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Cutting Edge: TLR2-Mediated Proinflammatory Cytokine and Chemokine Production by Microglial Cells in Response to Herpes Simplex Virus

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Recent studies indicate that TLRs are critical in generating innate immune responses during infection with HSV-1. In this study, we investigated the role of TLR2 signaling in regulating the production of neuroimmune mediators by examining cytokine and chemokine expression using primary microglial cells obtained from TLR2−/− as well as wild-type mice. Data presented here demonstrate that TLR2 signaling is required for the production of proinflammatory cytokines and chemokines: TNF-α, IL-1β, IL-6, IL-12, CCL7, CCL8, CCL9, CXCL1, CXCL2, CXCL4, and CXCL5. CXCL9 and CXCL10 were also induced by HSV, but their production was not dependent upon TLR2 signaling. Because TLR2−/− mice display significantly reduced mortality and diminished neuroinflammation in response to brain infection with HSV, the TLR2-dependent cytokines identified here might function as key players influencing viral neuropathogenesis.


Microglial cells, the resident macrophages of the brain, are sensors of viral infection within the CNS and play a pivotal role in generating innate neuroimmune responses (1, 2). During the early stages of infection, microglial cells act as key cellular mediators of neuroinflammatory processes and contribute to the first line of defense before lymphocyte infiltration into the brain by producing and secreting a number of immune modulators. Secreted cytokines and chemokines, in turn, activate immune responses of cells that infiltrate the CNS to counter invading pathogens. Although significant progress has been made in understanding neuroimmune responses to viral infections, the exact brain cell-specific molecular mechanisms that lead to the production of proinflammatory immune mediators remain to be elucidated.

In recent years, it has become clear that TLRs, a class of pattern recognition receptors, are critical in recognizing a wide range of pathogens (3). These reports suggest that viruses may trigger inflammatory cytokine production via multiple TLRs (4–8). Murine microglia express mRNA for all identified TLRs (8), and several studies have implicated TLRs as important players during a number of herpesvirus infections, depending upon the cell type. For example, HSV infection of dendritic cells induces secretion of a number of cytokines and chemokines, which then activate NK cells. Additionally, plasmacytoid dendritic cells produce IFN-α in response to HSV-1 and HSV-2 infection via TLR9-dependent and -independent pathways (6, 9–11). HSV-1 has also been shown to activate IFN-producing cells in vitro through TLR9 (8). TLR9 recognizes abundant CpG motifs in HSV-1 DNA and initiates a signaling cascade leading to NF-κB activation and cytokine secretion (12).

In addition to TLR9, TLR2 has been demonstrated to play a major role in the pathogenesis of HSV-induced encephalitis (5, 13). In support of the pivotal role for TLR2 in HSV neuropathogenesis, infected TLR2−/− mice had significantly lower mortality rates than wild-type mice, and they did not show a neuroinflammatory infiltrate (13). Both HSV-1 and HSV-2 were shown to induce IL-6 and IL-8 expression in human peripheral-blood mononuclear cells in a dose-dependent manner and to activate NF-κB. This cytokine response was found to be mediated through TLR2 and was not dependent upon virus replication.

Previous studies from our laboratory have demonstrated that HSV-1 infection of primary human astrocytes and neurons leads to robust virus growth and replication, but neither cell type produced cytokines or chemokines in response to infection. In contrast, human microglia produced significant amounts of TNF-α, IL-1β, CXCL-10, and CCL5 but did not permit productive viral replication (14). Because microglial cells produce inflammatory cytokines and chemokines in response to HSV, the role of TLR2 signaling in the generation of these mediators needs to be examined. To gain insights into the innate neuroimmune responses generated early during HSV infection and to understand the role of TLR2 signaling in facilitating these responses, we studied the production and secretion of proinflammatory immune mediators using isolated murine...
microglial cells from wild-type and TLR2<sup>−/−</sup> mice. In this study, we show for the first time that HSV infection of microglia induces a unique cytokine expression profile and that TLR2 signaling is required for robust production of a number of major proinflammatory cytokines.

**Materials and Methods**

**Mice and virus**

Wild-type C57BL/6 and TLR2<sup>−/−</sup> (Tlr2<sup>−/−</sup>) mice were purchased from The Jackson Laboratory. Purified microglial cell cultures were prepared as described previously with minor modifications (15). A total of 1 × 10<sup>6</sup> cells/well was used for the microarrays, and 2 × 10<sup>5</sup> cells/well were used for protein assays. Microglial cell cultures used in these experiments were >99% pure, as determined by MAC-1 Ab staining (Roche Applied Science). A highly neurovirulent HSV-1 17 syn strain, propagated and purified from rabbit skin fibroblasts by the standard sucrose-gradient procedure, was used for infection studies at a multiplicity of infection of 2. After adding the virus, culture plates were incubated at 37°C for 5 h.

**Oligonucleotide microarray**

The Oligo GEArray Mouse Inflammatory Cytokines and Receptors Microarray (OMM-11) (SuperArray) was used for our studies, and hybridization procedures were performed per manufacturer’s instructions. Wild-type and TLR2<sup>−/−</sup> microglial cells were treated with HSV, and total RNA was extracted after 5 h postinfection (p.i.)<sup>3</sup> using the RNeasy Mini Kit (Qiagen). Chemiluminescent detection steps were performed, and positive spots on the arrays were scanned using a Kodak Image Station and were quantified using GEArray Analysis Suite software (SuperArray). Data are presented as relative induction of each cytokine and chemokine, normalized to GAPDH, and are representative of two independent experiments.

**Real-time PCR**

The cDNA from wild-type and TLR2<sup>−/−</sup> microglial cells was synthesized using 1 μg of total RNA using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies) and oligo(dT)<sub>12-18</sub> primers (Sigma-Genosys). Quantitative real-time PCR was performed with LightCycler 2.0 thermocycler (Roche Applied Science) using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science) and oligo(dT)<sub>6–12</sub> primers (Sigma-Genosys). Quantitative real-time PCR was performed with LightCycler 2.0 thermocycler (Roche Applied Science) using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science) following the manufacturer’s specifications. The PCR conditions were as follows: 40 denaturation cycles of 95°C for 10 s, annealing at 58°C for 5 s, and elongation at 72°C for 10 s. The relative product levels were quantified using the 2<sup>−ΔΔCT</sup> method (16). Primer sequences are available upon request. Data are presented as relative induction of each cytokine and chemokine, normalized to β-actin, and are representative of two independent experiments.

**ELISA**

A sandwich ELISA-based system (17) was used to quantify cytokine and chemokine levels from microglial cell culture supernatants. In brief, purified rat anti-mouse TNF-α, IL-6, and IL-12 Abs (BioSource International), CCL2 Ab (BD Biosciences), and goat anti-mouse CXCL9 and CXCL10 Abs (R&D Systems) were coated onto microtiter plates at 2–4 μg/ml. Plates (96-well) were blocked with 1% BSA, and after adding 50 μl each sample and standard cytokines and chemokines plates were incubated for 2 h at 37°C. The wells were washed with PBS/Tween 20 (0.5%) and incubated with primary Abs followed by biotin-labeled secondary Abs for an additional 1 h at 37°C. After an extensive wash, they were treated with streptavidin for color development and were read at 450 nm. Data are presented as relative induction of each cytokine, and bars represent the mean ± SD of triplicate samples, which are representative of at least two (TLR2<sup>−/−</sup>) or three (wild-type) independent experiments.

**Immunocytochemical analysis**

At 4 h p.i., microglial cells were fixed with 4% paraformaldehyde for 20 min and washed with PBS. They were then stained with mouse anti-ICP4 Ab (Advanced Biotechologies) at a concentration of 10 μg/ml, Vector M.O.M. immunodetection kit (Vector Laboratories) containing the secondary Ab and detection reagents was used according to the manufacturer’s instructions to detect the localization of ICP4 within the nucleus of infected microglial cells.

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<sup>3</sup> Abbreviation used in this paper: p.i., postinfection.

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**Results**

Expression of immune mediators by HSV-infected murine microglia

HSV-1 infection of human microglia results in the production of proinflammatory cytokines and chemokines (14), and IL-1β, TNF-α, CCL5, IL-6, IFN-γ, and CXCL10 have been shown to be up-regulated in other types of HSV-infected primary cells as well as cell lines (5, 13, 14, 18). To investigate the role of TLR2 signaling in proinflammatory cytokine and chemokine production by microglia, we assessed the expression profiles with a mouse-specific oligonucleotide array using RNA from infected and uninfected microglial cells from both wild-type and TLR2<sup>−/−</sup> mice at 5 h p.i. The array contained pro- and anti-inflammatory cytokines and chemokines, their receptors, and signaling pathway-related molecules. Interestingly, TLR2 mediated the expression of a number of major cytokines and chemokines in wild-type cells upon infection (Table I).

**Table I. Induced expression of proinflammatory cytokines, chemokines, and related genes in HSV-infected C57BL/6 vs TLR2<sup>−/−</sup> microglial cells at 5 h p.i.**

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<thead>
<tr>
<th>Gene</th>
<th>Common Name</th>
<th>Fold Δ</th>
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<tbody>
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<td>IL-17E</td>
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</tr>
<tr>
<td>TNF-α</td>
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These immune mediators, with the exception of TNF-α, were not expressed in TLR2−/− cells showing that TLR2 signaling was required for their expression. As shown, TLR2 triggered the expression of important inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12, CCL7, CCL8, CCL9, CXCL1, CXCL2, CXCL4, and CXCL5, and the levels of others such as macrophage migration inhibitory factor were elevated in HSV-infected wild-type cells when compared with infected TLR2−/− cells (Table I). Taken together, these findings indicate that TLR2 signaling was required for the production of major inflammatory cytokines and chemokines during the early stages of HSV-1 infection.

Cytokines and chemokines induced by TLR2 signaling

To validate the differences identified in the microarray expression profiles of wild-type and TLR2−/− microglia and to confirm that the induced expression of cytokines and chemokines is indeed mediated by TLR2, we tested the expression of a smaller number of select proinflammatory cytokines and chemokines using quantitative real-time PCR. As shown in Fig. 1, the expression of TNF-α, IL-1β, IL-6, IL-12B, CXCL1, and CXCL2 was highly induced by HSV-1 in microglia from wild-type mice, but was not detectable in HSV-infected microglia from TLR2−/− mice, clearly demonstrating that the production of these immune mediators required TLR2 signaling. Differences in cytokine induction levels observed between microarray and real-time PCR assay are most likely due to the sensitivities of the methods used. The expression of two other chemokines (CXCL9 and CXCL10) and IL-18, which was not dependent upon TLR2 signaling, was also validated (Fig. 1). Although higher expression of IL-18 and CXCL10 was found in infected wild-type cells, CXCL9 was expressed at a higher level in TLR2−/− cells. Thus, CXCL9 is produced in TLR2−/− cells in response to HSV-1, possibly through other TLRs.

To further examine whether the expression patterns of selected proinflammatory cytokines and chemokines identified by array and real-time PCR studies correlated with protein levels, ELISA were performed using supernatants of HSV-treated and untreated microglial cells. For ELISA, 8-, 24-, and 48-h time points were chosen because protein production is known to appear after mRNA (14). ELISA clearly demonstrated the induction of TNF-α, IL-6, IL-12, CCL2, CXCL9, and CXCL10 in HSV-infected wild-type cells (Fig. 2A). HSV-infected TLR2−/− microglia did not produce these cytokines and chemokines at the 8-h time point. This result also confirmed that their expression is mediated through TLR2. Interestingly, the production of chemokines CCL2, CXCL9, and CXCL10 was induced in both TLR2−/− and wild-type microglia. In addition, when ELISA were performed with LPS-stimulated uninfected microglial cells, we observed no differences in the production of TNF-α between wild-type and TLR2−/− cells (Fig. 2B). Thus, LPS-stimulated signaling through TLR4 is intact in TLR2−/− microglia.
Viral entry and immediate-early gene expression in TLR2 

HSV binding to host cells takes place through the interaction of viral glycoproteins (gB or gC or both) with cell surface glycosaminoglycan chains of heparin sulfate proteoglycans (19). However, binding of virus to the cell is not sufficient to mediate penetration. In the case of human CMV, mere contact by CMV virions was found to be sufficient to trigger the production of proinflammatory cytokines and IFN-stimulated genes in PBMCs through TLR2-dependent pathway (4). To test whether production of cytokines and chemokines seen in mouse microglia was the effect of inefficient viral infection, we examined HSV entry into microglia from wild-type and TLR2 

microglial cells. Uninfected wild-type microglia (A), HSV-infected wild-type cells (B), uninfected TLR2 

microglia (C), and HSV-infected TLR2 

cells (D) were probed using Abs against ICP4 and stained following 4-h infection with HSV.

**FIGURE 3.** HSV entry and expression of ICP4 in wild-type and TLR2 

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**FIGURE 2.** TLR2-mediated expression of cytokines and chemokines in murine microglia. A, ELISA were performed for cytokines and chemokines in wild-type and TLR2 

microglial cells following exposure to HSV for 8, 24, and 48 h. B, LPS-induced expression of TNF-α in infected wild-type and TLR2 

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**Viral entry and immediate-early gene expression in TLR2 

microglial cells**

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Discussion

HSV infection of the CNS may result in severe focal, necrotizing encephalitis accompanied by swelling of the brain, leading to the death of the infected individual. However, the precise cellular and molecular mechanisms that cause mortality are unclear. It has recently been reported that wild-type adult mice succumbed to infection when a very high dose (5 \times 10^9 PFU) of HSV was administered, whereas TLR2 

mice had a lower mortality rate in response to the same viral challenge (20). In contrast, both wild-type and TLR4 

neonatal mice died after challenge with only 10^3 PFU, whereas TLR2 

animals survived at the same dose of HSV (13). Furthermore, pathological assessment showed no signs of inflammatory infiltrates in the brains of TLR2 

mice as opposed to the wild-type mice, although viral titers were the same in brains of both groups.

In the present study, we showed for the first time that TLR2 plays a pivotal role in the production of a number of proinflammatory cytokines and chemokines including TNF-α, IL-1β, IL-6, IL-12, CCL2, CCL7, CCL8, CCL9, CXCL1, CXCL4, and CXCL5. These findings clearly demonstrate that TLR2 is involved in generating neuroimmune responses through the production of these important mediators. In another study using murine microglia, it was recently reported that *Staphylococcus aureus* peptidoglycan induced TNF-α, IL-12B, CCL2,
and CCL2 production through TLR2 (21). As our results show, TLR2 does mediate the production of a number of important proinflammatory cytokines and chemokines in response to HSV infection. However, production of some viral-induced cytokines and chemokines, such as IL-18, CCL2, CXCL9, and CXCL10, does not appear to be under control of TLR2.

It has recently been reported that the expression of the IFN-γ-inducible chemokines CXCL9, CXCL10, and CXCL11, as well as CCL5, was highly elevated in the brains of HSV-infected Sjl mice (22). Induced expression of these chemokines was also observed in HSV-infected wild-type mice, but not in mice lacking CXCR3 (23). Our data presented in Fig. 1 are in agreement with these reports indicating that some chemokines (e.g., CXCR3 ligands) are produced in response to HSV-1 infection, but their expression is not dependent on TLR2.

In this study, we show that the robust production of proinflammatory cytokines seen in microglial cells was not mediated by ICP4 because this viral protein is expressed in TLR2 and TLR4 double-knockout mice. Furthermore, it has been reported that a biphasic production of cytokines takes place in some infected cell types, one that is dependent upon virus replication and one that is not (24). To examine this possibility, we studied cytokine expression profiles in TLR2−/− microglia by microarray at 16 h p.i., in addition to 5 h p.i., and no significant difference was observed between the two time points (data not shown).

Thus, it appears that, at least in murine microglia, HSV-infection does not induce a second wave of cytokine production other than that seen at early phase of infection.

In this study we focused on the early events during HSV-1 infection of microglial cells. HSV-1 was found to infect TLR2−/− microglia with equal efficiency to wild-type cells, and TLR2 itself was highly elevated in response to viral infection of wild-type microglia. This newly up-regulated TLR2 may promote additional expression of proinflammatory cytokines, further amplifying the innate immune response. Thus, inhibition of TLR2 signaling might prove to be an effective approach in the treatment of overzealous neuroimmune responses seen during HSV-induced encephalitis.

Disclosures

The authors have no financial conflict of interest.

References