6 and MCP-1 were induced in a dose-dependent, TLR2-dependent manner (Fig. 3B). Similarly, microglial cells from wild-type and TLR9- and UNC93B1-deficient mice responded with TNF production following HSV-1 challenge while those from TLR2- and TLR2/TLR9-deficient mice had minimal cytokine production (Fig. 3C). Despite the lack of TLR9 dependence in their response to HSV-1, astrocytes and microglial cells were capable of producing TLR9-dependent cytokine responses to CpG. Together, these results suggest that HSV-1 cytokine production in astrocytes and microglial cells is mediated predominantly by TLR2 rather than TLR9.

**TLR2 signaling predominates in inflammatory cytokine responses by PEC, peripheral blood neutrophils, and monocytes following HSV-1 infection.** Macrophages and neutrophils are important inflammatory cells recruited to the CNS during viral infection and can mediate destructive inflammatory responses in HSV encephalitis (18). We therefore examined these specific leukocyte populations, assessing the roles of TLRs during HSV challenge. To investigate TLR-mediated responses to HSV-1 in macrophages, we utilized murine PEC and found that IL-6 and MCP-1 are induced in a dose-dependent, TLR2-dependent manner (Fig. 4A). Using intracellular cytokine staining, we demonstrated that TNF production in PEC from wild-type and knockout mice following HSV-1 infection is substantially TLR2 dependent (Fig. 2C). The high IFN-β production likely represents a feedback response by the host following a viral infection in which protective IFN responses are typically critical for survival. We have made similar observations with IFN-α/IFN-βR knockout mice infected with coxsackie B virus (29). IFN-α was below the threshold of detection by ELISA in the infected brain homogenates (data not shown). Thus, deficiency in TLR2, TLR9, TLR2/TLR9, or UNC93B1 does not appear to directly influence HSV-1 replication in the brain in the direct intracranial injection model of HSV encephalitis.

**Astrocytes and microglial cells produce inflammatory cytokines following HSV-1 infection in a TLR2-dependent manner.** Astrocytes and microglial cells are resident cells of the CNS that mediate innate immunity. We previously employed primary mouse CNS astrocytes and glial cells to study neurotropic viral activation of TLRs (31). In order to explore the potential roles of both TLR2 and TLR9 in cytokine induction during HSV-1 infection of both microglial cells and astrocytes, we challenged murine CNS mixed glial cells with HSV-1 or TLR agonists. Subsets of cells present in the primary mixed glial cell cultures were identified by flow cytometry: microglial cells were defined as CD11b+ cells and astrocytes were defined as CD11b− cells, as shown in Fig. 3A and as described previously (31). HSV-1 challenge of wild-type cells led to robust production of MCP-1 in astrocytes and production of TNF in microglial cells as detected by single-cell-based intracellular cytokine staining (Fig. 3B). The percentage of cells producing cytokines correlated with the multiplicity of infection (MOI), i.e., a higher MOI resulted in a higher percentage of responding cells. While the exact percentage of HSV-infected cells was not established in these studies, some cytokine-producing cells perhaps were not infected but instead responded to products of infected cells. We determined the specific roles of TLR2, TLR9, and UNC93B1 using cells obtained from deficient mice. We also examined cytokine production in astrocytes and microglial cells from TLR2/TLR9 double knockout mice. Challenge with HSV-1 resulted in equivalent levels of production of MCP-1 in astrocytes from wild-type, TLR9-deficient, and 3d mice but negligible production in astrocytes from TLR2- and TLR2/TLR9-deficient mice (Fig. 3B). Similarly, microglial cells from wild-type and TLR9- and UNC93B1-deficient mice responded with TNF production following HSV-1 challenge while those from TLR2- and TLR2/TLR9-deficient mice had minimal cytokine production (Fig. 3C).
FIG 3 Murine astrocytes and microglial cells produce inflammatory cytokines predominantly through TLR2 following infection with HSV-1. Primary brain mixed glial cultures, which are a mixed population of astrocytes and microglial cells, were obtained from wild-type and TLR2-, TLR9-, TLR2/TLR9- and UNC93B1-deficient mice and cultured with TLR ligands, virus, or medium alone for 1 h and then with brefeldin A for 18 h. (A) Flow cytometry shows that populations of primary brain mixed glial cells produce cytokines in response to stimulation with HSV-1 (MOI of 3). Cells were fixed, permeabilized, stained for a surface marker (CD11b) and intracellular cytokines (TNF [upper panels] and MCP-1 [lower panels]), and analyzed by flow cytometry. Data shown are percentages of cells expressing cytokines. Microglial cells (CD11b+/H11001) make TNF (upper right panel), while astrocytes (CD11b/H11002) make MCP-1 (lower right panel) when infected with HSV. APC, allophycocyanin; PE, phycoerythrin. (B) Astrocytes (CD11b-H11002) produce MCP-1 in a TLR2-dependent but TLR9-independent manner following challenge with HSV (MOI of 10 and 3) or Pam2CSK4 (100 ng/ml). Astrocytes respond to CpG 1826 (10 µM) in a TLR9- and UNC93B1-dependent manner. Astrocytes respond to poly(IC) (25 µg/ml) in a UNC93B1-dependent manner. LPS serves as a TLR2-, TLR9-, and UNC93B1-independent control. (C) Microglial cells (CD11b+/H11001) produce TNF in a TLR2-dependent but TLR9-independent manner following challenge with HSV (MOI of 10 and 3) or Pam2CSK4 (100 ng/ml). Microglial cells respond to CpG 1826 (10 µM) in a TLR9-dependent manner. Microglial cells produce minimal TNF in response to poly(IC) (25 µg/ml). LPS serves as a TLR2-, TLR9-, and UNC93B1-independent control.
While PEC can generate cytokines in response to the TLR9 ligand CpG, we did not observe TLR9-dependent MCP-1 production following HSV-1 challenge (Fig. 4C). Thus, we concluded that murine macrophages generate inflammatory cytokines through a TLR2-dependent, TLR9-independent means.

We then examined the responses of murine peripheral blood neutrophils to infection with HSV-1. To directly examine inflammatory cytokine production by murine neutrophils, we used single-cell-based intracellular cytokine staining of wild-type murine peripheral blood samples. TNF production by neutrophils (defined as cells staining 7/4+ and F4/80−), as well as that by monocytes/macrophages (defined as cells staining 7/4− and F4/80+), was confirmed following challenge with HSV-1 or the TLR agonist Pam3CSK4 or LPS (Fig. 5A). When comparing intracellular responses of wild-type and TLR2 knockout mouse blood cells to challenge with various doses of HSV-1, we found a predominant contribution of TLR2 toward TNF production in neutrophils (Fig. 5B), as well as in monocytes/macrophages (Fig. 5C). Murine neutrophils had negligible cytokine production in response to the TLR9 agonist CpG (Fig. 5D), so we did not examine the effects of HSV-1 infection in blood samples from TLR9 knockout mice.

**DISCUSSION**

HSV-1 encephalitis causes significant morbidity, so gaining a better understanding of the genes associated with susceptibility or resistance is of high priority. Using an intracranial model of infection, we monitored the development of lethal HSV disease in mice deficient in specific innate immunity genes. We used direct intracranial inoculation of HSV-1 into the brain rather than peritoneal or ocular delivery for these encephalitis studies. While HSV is not usually introduced by direct inoculation of brains in humans and is rather spread from peripheral tissues via neuronal transport, we found this approach to be a practical method of viral delivery in mice because it allows analysis of CNS infection solely, separate from the effects in other tissues. Relatively small amounts of virus are needed for rapid and reproducible onset of HSV-related disease by i.c. inoculation, which does not rely on peripheral neuronal uptake and transport. This allows for assessment of the innate immune system as the primary means for controlling viral replication, as well as for assessment of the cellular expression of innate immune receptors in disease pathogenesis.

The aim of these studies was to define the innate immune pathways responsible for protective and detrimental cytokine responses during HSV-1 encephalitis. The results demonstrate that expression of TLR2 can be detrimental to the host and that IFN-α/IFN-βR expression is protective in the setting of HSV-1 encephalitis. Not surprisingly, IFN-α/IFN-βR knockout mice had extremely high levels of virus in the brain. Several studies have implicated TLR9 in HSV responses based on findings with plasmacytoid dendritic cells in vitro (12, 17), but our studies demonstrate that TLR9 deficiency in itself does not negatively impact survival during intracranial infection. The lack of a mutant phenotype in HSV-1-infected TLR9 knockouts is consistent with findings of Rasmussen et al., who reported no differences in mortality or viral titers between wild-type and TLR9 knockout mice infected with HSV-2 by intraperitoneal injection (21).

Interestingly, a previous study demonstrated that deficiency of MyD88, the adaptor molecule for both TLR2 and TLR9, leads to increased dissemination of encephalitis following intracorneal in-
Peripheral blood neutrophils and monocytes/macrophages produce inflammatory cytokines in response to HSV-1 in a predominantly TLR2-dependent manner. (A) Wild-type murine peripheral blood neutrophils and monocytes/macrophages were assessed for their ability to produce TNF in response to HSV-1 and other stimulants as measured by single-cell intracellular staining. The upper panel shows results for intracellular TNF staining for neutrophils (7/4) or monocytes/macrophages (F4/80) challenged with LPS (1μg/ml), Pam2CSK4 (1μg/ml), loxoribine (1mM), or HSV-1 (MOI of 10). The left lower panel shows distributions of cells stained for neutrophil (y axis; 7/4) and monocyte/macrophage (x axis; F4/80) markers. The right lower panel bar graph shows the geometric mean fluorescence of TNF from gated neutrophils or monocytes/macrophages following challenge with LPS, Pam2CSK4, or HSV-1. (B) Blood from wild-type or TLR2 knockout mice was challenged with HSV-1 or Pam2CSK4 (1μg/ml), and intracellular TNF was assessed for gated cells. The geometric mean fluorescence for neutrophils is shown. (C) Blood from wild-type or TLR2 knockout mice was challenged with HSV-1 or Pam2CSK4 (1μg/ml), and intracellular TNF was assessed for gated cells. The geometric mean fluorescence for monocytes/macrophages is shown. (D) Murine neutrophils had a relatively poor TNF response to challenge with CpG 1826 (20μM) in comparison to Pam2CSK4 (1μg/ml), LPS (1μg/ml), or loxoribine (1mM), with data shown as geometric means.
occlusion with HSV-1 but that in the context of TLR2 or TLR9 deficiency alone, intracorneal inoculation with HSV-1 does not lead to encephalitis and results in a localized corneal infection (24). Others reported that TLR2 and TLR9 act synergistically to control HSV-2 replication during systemic infection (26). Likewise, we found that TLR2/TLR9 dual deficiency appears to diminish the protective effects conferred by TLR2 deficiency, which may reflect a cooperative role of TLR2 and TLR9. Our results differ from those reported by Lima et al. and Mansur et al., who infected wild-type C57BL/6 or MyD88, TLR2, TLR9, or TLR2/TLR9 knockout mice with HSV-1 by the intranasal route and observed significant increases in susceptibility and increased viral titers in MyD88, TLR9, and TLR2/TLR9 knockout mice (16, 19). The differences between their findings and ours most likely relate to the route of infection and the relative degrees of expression of the specific TLRs in specialized tissues involved in the route of infection (i.e., intracranial versus intranasal). We directly compared profiles of inflammatory cytokine production in astrocytes and microglial cells from TLR2-deficient and TLR2/TLR9-deficient mice and found them to be similar following challenge with HSV-1, so differences in inflammatory cytokine production did not explain the differences in survival.

Detrimental consequences of innate immune responses are illustrated by the finding of excess TLR2-mediated inflammation in HSV-infected brains. These TLR2-mediated inflammatory responses in the central nervous system are likely driven by astrocytes and microglial cells at early time points following infection and then later by recruited macrophages and/or neutrophils, as supported by our in vitro data. Indeed, Aravalli et al. challenged primary murine microglial cells from wild-type and TLR2 knockout mice with HSV-1 and found that multiple inflammatory cytokines were driven in a TLR2-dependent manner (1). Although murine neutrophils (2), microglial cells (resident macrophages of the central nervous system) (7, 15), and astrocytes (3) each express both TLR2 and TLR9, TLR2 is the predominant mediator of inflammatory cytokines following HSV-1 infection. The absence of TLR2-mediated induction of inflammatory cytokines does not diminish viral replication.

The pathways required for regulation of viral replication remain incompletely defined. Deficiency of UNC93B1, which affects signaling by the endosomal TLRs TLR3, TLR7, and TLR9, did lead to increased mortality of mice infected with HSV-1. Through human studies, Casrouge and colleagues identified UNC93B1 as important in susceptibility to HSV encephalitis, as polymorphisms of the corresponding gene are associated with familial adult-onset herpes encephalitis (6). Deficiencies in UNC93B1 have also been linked with impaired type I IFN responses (6, 27). Our survival data for HSV-1 intracranial infection in 3d mice confirm the essential role of UNC93B1 as a protective factor in HSV encephalitis. However, we were not able to establish differences in viral replication or type I IFN induction, as viral burdens were not elevated and IFN-β levels were not diminished in the brains of 3d mice. The reason for the decreased survival of UNC93B1-deficient mice is not apparent. While these findings do not diminish the significance of UNC93B1 in HSV-1 disease in humans, they suggest that other signaling pathways may be crucial for protective CNS type I IFN responses.

The innate immune pathways activated by HSV-1 are both complex and intriguing. The interplay between virus and various host sensors that leads to the activation of cytokines may lead to the suppression of virus but with the added cost of excessive inflammation. As summarized in Tables 1, 2, and 3, our data show that numerous host cells involved in CNS infection, including microglial cells, astrocytes, neutrophils, and monocytes/macrophages, respond to HSV-1 in a TLR2-dependent, TLR9-independent manner to produce inflammatory cytokines and chemokines. The absence of TLR2 confers a survival advantage in mice during HSV encephalitis, which is consistent with our previous observations (14). In contrast, the absence of type I IFN production negatively impacts survival, but we did not find that type I IFN production in the brain required TLR9 or UNC93B1. Other innate immune factors that could affect type I IFN production in the brain during HSV encephalitis include the cytosolic DNA sensor IFI16 (28) and TLR3/TRIF (8, 23, 30). Future studies examining the roles of inflammasomes and IFI16 may reveal additional information

### Table 1: Inflammatory cytokine production after in vitro challenge of murine cells with HSV-1

<table>
<thead>
<tr>
<th>Cell type</th>
<th>TLR2 dependent</th>
<th>TLR9 independent</th>
<th>UNC93B1 independent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microglial cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astrocyte</td>
<td>TLR2 dependent</td>
<td>TLR9 independent</td>
<td>UNC93B1 independent</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>TLR2 dependent</td>
<td>TLR9 independent</td>
<td></td>
</tr>
<tr>
<td>Monocyte/macrophage</td>
<td>TLR2 dependent</td>
<td>TLR9 independent</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2: Innate immunity profiles for total brain homogenates following mouse challenge with HSV-1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory cytokine production</td>
<td>TLR2 dependent</td>
</tr>
<tr>
<td>Type I IFN production</td>
<td>TLR2 independent</td>
</tr>
<tr>
<td>Control of viral replication</td>
<td>TLR2 independent</td>
</tr>
</tbody>
</table>

### Table 3: Summary of important innate immune factors in HSV encephalitis

<table>
<thead>
<tr>
<th>Factor(s)</th>
<th>Impact on disease</th>
<th>Receptor(s) involved (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory cytokines</td>
<td>Detrimental in excess</td>
<td>TLR2</td>
</tr>
<tr>
<td>Type I IFN</td>
<td>Protective</td>
<td>DNA sensor? TRIF (23) UNC93B1 (6)</td>
</tr>
</tbody>
</table>

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Wang et al.
REFERENCES


