

Lethal Encephalitis in Myeloid Differentiation Factor 88-Deficient Mice Infected with Herpes Simplex Virus 1

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Herpes simplex virus 1 (HSV-1), a large DNA virus from the *Herpesviridae* family, is the major cause of sporadic lethal encephalitis and blindness in humans. Recent studies have shown the importance of Toll-like receptors (TLRs) in the immune response to HSV-1 infection. Myeloid differentiation factor 88 (MyD88) is a critical adaptor protein that is downstream to mediated TLR activation and is essential for the production of inflammatory cytokines. Here, we studied the relationship between MyD88 and HSV-1 using a purified HSV-1 isolated from a natural oral recurrent human infection. We observed the activation of TLR-2 by HSV-1 *in vitro* using Chinese hamster ovary cells stably transfected with a reporter gene. Interestingly, we found that only peritoneal macrophages from MyD88^{-/-} mice, but not macrophages from TLR2^{-/-} or from wild-type mice, were unable to produce tumor necrosis factor- α in response to HSV-1 exposure. Additionally, although TLR2^{-/-} mice showed no enhanced susceptibility to intranasal infection with HSV-1, MyD88^{-/-} mice were highly susceptible to infection and displayed viral migration to the brain, severe neuropathological signs of encephalitis, and 100% mortality by day 10 after infection. Together, our results suggest that innate resistance to HSV-1 is mediated by MyD88 and may rely on activation of multiple TLRs. (*Am J Pathol* 2005, 166:1419–1426)

Herpes simplex virus 1 (HSV-1), from the *Herpesviridae* family, is a complex virus containing a large 140-kb DNA, which encodes 84 proteins and is the ubiquitous neurotropic human pathogen most commonly associated with oro-labial and ocular infections.¹ The most serious infection caused by HSV-1 is sporadic encephalitis,² which has a mortality rate of ~70%, when not treated.³ HSV-1 is transmitted primarily by contact with oral secretions. On oral entry into skin and mucosal sites, HSV-1 replicates locally in epithelial cells, resulting in cell lysis and local inflammatory response. After primary infection, HSV-1 can travel along sensory nerve pathways and may become latent in the sensory ganglia, where it can eventually be reactivated.³ Animal models of human HSV encephalitis in mice using intranasal inoculation have been described.² This inoculation pathway leads to an inflammatory response that can be dangerous to the host. However, the precise mechanisms by which HSV-1 causes death are not clear.

Toll-like receptors (TLRs) are innate immunity receptors linked with the response to pathogen-associated molecular patterns. Since the first description of TLRs in mammals, many TLR agonists have been described: peptidoglycans⁴ and *Trypanosoma cruzi* GPI anchor for TLR2,⁵ lipopolysaccharide (LPS) for TLR4,^{6–8} dsRNA for TLR3,⁹ flagellin for TLR5,¹⁰ and CpG DNA for TLR9.¹¹ TLRs activate inflammatory responses and modulate immunity by several different signal transduction pathways. The most well known pathway involves myeloid differentiation factor 88 (MyD88), an adapter molecule composed of a Toll-interleukin-1 receptor domain and a death

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domain.¹² MyD88 recruits the serine threonine kinase, interleukin receptor associated kinase-4, that activates tumor necrosis factor- α receptor-associated factor-6 (TRAF-6) which in turn phosphorylates I κ B, causing it to dissociate from and leave nuclear factor (NF)- κ B free in the cytoplasm. NF- κ B then translocates to the nucleus and acts as a transcription factor of innate immunity-associated genes.^{12,13} In addition, TLR3 appears to activate the inflammatory response through another adapter molecule, named Toll-interleukin-1 receptor domain-containing adaptor-inducing interferon- β .¹³ This pathway is MyD88-independent, and culminates with the translocation of interferon regulatory factor 3 (IRF-3) to the nucleus, leading to production of interferon (IFN)- β and IFN-inducible genes.¹³

A role for the TLR2, TLR3, TLR4, and TLR9 in the response to viruses has been previously established.^{9,12,14-18} Lund and colleagues¹⁷ showed that genomic HSV-2 DNA, which is closely related to HSV-1, was recognized by TLR9 and mediated activation through an MyD88-dependent endocytic pathway leading to type I IFN response. Using a recombinant HSV-1 KOS strain, Krug and colleagues¹⁴ confirmed the involvement of TLR9 in type I IFN response. Lundberg and colleagues¹⁸ also showed that HSV-1 DNA is stimulatory both *in vitro* and *in vivo*. Recently, Kurt-Jones and colleagues¹⁶ demonstrated that TLR2 mediates the induction of inflammatory cytokines in response to intravenous inoculation with the HSV-1 KOS strain, whereas in mice lacking functional TLR2, they detected a reduction in encephalitis symptoms.

Here we used a HSV-1 isolated from a natural oral recurrent human infection, expanded in Vero cells, and purified in sucrose gradient.¹⁹ We demonstrate the activation of TLR2 by HSV-1 *in vitro* using Chinese hamster ovary (CHO) cells stably transfected with human TLR2 and a reporter gene. We also show for the first time, using an *in vivo* mouse model of intranasal inoculation,³ which is a natural route of infection, that HSV-1 leads to lethal encephalitis in 100% of the mice lacking the functional MyD88 protein. These results further suggest the importance of TLRs and innate immunity in host resistance to HSV-1.

Materials and Methods

Viruses, *Staphylococcus aureus*, and LPS

HSV-1 strain EK,²⁰ isolated from a human case of recurrent oral herpes with blisters and *Vaccinia virus* Western Reserve (VV) were allowed to multiply in Vero cells, maintained with minimal essential medium (GIBCO, Grand Island, NY) containing 5% fetal bovine serum (FBS) (GIBCO) and 25 μ g/ μ l of ciprofloxacin (Fesenius, Pune, India) at 37°C in a 5% CO₂ atmosphere. HSV-1 and VV were purified in sucrose gradients,¹⁹ and the titers determined in Vero cells as previously described.²¹ The virus titers obtained were: 1.1×10^8 PFU/ml for HSV-1 and 2×10^{10} PFU/ml for VV. LPS from *Escherichia coli* O55:B5 was

obtained from Sigma (St. Louis, MO) and UV-inactivated *S. aureus* was described before.⁵

Vero Cells

Vero cells were maintained in minimal essential medium supplemented with 5% heat-inactivated FBS and antibiotics in 5% CO₂ at 37°C. These cells were used for multiplication and titration of virus and in neutralization tests.

CHO Cell Lines

The CHO reporter cell lines,^{22,23} a kind gift from Douglas T. Golenbock (University of Massachusetts Medical School, Worcester, MA), were maintained as adherent monolayers in Ham's F-12/Dulbecco's modified Eagle's medium supplemented with 5% FBS and antibiotics at 37°C, 5% CO₂. All of the cell lines were derived from clone 3E10, a CHO/CD14 cell line that has been stably transfected with a reporter construct containing the structural gene for CD25 under the control of the human E-selectin promoter. This promoter contains a NF- κ B binding site; CD25 expression is completely dependent on NF- κ B translocation to the cell nucleus.²³ Cells expressing TLRs were constructed by stable transfection of the CHO/CD14 reporter cell line with the cDNA for human TLR2 or expressing endogenous TLR4 as described.²² In addition to the LPS-responsive cell lines described above, we also tested a LPS nonrespondent cell line derived from 3E10²² designated clone 7.19, as well as a clonal line derived from this mutant that was transfected with CD14 and TLR2 (7.19/CD14/TLR2). The LPS nonresponsive phenotype of the 7.19 cell lines is due to a mutation in the *MD-2* gene, and thus is defective in signaling via TLR4.²² These cell lines report NF- κ B activation via surface expression of CD25, similarly to the other CHO cell lines described.

Flow Cytometry Analysis

CHO reporter cells were plated at a density of 1×10^5 cells/well in a 24-well tissue culture dish. After 20 hours, UV-inactivated bacteria, HSV-1 or VV were added in a total volume of 250 μ l of medium/well for 18 hours. The cells were then harvested with trypsin-ethylenediaminetetraacetic acid (Sigma, St. Louis, MO) and washed once with medium containing 5% FBS and then with phosphate-buffered saline (PBS). Cells harvested physically without trypsin displayed similar results. Subsequently, the cells were counted and 1×10^5 cells stained with phycoerythrin-labeled anti-CD25 (mouse monoclonal antibody to human CD25, R-PE conjugate; Caltag Laboratories, Burlingame, CA) 1:200 in PBS, on ice in the dark, for 30 minutes. After labeling, the cells were washed twice with PBS containing 1 mmol/L sodium azide (Sigma), and 10,000 cells were examined by flow cytometry (BD Biosciences, San Jose, CA) for the expression of surface CD25 as described.^{5,8,22} After excluding dead cells by gating with forward and side scatter parameters,

an average of 8750 ± 312 live cells, were analyzed for the expression of CD25. Analysis was performed using CellQuest software (BD Biosciences).

Animals

TLR2^{-/-} and MyD88^{-/-} mice were generated at Osaka University (Osaka, Japan) and backcrossed in the C57BL/6 background for eight generations. IFN γ ^{-/-} mice in the C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). The knockout mice were transferred to the Federal University of Minas Gerais, Institute of Biological Sciences (Belo Horizonte, Minas Gerais, Brazil) and maintained in a pathogen-free, barrier environment. C57BL/6 mice, used as wild-type (WT) control, were obtained from the Centro de Pesquisas René Rachou, Oswaldo Cruz Foundation (Belo Horizonte, Minas Gerais, Brazil). Four-week-old male mice were anesthetized with ketamine (Agribands do Brasil Ltda, Paulinia, Brazil), and 10^4 PFU of the purified HSV-1 contained in $10 \mu\text{l}$ were inhaled by mice as described previously.⁵ Control mice inhaled PBS. Nine mice from each knockout or WT group were used in the survival experiments shown in Figure 2. Eight days after infection, brain, lung, liver, and spleen were removed from three animals per group and either frozen or fixed in formalin (Sciavicco Comercio e Industria Ltda, Belo Horizonte, Brazil). Each experiment was repeated three times. Mice presenting symptoms such as total paralysis and/or seizures were sacrificed. The mouse colonies and all experimental procedures were performed according to the institutional animal care and use guidelines from the Centro de Pesquisas René Rachou, Oswaldo Cruz Foundation.

Murine Macrophage Preparation and Tumor Necrosis Factor (TNF)- α Measurement

Thioglycollate-elicited peritoneal macrophages were obtained from either C57BL/6, TLR2^{-/-}, or MyD88^{-/-} mice by peritoneal washing. Adherent peritoneal macrophages were cultured in 96-well plates (2×10^5 cells/well) at $37^\circ\text{C}/5\% \text{CO}_2$ in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) supplemented with 5% heat-inactivated FBS (Life Technologies), 2 mmol/L L-glutamine (Sigma) and 40 $\mu\text{g}/\text{ml}$ of gentamicin (Schering do Brasil, Rio de Janeiro, Brazil). Cells were then stimulated with HSV-1 (multiplicity of infection, 40), LPS, or *S. aureus* for 24 hours to evaluate TNF- α production. TNF- α was quantified using a DuoSet ELISA kit from R&D Systems (Minneapolis, MN).

Virus Detection by Nested Polymerase Chain Reaction (PCR) and Determination of Virus Concentration in Mice Tissues

Frozen mice tissues were ground with sterile sand and $200 \mu\text{l}$ of minimal essential medium, centrifuged, and the supernatant was used for titration in a standard tissue

culture infectious dose (TCID₅₀) assay²⁴ and for nested PCR. The primers and conditions used for the first reaction of PCR were described previously by Nogueira and colleagues.²⁰ The nested PCR was developed using the primers specific for HSV-1 thymidine kinase: TK13 CCA GCA TAG CCA GGT CAA GC and TK15 GCG AAC ATC TAC ACC ACA CAA CA. The reaction was performed at 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute for 40 cycles.

Immunohistochemistry

Brain samples were fixed with 10% formaldehyde in phosphate buffer and then embedded in paraffin. Sections were mounted on glass slides, deparaffinized, and then treated with 3.5% H₂O₂ in PBS. Tissue sections were blocked with 7% normal goat serum in PBS for 30 minutes at room temperature and incubated overnight with polyclonal rabbit anti-herpes simplex 1 antibody p0175 (DAKO, NH) diluted 1:50 or monoclonal anti-gC HSV-1 diluted 1:500 in PBS containing 0.4% Triton X-100. After incubation with primary antibody, tissue sections were washed three times with PBS and incubated for 90 minutes at room temperature with the secondary biotinylated antibody solution (DAKO, NH), washed with PBS, and treated with the tertiary solution, containing peroxidase-conjugated streptavidin (DAKO, NH), for 60 minutes at room temperature. Sections were then rinsed in PBS and with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) (0.05%) and hydrogen peroxide (0.03%). The sections were then rinsed in PBS and stained with hematoxylin and eosin (H&E) (Reagen, Rio de Janeiro, Brazil).

Neutralization Test

Sera of mice were serially diluted from 1:10 to 1:1280 in minimal essential medium in a total volume of $100 \mu\text{l}$. One hundred TCID₅₀ of HSV-1 was added to each dilution and incubated for 1 hour at 37°C in a 5% CO₂ atmosphere. The mixture was added to a 96-well plate containing Vero cells. The plates were incubated and observed during 5 days. All of the samples were titrated in duplicate. The titer was determined as the inverse of the highest dilution of serum that protected Vero cells from cytopathic effect of HSV-1.

Statistical Analysis

Statistical analyses were performed with Student's *t*-test using the software program Minitab (Minitab Inc., State College, PA).

Results

In Vitro Activation of TLR2 by HSV-1

We investigated if a recently isolated strain of HSV-1, purified by sucrose gradient, induces activation of TLR2 *in vitro*. Flow cytometry analysis of the expression of a

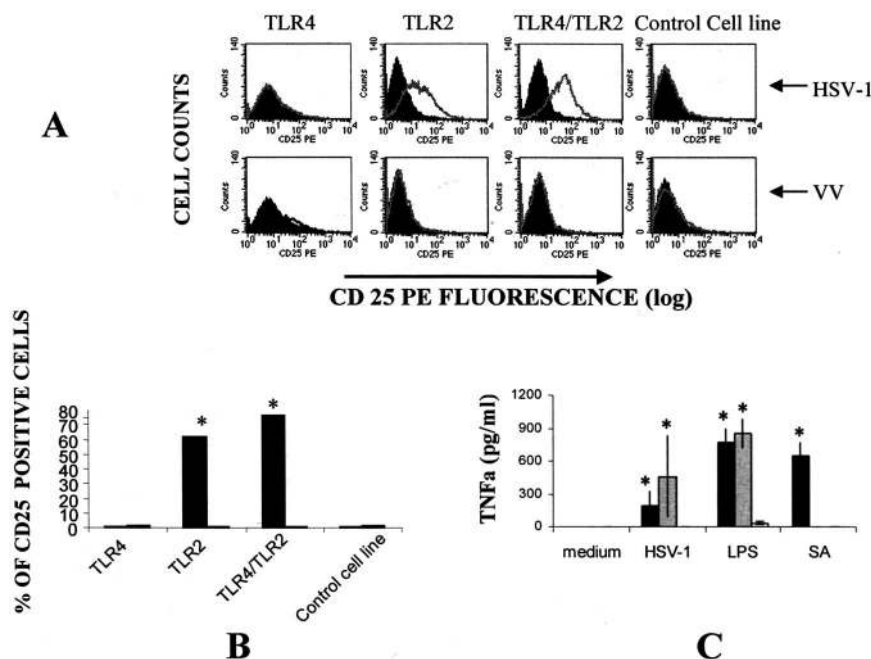


Figure 1. TLR2 mediates cellular activation after exposure to HSV-1. **A:** CHO/CD14 (expressing endogenous TLR4), 7.19/CD14/TLR2 (expressing TLR2), CHO/CD14/TLR2 (expressing TLR4/TLR2), or 7.19 (LPS nonresponder control) cells were left untreated (**black area**) or exposed (**gray lines**) to 10^4 PFU of HSV-1 (**top**) or to 10^4 PFU of VV (**bottom**), and the expression of the reporter gene (CD25) was measured 18 hours later by flow cytometry. The data are representative of three experiments. **B:** The cell lines were exposed to 10^4 PFU of HSV-1 (**black bars**) or of VV (**gray bars**) and the expression of the reporter transgene CD25 was measured by flow cytometry. The percentage of CD25-positive cells was obtained by subtracting the percentage of stimulated cells expressing CD25 from the percentage of nonstimulated cells expressing CD25. An average of 8750 ± 312 cells were analyzed in each experiment. This experiment is representative of three performed. **Asterisks** indicate that differences in reporter gene expression on TLR2 or TLR4/TLR2 cells is statistically significant ($P < 0.01$) when compared to TLR4 or control cell lines. **C:** Macrophages derived from WT (**black columns**), TLR2^{-/-} (**gray columns**), or MyD88^{-/-} (**white columns**) mice were exposed to HSV-1 (multiplicity of infection, 40), LPS, and *S. aureus* and the levels of TNF- α were measured in the culture supernatants at 24 hours after macrophage stimulation. **Asterisks** indicate that differences are statistically significant ($P < 0.05$), when comparing cytokine levels produced by macrophages from WT or TLR2^{-/-} mice to macrophages from MyD88^{-/-} mice. The experiment was performed in triplicates and the results shown are one representative of two experiments that yielded the same results.

CD25 reporter gene in CHO cells, stably transfected with the TLR constructions described previously^{22,23} is shown in Figure 1A. We used cells stably transfected with CD14 alone (CHO/CD14) or with CD14 and TLR2 (CHO/CD14/TLR2), both expressing endogenous TLR4, as well as the clone 7.19, which does not have a functional TLR4 signaling pathway, but are stably transfected with CD14 (7.19) or CD14 and TLR2 (7.19/CD14/TLR2). These cells were exposed for 18 hours to 10^4 PFU of HSV-1 or *Vaccinia virus* (VV). VV served as a negative control,²⁵ because it was produced in cell cultures and purified by the same process¹⁹ used to purify HSV-1. We observed that the cells stimulated with HSV-1 were activated through TLR2, but not through TLR4. Figure 1B shows an increased percentage of CD25-positive cells in TLR2/CHO or TLR2/TLR4/CHO cells stimulated with HSV-1. These data indicate that purified HSV-1 triggers NF- κ B through TLR2/CD14, but not through TLR4/CD14.

To compare the host innate immune response after HSV-1 challenge *ex vivo*, we measured the levels of TNF- α in culture supernatants of macrophages from WT, TLR2^{-/-}, and MyD88^{-/-} mice (Figure 1C). Both WT and TLR2^{-/-} produced significant amounts of this cytokine when challenged by HSV-1, whereas the production in MyD88^{-/-} was totally abrogated. LPS and *S. aureus* were used as controls. LPS,^{6,7} differently from *S. aureus*,²² still activated macrophages from TLR2^{-/-} mice, whereas

none of the microbial stimuli were effective on macrophages from MyD88^{-/-} mice.¹³

MyD88^{-/-} Mice Are Highly Susceptible to HSV-1 Infection

Our next step was to evaluate the importance of TLR2 and MyD88 during infection with HSV-1 in an *in vivo* model. We used the intranasal model² because it is a natural route of infection with HSV-1. Four-week-old C57BL/6, TLR2^{-/-}, and MyD88^{-/-} mice were inoculated with 10^4 PFU of HSV-1 intranasally. Because it has been previously demonstrated that IFN- γ receptor-deficient mice are more susceptible to infection with HSV-1²⁶⁻²⁹ and that mice with herpetic stromal keratitis produce high levels of IFN- γ ,³⁰ IFN γ ^{-/-} mice were also tested.

In our initial studies we used different isogenic mouse strains, such as the C57BL/6, BALB/c, and 129 strains. All of these strains were found to be resistant to intranasal infection and showed 100% survival at 4 weeks of age, after infection with 10^4 PFU of HSV-1 (data not shown). The TLR2^{-/-} mice did not show any observable clinical symptoms, and were as resistant as C57BL/6 mice to infection with HSV-1. In contrast, 100% of MyD88^{-/-} mice died between 6 to 10 days after infection (Figure 2A). The IFN γ ^{-/-} mice were also more susceptible to

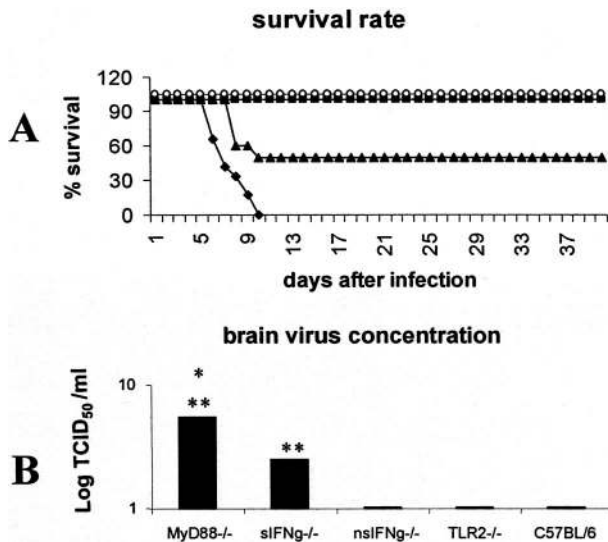


Figure 2. MyD88^{-/-} mice are highly susceptible to infection with HSV-1. **A:** Nine MyD88^{-/-} (diamonds), IFN γ ^{-/-} (triangles), TLR2^{-/-} (squares), and C57BL/6 (circles) 4-week-old mice were intranasally inoculated with 10⁴ PFU of HSV-1 or PBS, and survival was assessed daily. **B:** Eight days after intranasal infection with 10⁴ PFU of HSV-1 the brains from nine mice of each group, or from animals that had died from infection, were collected after sacrifice, macerated, and inoculated into Vero cell cultures to perform the titration procedure in triplicate. sIFN^{-/-}, IFN^{-/-} mice with clinical symptoms; nsIFN^{-/-}, IFN^{-/-} mice with no clinical symptoms. This experiment is representative of three performed. *, Virus brain concentration in MyD88^{-/-} was statistically higher ($P < 0,05$) as compared to virus brain concentration in sIFN^{-/-}, nsIFN^{-/-}, TLR2^{-/-}, or in C57BL/6 mice. **, Virus brain concentrations in MyD88^{-/-}, or in sIFN^{-/-}, mice were statistically higher ($P < 0001$) as compared to virus brain concentration in nsIFN^{-/-}, TLR2^{-/-}, or in C57BL/6 mice.

HSV-1 infection (Figure 2A). After brain tissues from mice sacrificed on the 8th day after infection were inoculated into Vero cell cultures, the samples from MyD88^{-/-} and from IFN γ ^{-/-} mice with symptoms of infection were determined to have high TCID₅₀ (Figure 2B), as compared to brain tissues of TLR2^{-/-}, C57BL/6, or IFN γ ^{-/-} mice that did not display clinical symptoms of infection. Attempts to recover virus from lung, spleen, and liver, from these mice, either by nested PCR or isolation in Vero cells were unsuccessful (data not shown).

The brains of mice sacrificed at 8 days after infection were processed for nested PCR reactions, as previously described,²⁰ with specific primers for HSV-1 thymidine kinase (TK) gene, and the results are shown in Table 1. Only the brains from MyD88^{-/-} and from IFN γ ^{-/-} mice with symptoms were positive for HSV-1 TK. To confirm that all mice were infected, a neutralization test was performed (Table 1) using sera from C57BL/6 and TLR2^{-/-}

mice obtained at 30 days after infection. Our results show that all mice were seropositive (Table 1). Of note, the neutralization test in MyD88^{-/-} and symptomatic IFN γ ^{-/-} was performed after 8 days of infection, because of their early death (Figure 2). No mice (ie, WT, TLR2^{-/-}, MyD88^{-/-}, or IFN γ ^{-/-}) presented seropositive results (data not shown) at this time. Together, these results indicate that the absence of IFN- γ or MyD88 enhances the entry of HSV-1 into the brain and results in 50% or 100% mortality, respectively.

Lethal Encephalitis in MyD88^{-/-} and IFN γ ^{-/-} Mice Infected with HSV-1

Macroscopic observation of brains from MyD88^{-/-} and IFN γ ^{-/-} mice with clinical symptoms revealed hemorrhagic and necrotic areas, differing substantially from TLR2^{-/-}, C57BL/6, or IFN γ ^{-/-} without clinical symptoms that failed to show these gross changes. To further confirm the effects of the infection *in vivo*, we used immunohistochemical and histopathological methods on sections of brain and trigeminal ganglia of mice.

Microscopic examination of the brains stained with H&E revealed focal encephalitis characterized by mononuclear cell infiltrates and activated glial cells associated with necrosis and vascular congestion in some areas of cortex tissue of MyD88^{-/-} and of IFN γ ^{-/-} mice presenting clinical symptoms (Figure 3A and Table 2), while TLR2^{-/-} mice showed only mild vascular congestion (Figure 3A and Table 2). In contrast, brains of IFN γ ^{-/-} without clinical symptoms (data not shown) or C57BL/6 mice did not show morphological alterations. Immunoreactivity to mouse polyclonal anti-HSV-1 was observed in MyD88^{-/-} and in IFN γ ^{-/-} mice with clinical symptoms, but not in TLR2^{-/-} or IFN γ ^{-/-} mice without clinical symptoms, or C57BL/6 mice (Figure 3C). Viral infection was confirmed in trigeminal ganglia in all experimental groups, including C57BL/6, TLR2^{-/-}, IFN γ ^{-/-}, and MyD88^{-/-} mice, at the 8th day after infection, using immunohistochemistry with monoclonal antibody anti-HSV-1 (Figure 3B).

Discussion

Immune response against infection with HSV-1 is very complex. Using the murine experimental model, it has been reported that type I and type II IFNs as well as TNF- α are the main elements activated in the innate

Table 1. HSV-1-Specific PCR and Serum Neutralization to Confirm Mice Infection with HSV-1

	MyD88 ^{-/-}	TLR2 ^{-/-}	C57BL/6	nsIFN γ ^{-/-}	sIFN γ ^{-/-}
Brain PCR using HSV-1TK gene primers	+	-	-	-	+
Abs neutralization*	ND	20	60	160	ND

The brains from mice sacrificed 8 days after infection, or from animals that had died from infection, were processed to PCR reactions with specific primers for TK HSV-1 gene. PCR reactions run on brain from uninfected mice were negative. The sera from surviving animals were obtained 30 days after infection and used in the neutralization test.

*The titers are correspondent of the inverse values of the sera dilution that protects Vero cells from cytopathic effect of HSV-1, calculated as the median titer from sera from four animals. The neutralization test was performed in duplicate. sIFN^{-/-}, IFN^{-/-} mice with clinical symptoms; nsIFN^{-/-}, IFN^{-/-} mice with no clinical symptoms; ND, not done.

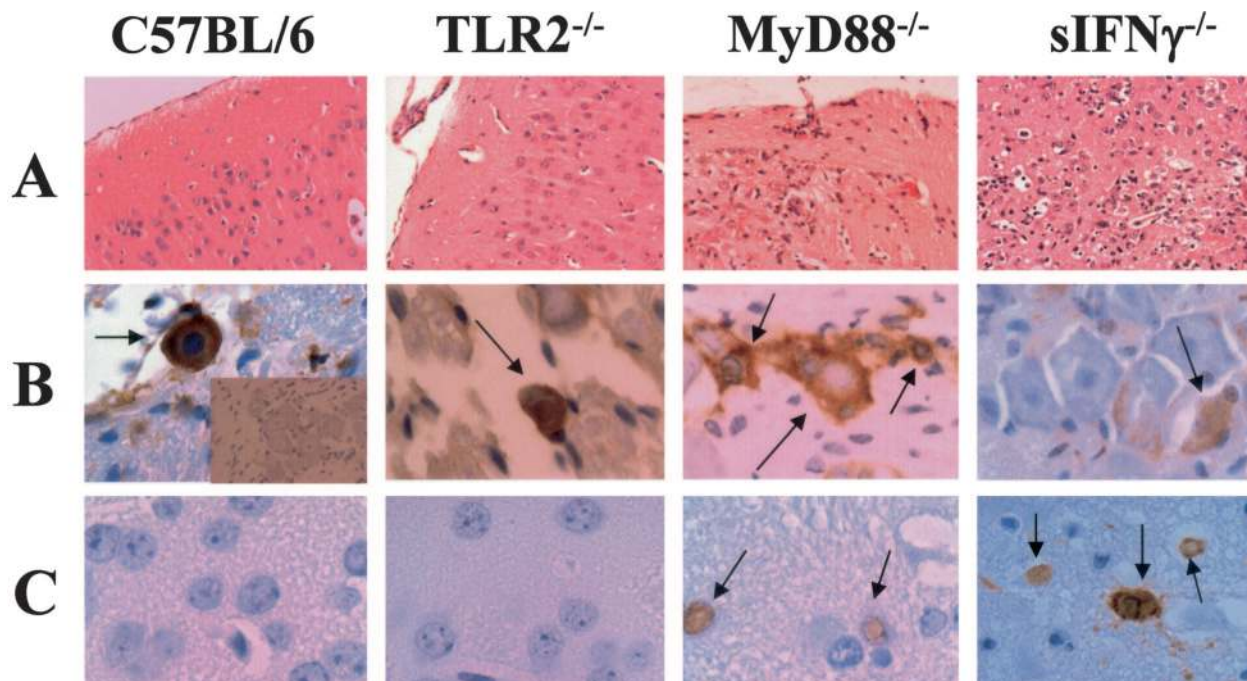


Figure 3. HSV-1 is able to enter in *MyD88*^{-/-} and *IFN* γ ^{-/-} brains and cause brain degenerative changes and necrosis. Three *TLR2*^{-/-}, *MyD88*^{-/-}, or *IFN* γ ^{-/-} mice were sacrificed 8 days after intranasal infection with 10⁴ PFU HSV-1, and representative sections of cerebral cortex (**A** and **C**) or trigeminal ganglia (**B**) were made. **A:** H&E-stained sections (see semiquantitative analysis of encephalitis in Table 2). **B:** Immunohistochemical staining for anti-gC protein from HSV-1 showing reactions against the virus (**arrows**). **C:** Anti-HSV-1 (DAKO)-immunoreactive cells (**arrows**) in affected areas of *Myd88*^{-/-} and *sIFN* γ ^{-/-} mice. The **inset in B (left)** is a negative control from the trigeminal ganglion of a noninfected WT control animal. Original magnifications: $\times 200$ (**A**); $\times 400$ (**B**); $\times 1000$ (**C**).

immune response against infection with HSV-1.²⁷⁻³² It has also been shown that HSV-1 activates both TLR2 and TLR9 in a *MyD88*-dependent manner, suggesting the importance of TLRs in encephalitis development and host resistance to this viral infection.^{14,16,17} Although we confirmed activation of TLR2 by HSV-1 in transfected CHO cells, the lack of functional *MyD88*, but not functional TLR2, resulted in severely impaired cytokine synthesis by inflammatory macrophages exposed to HSV-1. Consistently, *MyD88* knockout, but not *TLR2* knockout mice, displayed enhanced susceptibility to experimental infection with HSV-1. We favor the hypothesis that a combined effort of different TLRs is implicated in the activation of the innate immune system and host resistance to infection with HSV-1. HSV-1 is a complex enveloped virus, which has 140-kb DNA and expresses 84 proteins.^{1,31} Therefore, we speculate that HSV-1 is recognized by multiple TLRs, such as TLR2 and TLR9, that may have additive effects in activation of *MyD88* on infection with HSV-1.

Thus, simultaneous blocking of the function of multiple TLRs may be required to yield the same phenotype as seen in *MyD88*^{-/-} mice on infection with HSV-1. Nevertheless, our findings provide new information that corroborates the hypothesis that *MyD88* and possibly TLRs have an important role in host resistance to viral infection and pathogenesis observed during infection with HSV-1.

In a recent report, Boivin and colleagues² described the enhanced expression of TLR2 in the hindbrain of mice infected with HSV2. More importantly, Kurt-Jones and colleagues¹⁶ demonstrate that HSV-1 activates TLR2 *in vitro* in CHO-transfected cells. In this study, infection of adult mice with 10⁹ PFU of HSV-1 KOS strain, showed that WT mice were more susceptible to the virus infection as compared to *TLR2*^{-/-} mice. When Kurt-Jones and colleagues¹⁶ infected neonates (4-day-old mice) with 10⁴ PFU of HSV-1 KOS strain, they also observed that *TLR2*^{-/-} mice, with a mortality of 30%, were more resistant than *TLR4*^{-/-} or WT mice, which presented more

Table 2. Semiquantitative Histopathological Analysis of Encephalitis in HSV-1-Infected Animals

Parameters*	Degenerative changes and necrosis	Perivascular cuffing and congestive changes	Mononuclear cell infiltrates
Groups			
WT	-(3/3)	-(3/3)	-(3/3)
<i>TLR2</i> ^{-/-}	-(3/3)	+(3/3)	-(3/3)
<i>MyD88</i> ^{-/-}	+++ (3/3)	+++ (3/3)	+++ (3/3)
<i>sIFN</i> γ ^{-/-}	+++ (3/3)	+++ (3/3)	+++ (3/3)

*The histopathological evaluation criteria for quantification of changes was based on the alterations observed in the brain tissues collected from the different groups of mice as compared to WT; see Figure 3A. -, Absence of significant alterations; +, mild alterations; +++, intense alterations; (3/3), three of three examined animals presented the degree of changes indicated. This experiment is representative from three experiments performed. *sIFN*^{-/-}, *IFN*^{-/-} mice with clinical symptoms.

than 90% mortality. They demonstrated in their model that HSV-1-induced encephalitis and lethality was primarily mediated by TLR2.

Our *in vitro* data further confirmed that an earlier interaction from HSV-1 with the innate immunity could happen through TLR2. However, when measuring TNF- α , a critical cytokine for host resistance against HSV-1³² we found that induction of TNF- α production by inflammatory macrophages exposed to HSV-1 was abolished in cells from MyD88^{-/-}, but not in cells from TLR2^{-/-} mice. Further, we found that all MyD88^{-/-} mice died after HSV-1 intranasal inoculation of 10⁴ PFU. Conversely, the mice lacking the TLR2 functional gene have the same survival rate of WT mice, suggesting that other TLRs could be involved in the response against HSV-1. In summary, our study indicates a critical role of MyD88 in anti-viral defense, whereas Kurt-Jones and colleagues¹⁶ demonstrate that activation of TLR2 by HSV-1 will lead to detrimental inflammatory response and lethal encephalitis. Thus, one important goal of our future studies will be the identification of the TLR involved in host anti-viral defenses against HSV-1. In any case TLRs are not evenly distributed in the different organ tissues and cells and the difference in the results obtained in these studies, could be explained by the different HSV-1 strains, the size of HSV-1 inoculum, and/or the route of infection. The Kurt-Jones group¹⁶ used the intraperitoneal route and gave 10⁹ PFU of HSV-1 KOS in adults mice or 10⁴ PFU in neonates, whereas we used a clinically isolated strain, with a concentration of 10⁴ PFU in 4-week-old mice. Further, the genetic background of mice used in these studies may also have influenced the outcome of infection. We used MyD88^{-/-} and TLR2^{-/-} mice, which have been backcrossed eight times into the C57BL/6 background, and C57BL/6 as control. Kurt-Jones and colleagues¹⁶ used a F2 TLR2^{-/-} mice, and the interbred 129 \times C57BL/6 as control.

Consistent with the mortality results, we found HSV-1 replication in the brain of mice lacking the MyD88, but not in brain of TLR2^{-/-} mice. As previously shown,²⁶⁻³⁰ we also observed an enhanced susceptibility of IFN γ ^{-/-} mice infected with HSV-1 (50% of mortality). Further, we showed that MyD88^{-/-} and symptomatic IFN γ ^{-/-} presented severe neuropathological signs of encephalitis, whereas TLR2^{-/-} presented only mild neuropathological signs and the WT showed no signals in the histopathology analysis of the brain. Because HSV-1 remains in trigeminal ganglia after infection, we performed immunohistochemistry against gC protein of HSV-1 in trigeminal ganglia, and showed that all mice (WT, TLR2^{-/-}, MyD88^{-/-}, and IFN γ ^{-/-}) were efficiently infected. Additionally, we demonstrated that after 30 days of infection, all mice that survived infection produced neutralizing antibodies against HSV-1. The neutralization test was also performed at day 8 day after infection but no mice (WT, TLR2^{-/-}, MyD88^{-/-}, or IFN γ ^{-/-}) presented seropositive results. The early death from MyD88^{-/-} and IFN γ ^{-/-}, when the acquired defense was not yet established, further indicates that in our model innate immune response has a critical role in host defense against HSV-1 infection.

Finally, Lundberg and his colleagues¹⁸ described the immunostimulatory role of HSV-1 genome, which is unmethylated and rich in G+C. They showed that mouse splenocytes treated with HSV-1-derived oligonucleotides produced IFN- γ , TNF- α , and interleukin-6, and possessed a potent adjuvant activity *in vivo*, leading to TH1 response after immunization and restimulation with ovalbumin. Krug and colleagues¹⁴ also demonstrated in plasmacytoid dendritic cells, that HSV-1 activates murine cells through TLR9. They showed that these highly specialized IFN producer cells responded *in vitro* to stimulus with HSV-1 through TLR9 and MyD88. Further, Lund and colleagues¹⁷ described that activation of plasmacytoid dendritic cells by HSV-2 also occurs via TLR9. However, *in vivo* experiments¹⁴ showed that mice deficient in either MyD88 or in TLR9, although presenting impaired the response from plasmacytoid dendritic cells, could still control corneal infection with HSV-1. The 100% lethality observed in infected MyD88^{-/-} mice in this study, in comparison with the controlled infection in the mice infected by scarring of cornea, further suggest that the inoculation route and/or the strain of the virus could play an important role in the outcome of the experimental infection. Therefore, additional studies will be necessary to define what is (are) the critical TLR(s) in controlling viral replication in the brain and host resistance to infection with HSV-1.

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